Metiram

Document M-CA, Section 1

IDENTITY OF THE ACTIVE SUBSTANCE

Compiled by:

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 1 IDENTITY OF THE ACTIVE SUBSTANCE

CA 1.1 Applicant

BASF SE
D-67056 Ludwigshafen
Germany

Crop Protection Division:
P.O. Box 120
D-67114 Limburgerhof
Germany

Contact:

Alternative:

CA 1.2 Producer

Manufacturer of metiram (legal entity):

BASF SE
D-67056 Ludwigshafen
Germany

Crop Protection Division:
P.O. Box 120
D-67114 Limburgerhof
Germany

Contact:

Alternative:

Location of manufacturing plant of metiram:

CONFIDENTIAL information – data provided separately (Document J)

CA 1.3 Common Name Proposed or ISO-accepted and synonyms

There is no ISO common name for this substance. The name "metiram" is approved by several authorities (EU, USA, Japan, New Zealand, etc.). The name "metiram-zinc" has been used in the literature, but it has no official status.

CA 1.4 Chemical Name (IUPAC and CA nomenclature)

IUPAC nomenclature
Zinc ammoniate ethylenebis(dithiocarbamate)poly[ethylenebis(thiuramdisulphide)]

CA nomenclature
Tris[amine[ethylenebis(dithiocarbamato)zinc(II)][tetrahydro-1,2,4,7-dithiadiazocine-3,8-dithione] polymer

Further information
Metiram is not stable in its pure form, and therefore Metiram PAS as well as Metiram TGAI are inaccessible. The highest concentrated form available is Metiram TK (BAS 222 29 F). In the past this form was named differently like Metiram-Complex 95%, Metiram Premix 95 or Metiram TK 85. These historical and current names describe all the same form of metiram with the same composition:

Metiram TK = Metiram TK 85 = Metiram Premix 95 = Metiram–Complex 95% = BAS 222 29 F

CA 1.5 Producer's Development Code Numbers

BAS 222 F
BAS 222 29 F
Reg.No. 250248

CA 1.6 CAS, EC and CIPAC Numbers

CAS: 9006-42-2
EINECS: not assigned
CIPAC: 478

CA 1.7 Molecular and Structural Formula, Molar Mass

Molecular formula: $(C_{16}H_{33}N_{11}S_{16}Zn_3)_x$
Molar mass: $(1088.6)_x$

Structural formula:

CA 1.8 Method of Manufacture (synthesis pathway) of the active substance

CONFIDENTIAL information - data provided separately (Document J)

CA 1.9 Specification of Purity of the Active Substance in g/kg

Minimum content: 840 g/kg

CA 1.10 Identity and Content of Additives (such as Stabilisers) and impurities

CA 1.10.1 Additives

CONFIDENTIAL information - data provided separately (Document J)

CA 1.10.2 Significant impurities

CONFIDENTIAL information - data provided separately (Document J)

CA 1.10.3 Relevant impurities

Imidazolidine-2-thione (= ETU): max. 1 g/kg

CA 1.11 Analytical Profile of Batches

CONFIDENTIAL information - data provided separately (Document J)

Metiram

Document M-CA, Section 2

PHYSICAL AND CHEMICAL PROPERTIES OF THE ACTIVE SUBSTANCE

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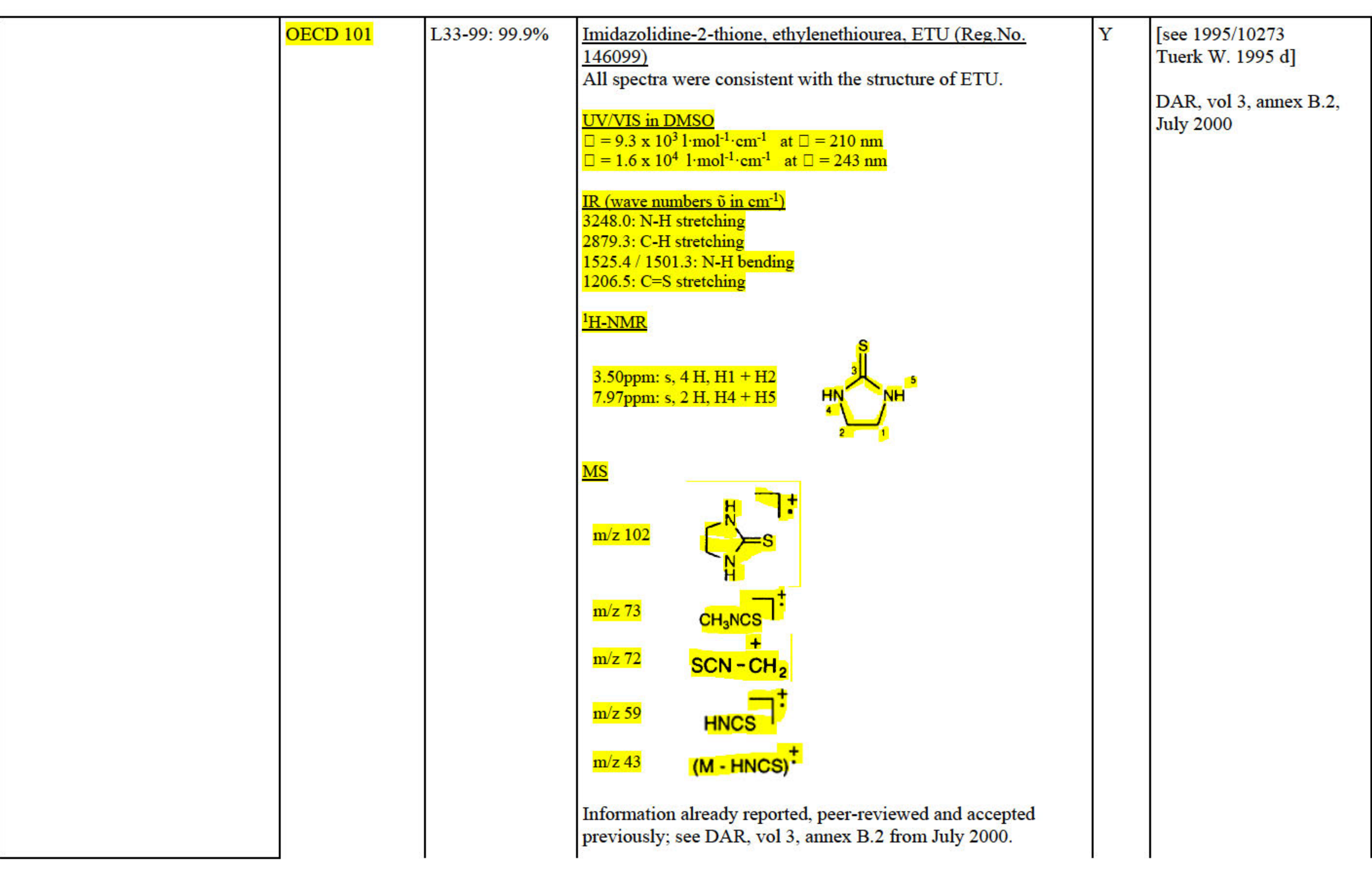
CA 2 PHYSICAL AND CHEMICAL PROPERTIES OF THE ACTIVE SUBSTANCE

Test or Study & Data Point	Guideline and method	Test material purity and specification	Findings	GLP Y/N	Reference
CA 2.1 Melting point and boiling point	OECD 102	CP015999: 90.1%	Melting point: 156.3 - 156.4 °C Boiling point: not applicable (decomposition before boiling) Information already reported, peer-reviewed and accepted previously; see DAR, vol 3, annex B.2 from July 2000.	Y Y	see 1994/11194 Turek W. 1994 a] [see 1995/10224 Turek W. 1995 a] DAR, vol 3, annex B.2, July 2000
CA 2.2 Vapour pressure, volatility	EEC A.4 OECD 104	300014: 92.7% CP015999: 90.1%	Vapour pressure $p = 7.4 \cdot 10^{-8}$ Pa (20 °C) $p = 2.4 \cdot 10^{-7}$ Pa (25 °C) Justification for new study: The old study included in the DAR (vol 3, annex B from July 2000) does not contain measurements at higher temperatures.	Y Y Y	[see 2013/1250884 Kroehl T. 2013 a] [see 1994/11194 Turek W. 1994 a] [see 1995/10224 Turek W. 1995 a]
	Calculation from water solubility and vapour pressure	not relevant	Volatility, Henry's law constant $H = 4.0 \cdot 10^{-5}$ Pa · m³ · mol⁻¹ Justification for new study: Necessary due to the new determination of the vapour pressure (2013/1250884)	N	[see 2014/1142747 Kroehl T. 2014 a]
CA 2.3 Appearance (Physical state, colour)	Visual inspection	CP015999: 90.1%	Solid consisting of a light-yellow powder. Information already reported, peer-reviewed and accepted previously; see DAR, vol 3, annex B.2 from July 2000.	Y Y	[see 1994/11194 Turek W. 1994 a] [see 1995/10224 Turek W. 1995 a] DAR, vol 3, annex B.2, July 2000

Test or Study & Data Point	Guideline and method	Test material purity and specification	Findings	GLP Y/N	Reference
CA 2.4 Spectra (UV/VIS, IR, NMR, MS), molar extinction at relevant wavelengths, optical purity	OECD 101	CP015999: 90.1%	<u>Metiram</u> All spectra were consistent with the structure of metiram. <u>UV/VIS in DMSO</u> □ = 6.4 x 10^4 $l \cdot mol^{-1} \cdot cm^{-1}$ at □ = 259 nm □ = 4.8 x 10^4 $l \cdot mol^{-1} \cdot cm^{-1}$ at □ = 281 nm □ = 3.0 x 10^4 $l \cdot mol^{-1} \cdot cm^{-1}$ at □ = 295 nm <u>IR (wave numbers ῦ in cm^{-1})</u> 3242.7: N-H stretching 3008.7: overtone of N-H bending at 1504.7 cm^{-1} 2933.5: C-H stretching 1521.1 / 1504.7: N-H bending 1106.5: C-N stretching 626.0: C-S stretching <u>^{1}H-NMR</u> Metiram is a mixture of various polymers $(C_{16}H_{33}N_{11}S_{16}Zn_3)_x$ which does not allow an unambiguous assignment of the NMR-signals. <u>MS</u> m/z 144 $[SCN-CH_2-CH_2-NCS]^{+\bullet}$ m/z 102 [imidazolidine-2-thione, N-H, N-H, =S]$^{+\bullet}$ m/z 76 $[CS_2]^{+\bullet}$ m/z 72 $SCN-CH_2^{+}$ Information already reported, peer-reviewed and accepted previously; see DAR, vol 3, annex B.2 from July 2000.	Y	[see 1995/10069 Tuerk W. 1995 b] [see 1995/10211 Tuerk W. 1995 c] DAR, vol 3, annex B.2, July 2000

Test or Study & Data Point	Guideline and method	Test material purity and specification	Findings	GLP Y/N	Reference
	OECD 101	300011: 91.7%	In the old study the UV/VIS is not covering the range between 400 – 700 nm. Therefore a new study was performed UV/VIS in DMSO (pH 9.0) ε = 74955 $l\cdot mol^{-1}\cdot cm^{-1}$ at λ = 259 nm ε = 61397 $l\cdot mol^{-1}\cdot cm^{-1}$ at λ = 280 nm ε = 49013 $l\cdot mol^{-1}\cdot cm^{-1}$ at λ = 290 nm ε = 4635 $l\cdot mol^{-1}\cdot cm^{-1}$ at λ = 349 nm UV/VIS in DMSO/water (10/90) (pH 6.4) ε = 64348 $l\cdot mol^{-1}\cdot cm^{-1}$ at λ = 259 nm ε = 61943 $l\cdot mol^{-1}\cdot cm^{-1}$ at λ = 283 nm ε = 54682 $l\cdot mol^{-1}\cdot cm^{-1}$ at λ = 290 nm ε = 7191 $l\cdot mol^{-1}\cdot cm^{-1}$ at λ = 341 nm UV/VIS in DMSO/1M HCl/water (10/5/85) (pH 1.5) ε = 41323 $l\cdot mol^{-1}\cdot cm^{-1}$ at λ = 241 nm ε = 28300 $l\cdot mol^{-1}\cdot cm^{-1}$ at λ = 280 nm ε = 26929 $l\cdot mol^{-1}\cdot cm^{-1}$ at λ = 290 nm UV/VIS in DMSO/1M NaOH/water (10/5/85) (pH 12.4) ε = 72968 $l\cdot mol^{-1}\cdot cm^{-1}$ at λ = 258 nm ε = 86688 $l\cdot mol^{-1}\cdot cm^{-1}$ at λ = 286 nm ε = 79009 $l\cdot mol^{-1}\cdot cm^{-1}$ at λ = 290 nm ε = 4670 $l\cdot mol^{-1}\cdot cm^{-1}$ at λ = 332 nm	Y	[see 2008/1086981 Kroehl T. 2008 a]



	OECD 101	L33-99: 99.9%	Imidazolidine-2-thione, ethylenethiourea, ETU (Reg.No. 146099) All spectra were consistent with the structure of ETU. UV/VIS in DMSO □ = 9.3 x 10^3 l·mol^{-1}·cm^{-1} at □ = 210 nm □ = 1.6 x 10^4 l·mol^{-1}·cm^{-1} at □ = 243 nm IR (wave numbers ῦ in cm^{-1}) 3248.0: N-H stretching 2879.3: C-H stretching 1525.4 / 1501.3: N-H bending 1206.5: C=S stretching ^{1}H-NMR 3.50ppm: s, 4 H, H1 + H2 7.97ppm: s, 2 H, H4 + H5 MS m/z 102 m/z 73 $CH_3NCS^{+\cdot}$ m/z 72 $SCN-CH_2^+$ m/z 59 $HNCS^{+\cdot}$ m/z 43 $(M - HNCS)^{+\cdot}$ Information already reported, peer-reviewed and accepted previously; see DAR, vol 3, annex B.2 from July 2000.	Y	[see 1995/10273 Tuerk W. 1995 d] DAR, vol 3, annex B.2, July 2000

	OECD 101	01815-177: 99.9%	In the old study the UV/VIS is not covering the range between 400 – 700 nm. Therefore a new study was performed. UV/VIS in Methanol (pH 6.8) ε = 9958 $l \cdot mol^{-1} \cdot cm^{-1}$ at λ = 204 nm ε = 15983 $l \cdot mol^{-1} \cdot cm^{-1}$ at λ = 238 nm ε = 107 $l \cdot mol^{-1} \cdot cm^{-1}$ at λ = 290 nm ε = 107 $l \cdot mol^{-1} \cdot cm^{-1}$ at λ = 295 nm	Y	[see 2014/1189178 Kroehl T. 2014 b]
CA 2.5 Solubility in water	CIPAC 3053/M (column elution) - comparable to A.6 and OECD 105- and analytical determination via CS_2 method	WH4264: 93.5%	Solubility ≤ 2 mg/l at 20 °C Information already reported, peer-reviewed and accepted previously; see DAR, vol 3, annex B.2 from July 2000.	N N	see 1985/10132 Pawliczek J.B., Keller W. 1985 a] [see 2000/1013264 Daum A. 2000 a] DAR, vol 3, annex B.2, July 2000
CA 2.6 Solubility in organic solvents	CIPAC MT 181	CP6169: 91.4%	Solubility < 0.1 g/l at 25 °C in n-hexane, toluene, dichloromethane, methanol, isopropanol, acetone, ethyl acetate. Information already reported, peer-reviewed and accepted previously; see DAR, vol 3, annex B.2 from July 2000.	Y	[see 1990/10892 Redeker J. 1990 a] DAR, vol 3, annex B.2, July 2000
CA 2.7 Partition co-efficient n-octanol/water	OECD 107) (shake-flask method, analyzed via ^{14}C scintillation counter)	Metiram (14C-labelled): 95.6%, specific activity: 41.05 mCi/m Mol	Metiram log Pow (at pH 5) = 1.9 log Pow (at pH 7) = 0.33 log Pow (at pH 9) = -0.37 Information already reported, peer-reviewed and accepted previously; see DAR, vol 3, annex B.2 from July 2000.	N N	[see 1985/10105 Keller W. 1985 a] [see 2000/1013277 Grote C. 2000 a] DAR, vol 3, annex B.2, July 2000
	Calculation (ACD/Labs)		Imidazolidine-2-thione, ethylenethiourea, ETU (Reg.No. 146099) log Pow (at pH 5.5) = -0.66 log Pow (at pH 7.0) = -0.66 log Pow (at pH 9.0) = -0.66 Justification for new study: New data, previously not available.	N	[see 2014/1321435 Anonymous 2015 a]
	Calculation (ACD/Labs)		Imidazolidine-2-one, ethylene urea, EU (Reg.No. 27270) log Pow (at pH 5.5) = -1.24 log Pow (at pH 7.0) = -1.24	N	[see 2014/1321434 Anonymous 2015 b]

			log Pow (at pH 9.0) = -1.24 Justification for new study: New data, previously not available.		
	OECD 107) (shake-flask method, analysed via Kjeldahl)	98.5%	Imidazolidine-2-one, ethylene urea, EU (Reg.No. 27270) log Pow = -1.16 Justification for new study: New data, previously not available.	N	[see 2015/1040811 Anonymous 2015 c]
	Calculation (ACD/Labs)		5,6-dihydro-3H-imidazo[2,1-c]-1,2,4-dithiazole-3-thione, EBIS (Reg.No. 243959) log Pow (at pH 5.5) = 1.77 log Pow (at pH 7.0) = 1.77 log Pow (at pH 9.0) = 1.77 Justification for new study: New data, previously not available.	N	[see 2014/1321432 Anonymous 2015 d]
	Calculation (ACD/Labs)		2,3,7,8-tetrahydrodiimidazo[2,1-b:1',2'-e][1,3,5]thiadiazine-5-thione, TDIT (Reg.No. 4670450) log Pow (at pH 5.5) = 0.75 log Pow (at pH 7.0) = 0.75 log Pow (at pH 9.0) = 0.75 Justification for new study: New data, previously not available.	N	[see 2014/1321433 Anonymous 2015 e]
CA 2.8 Dissociation in water - dissociation constant(s) (pKa values) - identity of dissociated species - dissociation constant(s) (pKa values) of the active principle			The insolubility of metiram in water and the decomposition of metiram in water prevents the existence of a dissociation constant of metiram.		
CA 2.9 Flammability and	EEC A.10	30014: 92.7%	Flammability Metiram is not considered highly flammable.	Y	[see 2012/1254517 Duerrstein 2013 a]

self-heating			New study available.		
	EEC A.15	30014: 92.7%	Self-ignition The Relative Self-Ignition Temperature is 395.0 °C. New study available.		
CA 2.10 Flash point			Metiram is a solid with a melting point > 40 °C. Therefore, the flash point has not been determined.		
CA 2.11 Explosive properties	EEC A.14 OECD 103	30014: 92.7%	Metiram is not considered explosive (energy release in DSC < 500 F/g). Justification for new study: No study was available, only expert statement.	Y	[see 2012/1254517 Duerrstein 2013 a]
CA 2.12 Surface Tension	EEC A.5 (ring method)	94-2	71.8 mN/m (0.5%, 20°C) 67.2 mN/m (2.0%, 20°C) Information already reported, peer-reviewed and accepted previously; see DAR, vol 3, annex B.2 from July 2000.	Y Y	[see 1994/10792 Kroehl T. 1994 a] [see 1994/11001 Kroehl T. 1994 b] DAR, vol 3, annex B.2, July 2000
CA 2.13 Oxidising properties	EEC A.17	30014: 92.7%	Metiram is not considered an oxidising substance. Justification for new study: No study was available, only expert statement.	Y	[see 2012/1254517 Duerrstein 2013 a]
CA 2.14 Other studies	EEC A.4 OECD 104	01815-177: 99.9 %	Vapour pressure of imidazolidine-2-thione, ethylenethiourea, ETU (Reg.No. 146099) p = 3.1 · 10^{-6} Pa (20 °C) p = 6.8 · 10^{-6} Pa (25 °C) Justification for new study: New data, previously not available.	Y	[see 2012/1206921 Kroehl T. 2012 a]
	EEC A.4 OECD 104	L32-228: 99.2 %	Vapour pressure of 5,6-dihydro-3H-imidazo[2,1-c]-1,2,4-dithiazole-3-thione, EBIS (Reg.No. 243959) p = 1.6 · 10^{-3} Pa (20 °C) p = 2.8 · 10^{-3} Pa (25 °C) Justification for new study: New data, previously not available.	Y	[see 2013/1126048 Kroehl T. 2013 b]

		42779588Q0: 98%	Properties of technical imidazolidine-2-one, ethylene urea, EU (Reg.No. 27270) Water solubility: 596 g/L (20 °C) Justification for new study: New data, previously not available.	Y	[see 2005/1043640 Dolich T. 2005 a]
		090/2012 (aus Charge 12-0048): 100% Approx.	Properties of dried imidazolidine-2-one, ethylene urea, EU (Reg.No. 27270) Vapour pressure: 1.6 x 10^{-5} hPa (20 °C), 3.1 x 10^{-5} hPa (25 °C) Justification for new study: New data, previously not available.	Y	[see 2013/1418420 Sametschek E. 2013 a]

Metiram

Document M-CA, Section 3

FURTHER INFORMATION ON THE ACTIVE SUBSTANCE

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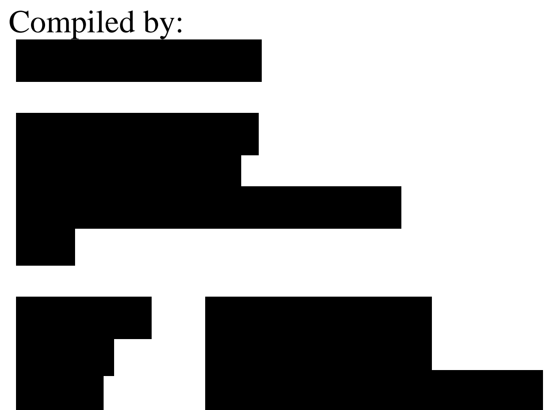

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CA 3 FURTHER INFORMATION ON THE ACTIVE SUBSTANCE

CA 3.1 Use of the Active Substance

Metiram belongs to the group of non systemic fungicide Ethylenebisdithiocarbamates (EBDCs). EBDCs are used on a wide range of crops world wide, including grape, pomefruit, leafy, root and fruiting vegetables. They control many fungal diseases such as blight, leaf spot, rust, downy mildew and scab.

CA 3.2 Function

Metiram is mainly used to protect perennial as well as annual crops from most important fungal diseases.

CA 3.3 Effects on Harmful Organisms

Metiram is a non systemic fungicide having protective action. It inactivates fungal proteins by interacting with essential thiol groups, becoming fungitoxic.

CA 3.4 Field of Use Envisaged

Agriculture

CA 3.5 Harmful Organisms Controlled and Crops or Products Protected or Treated

Metiram is used to control a broad range of important fungal diseases such as

- *Albugo spp*
- *Alternaria spp.*
- *Bremia spp.*
- *Colletotrichum spp.*
- *Diplocarpon rosea*
- *Erysiphe spp.*
- *Guignardia bidwellii*
- *Microsphaera spp.*
- *Phomopsis viticola*
- *Phytophthora infestans*
- *Plasmopara spp.*
- *Podosphaera spp.*
- *Pseudoperonospora cubensis*
- *Pseudopezicula tracheiphila*
- *Puccinia spp.*
- *Septoria spp.*
- *Sphaerotheca spp.*
- *Stemphylium vesicarium*
- *Uromyces spp.*
- *Venturia inaequalis*
- *Venturia pirina,*

Metiram is used in a wide range of crops including (representative uses in bold):

Vegetables

- Bulb vegetables
    - Onions
    - Shallot
    - Garlic
- Root and tuber vegetables
    - **Potato**
    - Celeriac
- Stem vegetables
    - Asparagus
- Fruiting vegetables
    - Cucurbits with edible peel (cucumber, gherkins, courgette, zucchini)
    - Cucurbits with inedible peel (melon, watermelon, pumpkin)
    - Tomato
    - Aubergine
- Leafy vegetables
    - Lettuce and salad plants
    - Fresh herbs and edible flowers

Perennial crops

- **Grapes**
- Pomefruit

Others

- Ornamentals

CA 3.6 Mode of Action

Metiram belongs to the group of fungicides known as the ethylene-bis-dithiocarbamates (dithiocarbamates). They are considered to have a multi-site, non-specific mode of action that disrupts many of the essential processes within the fungal cell. The exact biochemical mode of action of dithiocarbamates is not known. It is thought they act as fungicides by being metabolized to a radical which inactivates the sulphydryl groups in amino acids in fungal cells. The dithiocarbamate ion is able to form a complex with metal ions contained in some enzymes and in this way to deactivate them. These effects of metiram lead to a strong inhibition of fungal spores.

CA 3.7 Information on Occurrence or Possible Occurrence of the Development of Resistance and Appropriate Management Strategies

Despite widespread use of dithiocarbamates over the past fifty years, there are no cases of practical resistance to the dithiocarbamate group of fungicides: consequently no mechanism of resistance has been studied.

No reduction of field efficacy of metiram has been reported in numerous field trials in many crops against different pathogens over many years.

Strains of fungi that had developed resistance to other chemical classes of fungicides are not cross-resistant to dithiocarbamates.

CONCLUSION

The resistance risk for metiram is very low and no resistance management strategies are needed.

CA 3.8 Methods and Precautions Concerning Handling, Storage, Transport or Fire

Personal protection: Control parameters

Components with occupational exposure limits: CAS 100 – 97 -0: methenamine; hexamethylenetetramine

Exposure controls

Personal protective equipment

Respiratory protection:
Suitable respiratory protection for lower concentrations or short-term effect: Particle filter with medium efficiency for solid and liquid particles (e.g. EN 143 or 149, Type P2 or FFP2).

Hand protection:
Suitable chemical resistant safety gloves (EN 374) also with prolonged, direct contact (Recommended: Protective index 6, corresponding > 480 minutes of permeation time according to EN 374): E.g. nitrile rubber (0.4 mm), chloroprene rubber (0.5 mm), butyl rubber (0.7 mm) and other

Eye protection:
Safety glasses with side-shields (frame goggles) (e.g. EN 166)

Body protection:
Body protection must be chosen depending on activity and possible exposure, e.g. apron, protecting boots, chemical-protection suit (according to EN 14605 in case of splashes or EN ISO 13982 in case of dust).

General safety and hygiene measures
Wearing of closed work clothing is recommended. Handle in accordance with good industrial hygiene and safety practice. Keep away from food, drink and animal feeding stuffs.

Precautions for safe handling

No special measures necessary if stored and handled correctly. Ensure thorough ventilation of stores and work areas. When using do not eat, drink or smoke. Hands and/or face should be washed before breaks and at the end of the shift.

Protection against fire and explosion:
Avoid dust formation. Dust can form an explosive mixture with air. Prevent electrostatic charge – sources of ignition should be kept well clear – fire extinguishers should be kept handy.

Conditions for safe storage, including any incompatibilities

Segregate from foods and animal feeds.
Further information on storage conditions: Protect against moisture. Keep away from heat. Protect from direct sunlight.

Transport

Land transport

ADR

- Hazard class: 9
- Packing group: III
- ID number: UN 3077
- Hazard label: 9, EHSM
- Proper shipping name: ENVIRONMENTALLY HAZARDOUS SUBSTANCE, SOLID, N.O.S. (contains METIRAM)

RID

- Hazard class: 9
- Packing group: III
- ID number: UN 3077
- Hazard label: 9, EHSM
- Proper shipping name: ENVIRONMENTALLY HAZARDOUS SUBSTANCE, SOLID, N.O.S. (contains METIRAM)

Inland waterway transport

ADN

- Hazard class: 9
- Packing group: III
- ID number: UN 3077
- Hazard label: 9, EHSM
- Proper shipping name: ENVIRONMENTALLY HAZARDOUS SUBSTANCE, SOLID, N.O.S. (contains METIRAM)

Sea transport

IMDG

- Hazard class: 9
- Packing group: III
- ID number: UN 3077
- Hazard label: 9, EHSM
- Marine pollutant: YES
- Proper shipping name: ENVIRONMENTALLY HAZARDOUS SUBSTANCE, SOLID, N.O.S. (contains METIRAM)

Air transport

IATA/ICAO

- Hazard class: 9
- Packing group: III
- ID number: UN 3077
- Hazard label: 9, EHSM
- Proper shipping name: ENVIRONMENTALLY HAZARDOUS SUBSTANCE, SOLID, N.O.S. (contains METIRAM)

Fire-fighting measures

Extinguishing media

Suitable extinguishing media: water spray, foam, dry powder
Unsuitable extinguishing media for safety reasons: carbon dioxide

Special hazards arising from the substance or mixture

Carbon monoxide, carbon dioxide, hydrogen sulfide, nitrogen oxides, sulfur oxides might be released in case of fire.

Advice for fire-fighters

Special protective equipment: Wear self-contained breathing apparatus and chemical-protective clothing.

Further information: Keep containers cool by spraying with water if exposed to fire. In case of fire and/or explosion do not breathe fumes. Collect contaminated extinguishing water separately, do not allow to reach sewage or effluent systems.
Dispose of fire debris and contaminated extinguishing water in accordance with official regulations.

CA 3.9 Procedures for Destruction or Decontamination

Waste treatment methods

Must be sent to a suitable incineration plant, observing local regulations.

Contaminated packaging:
Contaminated packaging should be emptied as far as possible and disposed of in the same manner as the substance/product.

Methods and material for containment and cleaning up

For small amounts: Contain with dust binding material and dispose of.

For large amounts: Sweep/shovel up.

Dispose of absorbed material in accordance with regulations. Collect waste in suitable containers, which can be labeled and sealed. Clean contaminated floors and objects thoroughly with water and detergents, observing environmental regulations. Avoid raising dust.

CA 3.10 Emergency Measures in Case of an Accident

First-aid measures

Description of first aid measures

Remove contaminated clothing.

If inhaled: Keep patient calm, remove to fresh air, seek medical attention.

On skin contact: Wash thoroughly with soap and water.

On contact with eyes: Wash affected eyes for at least 15 minutes under running water with eyelids held open.

On ingestion: Immediately rinse mouth and then drink 200-300 ml of water, seek medical attention.

Most important symptoms and effects, both acute and delayed

The most important known symptom is skin sensitization.

Indication of any immediate medical attention and special treatment needed

Treatment: Treat according to symptoms (decontamination, vital functions), no specific antidote known.

Personal precautions, protective equipment and emergency procedures

Use personal protective clothing. Avoid contact with the skin, eyes and clothing. Avoid dust formation.

Environmental precautions

Do not discharge into the subsoil/soil. Do not discharge into drains/surface waters/groundwater.

Metiram

Document M-CA, Section4

ANALYTICAL METHODS

Compiled by:

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number
29/Feb/2016	Food of animal origin; General description modified CA 4.1.2/12; Additional Study, 2015/1000807 CA 4.2/2; Additional Study, 2016/1030250	BASF DocID 2016/1051891
31/Aug/2017	Soil, supplemental information on analytical methods used in efate studies CA 4.1.2/6 (already as 7.1.1.1/4, 2010/1090462), Analytical methods only CA 4.1.2/7 (already as 7.1.2.1.2/14, 2014/1189253), Analytical methods only CA 4.1.2/8 (already as 7.1.2.1.2/19, 2010/1056131), Analytical methods only CA 4.1.2/9 (already as 7.1.2.1.2/21, 2014/1189254), Analytical methods only CA 4.1.2/10 (already as 7.1.3.1.1/1, 2013/1095966), Analytical methods only CA 4.1.2/11 (already as 7.1.3.1.2/7, 2010/1065127), Analytical methods only CA 4.1.2/12 (already as 7.1.3.1.2/8, 2014/1000103), Analytical methods only	BASF DocID 2017/1161175

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 4 ANALYTICAL METHODS

Changes of update 1 were highlighted in yellow. Changes in update 2 are updated in light blue.

CA 4.1 Methods used for the generation of pre-approval data

CA 4.1.1 Methods for the analysis of the active substance as manufactured

(a)Determination of the pure active substance in the active substance as manufactured and specified in the dossier submitted in support of approval under Regulation (EC) No 1107/2009

Report:	*CA 4.1.1/1* *Daum A., 2009a* *Addendum to analytical method CF-A 633, BASF DocID 2001/1020544* *2009/1072428*
Guidelines:	*none*
GLP:	*no*

Report:	*CA 4.1.1/2* *Ziegler H., 2001a* *Quantitative determination of the active ingredient Metiram in BAS 222 28 F by titration* *2001/1020544*
Guidelines:	*none*
GLP:	*no*

Report:	*CA 4.1.1/3* *Ziegler H., 2002a* *Validation of the analytical method CF-A 633 - Determination of Metiram in water dispersible granules (WG) (BAS 222 28 F)* *2002/1004723*
Guidelines:	*Appendix 1 to § 19 a Section 1 Chemikaliengesetz of 25th July 1994*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Principle of the method

Metiram is determined according to method AFL0633/01. The new method number was assigned in the course of implementation of method CF-A 633 into an EDV based LIMS (Lab Information and Management System) and thus method AFL0633/01 is identical with method CF-A 633.

The content of the active ingredient is determined by CS2 liberation and titration according to analytical method AFL0633/01 (= CF-A 633). It is very unlikely that CS_2 is liberated from Reg.No. 251072, Reg.No. 146099 or Reg.No. 240586. Theoretically CS_2 may be liberated from Reg.No. 243959 and Reg.No. 247214. Due to the low content of these impurities determined by specific HPLC and GC methods the contribution to the total amount of CS_2 can be neglected.

The analytical method AFL0633/01 (= CF-A 633) is equivalent to CIPAC method 61/TC/M/1.2.

Specificity (selectivity)

The specificity of the titration method is given by CS_2 liberation. This reaction is typical for dithiocarbamates.

Linearity

Five samples were prepared with varying concentrations and analyzed in accordance with the analytical method. The linearity range evaluated is 35-105 %. The results prove the linearity in the investigated range.

Intercept:	5
Slope:	63
Correlation factor:	1.0000

Accuracy (recovery)

Known amounts of active ingredient were weighed six times and analyzed in accordance with the analytical method finding a mean recovery of 99.76% (RSD 0.269%)

Precision (repeatability)

A sample was weighed and analyzed six times in accordance with the analytical method finding a coefficient of variation of 0.174%. The RSD values meet the requirements given by the modified Horwitz equation (Horwitz RSDR * 0.67).

A sample BAS 222 28 F, Batch WF 18671 was weighed six times and the active ingredient content was determined.

Conclusion

The validation experiments demonstrate, that the herein described method AFL0633/01 (= CF-A 633) is suitable for the determination of the purity of technical Metiram (= Metiram TK).

(b) Determination of significant and relevant impurities and additives (such as stabilisers) in the active substance as manufactured

Report: CA 4.1.1/4
Daum A.,Machauer B., 2012a
Determination of ETU in formulations containing Metiram as active ingredient
2012/1015461
Guidelines: none
GLP: no
(certified by <none>)

Report: CA 4.1.1/5
Daum A., 2012b
Validation of analytical method AFL0845/01: Determination of ETU in WG formulations containing Metiram as active ingredient (BAS 222 28 F,BAS 222 29 F,BAS 652 00 F,BAS 642 00 F,BAS 518 01 F)
2012/1015462
Guidelines: EC 1107/2009 (14 June 2011), EPA 830.1000, EPA 830.1800, SANCO/3030/99 rev. 4 (11 July 2000), CIPAC 3807 (improved version), OECD Principles of Good Laboratory Practice, GLP Principles of the German Chemikaliengesetz (Chemicals Act)
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method
The content of Reg.No. 146099 (ETU) is determined by reversed phase HPLC with UV detection and external calibration according to analytical method AFL0845/01.

For the determination of ETU exists CIPAC method MT 162.1.

Identity
The identity was proved by comparison of the retention time and by individual fragmentation pattern of HPLC/MS.

Specificity (selectivity)
The specificity of the method was demonstrated by chromatographic and spectrometric examination, i.e. by HPLC-analysis of the species. The identity of the analytes was verified by comparing the particular retention times of the reference items with that of the test items, UVspectra within DAD and MS-Spectra within Mass Spectrometer. There were no indications of interferences due to other components.

Linearity
Five different concentrations of ETU were prepared and analyzed in each case in accordance with the present analytical method. The samples cover a concentration range of 0.008-0.5 %. The results prove the linearity in the investigated range.

Intercept:	9802
Slope:	3337678
Correlation factor:	1.0000

Accuracy (recovery)
Mean recoveries for the impurity ETU were found to be in a range of 96.12 % to 107.32 % at levels of approx. 0.01 % and 0.25 % of the concentrations of the test item. These values demonstrate that the observed results correspond in a high degree to the true values of the analytes in the samples.

Precision (repeatability)
A sample was weighed and analyzed six times in accordance with the analytical method finding a coefficient of variation of 8.427% at a mean value of 0.007%. The same experiment with fortified samples gave a coefficient of variation of 1.71% at a mean value of 0.108%. For the experiment with the fortified samples the RSD values meet the requirements given by the modified Horwitz equation (Horwitz RSDR * 0.67).

Component Analyte	Nominal Conc. [%]	corresp. conc. ' C '	RSD_R [%] ('inter' lab CV)	RSD_r [%] ('intra' lab CV)	% RSD analyzed	% RSD acceptable
ETU	0.108	0.00108	5.59	3.75	1.1711	yes

Limit of Quantification
The limit of quantification (LOQ) is 0.01%.

Conclusion
The validation experiments demonstrate, that analytical method AFL0845/01 is suitable for the determination of ETU in technical Metiram (= Metiram TK).

Further analytical methods contain CONFIDENTIAL information and are described in the confidential part (Document J).

CA 4.1.2 Methods for risk assessment

For the reviewers convenience a concordance list of naming and designations of reference compounds mentioned in CA 4.1.2 and 4.2 as well as the respective sections of Doc N is given below.

Table 4.1.2-1: List of Analytes

Common/Descriptive Name	BAS Code	Reg.No.	Actual Metabolite Code
Metiram	222 F	250284	M222F000
ETU		146099	M222F002
EU		27270	M222F003
EBIS		243959	M222F004
TDIT		4670450	M222F007
Hydantoin (HY)		132345	M222F008

The following methods cover the compounds necessary for the discussion on the residue definition for risk assessment for the environmental fate compartments as well as for the products of plant and animal origin as summarized in Document N.

(a) Methods in soil, water, sediment, air and any additional matrices used in support of environmental fate studies

Soil

Analytical methods for the determination of Metiram residues in soil were reviewed in the context of the inclusion in Annex I under Directive 91/414/EEC. These methods evaluated previously are summarized in Table 4.1.2-2 for the reviewer´s convenience.

Table 4.1.2-2: Summary of peer-reviewed analytical methods for determination of Metiram residues in soil

Method No.	Matrix	Method principle	Target analytes	LOQ mg/kg	Year	DocID	EU reviewed
M509 Zangmeister W. 2002 a	soil	CS2 as potassium-O-methyl-dithiocarbonate by UV-detection	Metiram	0.05	2002	2002/1005333	yes
MS270 (Gottschalk, R. 2002)	soil	residue method exists for mancozeb Due to the fact that this method measures CS2, such method is also adequate to address Metiram requirements	Metiram	n.a.	2002	(Letter of Access from Mancozeb Consortium)	yes
Gottschalk, R. 2000	soil	LC/MS/MS	ETU	n.a.	2000	(Letter of Access from Mancozeb Consortium)	yes
Novak R.A. 1986 a	soil	butyl-ETU by GC-FPD	ETU	0.01	1986	1986/10204	yes

Report: CA 4.1.2/1
Seibold A.,Class T., 2015a
Validation of analytical method L0241/01 for the determination of Metiram BAS 222 F as Methyl-EDBC in soil by LC-MS/MS
2014/1175671

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), EPA 860.1340: Residue Chemistry Test Guidelines - Residue Analytical Method

GLP: yes
(certified by Landesamt fuer Umwelt, Messungen und Naturschutz Baden Wuerttemberg, Karlsruhe, Germany)

Principle of the method

BASF method L0241/01, developed and validated at PTRL Europe, was developed for the determination of Metiram (BAS 222 F) after methylation with iodomethane as methyl-ethylenebis(dithiocarbamate) derivative (Me-EBDC) in soil by LC-MS/MS with a limit of quantification (LOQ) of 0.050 mg kg^{-1}.

5 g soil samples were extracted with EDTA buffer solution by shaking for 30 min on a horizontal shaker and sonication for 10 min, followed by centrifugation for 5 min at 4000 rpm. After decantation of the supernatant into a volumetric flask the extraction was repeated once and the extracts were combined and filled up with EDTA buffer solution. After addition of internal standard (100 ng mL^{-1} Nabam D4) to an aliquot of the raw extract and subsequent methylation with iodomethane, 2M calcium chloride hexahydrate solution was added to the sample and filled up with water. After centrifugation of the methylated sample for 3 min and 4000 rpm the soil extracts were analyzed by LC-MS/MS.

Recovery findings

The method proved to be suitable to determine residues of Metiram in soil. Samples were spiked with the analyte at the limit of quantification of 0.050 mg kg^{-1} and ten times higher (0.50 mg kg^{-1}). All average recovery values (mean of four or five replicates per fortification level and analyte) were between 70% and 110%. The detailed results are given in the table below (Table 4.1.2-3).

Table 4.1.2-3: Results of the method validation for the determination of Metiram in soil LUFA 2.2 and LUFA 5M

Soil	Analyte	m/z	Fortification level [mg kg^{-1}]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
LUFA 2.2	Metiram	241 → 134	0.050 0.50	5 5	78 82	7.4 3.6	80	6.3
		241 → 193	0.050 0.50	5 5	76 81	6.4 4.6	79	6.5
LUFA 5M	Metiram	241 → 134	0.050 0.50	5 4	99 74	18 7.0	88	21
		241 → 193	0.050 0.50	5 4	102 73	17 8.6	89	22

RSD = Relative standard deviation

Linearity
Good linearity (r > 0.99) was observed in the range of 0.025 or 0.026 ng mL^{-1} to 5.0 or 5.28 ng mL^{-1} for the two mass transitions using internal standard calibration.

Specificity
LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of Metiram in soil. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary.
Significant interferences (> 30% of LOQ) were not observed at the retention time and mass transitions of the analyte.

Matrix Effects
All final extracts contained as internal standard the stable deuterated isotope of the methylated monomer which compensates any observed matrix effects (differences < 20%).

Limit of Quantification
The method has a limit of quantification (LOQ) of 0.050 mg kg^{-1}, resulting from the lowest concentration level successfully tested within recovery experiments.

Limit of Determination
The method has a limit of determination (LOD) of 0.004 mg kg^{-1}, corresponding to the lowest calibration level used.

Repeatability
The relative standard deviations (RSD, %) for all fortification levels were < 20%.

Stability Working Solutions
Metiram and the internal standard Nabam D4 were not stable over the tested time period of 6 days in stock or fortification solutions (using water, EDTA buffer solution or methanol/water (20/80, v/v) as solvents) as well as in calibration solutions (using EDTA buffer solution as solvent) stored under refrigerated conditions. Thus, standard solutions have to be prepared freshly.

Extract Stability
Sample extracts of Metiram in EDTA buffer solution and after methylation were re-analyzed after storage between 2 to 7 days under refrigerated conditions and were found to be not stable.

Reproducibility
Reproducibility of the method was not determined within the validation study.

Conclusion

The method L0241/01 for analysis of Metiram in soil using LC-MS/MS for final determination, which is a highly specific technique.
It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries and is therefore applicable to correctly determine residues of Metiram in soils.

Report: CA 4.1.2/2
Walter W., 2015a
Validation of analytical method L0229/01 for the determination of the Metiram metabolites ETU, EU, EBIS, Hydanthoin and TDIT in soil by LC-MS/MS
2014/1000105

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), EPA 860.1340: Residue Chemistry Test Guidelines - Residue Analytical Method

GLP: yes
(certified by Landesamt fuer Umwelt, Messungen und Naturschutz Baden Wuerttemberg, Karlsruhe, Germany)

Principle of the method

BASF method L0229/01, developed and validated at PTRL Europe, was developed for the determination of Metiram metabolites ETU (Reg. No. 146099), EU (Reg. No. 27270), EBIS (Reg. No. 243959), Hydanthoin ([HY], Reg. No. 132345), and TDIT (Reg. No. 4670450) in soil by LC-MS/MS with a limit of quantification (LOQ) of 0.050 mg kg^{-1}.
10 g soil samples were extracted with methanol/water (50/50, v/v) containing 1% thiourea and 0.1% formic acid by shaking for 30 min on a horizontal shaker and sonication for 10 min in an ultrasonic bath, followed by centrifugation for 5 to 10 min at 4000 rpm. For analysis of ETU, EBIS, and TDIT, the supernatant was then decanted into a volumetric flask and the extraction was repeated twice with methanol containing 1% thiourea and 0.1% formic acid. The extracts were combined and filled up with methanol/water (20/80, v/v) to a final volume of 100 mL. The samples were diluted by a factor of 25 for LC-MS/MS analysis. For analysis of EU and HY an aliquot of the supernatant was centrifuged for 30 min at 15,000 rpm and transferred into a vial for LC-MS/MS analysis. Dilutions were made as appropriate.

Recovery findings

The method proved to be suitable to determine Metiram metabolites ETU, EU, EBIS, HY, and TDIT in soil. Samples were spiked with the analytes at the limit of quantification of 0.050 mg kg^{-1} and ten times higher (0.50 mg kg^{-1}). All average recovery values (mean of five replicates per fortification level and analyte) were between 70% and 110%. The detailed results are given in the table below (Table 4.1.2-4).

Table 4.1.2-4: Results of the method validation for the determination of ETU, EU, EBIS, HY, and TDIT in soils LUFA 5M and LUFA 2.2

Soil	Analyte	m/z	Fortification level [mg kg^{-1}]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
LUFA 5M	ETU	103 → 86	0.050 0.50	5 5	93 98	5.2 1.4	95	4.5
		103 → 60	0.050 0.50	5 5	96 98	2.7 0.6	97	2.0
	EU	87 → 44	0.050 0.50	5 5	102 105	5.9 0.9	103	4.3
		87 → 70	0.050 0.50	5 5	101 103	6.0 0.9	102	4.2
	EBIS	177 → 74	0.050 0.50	5 5	88 96	2.5 2.5	92	5.2
		177 → 43	0.050 0.50	5 5	86 94	4.3 1.8	90	5.7
	HY	101 → 73	0.050 0.50	5 5	104 101	3.2 2.5	103	3.1
		101 → 44	0.050 0.50	5 5	107 98	3.4 2.7	102	5.5
	TDIT	213 → 169	0.050 0.50	5 5	92 95	1.5 1.0	93	2.3
		213 → 86	0.050 0.50	5 5	91 93	0.5 0.5	92	1.5
LUFA 2.2	ETU	103 → 86	0.050 0.50	5 5	89 96	2.2 0.6	93	3.9
		103 → 60	0.050 0.50	5 5	90 97	3.5 1.1	94	4.3
	EU	87 → 44	0.050 0.50	5 5	99 101	9.1 6.4	100	7.5
		87 → 70	0.050 0.50	5 5	99 101	8.4 7.1	100	7.4
	EBIS	177 → 74	0.050 0.50	5 5	88 97	3.4 2.4	92	5.4
		177 → 43	0.050 0.50	5 5	87 97	6.1 1.8	92	7.4
	HY	101 → 73	0.050 0.50	5 5	80 83	5.5 9.3	81	7.6
		101 → 44	0.050 0.50	5 5	87 82	7.7 9.1	85	8.4
	TDIT	213 → 169	0.050 0.50	5 5	85 88	2.2 3.1	87	2.9
		213 → 86	0.050 0.50	5 5	85 87	2.1 3.8	86	3.2

RSD = Relative standard deviation

Linearity	Good linearity (r > 0.997) was observed in the range of 0.050 ng mL^{-1} to 10 ng mL^{-1} for the two mass transitions of ETU, TDIT, and EBIS, and 1.0 ng mL^{-1} to 100 ng mL^{-1} for the two mass transitions of EU and HY.
Specificity	LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of Metiram metabolites ETU, EU, EBIS, HY, and TDIT in soil. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary. Significant interferences (> 30% of LOQ) were not observed at the retention times and mass transitions of the analytes.
Matrix Effects	Matrix effects were tested preparing matrix-matched standards for each matrix and analyte. The matrix effect was significant for both soils and all analytes, so matrix-matched standards were used for the evaluation of the results (except EBIS and TDIT for the soil LUFA 2.2).
Limit of Quantification	The method has a limit of quantification (LOQ) of 0.050 mg kg^{-1}, resulting from the lowest concentration level successfully tested within recovery experiments.
Limit of Determination	The method has a limit of determination (LOD) of 0.015 mg kg^{-1}, corresponding to the lowest calibration level used.
Repeatability	The relative standard deviations (RSD, %) for all fortification levels were below 10%.
Stability Working Solutions	Stability of standard solutions was assessed for at least nine weeks at refrigerated conditions. All analytes, except TDIT, indicated sufficient stability in stock and spike solutions (in methanol), as well as in calibration solutions (in methanol/water, 20/80, v/v). TDIT indicated significant degradation of about 30% in both solvents.
Extract Stability	Sample extracts of ETU, EU, EBIS, and HY in methanol/water (20/80, v/v) were re-analyzed after at least six days of storage under refrigerated conditions and were found to be stable determined by complete recovery. Sample extracts of TDIT in methanol/water (20/80, v/v) were re-analyzed after at least six days of storage under refrigerated conditions. No decrease in stability in the stored extracts were observed when the results were evaluated with matrix-matched standards freshly prepared from aged calibration solutions (in methanol/water, 20/80, v/v). However, TDIT decreased in the calibration solutions (in methanol/water, 20/80, v/v), indicating that TDIT was not stable in the extracts.

Reproducibility Reproducibility of the method was not determined within the validation study.

Conclusion

The method L0229/01 for analysis of ETU (Reg. No. 146099), EU (Reg. No. 27270), EBIS (Reg. No. 243959), Hydanthoin (HY, Reg. No. 132345), and TDIT (Reg. No. 4670450) in soil used LC-MS/MS for final determination, which is a highly specific technique.

It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification, and recoveries and is therefore applicable to correctly determine residues of ETU, EU, EBIS, Hydanthoin [HY], and TDIT in soils.

Extractability of the soil methods

The extraction procedure of soil treated with Metiram must take into account the very special characteristics of this active substance. Intact polymeric Metiram itself will not be extracted since it is insoluble in all solvents. Therefore the approach of EDTA extraction has been used for the extraction procedure for Metiram within residue analytical Method L0241/01 (CA 4.1.2/1). This procedure is analyte specific, and has, in contrast to metabolism studies, not the intention to cover all potential analytes. Therefore a comparison with the metabolism extraction procedure is not useful. The efficiency of the EDTA procedure is proven by the presented results, covering the required criteria and showing that the chemical reagents used for the EDTA extraction guarantee a quantitative transformation of Metiram in the given matrix.

For the extraction procedure of the metabolites, described within residue analytical method L0229/01 (CA 4.1.2/2), the high degradability of the analytes had been taken into account. Therefore a procedure has been identified to bring the analytes into a stable environment for further treatment within the sample preparation and avoid further degradation caused by the extraction or preparation procedure. This is shown within the presented results, covering the required criteria and also showing good efficiency by high recoveries.

Water

Analytical methods for the determination of Metiram residues in water were reviewed in the context of the inclusion in Annex I under Directive 91/414/EEC. These methods evaluated previously are summarized in Table 4.1.2-5 for the reviewer´s convenience.

Table 4.1.2-5: Summary of peer-reviewed analytical methods for determination of Metiram residues in water

Method No.	Matrix	Method principle	Target analytes	LOQ µg/l	year	DocID	EU reviewed
Anonymous, 1988 a	water	GC/S-FPD	Metiram, ETU	0.1 (Metiram) 0.05 (ETU)	1988	1988/10265	yes
Kerkdijk H., Mol J.G.J. 2002 a	water	LC-MS/MS	ETU	0.05 (groundwater) 0.1 (surface water)	2002	2002/1012956	yes

Report: CA 4.1.2/3
Penning H., 2010a
Validation of analytical method L0155/01 for the determination of BAS 222 F (Metiram) in surface water and groundwater using LC-MS/MS
2009/1122341

Guidelines: SANCO/825/00 rev. 7 (17 March 2004), SANCO/3029/99 rev. 4 (11 July 2000)

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Since this method is also used for post-approval control and monitoring purposes, the corresponding ILV (BASF Doc ID 2014/1246507) is described in KCA 4.2/4.

Principle of the method
BASF method L0155/01, developed and validated at BASF, was developed for the determination of Metiram (BAS 222 F) as ethylene-bisdithiocarbamate (EBDC)-dimethyl in ground and surface water by LC-MS/MS with a limit of quantification (LOQ) of 0.050 µg L^{-1}.
The ethylene-bisdithiocarbamate (EBDC) moiety was formed by dissolving Metiram with a buffer solution consisting of EDTA, cysteine, methanol, and sodium hydroxide adjusted to pH 11. The formed EBDC analyte was methylated with iodomethane. The dimethylated EBDC was then quantified by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

Recovery findings

The method proved to be suitable to determine Metiram in ground and surface water. Samples were spiked at the LOQ of 0.050 µg L^{-1} and ten times higher (0.50 µg L^{-1}). All average recovery values (mean of five replicates per fortification level and analyte) were between 70% and 110%. The detailed results are given in the table below (Table 4.1.2-6).

Table 4.1.2-6: Results of the method validation for the determination of Metiram in ground and surface water

Matrix	Analyte	m/z	Fortification level [µg L^{-1}]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
Ground Water	**Metiram**	241 → 134	0.050	5	108	3.8	106	3.9
			0.50	5	103	2.2		
		241 → 193	0.050	5	100	1.6	102	2.5
			0.50	5	104	1.4		
Surface Water	**Metiram**	241 → 134	0.050	5	110	2.6	106	4.8
			0.50	5	101	1.1		
		241 → 193	0.050	5	109	1.2	105	4.4
			0.50	5	101	1.7		

RSD = Relative standard deviation

Linearity

Good linearity (r > 0.99) was observed in the concentration range of 0.01 ng mL^{-1} to 5.0 ng mL^{-1} for both mass transitions.

Specificity	LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of Metiram identified and quantified as EBDC-dimethyl in ground and surface water. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary. No significant interferences (> 30% LOQ) at the retention time and mass transitions of EBDC-dimethyl were observed.
Matrix Effects	Matrix effects have not been addressed in the original validation. But to fulfil the actual requirements, this has been checked in the corresponding independent laboratory validation, summarized in CA 4.2/3[BASF DocID 2014/1246507]. Within this ILV, matrix effects have been positively identified.
Limit of Quantification	The method has a limit of quantification (LOQ) of 0.050 µg L^{-1}, resulting from the lowest concentration level successfully tested within recovery experiments.
Limit of Determination	The method has a limit of determination (LOD) of 0.010 µg L^{-1}, corresponding to the lowest calibration level used.
Repeatability	The relative standard deviations (RSD, %) for all fortification levels were below 10%.
Standard Stability	Stability of standard solutions was assessed, but due to the instability of EBDC-dimethyl and the internal standard (Nabam-d4 hexahydrate), the standard solutions have to be prepared freshly for each analytical series.
Reproducibility	Reproducibility of the method is demonstrated by an Independent Laboratory Validation summarized in CA 4.2/3 [*BASF DocID 2014/1246507*].

Conclusion

The method L0155/01 for analysis of Metiram in ground and surface water used LC-MS/MS for final determination, which is a highly specific technique.

It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification, and recoveries and is therefore applicable to correctly determine residues of Metiram in ground and surface water.

Report: CA 4.1.2/4
Class T.,Walter W., 2015a
Validation of analytical method L0228/01 for the determination of the Metiram metabolites ETU, EU and EBIS in ground- and surface water by LC-MS/MS
2014/1000106

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), EPA 860.1340: Residue Chemistry Test Guidelines - Residue Analytical Method

GLP: yes
(certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

Since this method is also used for post-approval control and monitoring purposes, the corresponding ILV (BASF Doc ID 2014/1246508) is described in KCA 4.2/5.

Principle of the method

BASF method L0228/01, developed and validated at PTRL Europe, was developed for the determination of Metiram metabolites ETU (Reg. No. 146099), EBIS (Reg. No. 243959), and EU (Reg. No. 27270) in ground and surface water by LC-MS/MS with a limit of quantification (LOQ) of 0.050 µg L^{-1}.

For analysis of ETU and EBIS water samples were analyzed by LC-MS/MS with direct injection. For analysis of EU, 5 mL of a water sample were diluted with 40 mL acetonitrile. After concentration to a volume of about 1 mL using a rotary evaporator, the sample was diluted with acetonitrile to a final volume of 5 mL and an aliquot was analyzed by LC-MS/MS.

Recovery findings

The method proved to be suitable to determine Metiram metabolites ETU, EU, and EBIS in ground and surface water. Samples were spiked with the analytes at the limit of quantification (LOQ) of 0.050 µg L^{-1} and ten times higher (0.50 µg L^{-1}). All average recovery values (mean of five replicates per fortification level and analyte) were between 70% and 110%. The detailed results are given in the table below (Table 4.1.2-7).

Table 4.1.2-7: Results of the method validation for the determination of ETU, EU, and EBIS in ground and surface water

Matrix	Analyte	m/z	Fortification level [µg L^{-1}]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
Ground Water	ETU	103 → 86	0.050 0.50	5 5	79 92	6.7 2.7	86	9.2
		103 → 60	0.050 0.50	5 5	81 92	6.8 1.9	87	8.2
	EU	87 → 44	0.050 0.50	5 5	92 92	6.0 4.7	92	5.1
		87 → 70	0.050 0.50	5 5	95 93	8.6 5.4	94	6.9
	EBIS	177 → 74	0.050 0.50	5 5	83 84	2.6 3.0	83	2.7
		177 → 43	0.050 0.50	5 5	82 85	5.7 2.8	84	4.5
Surface Water	ETU	103 → 86	0.050 0.50	5 5	78 91	3.0 4.5	84	8.8
		103 → 60	0.050 0.50	5 5	76 94	3.5 3.4	85	11.8
	EU	87 → 44	0.050 0.50	5 5	107 83	2.6 10.3	95	14.6
		87 → 70	0.050 0.50	5 5	99 83	3.9 9.3	91	11.5
	EBIS	177 → 74	0.050 0.50	5 5	95 95	3.0 1.8	95	2.4
		177 → 43	0.050 0.50	5 5	92 89	2.8 4.1	91	3.7

RSD = Relative standard deviation

Linearity

Good linearity (r > 0.997) was observed in the range of 0.015 ng mL^{-1} to 5.0 ng mL^{-1} for the two mass transitions of ETU and EBIS, and 0.015 ng mL^{-1} to 1.0 ng mL^{-1} for the two mass transitions of EU.

Specificity

LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of Metiram metabolites ETU, EU, and EBIS in ground and surface water. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary.
Significant interferences (> 30% of LOQ) were not observed at the retention times and mass transitions of the analytes.

Matrix Effects

Matrix effects were tested preparing matrix-matched standards for each matrix and analyte. The matrix effect was significant for all matrices and analytes, so matrix matched standards were used for the evaluation of the results.

Limit of Quantification The method has a limit of quantification (LOQ) of 0.050 µg L^{-1}, resulting from the lowest concentration level successfully tested within recovery experiments.

Limit of Determination The method has a limit of determination (LOD) of 0.015 µg L^{-1}, corresponding to the lowest calibration level used.

Repeatability The relative standard deviations (RSD, %) for all fortification levels were below 20%.

Stability Working Solutions ETU and EBIS showed sufficient stability in stock and spike solutions (in methanol), as well as in calibration solutions (in methanol/water, 20/80, v/v) for at least 49 days under refrigerated conditions. EU indicated sufficient stability in stock, spike, and calibration solutions (in acetonitrile) for at least 28 days under refrigerated conditions [*verified within study BASF DocID 2014/1000105, CA 4.1.2/2*].

Extract Stability Sample extracts of ETU and EBIS in water and EU in water/acetonitrile were analyzed after at least eight days of storage under refrigerated conditions. It was found that ETU and EU were stable as demonstrated by complete recovery. Sample extracts of EBIS were found to be not stable as reduced recovery was obtained.

Reproducibility Reproducibility of the method is demonstrated by an Independent Laboratory Validation summarized in CA 4.2/~~6~~ 5 [*BASF DocID 2014/1246508*].

Conclusion

Based on the obtained results the described analytical method L0228/01 for analysis of ETU (Reg. No. 146099), EBIS (Reg. No. 243959), and EU (Reg. No. 27270) in ground and surface water fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification, and recoveries and is therefore applicable to correctly determine residues of ETU, EU, and EBIS in ground and surface water with a limit of quantification of 0.050 µg L^{-1}.

Air

Analytical methods for the determination of Metiram residues in air were reviewed in the context of the inclusion in Annex I under Directive 91/414/EEC. These methods evaluated previously are summarized in Table 4.1.2-8 for the reviewer´s convenience.

Table 4.1.2-8: Summary of peer-reviewed analytical methods for determination of Metiram residues in air

Method No.	Matrix	Method principle	Target analytes	LOQ µg/L	year	DocID	EU reviewed
418/1 and 418/2	air	HPLC-ECD	Metiram	0.004 (418/1) 0.0002 (418/2)	2002	2002/1005332	yes

To meet the actual requirements, a confirmatory methodology to the given methods has been validated.

Report:	*CA 4.1.2/5* *Zangmeister W., 2005a* *Validation of confirmatory method for analytical methods 418/1 and 418/2: Determination of BAS 222 28 F (Polyram DF) in air using Tenax adsorbers (418/1) or IOM sampler (418/2) by HPLC-ECD* *2005/1016485*
Guidelines:	*EEC 91/414, Residue analytical methods for post-registration control purposes (July 21 1998) Federal Biological Research Centre for Agriculture and Forestry Braunschweig Germany, SANCO/825/00 rev. 7 (17 March 2004)*
GLP:	*yes* *(certified by Landesamt fuer Umweltschutz und Gewerbeaufsicht, Mainz, Germany)*

Principle of the method

To cover actual requirements, a confirmatory methodology for analytical methods 418/1 and 418/2 was developed to confirm positive finding of BAS 222 28 F (Polyram DF) in air using HPLC-ECD, with a limit of quantification (LOQ) of the confirmatory method of 0.004 $\mu g\ L^{-1}$ air.

The analyte was spiked onto the Tenax adsorbers (method 418/1) or glass fiber filter surface (method 418/2). After 30 min of storage at room temperature, adsorbers and filters were eluted with EDTA solution (0.1 $mol\ L^{-1}$). The resulting EDTA complex of the released ethylene-bisdithiocarbamate (EBDC) was determined by HPLC with electrochemical detection (ECD) in an aliquot of the eluate.

The formulation BAS 222 28 F has been used, because of the bad solubility of the active ingredient Metiram. Nominal Content of the AI in the formulation is 70 %, this has to be taken into account as the results are expressed for the formulation.

Recovery findings

The method was validated at a fortification level of 0.004 µg L^{-1} air representing the limit of quantification (LOQ). The results from the fortification experiments are shown in Table 4.1.2-9. The recovery rates for BAS 222 28 F (Polyram DF) at LOQ are in the required range of 70% to 110%. The tested untreated matrix samples showed no relevant interfering peaks at the retention time of Metiram.

Table 4.1.2-9: Results of the method validation for the determination of BAS 222 28 F in air

Analyte	Matrix	Replicates	Mean Recovery [%] [a]	RSD [%]	Overall Recovery [%]	RSD [%]
Metiram	Tenax adsorbers Glass fiber filters	5 5	73 100	8.1 2.9	87	17.1

RSD = Relative standard deviation
[a] Blank corrected recovery

Linearity

Good linearity (correlation of 0.9991 for Tenax adsorbers and 0.9995 for glass fiber filters) was observed in the concentration range of 0.07 – 1.4 µg mL^{-1}.

Specificity

The described method can be used as confirmatory methodology of positive findings of BAS 222 28 F (Polyram DF) determined with analytical methods 418/1 and 418/2 described above. As confirmatory technique a second chromatographic system, using a HPLC column with different chemistry (synergy polar RP vs. J'sphere ODS H40), was used.
Significant interferences (> 30% of LOQ) were not observed at the retention time of the analyte.

Limit of Quantification

The method has a limit of quantification (LOQ) of 0.004 µ/L-1 air, covering the actual AOEL of Metiram. LOQ (C) is calculated to 0.004 µg L-1 air, if 540 L air is collected. This corresponds to a total amount of 2.1 µg BAS 222 28 F spiked to adsorbers and filters. Metiram content analyzed in BAS 222 28 F (Polyram DF) is 72.2%. So the theoretically spiked amount of Metiram is 1.52 µg.

Therefore LOQ of this method is 0.004 µg L^{-1} in air.

Repeatability

The relative standard deviations (RSD, %) was below 20%.

Standard Stability

Standard solutions used for calibration were stable for at least 14 days if stored refrigerated (already tested within original validation study, *BASF DocID 2002/1005332)*.

Reproducibility

Reproducibility of the method was not determined within the validation study.

Conclusion

Based on the obtained results the described confirmatory methodology is considered valid for the determination of BAS 222 28 F (Polyram DF) in air with a limit of quantitation (LOQ) of 0.004 µg L^{-1}, what means 0.003 µg L^{-1} based on the active ingredient Metiram.

Supplementary information

The studies describing adsorption behavior contain analytical information on soil and water phases. Emphasis is laid on information regarding analytical methods in soil.

Report:	*CA 4.1.2/6 (analytical methods only; summary of full report available in CA 7.1.1.1/4)* *Staudenmaier H.,Kuhnke G., 2011b* *Formation of CS2 from Metiram in soil* *2010/1090462*
Guidelines:	*OECD 307 (2002), EPA 835.4100, BBA IV 4-1, EPA 162-1, SETAC Procedures for assessing the environmental fate and ecotoxicity for pesticides (March 1995)*
GLP:	*yes* *(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)*

Principle of the method

The analytical methodology employed in this study follows BASF method M135 developed for plant matrices and adapted for processed foods [Tilting 2000, DocID 2000/1017115, II A 4.3/3] and BASF method M509 in soil [Zangmeister 2002, DocID 2002/1005333, II A 4.4/2]. Both methods were peer-reviewed during Annex I approval. The given information is provided as supplemental data to the validation of the CS_2 methodology for metiram.

In the current study, the determination of Metiram (Reg. No. 250284) in soil was based on an existing residue analytical method which was adapted to the purpose of a soil study. The soil sample was filled into an Erlenmeyer flask and connected to a decomposition apparatus with attached traps (methanolic KOH). The active substance was hydrolyzed under reductive conditions by boiling with stanneous hydrochloric acid. Carbon disulfide is quantitatively released under these conditions and is distilled with a stream of nitrogen. After passage through washing bottles filled with sulphuric acid and zinc acetate. Carbon disulfide is absorbed in a 0.5 N methanolic solution of potassium hydroxide. Quantitation is performed by analysis of xanthogenate (potassium-O-methyl-dithiocarbonate) formed using HPLC system LC 188 (without separation by a HPLC column) and UV detection at 302 nm (Dionex, UVD 170U). The limit of quantification (LOQ) is 0.05 mg kg^{-1} in soil. The study contains little information on the validation of the applied analytical methods.

Tilting (2000) and Zangmeister (2002) provide descriptions of the used analytical method that fulfil the requirements concerning specificity, repeatability, limit of quantification and recovery. Confirmatory techniques are described. Zangmeister (2002) shows that the described method is suitable for the determination of BAS 222 F in soil.

Conclusion

The method for analysis of Metiram was previously confirmed by two peer-reviewed validation reports.

Report:	*CA 4.1.2/7 (analytical methods only; summary of full report available in CA 7.1.2.1.2/14)* *Heinz N., 2014c* *ETU (metabolite of Metiram, BAS 222 F): Study on aerobic soil degradation* *2014/1189253*
Guidelines:	*OECD 307 (2002), BBA IV 4-1, EPA 835.4100, SANCO/3029/99 rev. 4 (11 July 2000)*
GLP:	*yes* *(certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)*

Principle of the method

The analytical methodology employed in this study follows closely BASF method L0229/01 developed and validated at PTRL Europe (Walter 2015a, DocID 2014/10000105, CA 4.1.2/2). It is provided here as supplemental data to the validation of method L0229/1. In the current study, ETU (Reg. No. 146099) was analyzed in six soils (LUFA 2.2, LUFA 2.3, LUFA 5M, Li10, Senozan, Am Fischteich) by LC-MS/MS with a limit of quantification (LOQ) of 0.050 mg kg^{-1}. The study was performed by PTRL Europe, Ulm, Germany.

The soil samples (50 g) were extracted with 125 mL of 0.1% formic acid and 1% thiourea in methanol/water (1/1, v/v) for 30 min on a horizontal shaker, followed by 10 min sonication in an ultrasonic bath. After centrifugation (5 min at 4000 rpm), the supernatant was decanted through a glass wool plug. The extraction was repeated twice and the extracts were combined and adjusted to 400 mL with 0.1% formic acid and 1% thiourea in water. After diluting with methanol/water (2/8, v/v), aliquots were analyzed by LC-MS/MS using an Thermo Aquasil C_{18} column and an acetonitrile/water gradient with 0.1% formic acid as modifier. Detection was accomplished in ESI+ mode at a mass transition of 103 m/z → 60 m/z for quantification and 103 m/z → 86 m/z for confirmation (the latter mass transition only evaluated for method pre-validation). The results were calculated by direct comparison of the sample peak responses to those of external matrix-matched standards.

Recovery findings	The method proved to be suitable to determine Metiram metabolite ETU in soil. Samples were spiked with the analyte at the limit of quantification of 0.050 mg kg^{-1} and fifteen times higher (0.75 mg kg^{-1}). All average recovery values (mean of three replicates per fortification level with LUFA 2.2, LUFA 2.3 and Senozan, mean of four replicates per fortification level with LUFA 5M, Li10 and Am Fischteich) were between 76% and 109%. The detailed results for pre-validation and validation are given in the tables below (Table 4.1.2-10 and Table 4.1.2-11).

Table 4.1.2-10: Results of the method pre-validation for the determination of ETU in six soils

Soil	Analyte	m/z	Fortification level [mg kg⁻¹]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
LUFA 2.2	ETU	103 → 86	0.050 0.75	2 2	87 87	2.9 0.4	87	1.7
		103 → 60	0.050 0.75	2 2	84 88	1.2 0.6	86	2.9
LUFA 5M	ETU	103 → 86	0.050 0.75	2 2	103 92	3.6 3.7	98	7.5
		103 → 60	0.050 0.75	2 2	104 90	1.6 1.9	97	8.5
LUFA 2.3	ETU	103 → 86	0.050 0.75	2 2	92 90	0.6 1.0	91	1.5
		103 → 60	0.050 0.75	2 2	90 93	2.7 0.2	92	2.3
Li10	ETU	103 → 86	0.050 0.75	2 2	89 86	3.7 4.5	88	3.9
		103 → 60	0.050 0.75	2 2	87 88	1.3 1.6	87	1.3
Senozan	ETU	103 → 86	0.050 0.75	2 2	85 80	5.3 0.2	82	4.8
		103 → 60	0.050 0.75	2 2	87 80	1.9 1.4	83	5.4
Am Fischteich	ETU	103 → 86	0.050 0.75	2 2	108 82	0.3 2.7	95	15.7
		103 → 60	0.050 0.75	2 2	93 76	6.2 5.6	84	12.5
All soils	ETU	103 → 86	0.050 0.75	12 12	94 86	9.8 5.5	90	9.1
		103 → 60	0.050 0.75	12 12	91 86	8.0 7.4	88	8.1

RSD = Relative standard deviation

Table 4.1.2-11: Results of the method validation for the determination of ETU in six soils

Soil	Analyte	m/z	Fortification level [mg kg⁻¹]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
LUFA 2.2	ETU	103 → 60	0.050 0.75	3 3	109 103	2.6 0.7	106	3.8
LUFA 5M	ETU	103 → 60	0.050 0.75	4 4	93 107	11.9 4.0	100	11.0
LUFA 2.3	ETU	103 → 60	0.050 0.75	3 3	101 100	6.6 2.2	100	4.4
Li10	ETU	103 → 60	0.050 0.75	4 4	89 104	11.5 3.0	96	10.9
Senozan	ETU	103 → 60	0.050 0.75	3 3	95 99	5.4 2.2	97	4.4
Am Fischteich	ETU	103 → 60	0.050 0.75	4 4	91 106	4.7 3.6	98	9.2
All soils	ETU	103 → 60	0.050 0.75	21 21	95 104	10.0 3.9	97	9

RSD = Relative standard deviation

Linearity	Good linearity (r > 0.999) was observed in the range of 0.050 ng mL^{-1} to 5 ng mL^{-1} (≥ six levels; standards in matrix) for the mass transition 103 m/z → 60 m/z of ETU (LUFA 2.3).
Specificity	LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of Metiram metabolite ETU in soil. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary.
	Significant interferences (> 30% of LOQ) were observed in some blank controls at the retention times and mass transitions of the analyte. For samples analyzed following a blank with a detection of the analyte (>LOD, i.e. >0.015 mg kg^{-1}), the measured sample value was corrected for the concentration found in the appropriate blank.
Matrix Effects	Matrix effects are negated by using matrix-matched standards, leading to acceptable recovery values.
Limit of Quantification	The method has a limit of quantification (LOQ) of 0.050 mg kg^{-1}, resulting from the lowest concentration level successfully tested within recovery experiments.
Limit of Determination	The method has a limit of determination (LOD) of 0.015 mg kg^{-1}, corresponding to 30% of the LOQ.
Repeatability	The relative standard deviations (RSD, %) for all fortification levels were below 20% in the pre-validation of the soil method and below 15% in the validation of the soil method.
Stability Working Solutions	Standard solutions were kept in a freezer when not in use. Stability was demonstrated by consistent LC-MS/MS results and acceptable recoveries for fortified samples throughout the study.
Extract Stability	Stability of the sample extracts was not assessed in this study. Samples were analyzed within one week following extraction.
Reproducibility	Reproducibility of the method was not determined within the study.

Conclusion

The method for analysis of ETU (Reg. No. 146099) in soil used LC-MS/MS for final determination, which is a highly specific technique. The limit of quantification was 0.050 mg kg^{-1}.

It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification, and recoveries and is therefore applicable to correctly determine residues of ETU in soils.

Report:	*CA 4.1.2/8 (analytical methods only; summary of full report available in CA 7.1.2.1.2/19)* *Class T., 2010c* *Ethylene Urea and Hydantoin (metabolites of Metiram, BAS 222 F): Study on aerobic soil degradation* *2010/1056131*
Guidelines:	*OECD 307 (2002), BBA IV 4-1, EPA 835.4100, SANCO/3029/99 rev. 4 (11 July 2000)*
GLP:	*yes* *(certified by Umweltministerium Baden-Wuerttemberg, Stuttgart)*

Principle of the method

The analytical methodology employed in this study follows BASF method L0229/01 developed and validated at PTRL Europe (Walter 2015a, DocID 2014/10000105, CA 4.1.2/2). It is provided here as supplemental data to the validation of method L0229/1. In the current study, Ethylene urea [EU] (Reg. No. 27270) and Hydanthoin [HY] (Reg. No. 132345) were analyzed in three soils (LUFA 2.2, Li10 and Bruch West) by LC-MS/MS with a limit of quantification (LOQ) of 0.020 mg kg^{-1} for EU and 0.040 mg kg^{-1} for HY. The study was performed by PTRL Europe, Ulm, Germany.

For soil extraction, soil aliquots (10 g or 25 g, dry weight) were weighed into PE centrifuge bottles and methanol/water (1/1, v/v) were added. Then the samples were shaken for about 30 minutes (at 300 rpm). Thereafter, the samples were centrifuged (10 min at 4000 rpm) and an aliquot of the supernatant was analyzed by LC-MS/MS using a Thermo Hypercarb column and a methanol/water gradient with 0.1% formic acid as modifier for EU and a methanol/water gradient with 0.05% acetic acid as modifier for HY. Detection is accomplished in ESI+ mode at a mass transition of 87 m/z → 44 m/z for quantification and 87 m/z → 70 m/z for confirmation with EU, and at a mass transition of 101 m/z → 44 m/z for quantification and 101 m/z → 73m/z for confirmation with HY. The results were calculated by direct comparison of the sample peak responses to those of external solvent-based standards.

Recovery findings	The method proved to be suitable to determine Metiram metabolites EU and HY in soil. Samples were spiked with the analyte at the limit of quantification of 0.020 mg kg^{-1} for EU and 0.040 mg kg^{-1} for HY and twenty times higher (0.40 mg kg^{-1} for EU and 0.80 mg kg^{-1} for HY). All average recovery values (mean of five or nine replicates per fortification level with LUFA 2.2, Li10 and Bruch West) were between 92% and 94% for EU and between 85% and 96% for HY. Detailed results for the validation are given in the tables below (Table 4.1.2-12 and Table 4.1.2-13).

Table 4.1.2-12: Results of the method validation for the determination of EU in three soils

Soil	Analyte	m/z	Fortification level [mg kg^{-1}]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
LUFA 2.2	EU	87 → 44	0.020	9	92	3.0	93	3.5
			0.40	5	94	3.9		
		87 → 70	0.020	9	93	6.9	94	5.7
			0.40	5	94	3.6		
Li10	EU	87 → 44	0.020	9	94	1.5	94	2.2
			0.40	5	93	3.3		
		87 → 70	0.020	9	92	3.6	93	3.4
			0.40	5	93	3.4		
Bruch West	EU	87 → 44	0.020	9	93	2.9	93	2.6
			0.40	5	93	2.1		
		87 → 70	0.020	9	94	4.0	94	3.5
			0.40	5	93	2.7		
All soils	EU	87 → 44	0.020	27	93	2.7	93	2.8
			0.40	15	94	3.0		
		87 → 70	0.020	27	93	4.9	93	4.3
			0.40	15	94	3.1		

RSD = Relative standard deviation

Table 4.1.2-13: Results of the method validation for the determination of HY in three soils

Soil	Analyte	m/z	Fortification level [mg kg^{-1}]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
LUFA 2.2	HY	101 → 44	0.040	5	85	6.1	90	7.1
			0.80	5	94	4.0		
		101 → 73	0.040	5	89	3.9	92	5.5
			0.80	5	94	5.4		
Li10	HY	101 → 44	0.040	5	87	5.3	92	6.7
			0.80	5	96	2.8		
		101 → 73	0.040	5	87	3.8	91	6.5
			0.80	5	95	5.4		
Bruch West	HY	101 → 44	0.040	5	89	3.9	92	5.3
			0.80	5	95	3.6		
		101 → 73	0.040	5	89	6.2	92	5.8
			0.80	5	95	4.0		
All soils	HY	101 → 44	0.040	15	87	5.0	91	6.2
			0.80	15	95	3.4		
		101 → 73	0.040	15	88	4.6	91	5.8
			0.80	15	95	4.7		

RSD = Relative standard deviation

Linearity

Good linearity (r >0.999) was observed using solvent-based standard solutions in the range of 1.0 ng mL^{-1} to 200 ng mL^{-1} for the mass transition 87 m/z → 44 m/z of EU. For HY, good linearity (r >0.999) was observed in the range of 4.0 ng mL^{-1} to 500 ng mL^{-1} for the mass transition 101 m/z → 44 m/z.

Specificity	LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of Metiram metabolites EU and HY in soil. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary. Significant interferences (> 30% of LOQ) were not observed at the retention times and mass transitions of the analyte (blank controls determined in pre-validation).
Matrix Effects	Matrix effects were not addressed within this study. However, good recoveries were obtained using solvent-based standards. These recoveries from the validation part were comparable to recoveries from the pre-validation exercise where matrix-matched standards were used.
Limit of Quantification	The method has a limit of quantification (LOQ) of 0.020 mg kg^{-1} for EU and 0.040 mg kg^{-1} for HY, resulting from the lowest concentration level successfully tested within recovery experiments.
Limit of Determination	The method has a limit of determination (LOD) of 0.004 mg kg^{-1} for EU and 0.008 mg kg^{-1} for HY, corresponding to the lowest calibration level used.
Repeatability	The relative standard deviations (RSD, %) for all fortification levels of EU and HY were below 10% in the validation of the soil method.
Stability Working Solutions	Standard solutions were kept in a freezer when not in use. Stability was demonstrated by consistent LC-MS/MS results and acceptable recoveries for fortified samples throughout the study.
Extract Stability	Stability of the sample extracts was not assessed in this study. Samples were analysed within one day following extraction.
Reproducibility	Reproducibility of the method was not determined within the study.

Conclusion

The method for analysis of EU (Reg. No. 27270) and HY (Reg. No. 132345) in soil used LC-MS/MS for final determination, which is a highly specific technique. The limits of quantification were 0.020 mg kg^{-1} and 0.040 mg kg^{-1} for EU and HY, respectively.
It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification, and recoveries and is therefore applicable to correctly determine residues of Ethylene urea (EU) and Hydantoin (HY) in soils.

Report:	*CA 4.1.2/9 (analytical methods only; summary of full report available in CA 7.1.2.1.2/21)* *Heinz N., 2014d* *TDIT (metabolite of Metiram, BAS 222 F): Study on aerobic soil degradation* *2014/1189254*
Guidelines:	*OECD 307 (2002), BBA IV 4-1, EPA 835.4100, SANCO/3029/99 rev. 4 (11 July 2000)*
GLP:	*yes* *(certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)*

Principle of the method

The analytical methodology employed in this study follows closely BASF method L0229/01 developed and validated at PTRL Europe [Walter 2015a, BASF DocID 2014/10000105, CA 4.1.2/2]. It is provided here as supplemental data to the validation of method L0229/01. In the current study, TDIT (Reg. No. 4670450) was analyzed in soil (LUFA standard soil LUFA 5M) by LC-MS/MS with a limit of quantification (LOQ) of 0.050 mg kg^{-1}. The study was performed by PTRL, Europe, Ulm, Germany.

Fifty grams of soil samples were extracted with 125 mL methanol/water (1/1, v/v) containing 1% thiourea and 0.1% formic acid by shaking for 30 min on a horizontal shaker and sonication for 10 min in an ultrasonic bath, followed by centrifugation for 5 min at 4000 rpm. The supernatant was then decanted through a glass wool plug and the extraction was repeated twice with 125 mL methanol containing 1% thiourea and 0.1% formic acid. The extracts were combined and filled up with water (containing 1% thiourea and 0.1% formic acid) to a final volume of 400 mL. The samples were diluted with methanol/water (2/8, v/v) by a factor of 100 for LC-MS/MS analysis using a Restek C_{18} column with a acetonitrile/water gradient with 0.05% formic acid as modifier. Detection was accomplished in ESI+ mode at mass transitions 213 m/z → 86 m/z for quantification and 213 m/z → 169 m/z for confirmation (confirmation mass transition only monitored for method pre-validation). The results are calculated by direct comparison of the sample peak responses to those of external solvent-based standards.

Recovery findings	The method proved to be suitable to determine Metiram metabolite TDIT in soil. Samples were spiked with the analyte at the limit of quantification of 0.050 mg kg^{-1} (one sample only at 0.025 mg kg^{-1}, i.e. half the LOQ) and fifteen times higher (0.75 mg kg^{-1}). All average recovery values (mean of eight or seven replicates for low and high fortification level, respectively) ranged from 88% to 92%. The detailed results are given in the table below (Table 4.1.2-14). Pre-validation results for the mass transition 213 m/z → 169 m/z (three replicates in total; two fortified at 0.050 mg kg^{-1} and one at 0.750 mg kg^{-1}) had an overall recovery of 97% and an RSD of 2%.

Table 4.1.2-14: Characteristics for the analytical method used for the determination of TDIT in soil LUFA 5M

Soil	Analyte	m/z	Fortification level [mg kg^{-1}]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
LUFA 5M	TDIT	213 → 86	0.050* 0.750	8 7	92 88	8.5 8.3	90	8.4

RSD = Relative standard deviation

* One sample fortified at 0.025 mg kg^{-1} only.

Linearity Good linearity (r > 0.999) was observed in the range of 0.010 ng mL^{-1} to 2.5 ng mL^{-1} (six calibration levels) for the mass transition 213 m/z → 86 m/z using solvent-based calibration solutions (methanol/water, 2/8, v/v).

Specificity LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of Metiram metabolite TDIT in soil. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary.

Significant interferences (> 30% of LOQ) were not observed at the retention times and mass transition 213 m/z → 86 m/z of the analyte.

Matrix Effects Matrix effects were not addressed within this study. However, good recoveries were obtained using solvent-based standards. These recoveries from the validation part were comparable to recoveries from the pre-validation exercise where matrix-matched standards were used.

Limit of Quantification The method has a limit of quantification (LOQ) of 0.050 mg kg^{-1}, resulting from the lowest concentration level successfully tested within recovery experiments.

Limit of Determination The method has a limit of determination (LOD) of 0.008 mg kg^{-1}, corresponding to the lowest calibration level used.

Repeatability The relative standard deviations (RSD, %) for all fortification levels were below 10%.

Stability Working Solutions Standard solutions were kept in a refrigerator or freezer when not in use. Stability was demonstrated by consistent LC-MS/MS results and acceptable recoveries for fortified samples throughout the study.

Extract Stability Stability of the sample extracts was not assessed in this study. Samples were promptly analyzed after sample preparation (on the same day; some samples after 2-3 days).

Reproducibility Reproducibility of the method was not determined within the study.

Conclusion

The method for analysis of TDIT (Reg. No. 4670450) in soil used LC-MS/MS for final determination, which is a highly specific technique. The limit of quantification was 0.050 mg kg^{-1}.

It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification, and recoveries and is therefore applicable to correctly determine residues of TDIT in soils.

Report:	*CA 4.1.2/10 (analytical methods only; summary of full report available in CA 7.1.3.1.1/1)* *Heinz N., 2015a* *Determination of adsorption behavior of Metiram (BAS 222 F) in 5 soils* *2013/1095966*
Guidelines:	*OECD 106 (2000)*
GLP:	*yes* *(certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)*

Principle of the method

The analytical methodology employed in this study for the soil fraction follows closely BASF method L0241/01 developed and validated at PTRL Europe [Seibold and Class, 2015a, DocID 2014/1175671, CA 4.1.2/1]. It is provided here as supplemental data to the validation of method L0241/01. In the current adsorption study, Metiram (Reg. No. 250284) was analyzed in five soils (LUFA 2.1, LUFA 2.3, Li 10, Nierswalde Wildacker, Poggio Renatico) and corresponding water fractions from the adsorption samples by LC-MS/MS (after methylation). The limit of quantification (LOQ) was 0.2 µg g^{-1} in soil and 1.0 ng mL^{-1} in water ($CaCl_2$ solution). The study was performed by PTRL, Europe, Ulm, Germany.

To the soil sample of one gram the internal standard (100 ng mL^{-1} Nabam D4) was added. Subsequently, the sample was extracted with EDTA buffer solution by shaking for 30 min on a horizontal shaker and sonication for 10 min, followed by centrifugation for 5 min at 4000 rpm. After decantation of the supernatant into a volumetric flask the extraction was repeated once and the extracts were combined and filled up with EDTA buffer solution.

The water phase from the adsorption experiments was filtered (0.2 µm) and internal standard was added to aliquots of each sample.

Aliquots of extracts from the soil and water phases were subsequently methylated with iodomethane. Calcium chloride hexahydrate solution (2M) was added to the samples and filled up with water. After centrifugation of the methylated sample for 3 min at 4000 rpm, the soil and water extracts were analyzed by LC-MS/MS using an Agilent Zorbax SB-C_8 column with a water/methanol gradient with 0.1% formic acid and 10 mM ammonium formate as modifier. Detection was accomplished in ESI+ mode at mass transitions 241 m/z → 193 m/z for quantification and 241 m/z → 134 m/z for confirmation. The results were calculated by comparing the peak area ratios of analyte area / internal standard area to those of external solvent-based standards.

Recovery findings

The method proved to be suitable to determine Metiram in soil and water. To assess recovery from soil extraction soil samples were spiked with the analyte at the limit of quantification of 0.20 µg g^{-1} and ten times higher (2.00 µg g^{-1}). Average recovery values over all five soils (one replicate per soil) ranged from of 83% to 87%. The detailed results are given in the table below (Table 4.1.2-15).

Recovery for the water phase was assessed with samples fortified at the limit of quantification (1.0 ng mL^{-1}) and ten times higher (10 ng mL^{-1}). Average recovery values over all five soil water phases ranged from 88% to 107%. The detailed results are given in the table below (Table 4.1.2-16).

Table 4.1.2-15: Characteristics for the analytical method used for the determination of Metiram in soil

Soil	Analyte	m/z	Fortification level [µg g^{-1}]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
LUFA 2.1	Metiram	241 → 193	0.20 2.00	1 1	77 70	n.a. n.a.	74	n.a.
		241 → 134	0.20 2.00	1 1	84 71	n.a. n.a.	78	n.a.
LUFA 2.3	Metiram	241 → 193	0.20 2.00	1 1	93 73	n.a. n.a.	83	n.a.
		241 → 134	0.20 2.00	1 1	99 74	n.a. n.a.	87	n.a.
Li 10	Metiram	241 → 193	0.20 2.00	1 1	82 77	n.a. n.a.	80	n.a.
		241 → 134	0.20 2.00	1 1	83 77	n.a. n.a.	80	n.a.
Nierswalde Wildacker	Metiram	241 → 193	0.20 2.00	1 1	77 108	n.a. n.a.	93	n.a.
		241 → 134	0.20 2.00	1 1	85 108	n.a. n.a.	97	n.a.
Poggio Renatico	Metiram	241 → 193	0.20 2.00	1 1	88 96	n.a. n.a.	92	n.a.
		241 → 134	0.20 2.00	1 1	86 95	n.a. n.a.	91	n.a.
All soils	Metiram	241 → 193	0.20 2.00	5 5	83 85	8.4 19.4	84	14.2
		241 → 134	0.20 2.00	5 5	87 85	7.5 18.7	86	13.4

RSD = Relative standard deviation

n.a. = not applicable; only one replicate per soil

Table 4.1.2-16: Characteristics for the analytical method used for the determination of Metiram in water

Soil	Analyte	m/z	Fortification level [ng mL^{-1}]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
LUFA 2.1	Metiram	241 → 193	1 10	1 1	52 90	n.a. n.a.	71	n.a.
		241 → 134	1 10	1 1	61 90	n.a. n.a.	76	n.a.
LUFA 2.3	Metiram	241 → 193	1 10	1 1	86 93	n.a. n.a.	90	n.a.
		241 → 134	1 10	1 1	92 90	n.a. n.a.	91	n.a.
Li 10	Metiram	241 → 193	1 10	1 1	150 74	n.a. n.a.	112	n.a.
		241 → 134	1 10	1 1	158 75	n.a. n.a.	117	n.a.
Nierswalde Wildacker	Metiram	241 → 193	1 10	1 1	113 92	n.a. n.a.	103	n.a.
		241 → 134	1 10	1 1	118 94	n.a. n.a.	106	n.a.
Poggio Renatico	Metiram	241 → 193	1 10	1 1	98 91	n.a. n.a.	95	n.a.
		241 → 134	1 10	1 1	104 92	n.a. n.a.	98	n.a.
All soils	Metiram	241 → 193	1 10	5 5	100 88	36.0 9.0	94	27.0
		241 → 134	1 10	5 5	107 88	33.4 9.6	97	26.8

RSD = Relative standard deviation
n.a. = not applicable; only one replicate per soil

Linearity Good linearity (r > 0.99) was observed in the ranges usually employed for standard solutions of 0.040 ng mL^{-1} to 15 ng mL^{-1} (≥ eight calibration levels). Calibration was done with solvent-based calibration solutions as well as using an internal standard.

Specificity LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of Metiram in soil and water. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary.

Significant interferences (> 30% of LOQ) were not observed at the retention time and mass transition (241 m/z → 193 m/z) of the analyte.

Matrix Effects All final extracts contained as internal standard the stable deuterated isotope of the methylated monomer which compensates any observed matrix effects.

Limit of Quantification The method has a limit of quantification (LOQ) of 0.20 µg g^{-1} for soil and 1.0 ng mL^{-1}, resulting from the lowest concentration level successfully tested within recovery experiments.

Limit of Determination The method has a limit of determination (LOD) of 0.02 µg g^{-1} for soil and 0.3 ng mL^{-1} for water, corresponding to the lowest calibration level used.

Repeatability The relative standard deviations (RSD, %) for all fortification levels were below 20% for soil (all five soils combined). For water samples, the high fortification level led to RSD values below 10%, while RSD values for the low fortification level were below 40% (water from all soil types combined).

Stability Working Solutions Solutions were kept in a freezer when not in use. Stability was demonstrated by consistent LC-MS/MS results.

Extract Stability Stability of the sample extracts was not assessed in this study and details on the storage time of the extracts was not provided.

Reproducibility Reproducibility of the method was not determined within the study.

Conclusion

The method for analysis of Metiram (Reg. No. 250284) in soil and water used LC-MS/MS for final determination, which is a highly specific technique. The limit of quantification was 0.20 µg g^{-1} for soil and 1.0 ng mL^{-1} for water.

It could be demonstrated that the method was suitable regarding linearity, specificity, repeatability, limit of quantification, and recoveries to determine the desired levels of Metiram in the soil and water fractions of the adsorption study. Adsorption coefficients were derived by direct method based on analyte recovered from soil.

Report: CA 4.1.2/11 (analytical methods only; summary of full report available in CA 7.1.3.1.2/7)
Class T., 2010d
Hydantoin (metabolite of Metiram, BAS 222 F): Determination of the adsorption / desorption behavior of Hydantoin on soils
2010/1065127

Guidelines: OECD Guidelines for the Testing of Chemicals - Volume 1 No 106 (21 January 2000)

GLP: yes
(certified by Umweltministerium Baden-Wuerttemberg, Stuttgart)

Principle of the method

The analytical methodology employed in this study for the soil fraction is similar, with a few exceptions, to BASF method L0229/01 developed and validated at PTRL Europe [Walter 2015a, DocID 2014/10000105, CA 4.1.2/2]. It is provided here as supplemental data to the validation of method L0229/01. In the current study, Hydantoin ([HY], Reg. No. 132345) was analyzed in three soils (LUFA 2.2, Li 10, Bruch West) and corresponding water fractions from the adsorption samples by LC-MS/MS with a limit of quantification (LOQ) of 0.50 µg g^{-1} in soil. The study was performed by PTRL, Europe, Ulm, Germany.

Five grams of soil samples were extracted with 5 mL water/methanol (1/1, v/v) by intensive shaking by hand, sonication for 10 min in an ultrasonic bath and finally shaking for 30 min on a horizontal shaker. The samples were centrifuged for 5 min at 4000 rpm. The supernatants from the soil extraction as well as the corresponding water phase were diluted with methanol/water (1/1, v/v) and analyzed by LC-MS/MS using a Thermo Hypercarb column with a water/methanol gradient with 0.05% acetic acid as modifier. Detection was accomplished in ESI+ mode at mass transitions 101 m/z → 44 m/z for quantification and 101 m/z → 73 m/z for confirmation (results of the confirmation mass transition were not reported in the study). The results are calculated by direct comparison of the sample peak responses to those of external solvent-based standards.

Recovery findings

The method proved to be suitable to determine Metiram metabolite HY in soil and water. To assess recovery from soil extraction soil samples were spiked with the analyte at the limit of quantification of 0.50 µg g^{-1} and ten times higher (5.00 µg g^{-1}). All average recovery values (mean of three to five replicates) ranged from 105% to 113%. When combining results of the three soils average recovery was 108% and 109% for the low and high fortification levels, respectively. The detailed results are given in the table below (Table 4.1.2-17).

Recovery for the water phase can be assessed from the dose control samples (dosed at 5.0 µg mL^{-1}), which had recoveries ranging from 95% to 124%.

Table 4.1.2-17: Characteristics for the analytical method used for the determination of HY in soil

Soil	Analyte	m/z	Fortification level [µg g^{-1}]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
LUFA 2.2	**HY**	101 → 44	0.50 5.00	5 5	106 109	6.1 3.4	107	4.9
Li 10	**HY**	101 → 44	0.50 5.00	3 3	109 113	4.7 3.2	111	4.0
Bruch West	**HY**	101 → 44	0.50 5.00	3 3	110 105	3.4 1.4	108	3.5
All soils	**HY**	101 → 44	0.50 5.00	11 11	108 109	5.0 3.8	108	4.4

RSD = Relative standard deviation

Linearity

Linearity (r > 0.999) was observed in the ranges usually employed for standard solutions of 25 or 50 ng mL^{-1} to 500 or 1000 ng mL^{-1} (≥ five calibration levels). Calibration was done with solvent-based calibration solutions (methanol/water, 1/1, v/v) and mass transition 101 m/z → 44 m/z was monitored.

Specificity

LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of Metiram metabolite HY in soil. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary. Validation data for the confirmatory mass transition is already presented for the original validation study 2014/1000105.

	Significant interferences (> 30% of LOQ) were not observed at the retention times and mass transition 101 m/z → 44 m/z of the analyte.
Matrix Effects	Matrix effects were not addressed within this study. However, good recoveries were obtained using solvent-based standards.
Limit of Quantification	The method has a limit of quantification (LOQ) of 0.50 µg g^{-1} for soil, resulting from the lowest concentration level successfully tested within recovery experiments.
Limit of Determination	The method has a limit of determination (LOD) of 0.025 µg g^{-1} for soil and 0.5 µg L^{-1} for water, corresponding to the lowest calibration level used.
Repeatability	The relative standard deviations (RSD, %) for all fortification levels were below 5% for soil and below 10% for water (for water only one concentration examined in the dose control samples).
Stability Working Solutions	Standard solutions were kept in a refrigerator when not in use. Stability was demonstrated by consistent LC-MS/MS results throughout the experimental phase (about two weeks).
Extract Stability	Stability of the sample extracts was not assessed in this study and details on the storage time of the extracts was not provided.
Reproducibility	Reproducibility of the method was not determined within the study.

Conclusion

The method for analysis of HY (Reg. No. 132345) in soil used LC-MS/MS for final determination, which is a highly specific technique. The limit of quantification for soil was 0.50 µg g^{-1}.

It could be demonstrated that the method in soil was suitable regarding linearity, specificity, repeatability, limit of quantification, and recoveries to determine the desired levels of HY in the soil fractions of the adsorption study.

Report:	*CA 4.1.2/12 (analytical methods only; summary of full report available in CA 7.1.3.1.2/8)*
	Sacchi R.R., 2014b
	Adsorption/desorption behavior of TDIT (metabolite of Metiram) on different European soils
	2014/1000103
Guidelines:	*OECD 106 (2000), EPA 835.1230, SOP-PA.1005*
GLP:	*yes*
	(certified by Instituto Nacional de Metrologia, Normalizacao e Qualidade Industrial - INMETRO, Rio de Janeiro, Brazil)

Principle of the method

In the current adsorption study, the Metiram metabolite TDIT (Reg. No. 4670450) was analyzed in four soils (LUFA 2.2, LUFA 5M, Speyerer Wald 2, Nierswalde Wildacker) and corresponding water fractions from the adsorption samples by LC-MS/MS. The limit of quantification (LOQ) was 0.0005 mg kg^{-1} in soil and 0.5 ng mL^{-1} in water ($CaCl_2$ solution). The study was performed by Global Environmental and Consumer Safety Laboratory, BASF S.A., Guaratinguetá, Brasil.

In addition to the samples from the adsorption experiments, fortified samples (water and soil samples; soil LUFA 5M) for method validation were prepared, extracted and analyzed in the same way as the samples of the main adsorption experiment. The fortified aqueous samples were diluted appropriately for LC-MS/MS determination. The fortified soil samples (1 g) were extracted three times with 3 mL methanol. The tubes were shaken at 250 rpm for 30 min at room temperature and then centrifuged at 9000 rpm for 10 min. The supernatants from soil extraction were decanted and made up to 10 mL. Aliquots were diluted in methanol and analyzed.

LC-MS/MS was performed using a Phenomenex Gemini C_{18} column and a water/methanol gradient with 0.1% formic acid as modifier. Detection was accomplished in ESI+ mode at mass transition 213 m/z → 86 m/z for quantification. The results were calculated by direct comparison of the sample peak responses to those of external solvent-based standards.

Recovery findings

The method proved to be suitable to determine TDIT in soil and water. To assess recovery from soil extraction soil samples (five to seven replicates) were spiked with the analyte at the limit of quantification of 0.0005 mg kg^{-1} and 2000 times higher (1.0 mg kg^{-1}). Recovery values ranged from 75% to 87%. Detailed results are given in the table below (Table 4.1.2-18).

Recovery for the water phase was assessed with samples (seven replicates) fortified at the limit of quantification (0.5 ng mL^{-1}) and 2000 times higher (1000 ng mL^{-1}). Recovery values ranged from 100% to 103%. Detailed results are given in the table below (Table 4.1.2-18).

Table 4.1.2-18: Characteristics for the analytical method used for the determination of TDIT in soil and water

Matrix	Analyte	m/z	Fortification level [soil: mg kg^{-1}] [water: ng mL^{-1}]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
Soil (LUFA 5M)	TDIT	213 → 86	0.0005	5	75	2.5	82	8.4
			1	7	87	4.2		
Water ($CaCl_2$)	TDIT	213 → 86	0.5	7	100	3.4	102	3.4
			1000	7	103	2.9		

RSD = Relative standard deviation

Linearity Good linearity (r > 0.99) was observed in the ranges usually employed for standard solutions of 0.025 ng mL^{-1} to 1.0 ng mL^{-1} (≥ six calibration levels). Calibration was done with solvent-based calibration solutions (methanol).

Specificity LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of TDIT in soil and water. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary.
Significant interferences (> 30% of LOQ) were not observed at the retention time and mass transition (213 m/z → 86 m/z) of the analyte.

Matrix Effects Matrix effects were not addressed within this study. However, good recoveries were obtained using solvent-based standards. These recoveries from the validation part were comparable to recoveries from the pre-validation exercise where matrix-matched standards were used.

Limit of Quantification The method has a limit of quantification (LOQ) of 0.0005 mg kg^{-1} for soil and 0.5 ng mL^{-1} for water, resulting from the lowest concentration level successfully tested within recovery experiments.

Limit of Determination The limit of determination (LOD) is not stated in the study report.

Repeatability The relative standard deviations (RSD, %) for all fortification levels were below 5% for soil and water.

Stability Working Solutions Standard solutions (TDIT in methanol) proved to be stable over a 30-day period (solutions stored over 1, 2, 3, 7, 10, 15 and 30 days gave results with less than 10% difference compared to freshly prepared solutions).

Extract Stability TDIT in 0.01 M $CaCl_2$ solution (without soil) was shown to be stable over 48 h of constant shaking. Stability of the soil extracts was not assessed. However, recovery rates below 90% from mass

	balances for the adsorption tests suggest that degradation might have occurred.
Reproducibility	Reproducibility of the method was not determined within the study.

Conclusion
The method for analysis of TDIT (Reg. No. 4670450) in soil and water used LC-MS/MS for final determination, which is a highly specific technique. The limit of quantification was 0.0005 mg kg^{-1} for soil and 0.5 ng mL^{-1} for water.
It could be demonstrated that the method used in this adsorption study fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries and is therefore applicable to correctly determine residues of TDIT the tested soils and water phases. Adsorption coefficients were derived by direct method based on analyte recovered from soil.

(b) Methods in soil, water and any additional matrices used in support of efficacy studies

No stand-alone validation of analytical methods were required in support of efficacy studies.

(c) Methods in feed, body fluids and tissues, air and any additional matrices used in support of toxicological studies

Analytical methods used in toxicological studies, are in general described and validated with the corresponding toxicological study. In addition, the following methods were developed and validated by stand-alone validation studies.

Report: *CA 4.1.2/13*
Schulz H., 2006a
Determination of Ethylenethiourea (Reg.No. 146 099) in urine - Validation of the BASF Method No. 373/3
2006/1009303

Guidelines: *SANCO/825/00 rev. 7 (17 March 2004), SANCO/3029/99 rev. 4 (11 July 2000)*

GLP: *yes*
(certified by Hessisches Ministerium fuer Umwelt, Laendlichen Raum und Verbraucherschutz)

Principle of the method

BASF method 373/3, developed and validated at SGS Fresenius, was developed for the determination of Metiram metabolite Ethylenethiourea ETU (Reg.No. 146099) in urine by LC-MS/MS with a limit of quantification (LOQ) of 0.01 mg kg^{-1}.
ETU was extracted from the urine with a mixture of sodium ascorbate, ammonium chloride and methanol-water. An aliquot was taken and the pH was adjusted to 8 before evaporating. The remaining water phase was cleaned by liquid/liquid partition (water/dichloromethane) on an Extrelut/Al_2O_3 column. After concentration of the eluate, the residue was determined by LC-MS/MS detection using two fragment ions for evaluation.

Recovery findings

The recoveries of ETU in fortified urine samples are summarised in Table 4.1.2-19. The mean recoveries of ETU ranged from 72% to 77% at the transition *m/z* 103 → *m/z* 44 and from 73% to 80% at the transition *m/z* 103 → *m/z* 86 over the two fortification levels. The RSD ranged from 4.8% to 5.9% and from 5.4% to 7.7%, respectively. The preferred transition for evaluation was *m/z* 103 → *m/z* 44. The second transition *m/z* 103 → *m/z* 86 was validated for confirmatory purposes.

Table 4.1.2-19: Recovery results from method validation of BF 222-ETU in human urine

Matrix	Fortification Level (mg kg^{-1})	Transition *m/z* 103 → *m/z* 44		Transition *m/z* 103 → *m/z* 86	
		Mean Recovery (%)	RSD (%)	Mean Recovery (%)	RSD (%)
Urine (n=7)	0.01	77	5.3	80	6.9
Urine (n=7)	0.1	72	4.8	73	5.4
Urine (n=14)	0.01 + 0.1	75	5.9	76	7.7

RSD = relative standard deviation

Linearity	Good linearity (r≥0.999) was observed in the range of 2.048 to 128.0 ng mL^{-1} for the two mass transitions of the ETU. Eight calibration points distributed over the concentration range were used. A stock solution in methanol was dissolved in methanol/ultra pure water (10/90, v/v) to create calibration solutions. A first order calibration curve (linear) was used.
Specificity	LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of the Metiram metabolites ETU in urine. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary. Significant interferences (> 30% of LOQ) were not observed for each mass transition and retention times and of the analyte.
Matrix effect	Matrix effects were tested preparing matrix matched calibration solutions for ETU. The matrix effect was significant for ETU; therefore, matrix mached calibration solution was used for the evaluation of the results.
Limit of Quantitation	The limit of quantitation is defined as 0.01 mg kg^{-1} resulting from the lowest concentration level successfully tested within recovery experiments.
Limit of Determination	The method has a limit of determination (LOD) of 0.002 mg kg^{-1}, corresponding to the lowest calibration level used.
Repeatability	The relative standard deviations (RSD, %) for all fortification levels were below 20%.
Reproducibility	The reproducibility of the method was not estimated as identical samples were not evaluated by an independent laboratory.

Conclusion

The method 373/3 for analysis of Metiram metabolite ETU (Reg. No. 146099) in urine used LC-MS/MS for final determination, which is a highly specific technique.
It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries, and is therefore applicable to correctly determine residues of Metiram metabolite ETU in urine with a limit of quantification of 0.01 mg kg^{-1}.

Report: CA 4.1.2/14
Class T., 2014a
Analytical method for the determination of the Metiram metabolites ETU, EU and EBIS in rat plasma
2014/1028663

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), EPA 860.1340, OECD Guidance Document on Pesticide Residue Analytical Methods (ENV/JM/MONO(2007)17 - 13-Aug-07

GLP: yes
(certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

Principle of the method

BASF method L0225/01, developed and validated at PTRL Europe, was developed for the determination of Metiram metabolites ETU (Reg. No. 146099), EBIS (Reg. No. 243959), and EU (Reg. No. 27270) in rat plasma by LC-MS/MS with a limit of quantification (LOQ) of 50 µg L^{-1}.

For determination of ETU, the internal standard (d4-ETU) is added to rat plasma. The sample is diluted with methanol with 160 mmol L^{-1} ammonium formate, vortexed and centrifuged. An aliquot of the supernatant is mixed with aqueous 0.2% formic acid for LC/MS/MS analysis. Final extracts from rat plasma samples with higher concentrations are diluted with blank control plasma final extracts (d4-ETU IS present). The analyte is monitored using two mass transitions (*m/z* 103 → 44 and *m/z* 103 → 60) for quantitation and for confirmation, the internal standard d4-ETU is monitored with its *m/z* 107 → 48 mass transition.

For determination of EU, rat plasma is diluted with methanol with 160 mmol L^{-1} ammonium formate, vortexed and centrifuged. The supernatant is transferred to an autosampler vial, diluted with water and acidified with formic acid for LC/MS/MS analysis. The analyte is monitored using two mass transitions (*m/z* 87 → 70 and *m/z* 87 → 44) for quantitation and confirmation.

For determination of EBIS, rat plasma is diluted with acidified methanol, vortexed and centrifuged. An aliquot of the supernatant is transferred to an autosampler vial and diluted with acidified methanol for LC/MS/MS analysis. The analyte is monitored using two mass transitions (*m/z* 177 → 42 and *m/z* 177 → 74) for quantitation and confirmation.

Recovery findings

The recoveries of ETU, EU and EBIS in fortified rat plasma samples are summarised in Table 4.1.2-20. The average recoveries at each fortification level for ETU and EBIS are in the range of 70% to 110%, with relative standard deviations ≤ 20%. For EU, the method validation resulted in average recoveries in the range of 129% to 137%. This was presumably caused by a matrix effect (suppression) which was overcompensated by the matrix-matched standards prepared for the analyte. However, as the RSDs are < 7%, the method validation results should be considered acceptable.

Table 4.1.2-20: Recovery results from method validation of ETU, EU and EBIS in rat plasma

Matrix	Analyte	Transition *m/z*	Fortification level (µg L^{-1})	No. of tests n	Mean Recovery (%)	RSD (%)
Rat plasma	ETU	103 → 44	50	5	97	2.6
			500	5	93	3.6
			overall: 50 + 500	10	95	3.5
		103 → 60	50	5	90	4.6
			500	5	89	4.6
			overall: 50 + 500	10	90	4.4
	EU	87 → 70	50	5	129	6.9
			500	5	136	2.5
			overall: 50 + 500	10	133	5.6
		87 → 44	50	5	137	1.9
			500	5	136	4.5
			overall: 50 + 500	10	136	3.3
	EBIS	177 → 42	50	5	78	4.0
			500	5	78	3.7
			overall: 50 + 500	10	78	3.6
		177 → 74	50	5	73	2.4
			500	5	74	4.0
			overall: 50 + 500	10	73	3.2

RSD = Relative Standard Deviation

Linearity

Good linearity (r≥0.999) was observed in the range of 0.5 to 15 ng mL^{-1} for the two mass transitions of ETU. Six calibration points distributed over the concentration range were used. The calibration solutions were prepared with d4-ETU internal standard (always at 10 ng mL^{-1}) in 1.0 mL of solvent (water/methanol, 6/4 v/v, 0.1% formic acid). A first order calibration curve (linear) was used.

For EU good linearity was observed in the range of 1.0 to 100 ng mL^{-1} for the two mass transitions (*m/z* 87 → 70; r≥0.999 and m/z 87 → 44; r≥0.998). The response factor is linear over a concentration range from 1 to 60 ng mL^{-1} for the less intensive *m/z* 87 → 70 mass transition. For the more intensive *m/z* 87 → 44 mass transition, lower concentrations (1 and 5 ng mL^{-1}) are affected by interference present in matrix-matched standards. This leads to a positive intercept in the calibration function/line. Five calibration points distributed over the concentration range for the *m/z* 87 → 70 mass transition were used. For the *m/z* 87 → 44 mass transition 5 calibration points distributed over the concentration range were used. The EU stock solution in methanol was diluted with water/methanol (6/4 v/v) with 0.1% formic acid to obtain matrix-matched calibration solutions using mixed matrix extracts from blank control plasma. A first order calibration curve (linear) was used.

For EBIS good linearity (r≥0.998) was observed in the range of 0.1 to 10 ng mL^{-1} for the two mass transitions. Six calibration points distributed over the concentration range were used. The EBIS stock solution in methanol was diluted with acidified methanol (0.15% formic acid) to obtain matrix-matched calibration solutions using mixed matrix extracts from blank control plasma. A first order calibration curve (linear) was used.

Specificity

LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of the Metiram metabolites ETU, EU and EBIS in rat plasma. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary.
Significant interferences (> 30% of LOQ) were not observed for each mass transition and retention times and of the analytes.

Matrix-effect

For ETU the use of d4-ETU as internal standard what compensates in general matrix effects, thus no separate test for matrix effects on ETU was performed. This is confirmed by comparison of peak areas response obtained for the internal standard d4-ETU, where a no relevant matrix effect was observed.
Negative matrix effect indicating suppression of MS/MS response by matrix components in final extracts were observed for EU (about - 40% suppression) and EBIS (about - 20% suppression), thus matrix-matched standards were required for quantitation.

Limit of Quantitation

The limit of quantitation is defined as 50 µg L^{-1} per analyte resulting from the lowest concentration level successfully tested within recovery experiments.

Limit of Determination

The method is covering a limit of determination (LOD) of 10 µg L^{-1} for all analytes, given by the individual calibration ranges.

Repeatability

The relative standard deviations for ETU, EU and EBIS were ≤ 20%. The values obtained are indicative of the method having satisfactory repeatability.

Standard stability

The data obtained demonstrate that all three analytes are stable in their stock solutions in methanol for at least 2 weeks when stored refrigerated or frozen.

Extract stability

All three analytes give recoveries comparable to initial analyses when re-analysed after refrigerated storage. The results indicate that the analytes are stable in extracts for at least 5 days when stored refrigerated.

Reproducibility

The reproducibility of the method was not estimated as identical samples were not evaluated by an independent laboratory. However, based on the performance of the method, its reproducibility is expected to be good.

Conclusion

The method L0225/01 for analysis of Metiram metabolites ETU (Reg. No. 146099), EBIS (Reg. No. 243959), and EU (Reg. No. 27270) in rat plasma used LC-MS/MS for final determination, which is a highly specific technique.

It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries, and is therefore applicable to correctly determine residues of Metiram metabolites ETU, EU and EBIS in rat plasma with a limit of quantification of 50 µg L^{-1}.

Report:	CA 4.1.2/15 Mallat E., 2015a The validation of the determination of Ethylenethiourea in rat urine samples using LC-MS/MS 2014/1280635
Guidelines:	U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), May 2001, BP. European Medicines Agency (EMA), Committee for Medicinal Products for Human Use (CHMP), EMEA/CHMP/EWP/192217/2009, 21 July 2011.
GLP:	yes (certified by Inspectorate for Health Protection and Veterinary Public Health, Den Haag, Netherlands)

Principle of the method

An LC/MS-MS bioanalytical method, developed and validated at ABL Netherlands, was developed for the determination of Metiram metabolite ETU (Reg. No. 146099) in rat urine by LC-MS/MS with a limit of quantification (LOQ) of 150 ng mL^{-1}. The method is based on the derivatization of ETU and its internal standard and subsequent analysis by LC-MS/MS.

For determination of ETU, the internal standard (d4-ETU) was used. Samples containing ETU were subjected to derivatization using pentafluorobenzylbromide and tetrabutylammonium bisulfate under alkalic conditions. After derivatization, a liquid-liquid extraction was performed using pentane. From the extract an aliquot is dried under nitrogen and reconstituted in acetonitrile. The purified samples were analyzed using an LC/MS-MS system. ETU is monitored using mass transition *m/z* 463 → 181 and the internal standard d4-ETU is monitored with its mass transition m/z 467 → 181.

Recovery findings	The recoveries of ETU in fortified rat urine samples are summarized in Table 4.1.2-21. The average recoveries at each fortification level for ETU are in the range of 70% to 110%, with relative standard deviations (RSD) ≤ 20%.

Table 4.1.2-21: Recovery results from method validation of ETU in rat urine

Matrix	Analyte	Transition *m/z*	Fortification level (ng L^{-1})	No. of tests n	Mean Recovery (%)	RSD (%)
Rat Urine	ETU	463 → 181	50*)	18	95	5.5
			150	18	99	3.0
			1500	18	97	1.7
			15000	18	97	1.6

*) LOD level
RSD = Relative Standard Deviation

Linearity

Good linearity (r≥0.999) was observed in the range of 50 to 20000 ng mL^{-1} for the mass transition of ETU. Ten calibration points distributed over the concentration range were used. The calibration stock solution was dissolved in methanol (final concentration 1000 µg mL^{-1}. A first order calibration curve (linear) was used.

Specificity

LC-MS/MS is a highly specific self-confirmatory technique. The following mass transitions were used for quantification:
ETU: m/z 463 -> 181
d4-ETU: m/z 467 -> 181
Significant interferences (> 20% of LOQ) were not observed for mass transition and retention times and of the analyte.

Matrix-effect

For the evaluation of the matrix effect, ETU (at concentrations of QC-Low and QC-High) and internal standard (at assay concentration) were spiked into six different lots of rat urine before the derivatization procedure. The mean peak areas of these double blank samples were compared to the mean peak areas of samples spiked with ETU and internal standard (IS) in neat (matrix free) solutions at equivalent concentrations.
The RSD values of IS-normalised matrix effect were 2.4% (QC-Low) and 1.4% (QC-High), respectively. These values indicate that no significant matrix effect was observed.

Limit of Quantitation

The limit of quantitation is defined as 150 ng mL^{-1} for ETU resulting from the lowest concentration level successfully tested within recovery experiments.

Limit of Determination

The method has a limit of determination (LOD) of 50 ng mL^{-1}, corresponding to the lowest calibration level used.

Repeatability

The relative standard deviations (RSD) for ETU were ≤ 20%. The values obtained are indicative of the method having satisfactory repeatability.

Standard stability

The data obtained demonstrate that the analyte is stable in the stock solution in methanol for at least 66 days when stored at ≤ -18°C.

Extract stability The analyte give recoveries comparable to initial analyses when re-analysed after storage. The results indicate that the analyte is stable in extracts for at least 75 hours when stored in an autosampler set at 2-8°C.

Reproducibility The reproducibility of the method was not estimated as identical samples were not evaluated by an independent laboratory. However, based on the performance of the method, its reproducibility is expected to be good.

Conclusion

The LC-MS/MS bioanalytical method for analysis of Metiram metabolite ETU (Reg. No. 146099) in rat urine used LC/MS-MS for final determination, which is a highly specific technique.

It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries, and is therefore applicable to correctly determine residues of the Metiram metabolite ETU in rat urine with a limit of quantification of 150 ng mL^{-1}.

(d) Methods in body fluids, air and any additional matrices used in support of operator, worker, resident and bystander exposure studies

Analytical methods used in OPEX studies, are in general described and validated with the corresponding OPEX study. In addition, the following method was developed and validated by a stand-alone validation study.

Report:	CA 4.1.2/16 Knoch E., 2015a Validation of an analytical method for the determination of Metiram and its metabolites in OPEX matrices 2015/1084023
Guidelines:	SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), Chemikaliengesetz der Bundesrepublik Deutschland in its current version, OECD-DOC ENV/MC/CHEM(98)17 Paris 1999
GLP:	yes (certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Principle of the methods

The used analytical method for the determination of Metiram (BAS 222 F, Reg. No. 250284) and its metabolites ETU (Reg. No. 146099), EU (Reg. No. 27270) and EBIS (Reg. No. 243959) in OPEX matrices are based on the validated methods L0089/01 and L0176/01.

Method L0089/01 was adapted by SGS Fresenius for the determination of Metiram in OPEX matrices by LC-MS/MS with different limits of quantification (LOQ) for each OPEX matrix (see Table 4.1.2-23). In principle the ethylene-bisdithiocarbamate (EBDC) moiety is formed out of BAS 222 F and extracted from the respective specimen with a buffer solution consisting of EDTA, cysteine, methanol and sodium hydroxide adjusted to pH 11.0. Afterwards the formed ethylene-bisdithiocarbamate (EBDC) analyte is methylated with iodmethane and further diluted. Specimens are quantified by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). Two parent-daughter ion transitions per analyte were determined. One transition was used for evaluation, the other one for confirmation.

Method L0176/01 was adapted by SGS Fresenius for the determination of the Metiram metabolites ETU (Reg. No. 146099), EU (Reg. No. 27270) and EBIS (Reg. No. 243959) in OPEX matrices by LC-MS/MS with different limits of quantification (LOQ) for each OPEX matrix and analyte (see Table 4.1.2-23). The OPEX specimens is extracted by shaking with their respective extraction solvent. Further filtration takes place by using a syringe filter. An aliquot is further diluted and prepared for analysis. Specimens are quantified by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). Two parent-daughter ion transitions per analyte were determined. One transition was used for evaluation, the other one for confirmation.

Recovery findings

The method proved to be suitable to determine residues of Metiram and its metabolites ETU, EU and EBIS in the OPEX matrices cotton coverall (cotton/polyester blend coverall) used as outer dosimeter material, cotton underwear used as inner dosimeter material, hand wash solution (0.01% Aerosol OT), face/neck wipes (cotton swabs), air filters (Tenax) and DFR solutions (0.01% Aerosol OT).

Taking into account the approved recovery range of 70 - 110% for each fortification level and a relative standard deviation (RSD) of ≤ 20%, the validation of the analytical methods for the determination of residues of Metiram, ETU, EU and EBIS in OPEX matrices was successful in inner dosimeter, outer dosimeter, hand wash solution and DFR dislodging solution. For Metiram in air filters the average recoveries ranged between 9% and 90% in case of spiking in acetone solution. The average recoveries for Metiram in air filters ranged between 90% and 100% when spiking was done in EDTA solution.

The detailed results are given in Table 4.1.2-22.

Table 4.1.2-22: Recovery results of Metiram, ETU, EU and EBIS in OPEX matrices

Matrix	Analyte	Transition *m/z*	Fortification level (µg/sample) (µg mL^{-1})	No. of tests	Mean Recovery (%)	RSD (%)
Inner dosimeter	Metiram	241 → 193	0.124	5	83	3.3
			1.24	5	97	1.6
			overall	10	90	8.4
		241 → 134	0.124	5	83	5.4
			1.24	5	97	1.9
			overall	10	90	8.8
	ETU	103 → 44	0.50	5	88	4.1
			4.99	5	88	2.0
			overall	10	88	3.0
		103 → 86	0.50	5	95	8.1
			4.99	5	86	2.3
			overall	10	91	7.8
	EU	87 → 44	0.50	5	104	1.7
			5.00	5	103	0.5
			overall	10	104	1.5
		87 → 70	0.50	5	106	5.3
			5.00	5	104	0.9
			overall	10	105	3.7
	EBIS	177 → 42	0.50	5	73	5.0
			5.04	5	70	2.8
			overall	10	72	4.7
		177 → 86	0.50	5	74	7.7
			5.04	5	70	2.1
			overall	10	72	6.1

Matrix	Analyte	Transition *m/z*	Fortification level (µg/sample) (µg mL^{-1})	No. of tests	Mean Recovery (%)	RSD (%)
Outer dosimeter	Metiram	241 → 193	0.124	5	87	3.8
			1.24	5	94	3.3
			overall	10	90	5.0
		241 → 134	0.124	5	87	3.7
			1.24	5	94	1.4
			overall	10	91	4.9
	ETU	103 → 44	0.50	5	107	5.0
			4.99	5	102	1.2
			overall	10	105	4.3
		103 → 86	0.50	5	108	4.0
			4.99	5	102	3.1
			overall	10	105	4.4
	EU	87 → 44	0.50	5	111	3.1
			5.00	5	104	1.2
			overall	10	108	4.2
		87 → 70	0.50	5	102	12.1
			5.00	5	97	3.3
			overall	10	99	8.9
	EBIS	177 → 42	0.50	5	93	6.5
			5.04	5	91	8.0
			overall	10	92	7.0
		177 → 86	0.50	5	97	4.8
			5.04	5	92	7.0
			overall	10	95	6.2
Hand wash solution	Metiram	241 → 193	0.01	5	87	3.8
			0.1	5	94	1.4
			overall	10	91	5.0
		241 → 134	0.01	5	84	4.0
			0.1	5	94	1.4
			overall	10	89	6.6
	ETU	103 → 44	0.04	5	91	4.0
			0.40	5	91	2.7
			overall	10	91	3.2
		103 → 86	0.04	5	95	6.8
			0.40	5	91	3.3
			overall	10	93	5.7
	EU	87 → 44	0.04	5	93	5.0
			0.40	5	92	2.8
			overall	10	93	3.9
		87 → 70	0.04	5	88	4.4
			0.40	5	90	3.2
			overall	10	89	3.8
	EBIS	177 → 42	0.04	5	89	17.5
			0.40	5	90	5.8
			overall	10	90	12.2
		177 → 86	0.04	5	91	16.3
			0.40	5	89	7.2
			overall	10	90	12.1

Matrix	Analyte	Transition *m/z*	Fortification level (µg/sample) (µg mL^{-1})	No. of tests	Mean Recovery (%)	RSD (%)
DFR dislodging solution	Metiram	241 → 193	0.01	5	92	12.4
			0.1	5	95	0.6
			overall	10	94	8.3
		241 → 134	0.01	5	86	15.2
			0.1	5	96	1.0
			overall	10	91	11.1
	ETU	103 → 44	0.04	5	94	0.8
			0.42	5	98	4.6
			overall	10	96	3.9
		103 → 86	0.04	5	95	5.7
			0.42	5	97	5.9
			overall	10	96	5.6
	EU	87 → 44	0.04	5	98	1.3
			0.42	5	99	4.6
			overall	10	98	3.2
		87 → 70	0.04	5	92	8.3
			0.42	5	97	5.2
			overall	10	95	7.1
	EBIS	177 → 42	0.04	5	108	4.7
			0.42	5	103	4.3
			overall	10	106	4.9
		177 → 86	0.04	5	106	3.2
			0.42	5	104	2.8
			overall	10	105	3.0
Face/neck wipes	Metiram	241 → 193	0.12	5	92	4.1
			1.24	5	98	1.6
			overall	10	95	4.5
		241 → 134	0.12	5	93	2.0
			1.24	5	98	2.4
			overall	10	95	3.7
Air filters (tenax) (spiked in aceton solution)	Metiram	241 → 193	0.05	5	37	10.5
			0.5	5	90	5.5
			overall	10	63	44.1
		241 → 134	0.05	5	51	6.9
			0.5	5	89	4.6
			overall	10	70	29.4
Air filters (tenax) (spiked in EDTA solution	Metiram	241 → 193	0.05	5	96	5.7
		241 → 134	0.05	5	101	3.7
Air filters (tenax) (spiked in aceton solution)*	Metiram	241 → 193	0.05	5	9	18.4
			0.5	5	35	9.5
			overall	10	22	65.0
		241 → 134	0.05	5	12	10.4
			0.5	5	34	9.6
			overall	10	23	52.6
Air filters (tenax) (spiked in EDTA solution)*	Metiram	241 → 193	0.05	5	74	5.9
			0.5	5	99	1.6
			overall	10	87	15.3
		241 → 134	0.05	5	72	5.3
			0.5	5	99	1.8
			overall	10	85	16.4

Matrix	Analyte	Transition *m/z*	Fortification level (µg/sample) (µg mL^{-1})	No. of tests	Mean Recovery (%)	RSD (%)
Air filters (tenax)	ETU	103 → 44 103 → 86	0.20	5	96	1.0
			2.00	5	95	1.6
			overall	10	95	1.5
			0.20	5	104	4.1
			2.00	5	92	2.6
			overall	10	98	7.3
	EU	87 → 44	0.20	5	100	2.5
			2.00	5	98	2.2
			overall	10	99	2.3
		87 → 70	0.20	5	104	7.3
			2.00	5	98	2.3
			overall	10	101	6.0
	EBIS	177 → 42	0.20	5	82	11.1
			2.00	5	73	5.2
			overall	10	77	10.5
		177 → 86	0.20	5	84	7.9
			2.00	5	74	6.2
			overall	10	79	9.6
Air filters (tenax)*	ETU	103 → 44	0.20	5	84	11.2
			2.00	5	90	3.7
			overall	10	87	8.4
		103 → 86	0.20	5	84	12.8
			2.00	5	91	3.5
			overall	10	87	9.4
	EU	87 → 44	0.20	5	104	0.8
			2.00	5	103	1.4
			overall	10	104	1.1
		87 → 70	0.20	5	105	1.7
			2.00	5	104	1.0
			overall	10	104	1.4
	EBIS	177 → 42	0.20	5	65	4.7
			2.00	5	75	4.3
			overall	10	70	4.9
		177 → 86	0.20	5	60	5.8
			2.00	5	74	9.4
			overall	10	67	13.3

* after 6 hours airflow at 1L/min (ambient temperature and air humidity in the laboratory)
sample = inner and outer dosimeter: 1 patch of 10 x 10 cm
face/neck wipes: 2 pads
air filters: 1 tube

Linearity

Considering all matrices, good linearity was observed in the range of 0.0126 to 0.7065 ng mL^{-1} (both transitions) for Metiram, 0.504 to 30.24 ng mL^{-1} (both transitions) for ETU, 0.5048 to 30.29 ng mL^{-1} (both transitions) for EU and 0.5045 to 25.23 ng mL^{-1} (both transitions) for EBIS. The correlation coefficients of the calibrations on the basis of the two detected ion transitions per analyte were above 0.999. Eight calibration points distributed over the concentration range were used for Metiram and EBIS, nine calibration points were used for ETU and EU.
To obtain samples for calibration purposes a diluted stock solution of Metiram was dissolved in EDTA solution. The diluted stock solution of ETU and EU was dissolved in methanol/water (10/90; v/v). And for EBIS, the diluted stock solution was dissolved in methanol/water (40/60; v/v). A first order calibration curve (linear) was used for all analytes.

Specificity

LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of the Metiram and its metabolites ETU, EU and EBIS in OPEX matrices. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary.
The specificity of the analytical method is acceptable, since no significant interferences (> 30 %) from the specimen matrices were detected at the retention time of interest.

Matrix-effect

No separate tests on matrix effects have been conducted. But as all recovery and control samples showed acceptable results, it can be concluded that the given methodology has no relevant matrix effects.

Limit of Quantification

The limit of quantitation (LOQ) of the methods was as follows for each analyte and matrix.

Table 4.1.2-23: Limit of quantitation for Metiram and its metabolites in OPEX matrices

Material	Metiram	ETU	EU	EBIS
Inner dosimeter	0.125 µg/patch[1]	0.5 µg/patch[1]	0.5 µg/patch[1]	0.5 µg/patch[1]
Outer dosimeter	0.125 µg/patch[1]	0.5 µg/patch[1]	0.5 µg/patch[1]	0.5 µg/patch[1]
Face/neck wipes	0.125 µg/2 pads[1]	-	-	-
Hand wash solution	0.5 µg/100 mL	4 µg/100 mL	4 µg/100 mL	4 µg/100 mL
Air filters (tenax)	0.05 µg/tube	0.2 µg/tube	0.2 µg/tube	0.2 µg/tube
DFR Dislodging Solution	0.25 µg/50 mL	4 µg/100 mL	2 µg/50 mL	2 µg/50 mL

[1] patch/pad: 10 cm x 10 cm

The limit of quantification resulting from the lowest concentration level successfully tested within recovery experiments.

Limit of Determination

The method has a limit of determination (LOD) of 30 % of the LOQ for each analyte in each matrix, respectively.

Repeatability

The relative standard deviations for Metiram, ETU, EU and EBIS were ≤ 20% for all transitions. The values obtained are indicative of the method having satisfactory repeatability. The detailed values are shown in Table 4.1.2-22.

Standard stability

Stability of stock solutions of ETU (in methanol), EU (in methanol) and EBIS (in ethyl acetate) was demonstrated by comparison of old and freshly prepared solutions.
The data obtained demonstrate that ETU, EU and EBIS are stable in their stock solutions for the duration of the laboratory work (117 days for ETU and EU and 119 days for EBIS). No stability test for Metiram was performed as these working solutions were prepared fresh daily.

Extract stability

No extract stability investigation were performed.

Reproducibility

The reproducibility of the method was not estimated as identical samples were not evaluated by an independent laboratory. However, based on the performance of the method, its reproducibility is expected to be good.

Conclusion

The used analytical method for the determination of Metiram (Reg. No. 250284) and its metabolites ETU (Reg. No. 146099), EU (Reg. No. 27270) and EBIS (Reg. No. 243959) in OPEX matrices are based on the validated methods L0089/01 and L0176/01. Both methods for analysis of Metiram and its metabolites ETU, EU and EBIS in OPEX matrices (cotton/polyester blend coverall) used as outer dosimeter material, cotton underwear used as inner dosimeter material, hand wash solution (0.01 % Aerosol OT), face/neck wipes (cotton swabs), air filters (Tenax) and DFR solutions (0.01 % Aerosol OT) used LC-MS/MS for final determination, which is a highly specific technique.

It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries, and is therefore applicable to correctly determine residues of Metiram metabolite ETU in OPEX matrices with different limit of quantification for each matrix and analyte (see Table 4.1.2-23).

(e) Methods in or on plants, plant products, processed food commodities, food of plant and animal origin, feed and any additional matrices used in support of residues studies

The following recovery and repeatability criteria are required according to the OECD Guidance document on analytical methods (ENV/JM/MONO(2007)17), depending on the fortification levels:

≤1 µg/kg	50 - 120 ± 35%
>1 µg/kg ≤0.01 mg/kg	60 - 120 ± 30%
>0.01 mg/kg ≤0.1 mg/kg	70 - 120 ± 20%
>0.1 mg/kg ≤1.0 mg/kg	70 - 110 ± 15%
>1 mg/kg	70 - 110 ± 10%

Food of animal origin

Analytical methods for the determination of Metiram residues in animal matrices reviewed in the context of the inclusion in Annex I under Directive 91/414/EEC. These methods evaluated previously are summarized in Table 4.1.2-24 for the reviewer´s convenience.

Table 4.1.2-24: Summary of peer-reviewed analytical methods for determination of Metiram residues in animal matrices

Method No.	Matrix	Method principle	Target analytes	LOQ µg/l	year	DocID	EU reviewed
BASF method No. 135/2, Weber H., 1999a	liver, muscle	GC/FPD	Metiram (CS2)	0.02 mg/kg	1999	1999/11785	yes
BASF method No. 525/0, Tilting N. 2002	meat, milk and eggs	GC/MS	Metiram (CS2)	0.02 mg/kg	2002	2002/1014144	yes
BASF method No. 159/1, Keller W. 1985	cattle meat and kidney, chicken eggs, meat, bones, skin, fat, cattle urine, molasses, chicken feed	GC/S-FPD	ETU	0.02 mg/kg (cattle meat and kidney, chicken eggs, meat, bones, skin, fat) 0.1 mg/kg (cattle urine) 0.05 mg/kg (molasses, chicken feed)	1985	1985/10155	yes

~~For the determination of Metiram residues in animal matrices based on the CS2 approach, a new method development, according to the actual guidelines, was planned and already initiated. Unfortunately, this study could not be finalized in time to present it within this dossier. Actually, it is scheduled to have this study finalized in October 2015 latest for submission.~~
Since the validation of a new method for the determination of Metiram residues in animal matrices based on the CS2 approach, was not completed for the original submission of the dossier ~~For that reason~~, the already reviewed methods, 135/2 and 525/0 are presented in detail for the reviewers convenience.

Report:	*CA 4.1.2/17* *Weber H., 1999a* *Validation of BASF method 135/2 for the determination of Metiram in animal matrices (liver, muscle)* *1999/11785*
Guidelines:	*OECD principle of Good Laboratory Practice (EMV/MC/CHEM(98))*
GLP:	*yes* *(certified by Behoerde fuer Arbeit, Gesundheit und Soziales, Freie und Hansestadt Hamburg, Hamburg)*

Principle of the method

Heating BAS 222 F with a solution of stannous chloride and hydrochloric acid yields carbon disulfide which was distilled in a stream of nitrogen. Volatile impurities were absorbed. The liberated carbon disulfide was absorbed in two traps filled with ice-cooled methanol. The content of carbon disulfide was determined by gas chromatography using flame photometric detector.

Recovery findings The recoveries of BAS 222 F in liver and muscle samples are summarized in Table 4.1.2-25. The results obtained were within the guideline requirements for all matrices with average recoveries after fortification with 0.02 and 0.2 mg/kg BAS 222 F between 80 and 112%.

Table 4.1.2-25: Recovery resu1ts from method validation of BAS 222 F in 1iver and muscle samples

Matrix	Fortification level (mg/kg)	n	Mean Recovery [%]	% RSD
Liver	0.02	5	100	15
	0.2	5	84	12
Muscle	0.02	5	112	18
	0.2	5	80	33

Linearity Good linearity was observed in the range of 0.02 to 1 .0 µg/ml of carbon disulfide standard solution.

Specificity No significant interferences from the sample matrix were reported.

Limit of Quantification The limit of quantification was defined by the lowest fortification level successfully tested which were 0.02 mg/kg (calculated as carbon disulfide) for liver and muscle.

Repeatability The coefficients of variation with respect to recoveries following fortifications at the limit of quantification were between 15 and 18% in all examined commodities. The values obtained are indicative of the method having satisfactory repeatability.

Reproducibility The reproducibility of the method was not estimated as identical samples were not evaluated by an independent laboratory. However, based on the performance of the method, its reproducibility is expected to be good.

Conclusion

The data presented demonstrate that the BASF method No. 135/2 permits the determination of BAS 222 F in liver and muscle samples. The limit of quantification was 0.02 mg/kg (calculated as carbon disulfide).

Report:	*CA 4.1.2/18* *Tilting N., 2002a* *Validation of analytical method 525/0 for the determination of BAS 222 F (Metiram) in muscle, milk and egg (confirmatory method)* *2002/1014144*
Guidelines:	*SANCO/825/00 rev. 6 (20 June 2000), EEC 91/414, EEC 96/46*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Principle of the method

The parent compound BAS 222 F was decomposed to carbon disulphide which was extracted with iso-octane. An aliquot of the isooctane phase was taken from the extract and was injected directly into the GC/MS for determination.

Recovery findings

The recoveries of BAS 222 F in meat, milk and eggs are summarized in Table 4.1.2-26. The results obtained were within the guideline requirements for all matrices with average recoveries after fortification with 0.02 and 0.2 mg/kg BAS 222 F between 71 and 86 %, except for meat with a recovery of 62 %.

Table 4.1.2-26: Recovery resu1ts from method validation of BAS 222 F in 1iver and muscle samples

Matrix	Fortification level (mg/kg)	n	Mean Recovery [%]	% RSD
Meat	0.02	5	62	3.0
	0.2	5	71	5.6
Milk	0.02	5	81	6.1
	0.2	5	86	2.3
Eggs	0.02	5	74	5.1
	0.2	5	75	2.3

Linearity

Good linearity was observed in the range of 2.5 to 100.0 ng/ml of carbon disulfide standard solution.

Specificity

No significant interferences from the sample matrix were reported.

Limit of Quantification

The limit of quantification was defined by the lowest fortification level successfully tested which were 0.02 mg/kg (calculated as carbon disulfide) for meat, milk and eggs.

Repeatability

The coefficients of variation with respect to recoveries following fortifications at the limit of quantification were between 3.0 and 6.1 % in all examined samples. The values obtained are indicative of the method having satisfactory repeatability.

Reproducibility

The reproducibility of the method was not estimated as identical samples were not evaluated by an independent laboratory. However, based on the performance of the method, its reproducibility is expected to be good.

Conclusion

The data presented demonstrate that the BASF method No. 525/0 permits the determination of BAS 222 F in meat, milk and eggs samples. The limit of quantification was 0.02 mg/kg (calculated as carbon disulfide).

Report: CA 4.1.2/19
Bendig P.,Maas X., 2015 a
Validation of the BASF analytical method L0256/01 for the determination of Metiram (BAS 222 F) in animal matrices using GC/MS
2015/1000807

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), EPA 860.1340 (1996), OECD Guidance Document on Pesticide Residue Analytical Methods (ENV/JM/MONO(2007)17 - 13-Aug-07

GLP: yes
(certified by Landesamt fuer Umwelt, Messungen und Naturschutz Baden Wuerttemberg, Karlsruhe, Germany)

Since this method is also used for post-approval control and monitoring purposes, the corresponding ILV (BASF Doc ID 2016/1030250) is described in KCA 4.2/2.

Principle of the method

Metiram residues were hydrolyzed by hot acidic digestion using aqueous $HCl/SnCl_2$ and trapping the released CS_2 in an isooctane layer using a closed/sealed vessel. After cooling, the isooctane was injected into a GC-MS for CS_2 determination. Two GC-MS methods were applied: the primary method used a DB-1701 column and the confirmatory method a CP-Sil 8 CB column. In both cases, the ESI+ mode was used and one fragment ion (76 m/z, corresponding to the molecular ion of CS_2) was monitored.
Subtraction of blank interferences was necessary, in case no CS_2 free matrix could be obtained.

Recovery findings In all matrices tested, the mean recovery values for GC-MS determination of metiram as CS_2 were between 70% and 110% for all fortification levels and all mass transitions. The detailed results are given in the table below.

Table 4.3-27: Validation results of method L0256/01: Metiram in animal matrices

Test substance	Matrix	Fortification level [a] (mg/kg)	No. of tests	Average recovery (%)		Relative standard deviation (%)	
Fragment ion				76 m/z (DB-1701)	76 m/z (CP-Sil 8 CB)	76 m/z (DB-1701)	76 m/z (CP-Sil 8 CB)
Metiram as CS_2	Bovine meat	0.010	5	81.7	92.6	17	13
		0.10	5	75.1	83.1	3.8	6.7
	Bovine liver	0.010	5	79.7[b]	83.0[b]	15	14
		0.10	5	82.8[b]	77.5[b]	11	9.4
	Bovine kidney	0.010	5	97.4[b]	88.9[b]	18	18
		0.10	5	83.0[b]	81.5[b]	4.3	4.4
	Bovine milk	0.010	5	76.1	85.3	6.3	12
		0.10	5	80.9	76.3	15	7.9
	Bovine fat	0.010	5	92.1	96.9[b]	5.3	18
		0.050	5	71.9	82.9[b]	3.0	8.9
		0.50	5	74.7	73.9	2.0	2.3
	Hen eggs	0.010	5	80.4	83.9	15	12
		0.050	5	83.1	80.0	2.3	5.0
		0.10	5	81.9	77.1	5.3	5.1

[a] Fortification level is expressed as CS_2, however metiram was fortified.
[b] Recovery samples were corrected for interferences / residues of the analyte detected in the respective control samples.

Linearity	Good linearity ($R^2 \geq 0.99$) was observed in the range of 0.40 ng/mL, 1.0 ng/mL or 2.0 ng/mL to 500 ng/mL for the m/z 76 ion on both capillary GC columns.
Specificity	GC-MS is a highly specific detection technique and therefore no confirmatory technique is required. Analysis is possible with two different chromatographic columns.
Matrix effects	The matrix effect was not significant (< 20%) for all matrices and both columns used.
Interferences	The method L0256/01 determines residues of metiram as CS_2 in animal matrices. The interferences/residues of the analytes measured in the control samples were on average below 20 % of the limit of quantitation (LOQ) for milk, egg and meat for both columns and for fat using the primary method. The interferences/residues of the analyte measured in the kidney, liver and the fat confirmatory method control samples were above 20 % on average of the limit of quantitation (LOQ). Thus the recoveries for CS_2 were corrected for the respective interferences.
Limit of quantitation	The limit of quantitation (LOQ) is defined by the lowest fortification level successfully tested. The LOQ is 0.010 mg/kg (calculated as carbon disulfide), corresponding to a concentration in the extract of 2.0 ng/mL (fat) or 10 ng/mL (meat, liver, kidney, milk and egg).
Limit of detection	The limit of detection (LOD) was defined as 20% of the LOQ, resulting in a LOD of 0.002 mg/kg of CS_2.
Repeatability	The relative standard deviations (RSD, %) for all commodities and all fortification levels were < 20%. The detailed values are shown in Table 4.3-27.
Reproducibility	An independent laboratory validation has been successfully conducted and is reported under [see KCA 4.2/2 2016/1030250].
Standard stability	In this study, CS_2 was shown to be stable in isooctane (stock and calibration solutions) for up to 43 days, when stored frozen in the dark. Stability of metiram formulation in stock and fortification solutions (water) was already shown in a previous study for at least 1 week, when stored frozen in the dark as aliquots in separate amber glass vials (BASF DocID 2014/1261097).
Extract stability	Sample extracts in isooctane were shown to be stable under frozen conditions for up to 48 days.

Conclusion

It could be demonstrated that the analytical method L0256/01 fulfills the requirements with regard to linearity, specificity, repeatability, limit of quantitation, and recoveries and is therefore applicable and suitable to correctly determine residues of metiram as CS_2 in animal matrices.

Report: CA 4.1.2/20
Class T.,Richter S., 2015a
BASF method L0254/01: Validation of an analytical method for the determination of the Metiram metabolite ETU in foodstuff of animal origin, using LC-MS/MS
2014/1223796

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), EPA 860.1340

GLP: yes
(certified by Landesamt fuer Umwelt, Messungen und Naturschutz Baden Wuerttemberg, Karlsruhe, Germany)

Report: CA 4.1.2/21
Class T.,Richter S., 2015b
Report Amendment No. 1: BASF method L0254/01: Validation of an analytical method for the determination of the Metiram metabolite ETU in foodstuff of animal origin, using LC-MS/MS
2015/1103828

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), EPA 860.1340

GLP: yes
(certified by Landesamt fuer Umwelt, Messungen und Naturschutz Baden Wuerttemberg, Karlsruhe, Germany)

Since this method is also used for post-approval control and monitoring purposes (BASF Doc ID 2015/1000809), the corresponding ILV is described in KCA 4.2/3.

Principle of the method

BASF method L0254/01, developed and validated at PTRL Europe, was developed for the determination of Metiram metabolite Ethylenethiourea ETU (Reg.No. 146099) in foodstuffs of animal origin (exemplified by whole milk and egg, bovine meat, liver and kidney and fat) by LC-MS/MS with a limit of quantification (LOQ) of 0.01 mg kg^{-1}.
Samples are extracted (adding sodium ascorbate, thiourea, ethylene urea and Celite filter aid) with methanol. Extracts are filtered, cleaned-up by liquid/liquid partition using a HM-N extraction column and concentrated for LC/MS/MS determination.

Recovery findings The results show that BASF method no. L0254/01 is suitable to determine residues of the metabolite ETU in foodstuffs of animal origin with an LOQ of 0.01 mg/kg. The total mean recovery values were between 81.7 and 98.1%. The relative standard deviations (RSD) for all fortification levels were ≤ 19%. Data are summarised in Table 4.1.2-28.

Table 4.1.2-28: Recovery results from method validation of the metabolite ETU in foodstuffs of animal origins

Matrix	Fortification Level (mg kg^{-1})	No of Fortified Samples	Transition 103 → 60		Transition 103 → 86	
			Mean %	RSD %	Mean %	RSD %
Milk	0.01	5	101	2.4	101	5.1
	0.1	5	95	2.8	92	2.6
	Total	10	98	4.1	97	6.2
Meat	0.01	5	96	6.6	92	11
	0.1	5	93	7.2	90	7.4
	Total	10	94	6.6	91	8.9
Egg	0.01	5	87	6.7	108	5.2
	0.1	5	77	3.9	75	4.2
	Total	10	82	8.2	92	19
Liver	0.01	5	84	7.1	92	8.3
	0.1	5	85	4.1	83	5.8
	Total	10	84	5.5	87	8.7
Kidney	0.01	5	88	8.1	101	12
	0.1	5	81	5.7	80	4
	Total	10	84	8.3	90	15
Fat	0.01	5	103	3.8	96	8.3
	0.1	5	89	3.9	85	4.2
	Total	10	96	8.5	91	9.3

RSD = Relative Standard Deviation

Linearity

Considering all matrices, good linearity (r≥0.99) was observed in the range of 0.1 to 10 ng mL^{-1} (for both transitions *m/z* 103 → 60 and *m/z* 103 -->86) for ETU. At least 5 calibration points distributed over the concentration range were used. The stock solution was dissolved in methanol/water (2/8, v/v). A first order calibration curve (linear) was used.

Specificity

LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of the Metiram metabolites ETU in foodstuffs of animal origin. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary.
Significant interferences (> 30% of LOQ) were not observed for each mass transition and retention times and of the analyte, except for egg blank controls, which showed a low interference at the confirmation transition.

Matrix-Effect

Significant matrix-effects (≥20 %) were determined by comparison of solvent vs matrix standards. Therefore quantitative determination of final extracts was carried out by internal standardization with d4-ETU (compensation of the matrix effect) using calibration solutions in solvent.

Limit of Quantification

The limit of quantitation (LOQ) is defined by the lowest fortification level successfully tested which was 0.01 mg kg^{-1} for milk, meat, egg, liver, kidney and fat.

Limit of Determination	The method has a limit of determination (LOD) of 20% of the LOQ, which corresponds to 0.002 mg/kg.
Repeatability	The relative standard deviations (RSD, %) for all commodities and all fortification levels were well below 20%. The values obtained are indicative of the method having satisfactory repeatability. The detailed values are shown in Table 4.1.2-28.
Standard stability	Stability of the analyte in stock, fortification and calibration solutions (in methanol) was demonstrated for up to 4 weeks when stored refrigerated. Stability of internal standard in solution (in methanol) was demonstrated for at least 2 months.
Extract stability	The stability of the final extracts was demonstrated for at least 2 days for kidney, for at least 7 days for egg and up to 21 days for milk, meat and fat by acceptable recoveries within 70 to 120% when stored refrigerated. ETU in final liver extracts is not stable after 2 days of refrigerated storage (after 2 days of storage already significant degradation of ETU and its internal standard demonstrated by low peak intensities). Thus final extracts of liver have to be analyzed immediately after preparation.
Reproducibility	The reproducibility of the method is demonstrated by an Independent Laboratory Validation (BASF DocID 2015/1000809) summarized in CA4.2/3.

Conclusion

The method L0254/01 for analysis of Metiram metabolite ETU (Reg. No. 146099) in foodstuffs of animal origin (exemplified by whole milk and egg, bovine meat, liver and kidney and fat) used LC-MS/MS for final determination, which is a highly specific technique.

It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries, and is therefore applicable to correctly determine residues of Metiram metabolite ETU in foodstuffs of animal origin with a limit of quantification of 0.01 mg kg^{-1}.

Food of plant origin

Analytical methods for the determination of Metiram residues in plant matrices were reviewed in the context of the inclusion in Annex I under Directive 91/414/EEC. These methods evaluated previously are summarized in Table 4.1.2-29 for the reviewer´s convenience.

Table 4.1.2-29: Summary of peer-reviewed analytical methods for determination of Metiram residues in plant matrices

Method No.	Matrix	Method principle	Target analytes	LOQ mg/kg	year	DocID	EU reviewed
BASF method No. 135, Tilting N., 2000	apple, grape, tomato, tobacco	Spectrophotometry (UV)	Metiram (CS2)	0.02 (apple) 0.06 (grape) 0.07 (tomato) 0.08 (tobacco)	2000	2000/1017115	yes
BASF method No. 159/2, Moellerfeld J., 1999	potato, tomato	HPLC using pulsed amperometric detection	ETU	0.05	1999	1999/10240	yes
BASF method No. 159/2, Sasturain J., 2000	tobacco	HPLC using pulsed amperometric detection	ETU	0.05	2000	2000/1000237	yes
DFG method S 15 Weber H., 1999	potato	Spectrophotometry (UV)	Metiram (CS2)	0.02	1999	1999/12032	yes
BASF method No. 373/0 Sasturain J., Mackenroth C., 2000	potato	HPLC/ECD	ETU	0.05	2000	2000/1000238	yes
BASF method No. 373/1, Moellerfeld J., 2001	potato, melon, grape, wine, apple, lettuce, tomato	HPLC using pulsed amperometric detection	ETU	0.05	2001	2001/1015054	yes
BASF method No. 373/2, Benz A., Mackenroth C., 2002	oilseed rape, grape, tomato, potato, wine, apple, lettuce, cherry	HPLC using pulsed amperometric detection	ETU	0.01	2002	2002/1004090	yes

Report: *CA 4.1.2/22*
Schulz H., 2006c
Determination of Metiram (Reg.No. 250 284) and Mancozeb (Reg.No. 292 855) in onions, cucumber, head lettuce and peas
2006/1021409

Guidelines: *SANCO/825/00 rev. 7 (17 March 2004), SANCO/3029/99 rev. 4 (11 July 2000), BBA Guideline Residue Analytical Methods for Post-Registration Control Purposes of July 21 1998, Guidance Document of Residue Analytical Methods 8064/VI/97 rev. 4 15.12.1998*

GLP: *yes*
(certified by Hessisches Ministerium fuer Umwelt, Laendlichen Raum und Verbraucherschutz)

Principle of the method

An analytical method, developed and validated at SGS Fresenius, was developed for the determination of Metiram (Reg. No. 250284) and Mancozeb (Reg. No. 292855) in onions and cucumber (Metiram) as well as in head lettuce and peas (Mancozeb) by GC-MS with a limit of quantification (LOQ) of 0.1 mg kg^{-1} for cucumber, lettuce and peas and 0.3 mg kg^{-1} for onions. Dithiocarbamate was degraded to carbon disulfide by means of ortho phosphoric acid and passed into isooctane by a stream of nitrogen. The final determination was carried out using GC-MS detection. The ion fragment m/z 76 was used for evaluation.

Recovery findings For lettuce and peas investigated during the study the mean recovery values for Mancozeb were between 78.6% and 89.6%. For onions and cucumbers the mean recovery values for Metiram were between 70.2% and 87.4%.The values were in the range of 70-110%. Detailed results are shown in Table 4.1.2-30.

Table 4.1.2-30: Recovery results from method validation of dithiocarbamates (Mancozeb/Metiram) in plant matrices

Crop	Commodity	Test substance	Fortification level (mg kg^{-1})	No. of tests	Mean Recovery (%)	RSD (%)
Lettuce	head	Mancozeb	0.1	5	79	6.1
		Mancozeb	1.0	5	87	6.8
			total	10	83	8.3
Peas	Peas w/o pods	Mancozeb	0.1	5	81	8.1
		Mancozeb	1.0	5	90	5.8
			total	10	86	8.6
Onion	bulb	Metiram	0.3	5	87	15.5
		Metiram	3.0	5	76	8.7
			total	10	82	14.2
Cucumber	Fruit with peel	Metiram	0.1	5	70	12.2
		Metiram	1.0	5	72	10.7
			total	10	71	10.9

RSD = relative Standard Deviation

Since Mancozeb and Metiram both belong to the group of EBDC fungicides and the method determines both active ingredients after degradation to CS_2, the validation data produced with Metiram should also be valid for Mancozeb and thus can be extrapolated.

Linearity Good linearity (r≥0.999) was observed for the CS_2 standard over the concentration range of 0.088 – 2.922 ng mL^{-1} for the mass transition *m/z* 76. Seven calibration points distributed over the concentration range were used.

Specificity The method is applicable for measuring residues of dithiocarbamates (Mancozeb/Metiram) in lettuce, peas, and cucumber down to a level of 0.1 mg kg^{-1} and in onions down to a level of 0.3 mg kg^{-1}. Blank signals or unspecific interferences in untreated control samples were not observed.
GC-MS was used for final determination with following column: Ultra 2 (HP 5), length 50 m, interior diamenter 0.2 mm, film thickness 0.33 µm.

Matrix Effect No separate tests on matrix effects have been conducted. But as all recovery and control samples showed acceptable results, it can be concluded that the given methodology has no relevant matrix effects.

Limit of Quantification The limit of quantitation (LOQ) is 0.1 mg kg^{-1} in all matrices except in onions, where the limit of quantitation is 0.3 mg kg^{-1}, resulting from the lowest concentration level successfully tested within recovery experiments.

Limit of Determination The method has a limit of determination (LOD) of 30 % of the LOQ for each analyte in each matrix, respectively.

Repeatability The relative standard deviations (RDS) for Metiram and Mancozeb were ≤ 20%. The values obtained are indicative of the method having satisfactory repeatability. The detailed values are shown in Table 4.1.2-30.

Reproducibility Reproducibility was not determined within this validation study.

Conclusion

An analytical method for analysis of Metiram (Reg. No. 250284) and Mancozeb (Reg. No. 292855) in onions and cucumber (Metiram) as well as in head lettuce and peas (Mancozeb) used GC-MS for final determination.

It could be demonstrated that the method fulfils the requirements, been in force during time of validation, with regard to linearity, specificity, repeatability, limit of quantification and recoveries, and is therefore applicable to correctly determine residues of Metiram and Mancozeb in onions and cucumber (Metiram) as well as in head lettuce and peas (Mancozeb) with a limit of quantification of 0.1 mg kg^{-1} for cucumber, lettuce and peas and 0.3 mg kg^{-1} for onions.

Nevertheless, an up to date validation of this method has been conducted and is presented below.

Report: CA 4.1.2/23
Meyer M., 2015a
Analytical method for the determination of residues of Metiram (BAS 222 F) in plant matrices by means of Carbondisulfide using GC/MS (BASF method L0234/01) - Validation of the method -
2015/1000804

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), OECD-DOC ENV/MC/CHEM(98)17 Paris 1998, SANCO/3029/99 rev. 4 (11 July 2000), EPA 860.1360, EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5)

GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

This method was originally developed and validated in 2006 (BASF DocID 2006/1021409), to meet current guidelines a new validation has been conducted.

Principle of the method

BASF method L0234/01, developed and validated at SGS Fresenius, was developed for the determination of Metiram (BAS 222 F, Reg.No. 146099) in lettuce, grapes, potatoes, dried peas and sunflower seeds by GC-MS with a limit of quantification (LOQ) of 0.1 mg kg^{-1} (0.056 mg kg^{-1} expressed as CS_2).
Metiram is transformed to CS_2 by means of orthophosphoric acid. Subsequently, the CS_2 is transferred to isooctane with a flow of nitrogen. The quantification was carried out using gas chromatography with mass spectrometric detection (GC-MS). Confirmation of the applied method was achieved using another GC column.

Recovery findings

The method proved to be suitable to determine residues of Metiram by means of the common moiety carbondisulfide (CS_2) in lettuce, grapes, potatoes, dry peas and sunflower. In all matrices tested, the mean recovery values were between 70% and 110% for Metiram by means of carbondisulfide (CS_2). The detailed results are given in Table 4.1.2-31.

Table 4.1.2-31: Recovery results of Metiram (expressed as CS_2) in plant matrices

Matrix	Analyte	Transition *m/z*	Fortification level (mg kg^{-1})	No. of tests	Mean Recovery (%)	RSD (%)
Lettuce (head)	Metiram	76 (primary measurement)	0.1	5	76	11
			1.0	5	84	10.6
			overall: 0.1 + 1.0	10	80	11
		76 (confirmatory measurement)	0.1	5	76	9.8
			1.0	5	94	9.4
			overall: 0.1 + 1.0	10	85	14
Grapes (bunches)	Metiram	76 (primary measurement)	0.1	5	80	19
			1.0	5	77	5.1
			overall: 0.1 + 1.0	10	79	14
		76 (confirmatory measurement)	0.1	5	73	14
			1.0	5	84	8.7
			overall: 0.1 + 1.0	10	79	12
Potato (tubers)	Metiram	76 (primary measurement)	0.1	5	88	7.2
			1.0	5	81	8.6
			overall: 0.1 + 1.0	10	85	8.5
		76 (confirmatory measurement)	0.1	5	75	6.7
			1.0	5	77	13
			overall: 0.1 + 1.0	10	76	10.1
Peas, dried (seed)	Metiram	76 (primary measurement)	0.1	5	87	8.5
			1.0	5	90	2.4
			overall: 0.1 + 1.0	10	89	6.0
		76 (confirmatory measurement)	0.1	5	75	13
			1.0	5	91	5.5
			overall: 0.1 + 1.0	10	83	13
Sunflower (seed)	Metiram	76 (primary measurement)	0.1	5	86	12
			1.0	5	87	7.1
			overall: 0.1 + 1.0	10	87	9.2
		76 (confirmatory measurement)	0.1	5	93	5.6
			1.0	5	95	9.8
			overall: 0.1 + 1.0	10	94	7.6

RSD = Relative Standard Deviation

Linearity

Considering all matrices, good linearity was observed in the range of 40.2 to 1809 ng mL^{-1} (both measurements) for CS_2. The correlation coefficients of the calibration curves on the basis of the two detected ion transitions per analyte were above 0.998.

Seven calibration points distributed over the concentration range were used. The diluted stock solution of CS_2 was dissolved in iso-octane, which was used for calibration purposes. A first order calibration curve (linear) was used.

Specificity

A highly specific chromatographic system was used (GC-MS; two different columns for primary and confirmatory measurement). The retention time of the reference items standard solutions matched the retention time in extracts from the fortified specimens.
The quantification was carried out using gas chromatography with mass spectrometric detection (GC-MS). Confirmation of the applied method was achieved using a GC column with different chemistry:
Column for primary measurement: Rxi-624Sil MS of Restek, length 32 m, interior diamenter 0.32 mm, film thickness 1.8 µm.
Column for confirmatory measurement: Rxi-1ms of Restek, length 32 m, interior diamenter 0.32 mm, film thickness 4.0 µm.

Matrix-effect

In this study no matrix effect tests were carried out.
This was found to be unnecessary due to the methodical approach of the validated method, in which the matrix is refluxed for two hours with highly concentrated acid and the analyte is expelled through concentrated sulphuric acid and captured in iso-octane.
A large number of analytical assays on various matrices using this method were conducted since its development back in 2005 and the mean concurrent recovery rates were within the required range without the use of matrix matched calibration solutions. Against the background of these experiences it was decided to omit the matrix tests in this study. In addition the good results for the recovery and control samples confirm this approach.
No significant interferences from the specimen matrices were detected at the retention time of interest.

Limit of Quantification

The limit of quantitation for Metiram is 0.1 mg kg^{-1} (0.056 mg kg^{-1} expressed as CS_2) for each matrix, resulting from the lowest concentration level successfully tested within recovery experiments.

Limit of Determination

The method has a limit of determination (LOD) of 30 % of the LOQ for each matrix.

Repeatability

The relative standard deviations (RSD) for Metiram by means of carbondisulfide (CS_2) were $\leq$ 20%. The values obtained are indicative of the method having satisfactory repeatability. The detailed values are shown in Table 4.1.2-31.

Standard stability

The data obtained demonstrate that carbondisulfide (CS_2) is stable in the stock solutions (in iso-octane) for 160 days for the duration of the laboratory work.

Extract stability The data obtained demonstrate that carbondisulfide (CS_2) is stable in the extracts of lettuce (head), grapes (bunches), potato (tubers), pead (dried) (seed) and sunflower (seed) for at least 7 to 8 days.

Reproducibility Reproducibility was not determined within this validation study.

Conclusion

The method L0234/01 for analysis of Metiram (BAS 222 F, Reg.No. 146099) in lettuce, grapes, potatoes, dried peas and sunflower seeds used GC-MS for final determination.

It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries, and is therefore applicable to correctly determine residues of Metiram in lettuce, grapes, potatoes, dried peas and sunflower seeds with a limit of quantification of 0.1 mg kg^{-1} (0.056 mg/kg expressed as CS_2).

In addition to the CS2-based residue analytical methods used for the determination of Metiram within residue studies, an additional method based on the EBDC approach has been developed and validated.

Report:	CA 4.1.2/24 Strobl M., 2010a Validation of the analytical method L0089: Determination of Metiram (BAS 222 F) in plant matrices using LC-MS/MS 2010/1007046
Guidelines:	SANCO/825/00 rev. 7 (17 March 2004), SANCO/3029/99 rev. 4 (11 July 2000), OECD Draft Guidance Document on Residue Analytical Methods (February 2007), EEC 96/46 (16.07.1996), EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Since this method is also used for post-approval control and monitoring purposes, the corresponding ILV (BASF Doc ID 2014/1261097) is described in KCA 4.2/1.

Principle of the method

BASF method L0089/01, developed and validated at BASF, was developed for the determination of Metiram (BAS 222 F, Reg. No. 250284) in rapeseed, potatoes, lettuce, grapes, wheat, lemon and onions by HPLC-MS/MS with a limit of quantification (LOQ) of 0.05 mg kg^{-1} for potatoes, lettuce, grapes, wheat, lemons and onions and 0.1 mg kg^{-1} for rapeseed.
The ethylene-bisdithiocarbamate (EBDC) moiety is formed out of BAS 222 F and extracted from plant material with a buffer solution consisting of EDTA, cysteine, methanol and sodium hydroxide adjusted to pH 11.0. The formed ethylene-bisdithiocarbamate (EBDC) analyte is methylated with iodmethane prior to C18 SPE clean up. Samples are quantified by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

Recovery findings	The results show that BASF method no. L0089/01 is suitable to determine residues of Metiram (BAS 222 F) in plant matrices. The mean recovery values were between 80 and 114%. The relative standard deviations (RSD) for all fortification levels were ≤ 18%. Data are summarised in Table 4.1.2-32.

Table 4.1.2-32: Recovery results from method validation of BAS 222 F in rapeseed, potatoes, lettuce, grapes, wheat grain, lemon fruit and onion bulbs

Matrix	Fortification Level (mg kg^{-1})	No of Fortified Samples	Transition 241 → 193		Transition 241 → 134	
			Mean %	RSD%	Mean %	RSD %
Rapeseeds	0.1	5	88	2.1	86	2.0
	1.0	5	88	1.8	86	3.6
	Total	10	88	1.8	86	2.8
Potato	0.05	5	101	3.7	91	4.6
	0.5	5	99	3.0	103	3.8
	Total	10	100	3.2	97	7.7
Head lettuce	0.05	5	95	9.1	99	7.6
	2.5	5	105	16.9	102	17.7
	Total	10	100	14.2	100	13.1
Grapes	0.05	5	96	5.3	98	7.5
	2.5	5	88	6.7	87	7.3
	Total	10	92	7.4	93	9.2
Wheat, grain	0.05	5	85	11.0	85	10.5
	0.5	5	81	4.1	80	2.6
	Total	10	83	8.3	82	8.1
Lemon, fruit	0.05	5	80	3.3	77	6.7
	0.5	5	109	2.2	103	11.5
	Total	10	94	16.7	90	17.7
Onion, bulb	0.05	5	114	1.9	107	0.8
	0.5	5	112	3.4	110	4.2
	Total	10	113	2.8	108	3.3

Linearity

The working interval, concentration between 0.05 ng mL^{-1} and 5.0 ng mL^{-1}, was considered linear with a correlation coefficient (r) ≥ 0.995. The concentration of the internal standard was always 1.0 ng mL^{-1}. At least 6 calibration points distributed over the concentration range were used. The calibration standard solutions were obtained by the combination of diluted stock solutions of the test item and the internal standard and were then further diluted in methanol, EDTA buffer solution and iodmethane. A first order calibration curve (linear) was used for all analytes, mass transitions and matrices, respectively.

Specificity

LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of Metiram in potatoes, lettuce, grapes, wheat, lemons and onions. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary.
Significant interferences (> 30% of LOQ) were not observed for each mass transition and retention times and of the analyte.

Matrix Effect

The recovery data was corrected for interference from the matrix compounds of the appropriate unfortified sample.

Limit of Quantification

The limit of quantitation (LOQ) is defined by the lowest fortification level successfully tested which is 0.05 mg kg^{-1} for potatoes, lettuce, grapes, wheat grain, lemon fruit and onion bulbs and 0.10 mg kg^{-1} for rapeseed.

Limit of Determination

The method has a limit of determination (LOD) of 30% of the LOQ for each matrix respectively.

Repeatability

The relative standard deviations (RSD) for Metiram were ≤ 20% for all transitions. The values obtained are indicative of the method having satisfactory repeatability. The detailed values are shown in Table 4.1.2-32.

Standard stability

Stability of Metiram in fortification solutions (in EDTA-buffer solution - methanol/water 20:80) and in calibration standard solutions (in EDTA-buffer solution - methanol/water 35:65) is about one week when stored in the freezer. Due to the presence of internal standard stability seems to be more than one week, but when comparing the absolute peak areas of Metiram stability is only one week.

Extract stability

The results indicate that Metiram is stable in extracts and in the final volume up to 1 week for grapes but only 3 days for lettuce.

Reproducibility

~~Reproducibility was not determined within this validation study~~.
An independent laboratory validation has been successfully conducted and is reported under [see KCA 4.2/1 2014/1261097].

Conclusion

The method L0089/01 for analysis of Metiram (BAS 222 F, Reg. No. 250284) in rapeseed, potatoes, lettuce, grapes, wheat, lemon and onions used LC-MS/MS for final determination, which is a highly specific technique.
It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries, and is therefore applicable to correctly determine residues of Metiram in rapeseed, potatoes, lettuce, grapes, wheat, lemon and onions with a limit of quantification of 0.05 mg kg^{-1} for all matrices, except for rapeseed the limit of quantification is 0.10 mg kg^{-1}.

Report: *CA 4.1.2/25*
Schulz H., 2006b
Determination of Ethylenethiourea (Reg.No. 146 099) in onions, cucumber, head lettuce and peas - Validation of the BASF method No. 373/3
2006/1008103

Guidelines: *SANCO/825/00 rev. 7 (17 March 2004), SANCO/3029/99 rev. 4 (11 July 2000), BBA Guideline Residue Analytical Methods for Post-Registration Control Purposes of July 21 1998, Guidance Document of Residue Analytical Methods 8064/VI/97 rev. 4 15.12.1998*

GLP: *yes*
(certified by Hessisches Ministerium fuer Umwelt, Laendlichen Raum und Verbraucherschutz)

Principle of the methods

BASF method 373/3, developed and validated at SGS Fresenius, was developed for the determination of Metiram metabolite Ethylenethiourea ETU (Reg.No. 146099) in onions, cucumber, head lettuce and peas by LC-MS/MS with a limit of quantification (LOQ) of 0.01 mg kg^{-1}.

The metabolite ETU (Ethylenethiourea) was extracted from the plant material with a mixture of sodium ascorbate, ethylene urea, ammonium chloride and methanol-water. After centrifugation, an aliquot is taken and the methanol is evaporated from the extract. The pH is adjusted to 8 before evaporating (in some cases after evaporation). The remaining water phase was cleaned by liquid/liquid partition (water/ dichloromethane) on an Extrelut/Al_2O_3 column. After concentration of the eluate, the final determination was carried out using LC-MS/MS detection. Two fragment ions (transition 103 → 44 and transition 103 → 86) were used for evaluation.

Recovery findings The method proved to be suitable to determine residues of ETU in cucumber, peas, onion and lettuce. In all matrices tested, the mean recovery values were between 70% and 110%. The detailed results are given in Table 4.1.2-33.

Table 4.1.2-33: Recovery results of ethylenethiourea (ETU) in plant matrices

Crop	Test Substance	No. of tests	Fort. level (mg kg^{-1})	1. transition (103 → 44)		2. transition (103 → 86)	
				Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)
Cucumber	ETU	5	0.01	83	14.1	83	13.4
		5	0.1	88	6.6	89	5.9
		mean		86	10.6	86	10.3
Pea	ETU	5	0.01	79	2.9	88	26.3
		5	0.1	87	2.7	86	4.0
		mean		83	5.9	87	18.0
Onion	ETU	5	0.01	99	4.5	101	18.1
		5	0.1	92	7.1	93	4.1
		mean		95	6.8	97	13.6
Head lettuce	ETU	5	0.01	82	3.7	91	7.4
		5	0.1	77	9.0	83	12.0
		mean		79	7.2	87	10.4

RSD = Relative Standard Deviation

Linearity Calibration was done with ETU standard solutions over the range 2.528-126.4 ng/mL. Good linearity was observed (r≥0.999). Eight calibration points distributed over the concentration range were used. The diluted stock solution was dissolved in methanol/ultra pure water (10/90, v/v). A first order calibration curve (linear) was used.

Specificity LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of the Metiram metabolites ETU in in cucumber, peas, onion and lettuce. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary.
Significant interferences (> 30% of LOQ) were not observed for each mass transition and retention times and of the analyte.

Matrix Effect As matrix effects were identified, matrix-matched calibration standards were used for calibration.

Limit of Quantification The limit of quantitation is 0.01 mg kg^{-1}, resulting from the lowest concentration level successfully tested within recovery experiments.

Limit of Determination The method has a limit of determination (LOD) of 30 % of the LOQ.

Repeatability The relative standard deviations for ETU were ≤ 20% for all transitions (exception: peas at LOQ, at transition 103 → 86). The preferred transition for evaluation was 103 → 44. The second transition 103 → 86 was validated for confirmatory purpose. The values obtained are indicative of the method having satisfactory repeatability. The detailed values are shown in Table 4.1.2-33.

Standard stability The stability of the calibration solutions was proved by comparing the concentrations of one old solution and one freshly prepared solution by means of a single injection.

Reproducibility Reproducibility of the method was not determined within this validation study.

Conclusion

The method 373/3 for analysis of Metiram metabolite ETU (Reg. No. 146099) in onions, cucumber, head lettuce and peas used LC-MS/MS for final determination, which is a highly specific technique.
It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries, and is therefore applicable to correctly determine residues of Metiram metabolite ETU in onions, cucumber, head lettuce and peas with a limit of quantification of 0.01 mg kg^{-1}.

Report: CA 4.1.2/26
Toledo F., 2011a
Determination of Ethyleneurea (Reg.No. 146 099) in plant matrices - Validation of the BASF analytical method No. L0176/01
2011/1062237
Guidelines: SANCO/825/00 rev. 7 (17 March 2004)
GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

This method L0176/01 is based on old method 373/3 (BASF DocID 2006/1008103), and can be seen as additional validation to cover current guidelines.

Principle of the methods
BASF method L0176/01, developed and validated at SGS Fresenius, was developed for the determination of Metiram metabolite Ethylenethiourea ETU (Reg.No. 146099) in onion, lettuce, potatoes and grapes by LC-MS/MS with a limit of quantification (LOQ) of 0.01 mg kg^{-1}.
ETU is extracted from the plant material with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol/water. After centrifugation, an aliquot is taken and the methanol is evaporated from the extract. The pH is adjusted to 8 before evaporating. The remaining water phase is cleaned on an Extrelut column following a liquid/liquid partition (water/dichlormethane). After concentration of the eluate, the residue is determined by LC-MS/MS.

Recovery findings The method proved to be suitable for analysis of ETU in onion bulbs, lettuce heads, potato tubers and grape bunches to a limit of quantitation of 0.01 mg kg^{-1}. In all matrices tested, the mean recovery values were between 77% and 95%. The detailed results are given in Table 4.1.2-34.

Table 4.1.2-34: Recovery results from method validation of ETU in onion, lettuce, potatoes and grapes

Matrix	Fortification Level (mg kg^{-1})	No of Fortified Samples	Transition 103 → 44		Transition 103 → 86	
			Mean %	RSD %	Mean %	RSD %
Onion bulb	0.01	5	94	7.5	95	11.5
	0.1	5	92	1.5	95	2.1
	Total	10	93	5.3	95	7.8
Lettuce head	0.01	5	84	11.1	89	16.1
	0.1	5	85	2.6	85	2.9
	Total	10	84	7.6	87	11.3
Potato tuber	0.01	5	77	8.0	83	9.4
	0.1	5	92	9.4	93	5.8
	Total	10	84	12.5	88	9.1
Grape bunches	0.01	5	94	1.3	92	6.0
	0.1	5	86	7.2	88	5.7
	Total	10	90	6.6	90	6.1

RSD = Relative Standard Deviation

Linearity	Considering all matrices, good linearity (r≥0.999) was observed in the range of 0.25 - 12.52 ng mL^{-1} for ETU. At least 7 calibration points distributed over the concentration range were used. The diluted stock solution was dissolved in methanol/purified water (1/9, v/v). A first order calibration curve (linear) was used.
Specificity	LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of the Metiram metabolites ETU in onion, lettuce, potatoes and grapes. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary. Significant interferences (> 30% of LOQ) were not observed for each mass transition and analyte at the retention times of interest.
Matrix Effect	As matrix effects were identified, matrix-matched calibration standards were used for calibration.
Limit of Quantification	The limit of quantitation (LOQ) is defined by the lowest fortification level successfully tested which was 0.01 mg kg^{-1} for onion bulbs, lettuce heads, potato tubers and grape bunches.
Limit of Determination	The method has a limit of determination (LOD) of 30% of the LOQ for each matrix.
Repeatability	The relative standard deviations (RSD, %) for ETU for all commodities and all fortification levels were well below 20%. The values obtained are indicative of the method having satisfactory repeatability. The detailed values are shown in Table 4.1.2-34.
Standard stability	The data obtained demonstrate that ETU is stable in its methanol based solutions for the duration of the laboratory work for at least 16 days. Stability of calibration solutions was proved by comparing the concentrations of an old solution with a freshly prepared solution by means of single injection.
Reproducibility	The reproducibility of the method was not estimated as identical samples were not evaluated by an independent laboratory. However, based on the performance of the method, its reproducibility is expected to be good.

Conclusion

The method L0176/01 for analysis of Metiram metabolite ETU (Reg. No. 146099) in onion, lettuce, potatoes and grapes used LC-MS/MS for final determination, which is a highly specific technique.

It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries, and is therefore applicable to correctly determine residues of Metiram metabolite ETU in onion, lettuce, potatoes and grapes with a limit of quantification of 0.01 mg kg^{-1}.

Report: CA 4.1.2/27
Meyer M., 2015a
Validation of analytical methods for the determination of Metiram metabolites in plant matrices (BASF method L0233/01)
2014/1167766

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), OECD-ENV/JM/MONO/(2007)17

GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Principle of the method

BASF method L0233/01, developed and validated at SGS Fresenius, was developed for the determination of Metiram metabolite Ethylenethiourea EU (Reg. No. 27270) and EBIS (Reg. No. 243959) in potato, lettuce, grapes, white beans, rape seed, wheat whole plant and wheat straw by LC-MS/MS with a limit of quantification (LOQ) of 0.01 mg kg^{-1}.
The metabolite EU is extracted from plant material with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol/water. After centrifugation, an aliquot is taken and the methanol is evaporated from the extract. The pH is adjusted to 8 before evaporating. The remaining water phase is cleaned by liquid/liquid partition (water/ethylacetate) on an Extrelut column. After concentration of the eluate, the residue is determined by LC-MS/MS.
The metabolite EBIS is extracted from plant material with a mixture of acetonitrile/formic acid (1000/1, v/v) in the presence of thiourea. After centrifugation, an aliquot is taken and cleaned by dispersive SPE against C18. The supernatant is used for the determination of EBIS by LC-MS/MS.
Two parent-daughter ion transitions per analyte were determined. One transition was used for evaluation, the other one for confirmation.

Recovery findings The method proved to be suitable to determine residues of EU and EBIS in potato, lettuce, grapes, white beans, rape seed, wheat whole plant and wheat straw. In all matrices tested, the mean recovery values were between 70% and 110% for both analytes. The detailed results are given in Table 4.1.2-35.

Table 4.1.2-35: Recovery results of EU and EBIS in plant matrices

Matrix	Analyte	Transition *m/z*	Fortification level (mg kg^{-1})	No. of tests	Mean Recovery (%)	RSD (%)
potato	EU	87 → 44	0.01	4	82	4.2
			0.1	5	85	20
			overall: 0.01 + 0.1	9	83	15
		87 → 70	0.01	4	85	8.2
			0.1	5	84	18
			overall: 0.01 + 0.1	9	84	13
	EBIS	177 → 42	0.01	5	83	4.0
			0.1	5	74	1.1
			overall: 0.01 + 0.1	10	78	6.6
		177 → 86	0.01	5	88	6.4
			0.1	5	74	2.6
			overall: 0.01 + 0.1	10	81	10

Matrix	Analyte	Transition *m/z*	Fortification level (mg kg^{-1})	No. of tests	Mean Recovery (%)	RSD (%)
lettuce	EU	87 → 44	0.01	5	78	4.0
			0.1	5	87	6.3
			overall: 0.01 + 0.1	10	82	7.8
		87 → 70	0.01	5	85	4.9
			0.1	5	86	6.4
			overall: 0.01 + 0.1	10	86	5.4
	EBIS	177 → 42	0.01	5	89	6.4
			0.1	5	86	2.5
			overall: 0.01 + 0.1	10	88	5.1
		177 → 86	0.01	5	95	5.3
			0.1	5	87	2.6
			overall: 0.01 + 0.1	10	91	6.1
grapes	EU	87 → 44	0.01	5	85	10
			0.1	5	78	6.9
			overall: 0.01 + 0.1	10	81	9.6
		87 → 70	0.01	5	81	8.6
			0.1	5	78	5.8
			overall: 0.01 + 0.1	10	79	7.3
	EBIS	177 → 42	0.01	5	100	4.2
			0.1	5	93	6.2
			overall: 0.01 + 0.1	10	97	6.3
		177 → 86	0.01	5	102	4.1
			0.1	5	90	5.0
			overall: 0.01 + 0.1	10	96	7.8
white beans	EU	87 → 44	0.01	5	101	5.9
			0.1	5	92	6.4
			overall: 0.01 + 0.1	10	96	7.7
		87 → 70	0.01	5	101	9.7
			0.1	5	92	6.2
			overall: 0.01 + 0.1	10	97	9.5
	EBIS	177 → 42	0.01	5	92	2.4
			0.1	5	92	2.0
			overall: 0.01 + 0.1	10	92	2.1
		177 → 86	0.01	5	96	3.2
			0.1	5	91	1.7
			overall: 0.01 + 0.1	10	94	3.5
rape seed	EU	87 → 44	0.01	5	78	5.1
			0.1	5	72	3.8
			overall: 0.01 + 0.1	10	75	6.2
		87 → 70	0.01	5	82	9.1
			0.1	5	75	2.9
			overall: 0.01 + 0.1	10	78	8.3
	EBIS	177 → 42	0.01	5	107	2.9
			0.1	5	109	1.4
			overall: 0.01 + 0.1	10	108	2.4
		177 → 86	0.01	5	104	2.7
			0.1	5	107	2.1
			overall: 0.01 + 0.1	10	106	2.8

Matrix	Analyte	Transition *m/z*	Fortification level (mg kg^{-1})	No. of tests	Mean Recovery (%)	RSD (%)
wheat whole plant	EU	87 → 44	0.01	5	81	8.7
			0.1	5	86	2.4
			overall: 0.01 + 0.1	10	84	6.4
		87 → 70	0.01	5	83	9.7
			0.1	5	85	3.0
			overall: 0.01 + 0.1	10	84	6.8
	EBIS	177 → 42	0.01	5	84	3.0
			0.1	5	82	2.0
			overall: 0.01 + 0.1	10	83	2.6
		177 → 86	0.01	5	84	1.4
			0.1	5	81	2.8
			overall: 0.01 + 0.1	10	82	2.6
wheat straw	EU	87 → 44	0.01	5	81	12
			0.1	5	81	16
			overall: 0.01 + 0.1	10	81	13
		87 → 70	0.01	5	81	12
			0.1	5	81	16
			overall: 0.01 + 0.1	10	81	13
	EBIS	177 → 42	0.01	5	83	2.8
			0.1	5	94	3.8
			overall: 0.01 + 0.1	10	88	7.1
		177 → 86	0.01	5	88	4.3
			0.1	5	95	2.7
			overall: 0.01 + 0.1	10	92	5.2

RSD = Relative Standard Deviation

Linearity

Considering all matrices, good linearity was observed in the range of 0.5 to 30 ng mL^{-1} (both transitions) for EU and 0.006 to 0.4 ng mL^{-1} (both transitions) for EBIS. The correlation coefficients of the calibration curves on the basis of the two detected ion transitions per analyte were above 0.998.

At least 6 calibration points distributed over the concentration range were used. The diluted stock solution of EU was dissolved in methanol/water (10/90, v/v) and the diluted stock solution of EBIS was dissolved in acetonitrile/water/HCOOH (90/10/0.1, v/v/v). A first order calibration curve was used.

Specificity

LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of the Metiram metabolites EU and EBIS in potato, lettuce, grapes, white beans, rape seed, wheat whole plant and wheat straw. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary.

Significant interferences (> 30% of LOQ) were not observed for each mass transition and retention times and of the analytes.

Matrix-effect

Even no relevant matrix effects were observed in the matrix lettuce for EU, matrix matched calibration standard solutions were used for the determination of EU in lettuce.
Also for EBIS no significant matrix effects were observed in any of the used matrices, but matrix matched calibration standard solutions were used for the determination of EBIS in all matrices.
Reason for using the matrix standards in these cases was, to accomplish a more stable chromatographic system by a continuous matrix saturation in the instrument.

Limit of Quantification

The limit of quantitation for EU and EBIS is 0.01 mg kg^{-1} for each analyte and each matrix, resulting from the lowest concentration level successfully tested within recovery experiments.

Limit of Determination

The method has a limit of determination (LOD) of 30% of the LOQ.

Repeatability

The relative standard deviations (RSD) for EU and EBIS were ≤ 20% for all transitions. The values obtained are indicative of the method having satisfactory repeatability. The detailed values are shown in Table 4.1.2-35.

Stability Working Solutions

The data obtained demonstrate that both analytes are stable in their stock solutions (in methanol for EU and EBIS and also in ethyl-acetate for EBIS) for the duration of the laboratory work (93 days for EU and 60 days for EBIS). The stability of the calibration solutions was proved by comparing the concentrations of one old solution and one freshly prepared solution by means of a single injection.

Reproducibility

The reproducibility of the method was not estimated as identical samples were not evaluated by an independent laboratory. However, based on the performance of the method, its reproducibility is expected to be good.

Conclusion

The method L0233/01 for analysis of Metiram metabolites EU (Reg. No. 27270) and EBIS (Reg. No. 243959) in potato, lettuce, grapes, white beans, rape seed, wheat whole plant and wheat straw used LC-MS/MS for final determination, which is a highly specific technique.
It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries, and is therefore applicable to correctly determine residues of Metiram metabolites EU and EBIS in potato, lettuce, grapes, white beans, rape seed, wheat whole plant and wheat straw with a limit of quantification of 0.01 mg kg^{-1}.

Report:	CA 4.1.2/28 Richter S., 2015b Metiram (BAS 222 F) and ETU: Bridging extractability of various extraction procedures from lettuce and potato tuber 2015/1000806
Guidelines:	SANCO/825/00 rev. 8.1 (16 November 2010)
GLP:	yes (certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

The objective of this study was to bridge for Metiram (BAS 222 F) and for ETU their extractability using various extraction procedures previously used in plant metabolism studies and comparing these residue findings to residues determined employing extraction procedures as described in commonly used residue methods.

Principle of the applied methods

Metabolism method (DocID 2009/1049027) for Metiram and ETU on Lettuce

Extraction procedure: The homogenized sample material is extracted 3 times with methanol and then 2 times with water. After each extraction step the mixture is centrifuged and decanted.

Final measurement for comparison: The combined extracts are analysed for Metiram (after adding EDTA buffer solution, nabam-d4 as internal standard, and CH3I for derivatization) by LC/MS/MS of the methyl derivative of EBDC (ethylene bis-dithiocarbamate) and for ETU by LC/MS/MS, using the corresponding residue method parameters.

Metabolism method (DocID 2009/1017248) for ETU on Potato

Extraction procedure: The homogenized sample material is extracted 3 times with 100 mL of methanol, after each extraction step the mixture is centrifuged and filtered (or decanted, to avoid losses on filters).

Final measurement for comparison: The combined extracts are analysed for ETU by LC/MS/MS, using the corresponding residue method parameters.

Metabolism method (DocID 1986/0523) for ETU on Potato

Extraction procedure: The homogenized sample material is extracted 3 times with methanol/water (3/1 v/v) and each 1 time with acetone and chloroform, using appropriate solvent volumes.

Final measurement for comparison: The combined extracts are then analysed for ETU by LC/MS/MS, using the corresponding residue method parameters.

Metabolism method (DocID 1986/0525) for ETU on Potato

Extraction procedure: For the analysis of ETU, the homogenized samples are extracted first with dichloromethane, then 5-times with methanol/water (4/1 v/v).

Final measurement for comparison: The combined extracts are then analysed for ETU by LC/MS/MS, using the corresponding residue method parameters.

Residue Method BASF L0089/01 (DocID 2010/1007047) for metriam, EBDC Method

Homogenized samples are extracted with EDTA buffer solution (containing EDTA, NaOH, cysteine, water and methanol), nabam-d4 is added as internal standard and CH3I for derivatization, Metiram is determined by LC/MS/MS of the methyl derivative of EBDC.

Residue Method BASF L0234/01 (DocID 2006/1021409) for Metiram, CS_2 Method

Homogenized samples are digested with 34 % H3PO4 to form CS2 from Metiram, for subsequent GC/MS determination.

Residue Method BASF L0176/01 (DocID 2011/1062237), ETU Method

For lettuce and potato tuber homogenized samples are extracted with methanol, water, thiourea and NH4Cl buffer, followed by centrifugation and ETU determination by LC/MS/MS.

Residue Method (PTRL in-house method) for Metiram CS_2 Method

The homogenized sample material was extracted with a mixture of water, concentrated hydrochloric acid and tin (II)-chloride in hydrochloric acid solution as well as isooctane by shaking for at least 90 minutes in a water bath (95-100°C). For extraction the bottle was tightly sealed and shaken for 2 hours in a shaking water bath at 80 °C. After cooling the isooctane-phase is used for GC/MS analysis.

The limit of quantification (LOQ) for Metiram by EBDC is 0.05 mg kg^{-1} (expressed as Metiram), for ETU 0.01 mg kg^{-1} and for Metiram determined as CS_2 it is 0.10 mg kg^{-1} (expressed as Metiram).

Residue results

A summary of residue results of treated samples is given in Table 4.1.2-36 for Metiram and in Table 4.1.2-37 for ETU.

Table 4.1.2-36: Extraction methods in plant materials; summary of residues results for Metiram

Extraction procedure based on:		Metabolism (2009/1049027)	Residue (L0089/01)	Rec. Corr. Residue (L0234/01)	Residue (PTRL method)
Specimen Type	Specimen No.	Metiram	Metiram	Metiram (determined as CS_2; expressed as Metiram)**	Metiram (determined as CS_2; expressed as Metiram)**
		Multiple extraction with MeOH / H_2O	Single Extraction with MeOH (EDTA,NaOH)	Single Extraction with 34% aqueous H_3PO_4	$HCl/SnCl_2$
		Residue R mg kg^{-1}	Residue R mg kg^{-1}	Residue R mg kg^{-1}*	Residue R mg kg^{-1}
Lettuce	L1306410005	32	26	78	63
	L1306420005	27	19	27	23
	L1306440005	27	21	29	41

* Residues are corrected for low recoveries.
** 1 mg of Metiram theoretically generates 0.56 mg CS_2 (conversion factor 1.79)

The extraction method L0089/01 with a single extraction with MeOH (EDTA, NaOH) gave slightly lower residue results compared to the residue results obtained with the metabolism method (2009/1049027), where 5 extractions were done.

Residue results for Metiram determined as CS_2 (L0234/01) were comparable to those obtained with the metabolism method (2009/1049027), except for sample L1306410005. As the method is not specific for Metiram, possibly other residues forming CS_2 were present in the sample which cannot be determined with the other 2 methods where Metiram is determined by EBDC.

Results obtained with BASF method L0234/01 were corrected for the low recovery results obtained from the concurrent fortified samples. Residues of Metiram determined as CS_2 found with the in-house method and confirmed with acceptable recovery results are comparable to the results found with BASF method L0234/01. Thus it is concluded, that corrected residue results for BASF method L0234/01 are suitable respectively valid.

In general it can be concluded with the exception on the high recoveries for L130641005 based on CS_2, that the extraction procedures are comparable as they are in the same order of magnitude.

Table 4.1.2-37: Extraction methods in plant materials; summary of residues results for ETU

Extraction procedure based on:		**Metabolism Study (2009/1049027)**	**Metabolism Study (1986/0523)**	**Metabolism Study (1986/0525)**	**Metabolism Study (2009/1017248)**	**Residue Method (L0176/01)**
Specimen Type	Specimen No.	ETU	ETU	ETU	ETU	ETU
		Multiple extraction with MeOH and water	3 times extraction with MeOH/water 3:1; 1 time Acetone 1 time Chloroform	Dichlorometha ne; 5 time extraction with MeOH/water 4:1	Multiple extraction with MeOH and water	Single extraction with MeOH, water, thiourea, NH_4Cl
		Residue R mg kg^{-1}	Residue R mg kg^{-1}	Residue R mg kg^{-1}	Residue R mg kg^{-1}	Residue R mg kg^{-1}
Lettuce	L1306410005	1.5	Not tested. Methods from metabolism studies were used for potatoes and not for lettuce samples.			0.56
	L1306420005	0.56				0.20
	L1306440005	0.60				0.18
Potato	L1301180005	not tested	<0.01	0.013	<0.01	<0.01
	L1301180006		<0.01	<0.01	<0.01	<0.01
	L1301180007		<0.01	<0.01	<0.01	0.010

For lettuce the method of the metabolism method (2009/1049027) resulted with the multiple extractions approx. 3 times higher residues of ETU compared to the single extraction of the residue method (L0176/01).

For potato residues with all methods were < LOQ except for 1 sample analyzed with residue method (L0176/01) which gave a residue result for ETU of 0.010 mg kg^{-1}. For the metabolism method (1986/0523) a residue for 1 sample with 0.013 mg kg^{-1} was found.

Concurrent Method Validation

For concurrent method validation, plant materials were fortified (3 replicates per LOQ and higher level and crop type) with solutions containing Metiram or ETU to obtain fortifications levels at the LOQ and at a higher level (10xLOQ, 100xLOQ, 500LOQ or 600xLOQ). Additionally, samples were kept untreated as blank controls to show that no significant chromatographic signal interference was observed.

Recovery findings Average recoveries for Metiram and ETU in lettuce and potato tuber were within the acceptable range of 70% to 110% with relative standard deviations (RSD) of ≤ 20% except for Metiram determined as CS_2 in lettuce extracted with extraction method L0234/01 with an average recovery of about 26% and a RSD of 34%.

Detailed results of recoveries are given in Table 4.1.2-38 to Table 4.1.2-40.

Table 4.1.2-38: Extraction methods in plant materials; summary of validation results of Metiram as EBDC

Matrix	Fortification Level (mg kg^{-1})	Metiram as EBDC (241 m/z -> 134 m/z)		
		Average (%)	RSD (%)	n
Metabolism Method 2009/1049027 (LC-MS/MS)				
Lettuce	0.05, 25	80	10	6
Matrix	**Fortification Level (mg kg^{-1})**	**Metiram as EBDC (241 m/z -> 193 m/z)**		
		Average (%)	**RSD (%)**	**n**
Residue Method L0089/01 (LC-MS/MS)				
Lettuce	0.05, 5.0, 25	83	17	7

RSD=Relative Standard Deviation
n=Number of results included in calculation

Table 4.1.2-39: Extraction methods in plant materials; summary of validation results of Metiram as CS_2

Matrix	Fortification Level (mg kg^{-1})	Metiram as CS_2 (76 m/z -> 44 m/z)		
		Average (%)	RSD (%)	n
Residue Method L0234/01 (GC-MS)				
Lettuce	0.10, 1.0, 60	26	34	6
Matrix	**Fortification Level (mg kg^{-1})**	**Metiram as CS_2 (76 m/z -> 44 m/z)**		
		Average (%)	**RSD (%)**	**n**
Residue Method PTRL in House Method (GC-MS)				
Lettuce	0.1, 60	76	12	6

RSD=Relative Standard Deviation
n=Number of results included in calculation

Table 4.1.2-40: Extraction methods in plant materials; summary of validation results of Metiram as EBDC

Matrix	Fortification Level (mg kg^{-1})	ETU (103 m/z -> 60 m/z)		
		Average (%)	RSD (%)	n
Metabolism Method 1049027 (LC-MS/MS)				
Lettuce	0.01, 1.0	82	9.4	6
Residue Method 2009/1017248 (LC-MS/MS)				
Potato	0.01, 1.0	93	10	6
Metabolism Method 1986/0523 (LC-MS/MS)				
Potato	0.01, 1.0	106	5.8	6
Metabolism Method 1986/0525 (LC-MS/MS)				
Potato	0.01, 1.0	83	19	6
Residue method L0176/01 (LC-MS/MS)				
Lettuce	0.01, 1.0	91	11	6
Potato	0.01, 1.0	90	14	6
	Overall	91	12	12

RSD=Relative Standard Deviation
n=Number of results included in calculation

Linearity

LC-MS/MS Analysis, Metiram as EBDC (lettuce)
Good linearity (r = 0.9995) was observed in the range of 0.125 to 10 ng mL^{-1} or 31.25 ng mL^{-1} (transitions m/z 241 -> 134 (quantitation) (method 2009/1049027) and m/z 241 -> 193 (quantitation) (method L0089/01).
Internal calibration standards in solvent (EDTA buffer solution) were used for quantitation of the analyte by LC-MS/MS, always with ≥ 5 concentration levels for evaluation of the final extracts. A first order calibration curve (linear) was used.

GC-MS Analysis, Metiram as CS_2 (lettuce):
Good linearity (r ≥ 0.99) was observed in the range of 10 to 100000 ng mL^{-1} (method L0234/01) (transition m/z 76 -> 44 (quantitation)).
External calibration using solvent standards (in isooctane) was used for quantitation of the analyte by GC-MS, always with ≥ 5 concentration levels for evaluation of the final extracts. A first order calibration curve (linear) was used.

LC-MS/MS Analysis, ETU (lettuce):
Good linearity (r = 0.9995 (method 2009/1049027) and r = 0.9985 (method L0176/01)) was observed in the range of 0.15 to 10 ng mL^{-1} (method 2009/1049027) and 0.20 to 15 ng mL^{-1} (method L0176/01) (transition m/z 103 -> 60 (quantitation)).
External calibration using matrix-mached standards (in methanol/water 1/9, v/v) was used for quantitation of the analyte by LC-MS/MS, always with ≥ 5 concentration levels for evaluation of the final extracts. A first order calibration curve (linear) was used.

LC-MS/MS Analysis, ETU (potato):
Good linearity (r = 0.9985 (method L0176/01), r = 0.9971 (method 2009/1017248), r = 0.9967 (method 1986/0523) and r = 0.9977 (method 1986/0525)) was observed in the range of 0.20 to 15 ng mL^{-1} (method L0176/01), 0.15 to 7.5 ng/mL (method 2009/1017248), 0.10 to 10 ng/mL (method 1986/0523) and 0.10 to 7.5 ng mL^{-1} (method 1986/0525) (transition m/z 103 -> 60 (quantitation)).
External calibration using matrix-mached standards (in methanol/water 1/9, v/v) was used for quantitation of the analyte by LC-MS/MS, always with ≥ 5 concentration levels for evaluation of the final extracts. A first order calibration curve (linear) was used.

Specificity Tested within original method validations.

Matrix-effect Tested within original method validations.

Limit of Quantification The limit of quantification (LOQ) for Metiram by EBDC is 0.05 mg kg^{-1} (expressed as Metiram), for Metiram determined as CS_2 0.10 mg kg^{-1} (expressed as Metiram) and for ETU it is 0.01 mg kg^{-1}.

Limit of Determination Limit of detection (LOD) derived from the different lowest calibration levels and the different multipliers and dilution factors for the methods/matrices was always 0.01 mg kg^{-1} for Metiram as EBDC.
Limit of detection (LOD) derived from the lowest calibration levels was 0.02 mg kg^{-1} for Metiram as CS_2.

Repeatability The relative standard deviations (RSD) for Metiram and ETU in lettuce and potato tuber were ≤ 20%, except for Metiram determined as CS_2 in lettuce extracted with extraction method L0234/01 with a RSD of 34%. The values obtained are indicative of the methods having satisfactory repeatability. The detailed values are shown in Table 4.1.2-38 to Table 4.1.2-40.

Stability in Solutions and Extracts

Standard solutions (in EDTA buffer solution (methods for Metiram by EBDC) and in isooctane (methods for Metiram by CS_2)) and final extracts were stored frozen for Metiram and refrigerated for ETU in the dark when not in use. Solutions for the Metiram analysis were prepared freshly every week. Solutions (in methanol/water 1/9, v/v) for ETU were used throughout the study (2 weeks) and gave consistent results and recoveries. Acceptable recoveries for Metiram and ETU in crop sample extracts demonstrate the stability of the analyte during the analysis of the samples.

Conclusion

For Metiram it can be concluded, that for the tested matrix lettuce the extraction procedures are comparable as they are in the same order of magnitude.

ETU shows good comparison for potato matrix, but a significant discrepancy for the matrix lettuce where a factor of approx. 3 has been identified between the extraction procedures of metabolism vs the residue method.

(f) Methods in soil, water, sediment, feed and any additional matrices used in support of ecotoxicology studies

Analytical methods used in ecotoxicological studies, are in general described and validated with the corresponding ecotoxicological study. In addition, the following methods were developed and validated within stand-alone validation studies.

Report:	CA 4.1.2/29 Meyer M., 2012a Validation of the BASF method L0089/01 for the determination of residues of Metiram (BAS 222 F) in arthropods using LC/MS/MS 2012/1287207
Guidelines:	EEC 91/414 Annex II (Part A Section 4), EEC 91/414 Annex III (Part A Section 5), SANCO/3029/99 rev. 4 (11 July 2000)
GLP:	yes (certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

This method L0089/01 was originally validated for the use in plant matrices, in addition it was used for the determination of Metiram in arthropods. Therefore an additional validation was conducted.

Principle of the method

BASF method L0089/01, developed and validated at SGS Fresenius, was developed for the determination of Metiram (BAS 222 F) in arthropods by LC-MS/MS with a limit of quantification (LOQ) of 0.050 mg kg^{-1}.
0.5 g of the arthropods specimen was weighed into a 50 mL centrifuge tube and internal standard Nabam-d4 hexahydrate was added. The ethylene-bisthiocarbamate (EBDC) moiety was formed out of Metiram and extracted from the specimen with a buffer solution consisting of EDTA, cysteine, methanol, and sodium hydroxide adjusted to pH 11. The formed EBDC analyte was methylated with iodomethane prior to C18 solid phase extraction (SPE) clean up. Final determination was performed by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS), monitoring two mass transitions.

Recovery findings: The method proved to be suitable to determine Metiram in arthropods. Samples were spiked with the analyte at the limit of quantification (LOQ) of 0.050 mg kg^{-1} and ten times higher (0.50 mg kg^{-1}). All average recovery values (mean of four or five replicates per fortification level and analyte) were between 70% and 110%. The detailed results are given in the Table 4.1.2-41.

Table 4.1.2-41: Results of the method validation for the determination of Metiram in arthropods

Matrix	Analyte	m/z	Fortification level [mg kg^{-1}]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
arthropods	Metiram	241 → 193	0.050 0.50	4[a] 5	98 86	5.3 1.8	92	7.8
		241 → 134	0.050 0.50	4[a] 5	86 86	6.2 4.3	86	4.8

RSD = Relative standard deviation
[a] One value was identified as outlier using Grubbs test.

Linearity Good linearity (r > 0.999) was observed in the range of 0.02021 ng mL^{-1} to 0.7073 ng mL^{-1} for the two mass transitions of Metiram.

Specificity LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of Metiram in arthropods. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique was not necessary.
Significant interferences (> 30% of LOQ) were not observed at the retention times of the analytes.

Matrix Effects No significant matrix effects were observed, therefore, no matrix-matched calibration standards were needed.

Limit of Quantification The method has a limit of quantification (LOQ) of 0.050 mg kg^{-1}.

Repeatability The relative standard deviations (RSD, %) for all fortification levels were below 20%.

Reproducibility Reproducibility of the method was not determined within the validation study.

Conclusion
The method L0089/01 for analysis of Metiram in arthropods used LC-MS/MS for final determination, which is a highly specific technique.
It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries and is therefore applicable to correctly determine residues of Metiram in arthropods.

Report:	*CA 4.1.2/30* *Heinz W., 2002a* *Validation of analytical method CP 402: Determination of BAS 222 28 F in water by GC/MS* *2002/1004292*
Guidelines:	*OECD Principles of Good Laboratory Practice, GLP Principles of the German Chemikaliengesetz (Chemicals Act)*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Principle of the method

Analytical method CP 402 (actual BASF method no. APL0384/01) was validated for the analysis of the formulation BAS 222 28 F (active substance Metiram, Reg. No. 250284) in aqueous matrices by GC/MS, for the support of tox/ecotox studies for dose verification.

The method is based upon the reductive hydrolysis of the active substance by a solution of $SnCl_2$ (15 g L^{-1}) in 15% hydrochloric acid at 70°C for 2 h. During the hydrolysis, the decomposition product carbon disulfide (CS_2) was extracted from the aqueous layer with 2 mL isooctane. After reaction completion, the CS_2 concentration in the isooctane phase was determined by gas chromatography/mass spectrometry (GC/MS) in selective ion monitoring (SIM) mode at m/z = 76 (quantifier ion). As qualifier ion m/z = 78 was recorded and used for control purposes in order to ensure an appropriate specificity.

The limit of quantification (LOQ) of the method is 0.1 mg L^{-1}.

Recovery findings The method proved to be suitable to determine BAS 222 28 F in water samples. Samples were fortified at concentrations of 0.1 mg L^{-1} (LOQ), 0.5 mg L^{-1}, and 2 mg L^{-1}. The analyses yielded acceptable mean recoveries between 102% and 111%. The detailed results are given in the table below (Table 4.1.2-42).

Table 4.1.2-42: Results of the method validation for the determination of BAS 222 28 F in water

Matrix	Analyte	Fortification level [mg L^{-1}]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
OECD Water	**BAS 222 28 F**	0.1	5	111	7.1	106	5.9
		0.5	5	102	3.5		
		2.0	5	104	3.7		

RSD = Relative standard deviation

Linearity The results proved good linearity (r > 0.999) of the detector response in the investigated concentration range of approximately 0.0632 mg L^{-1} to 2.528 mg L^{-1}.

Specificity GC/MS is highly specific for the analyte CS_2 generated from the test substance. The identification and quantification is based on the selected ion monitoring of EI MS molecular ion signals characteristic for the analyte (quantifier ion reported m/z = 76 and qualifier ion monitored m/z = 78). Under the described conditions the method is specific for the determination of BAS 222 28 F as CS_2 in water.
As the method is only used for dose verification of known substances and known nominal concentrations, no additional confirmatory technique is necessary.
No significant matrix interferences were observed in the investigated blank water samples.

Limit of Quantification The method has a limit of quantification (LOQ) of 0.1 mg L^{-1}.

Repeatability The relative standard deviations (RSD, %) for all fortification levels were below 20%.

Reproducibility Reproducibility of the method was not determined within the validation study.

Conclusion
The results of the analytical study proved that analytical method CP 402 is suitable for the determination of BAS 222 28 F in water. It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, accuracy, repeatability, and limit of quantification.

(g) Methods in water, buffer solutions, organic solvents and any additional matrices resulting from the physical and chemical properties tests

Where necessary, these methods are reported along with the respective studies

CA 4.2 Methods for post-approval control and monitoring purposes

Because of the special physical characteristics of the Metiram analytes, the commonly used multi residue methods are not useful, what can also be seen by the small amount of data available in the EURL-pesticides-datapool. Therefore the methods for risk assessment, already described in CA 4.1.2 Methods for Risk Management, are supposed to be used also for post-approval control and monitoring purposes. To fulfil the additional requirement of an independent laboratory validation for enforcement methods, ILV's have been conducted for the corresponding methods and are listed below.

The following recovery and repeatability criteria are required according to the OECD Guidance document on analytical methods (ENV/JM/MONO(2007)17), depending on the fortification levels:

≤1 µg/kg	50 - 120 ± 35%
>1 µg/kg ≤0.01 mg/kg	60 - 120 ± 30%
>0.01 mg/kg ≤0.1 mg/kg	70 - 120 ± 20%
>0.1 mg/kg ≤1.0 mg/kg	70 - 110 ± 15%
>1 mg/kg	70 - 110 ± 10%

(a) Methods for the analysis in food and feed of plant and animal origin

Based on the residue definition for MRL setting and enforcement, residue analytical methods are required for the parent molecule Metiram in food of plant and animal origin.

Food of plant origin

~~**For the determination of Metiram residues in plant matrices based on the CS_2 approach, an independent laboratory validation of analytical method L0234/01, presented in detail in section CA 4.1.2, Methods for Risk Management, was a planned and already initiated. Unfortunately, this study could not be finalized in time to present it within this dossier. Actually, it is scheduled to have these study finalized in December 2015 latest for submission.**~~

In addition to the CS_2-based residue analytical method for data generation, an additional method based on the EBDC approach has been developed and validated, and is described in detail in section CA 4.1.2, Methods for Risk Management. This method passed through a second validation study to cover actual guidelines. This additional validation study covers also the requirements of an ILV, therefore this method is supposed to be used for post-approval control and monitoring purposes.

Report:	CA 4.2/1 Richter S., 2015c Validation of BASF method L0089/01 for the determination of Metiram (BAS 222 F) as Methyl-derivative of Ethylen-bisdithiocarbamate (EBDC) in 5 crop types, using LC/MS/MS 2014/1261097
Guidelines:	SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000), EPA 860.1340 (1996)
GLP:	yes (certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

Principle of the method

BASF method L0089/01, validated at PTRL Europe, was developed for the determination of Metiram (BAS 222 F, Reg.No. 146099) after methylation (using CH_3I) as methyl-ethylenebis(dithiocarbamate) derivative (Me-EBDC) in apples, grapes, wheat grain, dry beans and sunflower seed by LC/MS/MS with a limit of quantification (LOQ) of 0.05 mg kg^{-1}, except for oily crops (sunflower seed) where the initial LOQ was 0.10 mg kg^{-1}, always expressed as Metiram.

The sample material just previously homogenized will be extracted after addition of nabam-D4 hexahydrate as internal standard with EDTA buffer solution (in water/methanol 8/2 v/v, cysteine present, pH 11 adjusted with NaOH). An aliquot of the supernatant is further diluted with EDTA buffer and methanol, then iodomethane is added to allow methylation of the free EBDC to form methyl derivatives. After a clean-up with C_{18}-SPE cartdriges, extracts are analysed by LC/MS/MS, monitoring two characteristic ion transitions (MRMs) for the di-methylated EBDC (*m/z* 241 -> 134 for quantitation and *m/z* 241 -> 193 for confirmation) and 1 MRM for the di-methylated EBDC-D4 internal standard (*m/z* 245 -> 197).

Recovery findings	The method proved to be suitable to determine residues of Metiram after methylation (using CH_3I) as methyl-ethylenebis(dithiocarbamate) derivative (Me-EBDC) in apples, grapes, wheat grain, dry beans and sunflower seed. Average recoveries for both MS/MS ion transitions were between 70 and 110% with the following exceptions: for wheat grain the quantification transition was slightly above 110% (114%). Relative standard deviations (RSD) were always ≤ 20%. The detailed results are given in Table 4.2-1.

Table 4.2-1: Recovery results of Metiram (as EBDC) in plant matrices

Matrix	Analyte	Transition *m/z*	Fortification level (mg kg^{-1})	No. of tests	Mean Recovery (%)	RSD (%)
Apples	Metiram	241 -> 134	0.05	5	98	2.3
			0.5	5	110	0.4
			overall: 0.05 + 0.5	10	104	6.3
		241 -> 193	0.05	5	108	2.8
			0.5	5	110	1.1
			overall: 0.05 + 0.5	10	109	2.3
Grapes	Metiram	241 -> 134	0.05	5	84	8.7
			0.5	5	98	2.5
			overall: 0.05 + 0.5	10	91	10
		241 -> 193	0.05	5	84	4.7
			0.5	5	96	2.5
			overall: 0.05 + 0.5	10	90	7.9
Wheat grain	Metiram	241 -> 134	0.05	5	99	4.2
			0.5	5	114	11
			overall: 0.05 + 0.5	10	106	11
		241 -> 193	0.05	5	97	7.0
			0.5	5	110	10
			overall: 0.05 + 0.5	10	103	11
Bean, dry	Metiram	241 -> 134	0.05	5	90	6.6
			0.5	5	108	1.8
			overall: 0.05 + 0.5	10	99	11
		241 -> 193	0.05	5	89	12
			0.5	5	108	1.5
			overall: 0.05 + 0.5	10	99	13
Sunflower seed	Metiram	241 -> 134	0.1	5	73	4.0
			1.0	5	78	2.6
			overall: 0.1 + 1.0	10	75	4.5
		241 -> 193	0.1	5	70	3.7
			1.0	5	78	2.6
			overall: 0.1 + 1.0	10	74	6.8

RSD = Relative Standard Deviation

Linearity

Good linearity (r≥0.999) was observed in the range of 0.125 to 10 ng mL^{-1} for the two mass transitions of Metiram (as EBDC). Seven calibration points distributed over the concentration range were used. The stock solution of the analytical standard Metiram (BAS 222 F) in EDTA buffer solution was used to prepare intermediate calibration solutions (in EDTA buffer solution) by volumetric dilutions. The intermediate calibration solutions were used to prepare methylated calibration solutions. A first order calibration curve (linear) was used.

Specificity

LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of the Metiram (as EBDC) in apples, grapes, wheat grain, dry beans and sunflower seed. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary.

Significant interferences (> 30% of LOQ) were not observed for each mass transition and retention times and of the analyte.

Matrix-effect

The matrix effect was tested for each matrix. Significant matrix effects (i.e. > 20% suppression or enhancement) on LC-MS/MS response were observed for grape and sunflower seed.
For the determination of the matrix effect, a methylated calibration solution was prepared and diluted either in a final extract of a blank sample or in methanol/water (60/40, v/v). For the evaluation of the results calibration solutions in solvent containing internal standards were used which compensates any matrix effects.

Limit of Quantification

The limit of quantification (LOQ) of the analytical method is 0.05 mg kg^{-1} except for sunflower seed with a LOQ of 0.10 mg kg^{-1}, resulting from the lowest concentration level successfully tested within recovery experiments.

Limit of Determination

The limit of determination (LOD) of the method is defined as the lowest analyte concentration injected as a calibration solution, resulting in a LOD of 0.01 mg kg^{-1} respectively 0.02 mg kg^{-1} for sunflower seed (corresponds each to 20 % of the LOQ).

Repeatability

The relative standard deviations (RSD) for Metiram (as EBDC) were ≤ 20%. The values obtained are indicative of the method having satisfactory repeatability.

Standard stability

Metiram indicated sufficient stability in stock solutions (water and EDTA buffer solution), spike solutions (water), in intermediate calibration solutions (EDTA buffer solution) as well as in methylated calibration solutions for at least 1 week when stored frozen in the dark as aliquots in separate amber glass vials.

Extract stability

Final sample extracts in methanol/water (60/40, v/v) were re-injected after at least 4 days of storage under frozen conditions. Re-injection of final extracts of grape, wheat grain, dry bean and sunflower seed resulted in recoveries within the acceptable range of 70 – 110%.
For apple, injection of final sample extracts in methanol/water (60/40, v/v) stored frozen for 8 days resulted in recovery results of about 200%. The ratio between the analyte and the internal standard changed during the storage, which resulted into high recovery results.
It is thus recommended to inject always final sample extracts immediately after preparation.

Reproducibility

In context of this laboratory validation study, the reproducibility of the method L0254/01 was estimated. The results confirm the results of the first validation study (BASF Doc ID 2010/1007046), therefore a high reproducibility is determined.

Conclusion

The method L0089/01 for analysis of Metiram as EBDC (BAS 222 F, Reg.No. 146099) in apples, grapes, wheat grain, dry beans and sunflower seed used LC-MS/MS for final determination.

It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries, and is therefore applicable to correctly determine residues of Metiram (as EBDC) in apples, grapes, wheat grain, dry beans and sunflower seed with a limit of quantification of 0.05 mg kg^{-1}, except for sunflower seed with a LOQ of 0.10 mg kg^{-1}.

The outcome of the ILV conforms the validity, already shown in the original validation study (BASF DocID 2010/1007046, KCA 4.1.2/24).

Food of animal origin

~~For the determination of Metiram residues in animal matrices based on the CS2 approach, a new method development, according to the actual guidelines, was planned and already initiated. Unfortunately, this study as well as the corresponding independent laboratory validation could not be finalized in time to present it within this dossier. Actually, it is scheduled to have these studies finalized in December 2015 latest for submission.~~
~~For that reason, the already reviewed methods, 135/2 and 525/0, are presented in detail for the reviewers convenience in section CA 4.1.2, Methods for Risk Management.~~

Report:	CA 4.2/2 Quarles L.,Hawkins A., 2016 a Independent laboratory validation of the BASF analytical method L0256/01 for the determination of Metiram (BAS 222 F) in animal matrices using GC/MS 2016/1030250
Guidelines:	EPA 860.1340, SANCO/825/00 rev. 8.1 (16 November 2010)
GLP:	yes (certified by United States Environmental Protection Agency)

Principle of the method
Metiram residues were hydrolyzed by hot acidic digestion using aqueous hydrochloric acid (HCl) and tin(II) chloride ($SnCl_2$) solution and trapping released CS_2 in an isooctane layer contained in a sealed vessel. After cooling, the isooctane was injected into a GC-MS for CS_2 determination. Two GC-MS methods were applied: the primary method used a DB-1701 column and the confirmatory method a CP-Sil 8 CB column. In both cases, the ESI+ mode was used and one fragment ion (76 m/z, corresponding to the molecular ion of CS_2) was monitored.
Subtraction of blank interferences was necessary, in case no CS_2 free matrix could be obtained.

Recovery findings	The method L0256/01 was proven to be suitable to determine residues of metiram as CS2 in different animal matrices at a limit of quantitation of 0.01 mg/kg using the primary analysis method. The overall mean recovery values of the validation experiments using the primary analysis method were between 70% and 76%, which meets the guidance criteria for mean recovery values. The detailed results are given in the tables below.

Table 4.2-2: Validation results of method L0256/01: Metiram in animal matrices

Test substance	Matrix	Fortification level[a] (mg/kg)	No of tests	Average recovery (%)		Relative standard deviation (%)	
Fragment ion				76 m/z (DB-1701)	76 m/z (CP-Sil 8 CB)	76 m/z (DB-1701)	76 m/z (CP Sil 8 CB)
Metiram as CS_2	Bovine meat	0.01	5	65.3 [c]	56.3 [c]	9.7	17
		0.10	5	86.7 [c]	73.3 [c]	3.3	2.7
		Overall	**10**	**76.0**	**64.8**	**6.5**	**9.8**
	Bovine liver	0.01	5	75.5[c]	72.6[c]	4.5	3.8
		0.10	5	76.1[c]	69.9[c]	2.2	3.5
		Overall	**10**	**75.8**	**71.2**	**3.3**	**3.6**
	Bovine kidney	0.01	5	68.5[c]	51.4[c]	10	8.5
		0.10	5	71.2[c]	69.2[c]	0.4	2.6
		Overall	**10**	**69.9 [b]**	**60.3 [b]**	**5.4 [b]**	**5.6 [b]**
	Bovine milk	0.01	5	68.2	71.0	1.5	2.7
		0.10	5	77.2	78.4	4.6	0.9
		Overall	**10**	**72.7**	**74.7**	**3.0**	**1.8**
	Bovine fat	0.01	5	73.4	62.6[c]	8.0	4.3
		0.10	5	71.6	65.0[c]	3.2	1.8
		Overall	**10**	**72.5**	**63.8**	**5.6**	**3.1**
	Hen eggs	0.01	5	77.8	71.5	0.9	1.4
		0.10	5	73.5	67.2	1.5	0.9
		Overall	**10**	**75.7**	**69.4**	**1.2**	**1.2**

[a] Fortification level is expressed as CS_2, however metiram was fortified.
[b] Different hydrolysis reagents used for LOQ and 10x LOQ fortification levels
[c] Recovery samples were corrected for interferences / residues of the analyte detected in the respective control samples.

Linearity

Good linearity ($R^2 \geq 0.98$) was observed for the m/z 76 ion on both capillary GC columns over the concentration range 0.40 ng/mL to 50 ng/mL (used for fat trials) and 2.0 ng/mL to 200 ng/mL (used for matrices other than fat).

Specificity

GC-MS is a highly specific detection technique and therefore no confirmatory technique is required. Analysis is possible with two different chromatographic columns.

Matrix effects

Based on findings of the method validation report (BASF DocID 2015/1000807), there were no significant matrix effects (< 20%) on reference substance response. Solvent calibration standards were used during this study for measurement of metiram as CS_2.

Interference

The method L0256/01 determines residues of metiram as CS_2 in animal matrices. The interferences/residues of the analytes measured in the control samples were on average below 20 % of the limit of quantification (LOQ) for milk and egg for both columns. The interferences/residues of the analyte measured in the kidney, liver, meat and the fat control samples were above 20 % on average of the limit of quantitation (LOQ). Thus the recoveries for CS_2 were corrected for the respective interferences.

Limit of quantitation The limit of quantitation (LOQ) is defined by the lowest fortification level successfully tested. The LOQ is 0.01 mg/kg of CS_2 corresponding to 0.0179 mg/kg of metiram.

Limit of detection The limit of detection (LOD) is defined as 20% of the LOQ, resulting in a LOD of 0.002 mg/kg of CS_2.

Repeatability The relative standard deviations (RSD, %) for all commodities and all fortification levels were < 20% for both primary and confirmatory analysis. The detailed values are shown in Table 4.3-2.

Reproducibility This ILV demonstrated that method L0256/01 is suitable for determination of metiram as CS_2 in the respective animal matrices using the primary analysis method.

Standard stability No significant change (<10% relative difference) in concentration of CS2 in isooctane was observed when stored frozen and in the dark for a period up to 96 days.

Extract stability Extract stability was not investigated within this study.

Conclusion

This ILV demonstrated that the primary analysis method for method L0256/01 fulfills the requirements with regard to specificity, repeatability, limit of quantification and recoveries and is therefore applicable to correctly determine residues of metiram as CS_2 in various animal matrix types.

The outcome of the ILV conforms the validity, already shown in the original validation study (BASF DocID 2015/1000807, KCA 4.1.2/19).

Report: CA 4.2/3
Benotti M.J., 2015a
Independent laboratory validation (ILV) of BASF analytical method L0254/01 for the determination of the Metiram metabolite ETU in foodstuff of animal origin, using LC-MS/MS
2015/1000809

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), OECD-ENV/JM/MONO/(2007)17

GLP: yes
(certified by United States Environmental Protection Agency)

Principle of the method

BASF method L0254/01 was developed for the determination of Metiram metabolite Ethylenethiourea ETU (Reg.No. 146099) in foodstuffs of animal origin (exemplified by whole milk and egg, bovine meat, liver and kidney and fat) by LC-MS/MS with a limit of quantification (LOQ) of 0.01 mg kg-1.
Samples are extracted (adding sodium ascorbate, thiourea, ethylene urea and Celite filter aid) with methanol. Extracts are filtered, cleaned-up by liquid/liquid partition using a HM-N extraction column and concentrated for LC/MS/MS determination.
The objective of this study was to independently validate BASF method L0254/01 (BASF DocID 2014/1223796, see MCA 4.1.2/12).

Recovery findings The results show that BASF method no. L0254/01 is suitable to determine residues of the metabolite ETU in foodstuffs of animal origin with an LOQ of 0.01 mg kg^{-1}. The total mean recovery values of ETU were between 72 and 90%. The relative standard deviations (RSD) for all fortification levels were ≤ 20%. Data are summarised in Table 4.2-3.

Table 4.2-3: Recovery results from method validation (ILV) of the metabolite ETU in foodstuffs of animal origin

Matrix	Fortification Level (mg kg^{-1})	Number of Replicates	Transition 103 → 60		Transition 103 → 86	
			Mean %	RSD %	Mean %	RSD %
Kidney	0.01	6	74	11	83	8
	0.1	6	75	4	72	4
	Total	12	74	8	78	10
Liver	0.01	6	86	5	90	15
	0.1	5	89	10	88	9
	Total	11	87	8	89	12
Eggs	0.01	5	77	13	85	20
	0.1	6	79	5	78	6
	Total	11	78	9	81	15
Meat	0.01	5	74	7	77	6
	0.1	6	73	8	72	8
	Total	11	74	7	74	8
Milk	0.01	5	78	8	72	2
	0.1	6	79	10	79	10
	Total	11	78	9	76	9
Fat	0.01	6	82	14	76	10
	0.1	6	75	6	75	6
	Total	12	78	12	75	8

RSD = Relative Standard Deviation

Linearity

Good linearity (r > 0.995) was observed in the range of 0.1 ng mL^{-1} to 10.0 ng mL^{-1} for the two mass transitions of ETU. At least 5 calibration points distributed over the concentration range were used. The ETU working solutions for calibrations were prepared in methanol/water (20/80, v/v). A first order calibration curve (linear) was used.

Specificity

LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of the Metiram metabolite ETU in foodstuff of animal origin. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary.
Significant interferences (> 30% of LOQ) were not observed for each mass transition and retention times and of the analyte for milk, fat, meat, liver, eggs and kidney.

Matrix-Effect

Matrix effects were investigated by comparing the peak areas of analyte in solvent to the same concentrations of analyte in matrix. The results demonstrate that matrix were significant (i.e. greater than positive or negative 20%) for both transitions necessitating the use of matrix-matched calibrations for determination of analyte concentration in fortified samples.

Limit of Quantification

The limit of quantitation (LOQ) is defined by the lowest fortification level successfully tested which was 0.01 mg kg^{-1} for all matrices.

Limit of Determination	The limit of determination (LOD) was set to 30% of the LOQ, which corresponds to 0.003 mg kg^{-1} for all matrices.
Repeatability	The relative standard deviations (RSD, %) for all commodities and all fortification levels were well below 20%. The values obtained are indicative of the method having satisfactory repeatability. The detailed values are shown in Table 4.2-3.
Standard stability	Already investigated within original validation study.
Extract stability	Already investigated within original validation study.
Reproducibility	In context of this independent laboratory validation study, the reproducibility of the method L0254/01 was estimated. The results of the ILV confirm the results of the validation study, therefore a high reproducibility is determined.

Conclusion

The method L0254/01 for analysis of Metiram metabolite ETU (Reg. No. 146099) in foodstuffs of animal origin (exemplified by whole milk and egg, bovine meat, liver, kidney and fat) used LC-MS/MS for final determination, which is a highly specific technique.

It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, limit of quantification and recoveries, and is therefore applicable to correctly determine residues of metiram metabolite ETU in foodstuffs of animal origin with a limit of quantification of 0.01 mg kg^{-1}.

~~**The results confirm the original validation study.**~~

The outcome of the ILV conforms the validity, already shown in the original validation study (BASF DocID 2014/1223796, KCA 4.1.2/20).

(b) Methods for the analysis in water

Report: CA 4.2/4
Jarrett H., 2015a
Independent laboratory validation of BASF method L0155/01 for the determination of Metiram in water by LC-MS/MS
2014/1246507

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4, EPA 850.6100, OECD-ENV/JM/MONO/(2007)17

GLP: yes
(certified by Department of Health of the Government of the United Kingdom, United Kingdom)

Principle of the method

BASF method L0155/01 was developed for the determination of Metiram (BAS 222 F) as ethylene-bisdithiocarbamate (EBDC)-dimethyl in ground- and surface water by LC-MS/MS with a limit of quantification (LOQ) of 0.050 µg L^{-1}.

The ethylene-bisdithiocarbamate (EBDC) moiety was formed by dissolving Metiram with a buffer solution consisting of EDTA, cysteine, methanol, and sodium hydroxide adjusted to pH 11. The formed ethylene-bisdithiocarbamate (EBDC) analyte was methylated with iodomethane. An aliquot of the sample was analyzed by LC-MS/MS.

The objective of this study was to independently validate BASF method L0155/01 (BASF DocID 2009/1122341, see MCA 4.1.2/3).

Recovery findings: The method proved to be suitable to determine Metiram in ground- and surface water. Samples were spiked at the limit of quantification of 0.050 µg L^{-1} and ten times higher (0.50 µg L^{-1}). All average recovery values (mean of at least five replicates per fortification level and analyte) were between 70% and 110%. The detailed results are given in the table below (Table 4.2-4).

Table 4.2-4: Results of the method validation for the determination of Metiram in ground- and surface-water

Matrix	Analyte	m/z	Fortification level [µg kg^{-1}]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
Ground Water	Metiram	241 → 134	0.050	6	77	7.9	91	16.6
			0.50	6	105	0.4		
		241 → 193	0.050	6	79	6.9	91	14.4
			0.50	6	103	1.4		
Surface Water	Metiram	241 → 134	0.050	5[a]	94	1.8	101	8.3
			0.50	6	107	6.0		
		241 → 193	0.050	5[a]	104	4.5	104	3.0
			0.50	6	104	1.4		

RSD = Relative standard deviation
[a] Sample (replicate 6) was excluded due to contamination in both transition.

Linearity	Good linearity (r > 0.9995) was observed in the range of 0.0104 ng mL^{-1} to 1.04 ng mL^{-1} for both mass transitions.
Specificity	LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of Metiram identified and quantified as ethylene-bisthiocarbamate (EBDC)-dimethyl in ground- and surface water. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary. No significant interferences (> 30% LOQ) at the retention time and mass transitions of EBDC-dimethyl were observed.
Matrix Effects	It was demonstrated that the matrix-load in the tested matrix-matched standards had a significant effect at low concentrations (response suppression > 20%) for ground- and surface water. Thus, quantitative determination was carried out using matrix-matched standards with nabam-d4 hexahydrate as internal standard.
Limit of Quantification	The method has a limit of quantification (LOQ) of 0.050 µg L^{-1}, resulting from the lowest concentration level successfully tested within recovery experiments.
Limit of Detection	The method has a limit of detection (LOD) of 0.005 µg L^{-1} for surface water and 0.002 µg L^{-1} for ground water, based on 3x height of baseline noise.
Repeatability	The relative standard deviations (RSD, %) for all fortification levels were below 20%.
Standard Stability	Standard stability for Metiram has not been established over the tested time period of 7 days stored refrigerated.
Extract Stability	Extracts were not stable over the tested time period of 7 days stored refrigerated. All samples were injected immediately after extraction.
Reproducibility	In context of this independent laboratory validation study, the reproducibility of the method L0155/01 was estimated. The results of the ILV confirm the results of the validation study, therefore a high reproducibility is determined.

Conclusion

The method L0155/01 for analysis of Metiram in ground- and surface-water used LC-MS/MS for final determination, which is a highly specific technique.

It could be demonstrated that the method fulfils the requirements with regard to linearity, specificity, repeatability, reproducibility, limit of quantification and recoveries and is therefore applicable to correctly determine residues of Metiram in ground- and surface-water.

~~The results confirm the original validation study.~~

The outcome of the ILV conforms the validity, already shown in the original validation study (BASF Doc ID 2009/1122341, KCA 4.1.2/3).

Report: CA 4.2/5
Ertunc T., 2015a
Independent laboratory validation of BASF Method L0228/01 for the determination of the Metiram metabolites ETU, EU and EBIS in ground- and surface-water by LC-MS/MS
2014/1246508

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), EPA 850.6100, OECD-ENV/JM/MONO/(2007)17

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Principle of the method

BASF method L0228/01, developed and validated at PTRL Europe, was developed for the determination of the Metiram metabolites ETU (Reg.No.146099), EBIS (Reg.No.243959) and EU (Reg.No.27270 in water by LC-MS/MS with a limit of quantification (LOQ) of 0.050µg L-1. Representative aliquots of water samples were used for direct injection into the analytical instrument. For Quantification matrix matched standards were used. Final determination was done by LC-MS/MS.

Recovery findings

The method proved to be suitable to determine metabolites ETU (Reg.No.146099), EBIS (Reg.No.243959) and EU (Reg.No.27270) in water. Samples were spiked at the limit of quantification of 0.050 µg L^{-1} and ten times higher (0.50 µg L^{-1}). All average recovery values (mean of at least five replicates per fortification level and analyte) were between 70% and 110%. The detailed results are given in the table below (Table 4.2-5).

Table 4.2-5: Results of the method validation for the determination of the Metiram metabolites ETU (Reg.No.146099), EBIS (Reg.No.243959) and EU (Reg.No.27270) in ground- and surface-water

Matrix	Analyte	m/z	Fortification level [µg L^{-1}]	Number of Replicates	Mean Recovery [%]	RSD [%]	Overall Recovery [%]	RSD [%]
Ground (Drinking) Water	ETU (Reg.No.146099)	103->60	0.050 0.50	6 6	80 103	2.1 2.4	91	13.3
		103->86	0.050 0.50	6 6	79 102	2.0 2.1	91	13.3
	EBIS (Reg.No.243959)	177->43	0.050 0.50	6 6	82 98	8.9 7.5	90	12.1
		177->74	0.050 0.50	6 6	87 97	4.9 8.6	92	9.1
	EU (Reg.No.27270)	87->44	0.05 0.5 5	6 6 6	96 93 102	6.1 5.1 3.3	97	5.9
		87->70	0.05 0.5 5	6 6 6	96 92 98	7.6 5.5 5.9	95	6.5

RSD = Relative standard deviation

Linearity	Good linearity (r > 0.99) was observed in the range of 0.015 ng/mL to 5.0 ng/mL for the two mass transitions of ETU and EBIS, 0.15 ngL up to 1.0 ng/L for EU (second mass transition EU 0.035-1.0 ng/L). At least seven calibration points distributed over the concentration range tested were used.
Specificity	LC-MS/MS is a highly specific self-confirmatory technique. Under the described conditions the method is specific for the determination of all three analytes ground (drinking) water. Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary. No significant interferences (> 30% LOQ) at the retention times and mass transitions observed.
Limit of Quantification	The method has a limit of quantification (LOQ) of 0.050 µg L^{-1}, resulting from the lowest concentration level successfully tested within recovery experiments.
Repeatability	The relative standard deviations (RSD, %) for all fortification levels were below 20%.
Reproducibility	These results of the independent laboratory validation confirm the results of the validation study and is reported in CA 4.1.2/4 [BASF DocID 2014/1000106].

Conclusion
The method L0228/01 for analysis of Metiram in ground- and surface-water used LC-MS/MS for final determination, which is a highly specific technique.
It could be demonstrated that method L0228/01 fulfils the legal requirements with regard to specificity, linearity, repeatability, limit of quantification and recoveries and is therefore applicable to correctly determine residues of ETU (Reg.No.146099), EBIS (Reg.No.243959) and EU (Reg.No.27270) in Ground (Drinking) Water.
The outcome of the ILV conforms the validity, already shown in the original validation study (BASF DocID 2014/1246508, KCA 4.1.2/4).

Metiram

Document M-CA, Section 5

TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

Compiled by:

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number
30/Jun/2015		Version 1 BASF DocID 2015/1029563
29/Feb/2016	The non QAU audited reports CA 5.7.1/2 BASF Doc Id 2014/1315300 and CA 5.8.2/2 BASF Doc Id 2014/1313072 have been substituted by the final report (QAU audited). No changes were necessary in the studies summaries	Version 2 BASF DocID 2016/1051892
31/Aug/2017	As requested by RMS, different studies have been added and summarised under chapters CA 5.3; 5.4; 5.5; 5.6. Under chapter 5.8, study BASF Doc Id 2013/1065851 has been replaced by BASF Doc Id 2016/1321628	Version 3 BASF DocID 2017/1161176

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

Annex II Dossier:
Studies on absorption, distribution, metabolism and excretion have been evaluated previously during the Annex I inclusion process. Regarding absorption, distribution, and excretion the data provided has been considered acceptable. A summary of the data is provided below (CA5.1.1/2 and CA5.1.3). Regarding metabolism, the notified has been requested to address the identity of unidentified metabolites.

AIR3 Dossier:
A new study in rat was conducted to investigate the excretion and identity of metabolites after oral application of ^{14}C-BAS222 F (metiram). A summary of the study is provided below (CA5.1.1/1).
A new study on *in vitro comparative metabolism* has been conducted with ^{14}C-BAS222 F. A summary of the study is provided below (CA5.1.1/4).

In support of the representative uses supported in the present dossier, and as previously requested, a new study was conducted to investigate the identity of ^{14}C-BAS222 F metabolites in rat. With the objective to provide a comprehensive elucidation of the metabolic profile in rats, radioactive metiram was orally administered at several different dosing regimes: the single dose administration (two different dose levels), and in addition a repeated dose administration (high dose). Metabolites then investigated in faeces, bile and urine (CA5.1.1). While providing detailed metabolite data both regarding structural identity and concentration, this study in principle confirms the results of previous investigations. To facilitate evaluation, a summary of the previously evaluated study is given below (CA5.1.2 and CA 5.1.3).

In addition, CA5.1.4 provides a summary of an in vitro comparative metabolism study using human hepatocytes to compare metabolic profiles of human and animal species. These investigations confirm absence of any detectable human-specific transformation product.

In summary, taking previous and new investigations together, the metabolism of ^{14}C-BAS222 F in rats is considered elucidated. The pathway is comparable to the metabolism found in ruminant and poultry (goat and hen). In addition, in vitro data indicates comparability with human metabolism. In conclusion, the metabolism data obtained are representative for animals and human metabolism.

CA 5.1 Studies on Absorption, Distribution, Metabolism and Excretion in Mammals

CA 5.1.1 Absorption, distribution, metabolism and excretion by oral exposure

Report: CA 5.1.1/1
2015a
Excretion and metabolism of 14C-BAS 222 F after oral administration in rats
2014/1000223

Guidelines: EPA 870.7485, EPA 860.1000, MAFF Testing Guidelines for Toxicology Studies: Metabolism Animals (Japan), OECD 417

GLP: yes
(certified by Ministry of Health, Welfare and Sport, The Hague, The Netherlands)

Executive Summary

The excretion and metabolism of BAS 222 F (metiram) was investigated in male rats after oral application of ^{14}C-labelled metiram. Dosing was done as (1) a single dose of 5 mg/kg bodyweight, (2) a single dose of 50 mg/kg bodyweight, (3) repeated doses (14 days) of 50 mg/kg body weight. In addition, administering to in bile-cannulated rats, (4) a single dose of 5 mg/kg bodyweight and (5) a single dose of 50 mg/kg bodyweight.
Sampling of urine and faeces were sampled at 6, 12 and 24 hours post application and thereafter in daily intervals until 168 hours. Urine, faeces and bile of the bile-cannulated animals were sampled 3, 6, 9, 12, 18 and 24 hours post application and thereafter in 6-hour intervals until 72 hours. From 72 hours onwards, urine, faeces and bile was collected in 12-hour intervals until 168 hours.
Reasonable efforts were made to identify all metabolites present 1% or greater of the administered dose. A proposed metabolic scheme of the rat in urine, bile and faeces is provided.

Excretion via faeces was high and amounted to proportions (of applied dose) of 55% (1), 68% (2), 67% (3), 62% (4) and 65% (5) over an 168 hour observation period. The major part of faecal excretion occurred within 12-48 hours post administration. Excretion of radioactivity via bile was lower and amounted for a 168 hour period to 10% (4) and 6% (5) of applied dose.

Excretion via urine amounted to proportions of applied dose of 43% (1), 23% (2), 28% (3), 38% (4) and 38% (5). The major part of urinary excretion occurred within 24 hours post administration.

Faeces of rats (early and late pools samples and individual time intervals) were extracted sequentially with methanol and water. The major part of radioactivity was extracted with methanol (up to 67.0% TRR). Water extraction released again significant amounts of radioactivity (up to 39.2% TRR). The sum of the solvent extracts ranged from at least 15.9% TRR (Group 2, 6-12 h) to a maximum of 85.3% TRR (Group 1, 0-6 h) indicating that a high proportion of the residue is not solvent extractable.
The residues after solvent extraction were subjected to a sequential solubilisation procedure with protease and lipase. For all early pools, protease solubilisation released the highest amounts of radioactivity (up to 30.5% TRR). Treatment with lipase resulted in release up to 10.0% of TRR and 1% ammonia released up to 14.9% TRR. All other treatments released relatively small portions of radioactivity, which accounted for up to 10% TRR. The sum of solubilized radioactive residues ranged from 43.1% TRR (early pool, 50 mg/kg) to 63.0% TRR (early pool, 5 mg/kg).

In urine, the metabolite patterns were comparable between groups, both qualitatively and quantitatively. The most abundant component was metabolite M222F002 (ETU, 9.6 – 17.8% dose). Further components present at significant amounts were M222F003 (EU, 3.5 - 5.8% dose) and M222F024 (≤ 3.4% dose).

Also in bile, the metabolite patterns were comparable between groups, both qualitatively and quantitatively. The most abundant component in bile was metabolite M222F024 (2.8 – 5.1% dose). In addition, two low level components were identified: M222F026 and M222F002 (ETU) (≤ 2.0% dose).

The metabolite patterns in faeces extracts (methanol and water) and protease solubiliates were comparable between groups both qualitatively and quantitatively. The most abundant component (up to 24% dose) was M222F002 (ETU). Further identified components were below or equal to 3.4% dose (M222F003 (EU), M222F005 (carbimid), M222F026, M222F025, M222F007 (TDIT), M222F004 (EBIS) and M222F024).

The metabolic pathway of BAS 222 F shows conversion of metiram to M222F002 (ETU) as the main pathway, presumably directly or indirectly via TDIT (M222F007) and EBIS (M222F004). M222F002 (ETU) is then further transformed into M222F003 (EU). Other transformations are postulated such as methylation of EBIS to generate M222F024, and formation of carbamid, M222F026 and M222F025. In addition, incorporation into natural cell constituents is indicated by the fact that a high proportion of the residue was not extractable by solvents however was released by treatment with protease and lipase.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: ^{14}C-labelled metiram (BAS 222 F)
- **Description:** ethylene-C14 label, unlabelled metiram
- **Chemical name:** Zinc ammoniate ethylenebis(dithiocarbamate)-poly(ethylenethiuramdisulphide)
- **BASF Registry No:** 250284
- **Lot/Batch #:** 153-6001 (^{14}C-labelled), CP052744 (unlabelled)
- **CAS#:** 9006-42-2
- **Development code:** Metiram: BAS 222 F
  ETU: M222F002
  EU: M222F003
  EBIS: M222F004
  Carbimid: M222F005
  TDIT: M222F007
  M222F024
  M222F025
  M222F026
- **Purity:** Radiochemical purity of labelled test item: not indicated
  Releasable carbondisulfide: 89%
  Purity of unlabelled test item: 93.6% (tolerance ± 1.0%)
- **Specific activity:** 7.64 MBq/mg
- **Stability of test compound:** The test item was stable over the test period.

2. Vehicle and/or positive control: 1% aqueous solution of carboxymethylcellulose

3. Test animals:
- **Species**: Rat
- **Strain:** Wistar Han rats (Crl: WI (Han))
- **Sex:** male
- **Age:** 9 weeks old at allocation
- **Weight at dosing:** Average 332 g
- **Source:**
- **Acclimation period:** 5 days
- **Diet:** SM R/M-Z diet from SSNIFF® Spezialdiäten GmbH, Soest, Germany
- **Water:** Tap water *ad libitum*
- **Housing:** During acclimatisation in macrolon cages containing sterilized sawdust bedding. In addition, paper was provided as nest material. During experiments in stainless steel metabolism cages with a grid to collect urine and faeces
- **Environmental conditions:**
- **Temperature:** 18.3 - 21.1°C
- **Humidity:** 29 - 71%
- **Air changes:** Not reported
- **Photo period:** 12 h light / 12 h dark

4. Preparation of dosing solutions

The ^{14}C-labelled test item (solid) was weighed into a suitable container. The unlabelled test item was weighed to the labelled test item, in such way to obtain a specific activity of about 5.09 MBq/mg (Group 1, 4) or 0.61 MBq/mg (Group 2, 3, 5). The blending ratio of labelled versus unlabelled compound was 2+1 (Group 1 and 4) or 2+23 (Group 2, 3 and 5).
Thereafter, the mixture of labelled and unlabelled test item was resuspended in 1% aqueous solution of carboxymethylcellulose and resuspended by stirring and sonication. Animals were dosed within 20 minutes after preparation of the formulation.
The application formulations were LSC measured and the measured value was taken for calculation of the concentration of the test item and as check for the homogeneity of the application formulation. The single oral doses were weighed into syringes and administered orally by gavage. The amount of the administered application formulation was calculated by weighing the syringes before and after application.
The radiochemical purity of the application formulations was not checked; the CS-method was not practical and a confirmation by this method was not performed.

The impurity check was performed using the formulation prepared for Group 1. The radioactive formulation (suspension in 1% CMC) was thawed, mixed and the radioactive concentration was determined in duplicate (10 µL) by liquid scintillation counting (LSC). An aliquot of this formulation was filtered by using Millex GV filters (0.22 µm). The radioactive concentrations in the filtered fractions were determined in duplicate (10 µL) by liquid scintillation counting (LSC). Afterwards the filtered formulation (1% CMC) was diluted with acetonitrile (1/1, v/v %). After mixing, the radioactive concentration of this diluted sample was determined in duplicate (10 µL) by liquid scintillation counting (LSC). The diluted filtered formulation was used to determine the column recovery which was performed by injection with and without the column in the LC-system and LSC of the LC-solvent collected during these runs. Afterwards the filtered formulation (1% CMC) diluted with acetonitrile (1/1, v/v %) was analysed with the LC-RAD and LC-MS method as used for the metabolite investigation. The presence of the following 4 compounds was checked in the filtered formulation samples: ETU, EU, Carbimid and EBIS.

B. STUDY DESIGN AND METHODS

1. Dates of experimental work September 16, 2013 – April 16, 2015

2. Study design

The aim of the study was to characterise and identify the metabolites of BAS 222 F (metiram, M222F000) in urine and bile samples as well as faeces extracts of Wistar rats, upon single oral dose of 5 mg/kg and 50 mg/kg bodyweight (groups 1 and 2), and repeated oral dose administration (14 days) of 50 mg/kg body weight (group 3) and in bile-cannulated animals after application of a single oral dose of 5 mg/kg and 50 mg/kg bodyweight (groups 4 and 5) exposure to BAS 222 F. An either LC-PDA-RAD or LC-PDA-MS method was developed for the analysis of the samples using a mobile phase consisting of milli-Q water and formic acid in methanol with an Ultra PFPP analytical column. MS detection was performed in the positive ionization mode.

Sampling and storage
Urine and faeces of Groups 1, 2 and 3 were sampled at predose (-24-0 h, except for Group 3), 6, 12 and 24 hours post application and thereafter in daily intervals until 168 hours. No faeces were collected from animal 1 and 10 after 12 hours, as there was no defecation in the respective time period. Samples from the animals of Groups 1, 2 and 3 were pooled and homogenized per matrix, group and time point, prior to radioactivity determination.
For metabolite investigations, time intervals were pooled per group, to obtain an early pool (0-6, 6-12, 12-24, 24-48 hours) and a late pool (48-72, 72-96, 96-120, 120-144, 144-168 hours) per group.
Urine, faeces and bile of Groups 4 and 5 (bile-cannulated animals) were sampled at predose (-24-0 h), 3, 6, 9, 12, 18 and 24 hours post application and thereafter in 6-hour intervals until 72 hours. From 72 hours onwards, urine, faeces and bile was collected in 12-hour intervals until 168 hours. No faeces was collected from animal 14 after 9 hours, animal 15 after 63, 132, 144, 156 and 168 hours and from animal 16 after 3, 6 and 54 hours, as there was no defecation in the respective time period.

Bile samples were analysed for radioactivity individually and were pooled afterwards per group and time point, and also over timepoints to obtain an early (0-3, 3-6, 6-9, 9-12 hours), mid (12-18, 18-24 hours) and late (24-30, 30-36, 36-42, 42-48 hours) bile pool per group. The bile of animal 19 (Group 5) was pooled separately, due to the clinical condition of animal.
The urine and faeces samples of Group 4 and 5 were not pooled and were not used for metabolite investigations, because in this group the focus was on bile metabolites.

Seven days after dosing with the radiolabelled compound the animals were deeply anaesthetized using isoflurane and blood was withdrawn causing the animals to be exsanguinated. After exsanguination, several tissues and organs were sampled. The metabolism cages were subsequently washed with methanol:water (1:1, v/v). All the samples (urine, faeces, bile, plasma, tissues, carcasses and cage wash) were frozen and stored at ≤ -15°C.

Table 5.1.1-1: Summary of dose groups and dosing parameters

Dose group	1	2	3	4	5
No. of doses and route of administration	Single oral dose	Single oral dose	Repeated (14-days) oral dose	Single oral dose	Single oral dose
Nominal dose (mg/kg bw)	5	50	50	5	50
Specific radioactivity used (MBq/mg)	4.95	0.567	0.548	5.12	0.568
Sampling	Urine Faeces	Urine Faeces	Urine Faeces	Urine Faeces Bile	Urine Faeces Bile

Determination of radioactivity
For the quantification of radioactivity in liquid samples, a liquid scintillation counter was used. Aliquots of liquid samples were mixed with a sufficient volume of a suitable scintillator prior to measurement and analysed for radioactivity without any additional treatment. All data were corrected using appropriate quench curves and are expressed in disintegrations per second (Bq).
In case of solid samples, aliquots of the homogenised samples were weighed into combustion cones and combusted by means of an automatic sample oxidizer. The $^{14}CO_2$ formed during combustion was trapped by absorption liquid and the collected radioactivity was measured by liquid scintillation counting (LSC).

HPLC analysis
For the detection of the different metabolites, the urine, bile and faeces samples were analysed by two analytical methods, one aiming at the generation of a radioactivity chromatogram used for quantitative determination, and the other aiming at the generation of mass spectrometry data (accurate MS/MS) using positive mode for structure elucidation. Based on retention time comparison known for the metabolite standards, the MS signals/peaks were assigned to the selected radioactive peaks. For confirmation, semi-quantitative comparison of the radioactive and MS peaks was performed, if feasible. Metabolite patterns (radioactive and MS) were compared between matrices.

An overview of the used methods:

1. HPLC-RAD for all samples, generating a radioactivity chromatogram for quantification of the metabolites.
2. HPLC-PDA-MS (accurate MS/MS) for selected samples, generating mass spectrometry data using positive mode for structure elucidation.
3. HPLC-MS as a confirmatory system for all samples.

The column used for LC-PDA-(RAD)MS analysis was a Ultra PFPP, the eluent system consisted of 2 mobile phases (A: water & B: 0.1% (v/v) formic acid in methanol) which were used applying linear gradient elution.

<u>Sample preparation for analysis</u>
Urine samples stored at < -15°C were thawed, vortex mixed and centrifuged for 5 min at 3300 g and room temperature. The amount of radioactivity in these samples was checked by LSC. In order to investigate the radioactive metabolic patterns, aliquots of urine samples of male Wistar rats (pooled per time interval up to 48 hours, and the early and late pool samples) were subjected to LC-RAD method. Additionally, to investigate the metabolic patterns, aliquots of urine samples of male Wistar rats (pooled per time interval up to 48 hours, and the early pool samples) were subjected to LC-MS method.
Blank urine samples (pre-dose urine samples of group 1 and 2) were treated exactly the same to obtain blank urine samples. These samples were used as blanks during the investigation of the metabolic patterns.
For structure elucidation of metabolites in urine of male Wistar rats, the early pool samples of group 1, 2 and 3, and urine of time interval 6-12h of group 3 were subjected to LC-MS analysis. The early pool urine samples of group 2 and group 3 were diluted (2.5 and 5 fold, respectively) with pre-dose urine of group 2, all other samples were subjected undiluted. The injection volume was 5 µL for all analysis.

Bile samples stored at < -15°C were thawed, vortex mixed and centrifuged for 5 min at 3300 g and room temperature. The amount of radioactivity in these samples was checked by LSC. For LC-RAD analysis, bile samples were injected undiluted. For MS analysis, the early pool samples of group 5 and group 5 (animal 19) were diluted (3 and 2 fold, respectively) with pre-dose bile of group 5, all other samples were injected undiluted. The injection volume was 5 µL for all analysis.
Blank bile samples (pre-dose bile samples of group 4 and 5) were treated exactly the same to obtain blank bile samples. These samples were used as blanks during the investigation of the metabolic patterns.

The late pool *faeces samples* were not extracted any further after the protease step, because the radioactive residue in the samples was low (< 0.7 % of the dose).

<u>Test extractions:</u> In order to set up the final extraction procedure, an investigation of extraction techniques was conducted. Finally, an extraction procedure was defined by considering the extractability results in order to achieve a high total extractability rate: methanol and water followed by physiological relevant enzymatic steps and as last steps ammonia and HCl.

<u>Main extractions:</u> The extraction procedure to investigate the metabolic patterns in faeces extracts of the rats was as follows: homogenised aliquots (about 5 gram for the early pools and 20 gram for the late pools) of faeces were extracted three times with 20 mL methanol. The residue was dried and extracted four times with water.
A sub sample of the residue after solvent extraction of the early pools was taken (2.5 gram), resuspended in Tris buffer (at ~ pH 7) and 25 mg protease (Sigma, from *Streptomyces griseus)* was added. This was incubated at 37 °C upon shaking for 3.5 h. Thereafter the same amount of protease was added again and the mixture was incubated overnight.
The protease incubation was repeated (since obtained mass-balances were incorrect) with the remaining residue after solvent extraction (1.7 to 3.2 g). The same procedure was followed with 25 mg protease.
The resulting residue was resuspended in a lipase solution (6.8 g KH_2PO_4, 190 mL 0.2 M NaOH and 10 g lipase (Sigma, from porcine pancreas), pH 7.5 in 1000 mL water) and incubated at 37 °C for about 4 h.

The residue after lipase incubation of the early pools was further extracted three times with 1% ammonia. Thereafter, the residue after ammonia extraction was extracted two times with 0.1 M HCl, and subsequently two times with 1 M HCl.
A subsample of the pellet was combusted after the methanol and water extraction and after the protease and lipase incubations, for mass-balance determinations.
The individual time intervals faeces pools (2 to 5 gram) were extracted with methanol and water as described above.

LC-RAD-MS: Methanol extracts
Faeces methanol extracts stored at ≤ -15°C were thawed and vortex mixed. The amount of radioactivity in these samples was checked by LSC. Aliquots of 1000 µL methanol extract of homogenized pooled faeces sample was transferred to an Eppendorf vial and evaporated to a final volume of 77-112 µL. After vortex mixing the extract was centrifuged. The amount of radioactivity in the supernatant was checked by LSC. In order to investigate the radioactive metabolic patterns, aliquots of the concentrated methanol extracts of faeces of male Wistar rats (pooled per time interval up to 48 hours, and the early and late pool samples) were subjected to LC-RAD method. Additionally, to investigate the metabolic patterns, aliquots of the concentrated methanol extracts of faeces of male Wistar rats (pooled per time interval up to 48 hours, and the early pool samples) were subjected to LC-MS method.

LC-RAD-MS: Water extracts
Faeces water extracts stored at ≤ -15°C were thawed and vortex mixed. The amount of radioactivity in these samples was checked by LSC. After vortex mixing the extract was centrifuged. The amount of radioactivity in the supernatant was checked by LSC. In order to investigate the radioactive metabolic patterns, aliquots of the water extracts of faeces of male Wistar rats (pooled per time interval up to 48 hours, and the early pool samples) were subjected to LC-RAD method. Additionally, to investigate the metabolic patterns, aliquots of the water extracts of faeces of male Wistar rats (pooled per time interval up to 48 hours, and the early pool samples) were subjected to LC-MS method.

LC-RAD-MS: Protease solubilizates
The protease solubilizates were homogenized directly after solubilisation. The amount of radioactivity in these samples was checked by LSC. The homogenized protease solubilizates were centrifuged. The amount of radioactivity in the supernatant was checked by LSC. In order to investigate the radioactive metabolic patterns, aliquots of the protease solubilizates of faeces of male Wistar rats (early pool samples) were subjected to LC-RAD method.
Blank faeces samples (pre-dose faeces samples of group 1 and 2) were treated exactly the same to obtain blank methanol and water extracts and blank protease solubilizates. These samples were used as blanks during the investigation of the metabolic patterns.
For structure elucidation of metabolites in faeces of male Wistar rats, the (concentrated) methanol extracts of early pool samples of group 2 and 3, and faeces methanol extract samples of time interval 12-24h of group 2 and 3 were subjected to LC-MS analysis. The injection volume was 5 µL for all analysis.

Determination of the total radioactive residue (TRR)
Total radioactivity in urine and bile samples (triplo analyses) and in the cage wash (single analysis) was determined by direct liquid scintillation counting (as described above). For solid samples total radioactivity was measured in triplo (as described above). Prior to LSC, faeces was homogenised by means of an ultraturrax in case of samples < 1 gram or a knife in case of samples ≥ 1 gram.

II. RESULTS AND DISCUSSION

1. Excretion and distribution of radioactivity

The amounts of radioactivity which were excreted via urine, bile and faeces after application of a single or repeated oral dose of ^{14}C-BAS 222 to Group 1 – 5 rats are summarised in Table 5.1.1-2 Table 5.1.1-3. The excretion via urine over an observation period of about 168 hours amounted to 43% of the dose for Group 1 (5 mg/kg), 23% of the dose for Group 2 (50 mg/kg), 28% of the dose for Group 3 (50 mg/kg, repeated administration), 38% of the dose for Group 4 (5 mg/kg, bile cannulation) and 38% of the dose for Group 5 (50 mg/kg, bile cannulation). For all groups the major part of urinary excretion occurred within 24 hours after test substance administration (> 80% for all dose groups).
Excretion of radioactivity via faeces was significantly higher and amounted for a period of about 168 hours to 55% of the dose for Group 1 (5 mg/kg), 68% of the dose for Group 2 (50 mg/kg), 67% of the dose for Group 3 (50 mg/kg, repeated administration), 62% of the dose for Group 4 (5 mg/kg, bile cannulation) and 65% of the dose for Group 5 (50 mg/kg, bile cannulation). Thereby, for Groups 1 and 2 the major part of faecal excretion occurred within 12-24 hours (> 80% for these dose groups). For Groups 3-5, the major part of faecal excretion occurred within 12-48 hours (> 80% for these dose groups).
Excretion of radioactivity via bile was lower and amounted for a period of about 168 hours to 10% of the dose for Group 4 (5 mg/kg, bile cannulation) and 6% of the dose for Group 5 (50 mg/kg, bile cannulation).

Table 5.1.1-2: Excretion balance after oral administration of ^{14}C-metiram - dose group 1, 2 and 3

		Group 1	Group 2	Group 3
Matrix	**Time interval [h]**	**% of the dose**	**% of the dose**	**% of the dose**
Urine	0-6	12.9	5.9	6.7
	6-12	12.0	6.1	6.5
	12-24	14.0	6.9	10.0
	24-48	2.5	2.6	4.0
	48-72	0.74	0.66	0.61
	72-96	0.27	0.38	0.19
	96-120	0.12	0.20	0.08
	120-144	0.08	0.08	0.08
	144-168	0.06	0.06	0.04
Total urine		***42.6***	***23.0***	***28.1***
Faeces	0-6	0.03	0.68	0.04
	6-12	3.4	4.2	7.5
	12-24	46.7	55.2	47.1
	24-48	4.6	5.8	11.3
	48-72	0.30	0.40	0.35
	72-96	0.13	1.60	0.09
	96-120	0.06	0.08	0.04
	120-144	0.05	0.07	0.04
	144-168	0.04	0.04	0.03
Total faeces		***55.3***	***68.1***	***66.5***
Total excreted		***97.9***	***91.1***	***94.6***
Cage wash		0.68	0.84	0.19
Total[1]		**98.6**	**91.9**	**94.8**

1 Carcasses were not analysed (mass balance >90%)

Table 5.1.1-3: Excretion balance after oral administration of ^{14}C-metiram - dose group 4 and 5

		Group 4	Group 5
Matrix	**Time interval [h]**	**% of the dose**	**% of the dose**
Urine	0-3	0.128	0.550
	3-6	8.24	5.07
	6-9	2.33	7.43
	9-12	8.71	5.91
	12-18	8.01	9.18
	18-24	3.29	3.50
	24-30	2.02	1.76
	30-36	1.53	1.61
	36-42	1.34	1.22
	42-48	0.466	0.449
	48-54	0.265	0.417
	54-60	0.175	0.205
	60-66	0.215	0.245
	66-72	0.113	0.130
	72-84	0.163	0.095
	84-96	0.218	0.086
	96-108	0.075	0.056
	108-120	0.109	0.058
	120-132	0.062	0.045
	132-144	0.060	0.065
	144-156	0.042	0.053
	156-168	0.041	0.063
Total urine		***37.6***	***38.1***

		Group 4	Group 5
Matrix	**Time interval [h]**	**% of the dose**	**% of the dose**
Faeces	0-3	0.007	0.002
	3-6	5.79	3.76
	6-9	0.029	0.033
	9-12	8.31	4.46
	12-18	30.3	30.1
	18-24	11.2	18.0
	24-30	2.68	5.25
	30-36	2.77	4.82
	36-42	1.00	2.00
	42-48	0.519	0.866
	48-54	0.167	0.436
	54-60	0.228	0.305
	60-66	0.149	0.201
	66-72	0.081	0.112
	72-84	0.091	0.062
	84-96	0.104	0.079
	96-108	0.041	0.171
	108-120	0.038	0.035
	120-132	0.030	0.034
	132-144	0.061	0.071
	144-156	0.025	0.021
	156-168	0.069	0.017
Total faeces		***62.2***	***65.1***

		Group 4	Group 5
Matrix	**Time interval [h]**	**% of the dose**	**% of the dose**
Bile	0-3	1.85	1.18
	3-6	3.20	1.53
	6-9	1.91	0.953
	9-12	0.941	0.742
	12-18	0.860	0.925
	18-24	0.321	0.356
	24-30	0.174	0.167
	30-36	0.106	0.116
	36-42	0.059	0.065
	42-48	0.033	0.037
	48-54	0.027	0.023
	54-60	0.023	0.018
	60-66	0.021	0.016
	66-72	0.015	0.013
	72-84	0.026	0.024
	84-96	0.025	0.020
	96-108	0.020	0.018
	108-120	0.013	0.016
	120-132	0.015	0.014
	132-144	0.014	0.013
	144-156	0.012	0.009
	156-168	0.008	0.009
Total bile		***9.66***	***6.23***
Total excreted		***109.4***	***109.4***
Cage wash		0.781	0.453
Total[1]		**110.2**	**109.9**

1 Carcasses were not analysed (mass balance >90%)

2. Extractability

In order to establish a suitable extraction protocol for faeces, a number of test extractions were carried out. Finally, faeces samples were sequentially extracted with methanol and water (see Table 5.1.1-4). For all dose groups and all time intervals, the major part of radioactivity was extracted by the methanol, whereby the amount ranged from at least 7.1% TRR (6-12 h, Group 2) to a maximum of 67.0% TRR (0-6 h, Group 1). In turn, extraction with water released again significant amounts of radioactivity. For all dose groups and all time intervals, the portions of radioactivity extracted with water were generally comparable. With water up to 39.2% TRR (24-48 h, Group 1) were extracted. The sum of the solvent extracts ranged from at least 15.9% TRR (Group 2, 6-12 h) to a maximum of 85.3% TRR (Group 1, 0-6 h).

Table 5.1.1-4: Extractability of faeces individual time interval pools

	Time interval [h]	TRR [% Dose] [% TRR]	Methanol* [% Dose] [% TRR]	Water* [% Dose] [% TRR]	ERR* [% Dose] [% TRR]
Group 1	0-6	0.03 100	0.021 66.98	0.006 18.30	0.027 85.28
	6-12	3.4 100	0.74 21.80	0.63 18.34	1.37 40.1
	12-24	46.7 100	24.47 52.44	11.82 25.33	36.29 77.77
	24-48	4.6 100	1.86 40.15	1.81 39.17	3.67 79.32
	Sum 0-48 h [% Dose]	**54.7**	**27.09**	**14.27**	**41.35**
Group 2	0-6	0.68 100	0.25 37.23	0.14 20.45	0.39 57.68
	6-12	4.2 100	0.30 7.13	0.37 8.79	0.68 15.92
	12-24	55.2 100	7.73 14.00	7.98 14.45	15.72 28.45
	24-48	5.8 100	0.67 11.54	0.93 16.01	1.59 27.55
	Sum 0-48 h [% Dose]	**65.9**	**8.95**	**9.42**	**18.38**

	Time interval [h]	TRR [% Dose] [% TRR]	Methanol* [% Dose] [% TRR]	Water* [% Dose] [% TRR]	ERR* [% Dose] [% TRR]
Group 3	0-6	0.04 100	0.03 66.66	0.007 16.90	0.034 83.56
	6-12	7.5 100	1.21 16.14	1.14 15.27	2.35 31.41
	12-24	47.1 100	13.92 29.57	6.26 13.29	20.18 42.86
	24-48	11.3 100	2.75 24.19	2.43 21.41	5.18 45.60
	Sum 0-48 h [% Dose]	**65.9**	**17.91**	**9.84**	**27.74**

* the percentage TRR and dose is given in the report, corrected for the procedural recovery.

3. Solubilisation of residues after solvent extraction of faeces

The residues after solvent extraction were subjected to a sequential solubilisation procedure with physiologically relevant enzymes (protease and lipase), 1% ammonia and HCl. The results of the solubilisation steps are summarised in
Table 5.1.1-5. For all early pools, protease solubilisation released the highest amounts of radioactivity (from 14.4% TRR to 30.5% TRR). Treatment with lipase resulted in release of 7.2 to 10.0% of TRR. Solubilisation with 1% ammonia released 8.5 to 14.9% of TRR. All other treatments released relatively small portions of radioactivity, which accounted for up to 10% TRR. The sum of solubilized radioactive residues ranged from 43.1% TRR (early pool, Group 2) to 63.0% TRR (early pool, Group 1).

Table 5.1.1-5: Solubilization of the residual radioactive residue (RRR)

	Pool sample	TRR [% Dose] [% TRR]	Protease [1)]* [% Dose] [% TRR]	Protease Repeat* [% Dose] [% TRR]	Lipase [% Dose] [% TRR]	RRR Interim[4)] [% Dose] [% TRR]	1% NH3 [% Dose] [% TRR]	0.1 M HCl [% Dose] [% TRR]	1M HCl [% Dose] [% TRR]	Sum of solubilizates [% Dose] [% TRR]	Recovery (ERR + solubili-zates) [% Dose] [% TRR]
Group 1	Early pool	54.7	11.64	61.39	4.49	n/a	n/a	n/a	n/a	65.7	88
		100	21.27	112.23	8.21	n/a	n/a	n/a	n/a	120	160.7[3)]
	Late pool	0.58	0.08	n/a	n/a	0.46	n/a	n/a	n/a	0.08	0.44
		100	14.5	n/a	n/a	0.8	n/a	n/a	n/a	14.5	76.8[5)]
Group 1 repeat	Early pool	54.7	16.7	n/a	3.92	17.0	8.2	4.6	1.0	34.4	54.6
		100	30.5	n/a	7.17	31.0	14.9	8.5	1.9	63.0	99.9
Group 2	Early pool	65.3	22.20	12.98[2)]	6.55	17.9	5.6	1.8	1.3	28.2	56.9
		100	34.00	19.89	10.04	27.4	8.5	2.7	2.0	43.1	87.1
	Late pool	2.19	0.28	n/a	n/a	0.07	n/a	n/a	n/a	0.28	1.28
		100	12.8	n/a	n/a	3.4	n/a	n/a	n/a	12.8	58.5[5)]
Group 3	Early pool	65.9	20.33	9.46[2]	5.12	20.2	8.6	5.3	0.92	29.4	62.2
		100	30.84	14.35	7.76	30.7	13.0	8.1	1.4	44.7	94.5
	Late pool	0.54	0	n/a	n/a	0.48	n/a	n/a	n/a	0	0.27
		100	0	n/a	n/a	89.1	n/a	n/a	n/a	0	49.8[5)]

* the percentage TRR and dose is given in the report, corrected for the procedural recovery.
1) The protease treatment for the early pools was repeated since the extraction/combustion mass-balance was insufficient; the solubilizate of the first treatment were used for structural identification and quantification
2) The protease repeat treatment was used for calculating the recovery.
3) Group 1 was repeated since the mass-balance of the extraction steps was incorrect
4) Interim combustion after the protease and lipase solubilisation (not used in calculating the recovery).
5) Recovery of late pools is low, due to the low amount of radioactivity present in these pools.

4. Metabolites in urine

Male rats: Structure elucidation of metabolites in urine of male rats was based on LC-MS analysis of urine samples of the early pools of groups 1, 2 and 3 and time interval 6-12 h of group 3, respectively. In urine of all early pools the following metabolites were identified (order of retention): M222F003 (EU), M222F002 (ETU), M222F005 (carbimid), and M222F024. In urine of time interval 6-12 h group 3 metabolites M222F003 (EU), M222F002 (ETU), and M222F024 were identified, all of which were also detected in all early pools.

Residues in urine samples of male rats were quantified with LC-(PDA)-RAD method. The results of the quantitative analysis of urine of male rats are summarised in
Table 5.1.1-6.

For all early pool samples, metabolite M222F002 (ETU) was identified as the main component (~44% ROI). As % Dose, the concentration was 17.8%, 9.6% and 12.1% in groups 1, 2 and 3, respectively. In all time intervals, metabolite M222F002 (ETU) was identified as the main component as well and the concentration increased almost continuously from 1.6% Dose (0-6 h, group 2) to 9.8% Dose (12-24 h, group 1), but decreased until 2.9% Dose (24-48 h, group 3) or not detectable levels (24-48 h, group 1).

The second most abundant metabolite was M222F003 (EU), accounting for 5.8%, 3.5% and 3.9% Dose in groups 1, 2 and 3 respectively. Metabolite M222F024 was also identified, although at lower concentrations (≤ 3.4% Dose). The metabolite pattern was similar for all time intervals and did not significantly change with time.

Out of the 42.6, 23.0 and 28.1% of the dose, which was excreted via urine from 0-168 h for groups 1, 2 and 3 respectively, 27.0, 14.0 and 16.9% dose was identified. Thereby, metabolite M222F002 (ETU) and M222F003 (EU) together accounted for 23.6, 13.2 and 16.0% Dose for groups 1, 2 and 3 respectively (see Table 5.1.1-11). The remaining 14.4, 5.8 and 10.2% Dose for groups 1, 2 and 3 respectively, were characterised by HPLC.

ETU and EU were also given as a percentage of the dose, when dose-normalised to molecular weight, see
Table 5.1.1-12. Calculated like this, EU accounted for 1.83%, 1.12% and 1.22% for Groups 1, 2 and 3 respectively and ETU accounted for 6.67%, 3.62% and 4.55% for Groups 1, 2 and 3 respectively.

Table 5.1.1-6: Composition of radioactivity in urine of male rats after oral administration of ^{14}C-metiram of group 1, 2 and 3

Metabolite Identity	Urine of Male Rats of Group 1, 2 and 3 – Composition of radioactivity in % of the dose														
	0-6 h			6-12 h			12-24 h			24-48 h			Sum		
Group	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Identified															
M222F002 (ETU)	2.67	1.59	1.82	4.52	2.51	2.46	9.84	4.52	4.97	n.d.	1.70	2.94	17.76	9.63	12.12
M222F003 (EU)	0.49	0.30	0.17	1.37	0.60	0.59	2.20	1.28	1.78	n.d.	n.d.	1.02	5.80	3.54	3.87
M222F024 ($C_5H_8N_2S_3$)	1.72	0.31	0.77	1.02	0.29	0.22	n.d.	n.d.	0.27	n.d.	0.45	n.d.	3.41	0.79	0.90
Total Identified	**4.88**	**2.20**	**2.76**	**6.91**	**3.40**	**3.27**	**12.04**	**5.80**	**7.02**	**0.00**	**2.15**	**3.96**	**26.97**	**13.96**	**16.89**
Total Characterised	**7.98**	**3.67**	**3.95**	**5.06**	**2.73**	**3.21**	**1.92**	**1.14**	**2.96**	**0.00**	**0.49**	**0.00**	**14.37**	**5.83**	**10.20**
Total Identified and Characterised	**12.86**	**5.87**	**6.71**	**11.97**	**6.13**	**6.48**	**13.96**	**6.94**	**9.98**	**0.00**	**2.64**	**3.96**	**41.34**	**19.79**	**27.09**

n.d. not detected (component could not be assigned to a specific peak)

<u>Bile of Male Rats:</u> Structure elucidation of metabolites in bile of male rats was based on LC-MS analysis of bile samples of the early pool of group 5 and group 5, animal 19, respectively. In bile of the early pool the following metabolites were identified (order of retention): M222F002 (ETU), M222F026, and M222F024.

Residues in bile samples of male rats were quantified with LC-(PDA)-RAD method. The results of the quantitative analysis of bile of male rats are summarised in
Table 5.1.1-7.

For all early pool samples, metabolite M222F024 was identified as the main component (65.0% and 60.9% ROI in groups 4 and 5 respectively). As % Dose, the concentration was 5.1% and 2.8% in groups 4 and 5, respectively. In all time intervals, metabolite M222F024 was identified as the main component as well and the concentration maintained quite constant over time from 1.4% Dose (0-3 h) to 0.5% Dose (9-12 h) for group 4 and from 0.6% Dose (0-3h) to 0.6% Dose (9-12h) for group 5.
Metabolite M222F026 and M222F002 (ETU) were also identified, although at lower concentrations (≤ 1.0% Dose). The metabolite pattern was similar for all time intervals and did not significantly change with time.

In the mid pools, only M222F002 (ETU) was detectable in group 4, at a concentration of 1.2% Dose.

Out of the 9.7 and 6.2% of the dose, which was excreted via bile from 0-168 h for groups 4 and 5 respectively, 7.6 and 3.7% Dose was identified. Thereby, metabolite M222F024 accounted for 5.1 and 2.8% Dose for groups 4 and 5 respectively (see Table 5.1.1-13). Another 1.5 and 0.95% Dose for groups 4 and 5 respectively, were characterised by HPLC.

ETU was also given as a percentage of the dose, when dose-normalised to molecular weight, see Table 5.1.1-14. Calculated like this, ETU accounted for 0.81% and 0.21% for Groups 4 and 5 respectively.

Table 5.1.1-7: Composition of radioactivity in bile of male rats after oral administration of ^{14}C-metiram of Group 4 and 5

Metabolite Identity	Bile of Male Rats of Group 4 and 5											
	Composition of radioactivity in % of the dose											
	0-3 h		3-6 h		6-9 h		9-12 h		Sum (early pools)		Sum (mid pools)	
Group	4	5	4	5	4	5	4	5	4	5	4	5
Identified												
M222F002 (ETU) 5.3-5.9	0.07	0.21	0.24	0.25	0.22	0.11	0.21	n.d.	0.98	0.57	1.18	n.d.
M222F003 (EU) 4.3-4.8									n.d.	n.d.	n.d.	n.d.
M222F005 (Carbimid) 15.7-16.2									n.d.	n.d.	n.d.	n.d.
M222F004 (EBIS) 17.0-17.4									n.d.	n.d.	n.d.	n.d.
M222F007 (TDIT) 16.1-17.0									n.d.	n.d.	n.d.	n.d.
M222F024 ($C_5H_8N_2S_3$) 18.1-18.5	1.39	0.59	1.89	1.12	1.09	0.59	0.51	0.64	5.14	2.84	n.d.	n.d.
M222F025 ($C_4H_5N_3S_2$) 16.1-17.0									n.d.	n.d.	n.d.	n.d.
M222F026 ($C_5H_8N_2OS_2$) 16.0-16.3 min	n.d.	0.10	0.14	n.d.	0.18	0.06	n.d.	n.d.	0.26	0.31	n.d.	n.d.
Total Identified	**1.46**	**0.90**	**2.27**	**1.37**	**1.49**	**0.76**	**0.72**	**0.64**	**6.38**	**3.72**	**1.18**	**0.00**
Total Characterised	**0.39**	**0.47**	**0.93**	**0.33**	**0.42**	**0.20**	**0.21**	**0.00**	**1.53**	**0.95**	**0.00**	**0.00**
Total Identified and Characterised	**1.85**	**1.37**	**3.20**	**1.70**	**1.91**	**0.96**	**0.93**	**0.64**	**7.91**	**4.67**	**1.18**	**0.00**

n.d. not detected (component could not be assigned to a specific peak

5. Metabolites in faeces

Male rats: Structure elucidation of metabolites in faeces of male rats was based on HPLC-MS analysis of the (concentrated) methanol extracts of early pool samples of group 2 and 3, and faeces methanol extract samples of time interval 12-24h of group 2 and 3. Analysis of the methanol extracts led to the identification of the following metabolites (in order of retention): M222F003 (EU), M222F002 (ETU), M222F005 (carbimid), M222F026, M222F025, M222F007 (TDIT), M222F004 (EBIS) and M222F024.

The summary of the residues in the solvent extracts over one time interval are shown in
Table 5.1.1-8 (0-6 h and 6-12h, group 1-3) and Table 5.1.1-9 (12-24 and 24-48 h, group 1-3). The summary of the residues in the early pools is shown in Table 5.1.1-10.

In the methanol extracts of the early pools, M222F002 (ETU) was identified at concentrations of up to 11% Dose. Metabolites M222F003 (EU), M222F005 (carbimid), M222F004 (EBIS), M222F007 (TDIT), M222F026, M222F024 and M222F025 were identified in the methanol extracts of the early pools as well, but in much lower concentrations (≤ 1.7% Dose). M222F002 (ETU) was also the main metabolite in all time intervals.
In the water extracts, M222F002 (ETU) was the main identified component (up to 3.8% Dose). M222F005 (carbimid) and M222F004 (EBIS) were present to some extent as well.

HPLC analysis of protease solubilizates of the early pools showed M222F002 (ETU) as the main metabolite, up to 9.1% Dose. M222F003 (EU) was present in group 3 only (0.7% Dose).

Out of 55.3%, 68.1% and 66.5% of the dose, which was excreted via faeces from 0-168 h, 8.5%, 29.0 and 30.7% Dose were identified, for groups 1, 2 and 3 respectively. Thereby, metabolite M222F002 (ETU) accounted for 6.0%, 24.2% and 23.8% Dose for groups 1, 2 and 3 respectively (see Table 5.1.1-11). Moreover, 3.3%, 4.7% and 7.6% of the dose in the solvent extracts were characterised by HPLC (see Table 5.1.1-10). In the residue after solvent extraction up to 6.5% Dose were solubilized with lipase (compare
Table 5.1.1-5). The remaining solubilizates accounted for 13.8%, 8.7% and 14.8 % of the dose, for groups 1, 2 and 3 respectively.

ETU and EU were also given as a percentage of the dose, when dose-normalised to molecular weight, see
Table 5.1.1-12. Calculated like this, EU accounted for 0.09%, 0.22% and 0.22% for Groups 1, 2 and 3 respectively and ETU accounted for 2.23%, 9.09% and 8.92% for Groups 1, 2 and 3 respectively.

Table 5.1.1-8: Composition of radioactivity in faeces (0-6 hours and 6-12 hours) of male rats after oral administration of ^{14}C-metiram of Group 1, 2 and 3

<table>
<tr><th rowspan="4">Metabolite Identity</th><th colspan="18">Faeces of Male Rats</th></tr>
<tr><th colspan="18">Composition of radioactivity in % of the dose</th></tr>
<tr><th colspan="9">0-6 hours</th><th colspan="9">6-12 hours</th></tr>
<tr><th colspan="3">Methanol</th><th colspan="3">Water</th><th colspan="3">Sum</th><th colspan="3">Methanol</th><th colspan="3">Water</th><th colspan="3">Sum</th></tr>
<tr><th>Group</th><th>1</th><th>2</th><th>3</th><th>1</th><th>2</th><th>3</th><th>1</th><th>2</th><th>3</th><th>1</th><th>2</th><th>3</th><th>1</th><th>2</th><th>3</th><th>1</th><th>2</th><th>3</th></tr>
<tr><td colspan="19">Identified</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>M222F002 (ETU)
5.2-5.5 min</td><td>0.02</td><td>0.11</td><td>0.06</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>0.02</td><td>0.11</td><td>0.06</td><td>0.31</td><td>0.16</td><td>0.71</td><td>0.26</td><td>0.08</td><td>n.d.</td><td>0.57</td><td>0.24</td><td>0.71</td></tr>
<tr><td>M222F003 (EU)
4.3-4.8 min</td><td>n.d.</td><td>0.03</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>0.03</td><td>n.d.</td><td>0.01</td><td>0.01</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>0.01</td><td>0.01</td><td>n.d.</td></tr>
<tr><td>M222F005 (Carbimid)
15.7-16.2 min</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>0.09[a)]</td><td>0.01</td><td>0.09[a)]</td><td>0.10[a)]</td><td>0.04[a)]</td><td>n.d.</td><td>0.19</td><td>0.05</td><td>0.09[a)]</td></tr>
<tr><td>M222F026 ($C_5H_8N_2OS_2$)
16.1-17.0 min</td><td>n.d.</td><td rowspan="3">0.05[a)]</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td rowspan="3">0.15[b)]</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td rowspan="3">0.15[b)]</td></tr>
<tr><td>M222F025 ($C_4H_5N_3S_2$)
16.1-17.0 min</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td rowspan="2">0.03</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td rowspan="2">0.03</td></tr>
<tr><td>M222F007 (TDIT)
16.1-17.0 min</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td rowspan="2">0.07</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td rowspan="2">0.07</td></tr>
<tr><td>M222F004 (EBIS)
17.0-17.4 min</td><td>n.d.</td><td>0.01</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>0.01</td><td>n.d.</td><td>n.d.</td><td>0.04</td><td>n.d.</td><td>0.05</td><td>n.d.</td></tr>
<tr><td>M222F024 ($C_5H_8N_2S_3$)
17.6-18.1 min</td><td>n.d.</td><td>0.01</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>0.01</td><td>n.d.</td><td>0.02</td><td>0.01</td><td>0.05</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>0.02</td><td>0.01</td><td>0.05</td></tr>
<tr><td>Total Identified</td><td>0.02</td><td>0.15</td><td>0.06</td><td>0.00</td><td>0.00</td><td>0.00</td><td>0.02</td><td>0.15</td><td>0.06</td><td>0.50</td><td>0.23</td><td>1.00</td><td>0.36</td><td>0.16</td><td>0.00</td><td>0.76</td><td>0.39</td><td>1.00</td></tr>
<tr><td>Total Characterised</td><td>0.00</td><td>0.054</td><td>0.00</td><td>0.00</td><td>0.00</td><td>0.00</td><td>0.00</td><td>0.054</td><td>0.00</td><td>0.15</td><td>0.06</td><td>0.19</td><td>0.05</td><td>0.04</td><td>0.00</td><td>0.20</td><td>0.10</td><td>0.19</td></tr>
<tr><td>Total Identified and Characterised</td><td>0.02</td><td>0.204</td><td>0.06</td><td>0.00</td><td>0.00</td><td>0.00</td><td>0.02</td><td>0.204</td><td>0.06</td><td>0.65</td><td>0.29</td><td>1.19</td><td>0.41</td><td>0.20</td><td>0.00</td><td>0.96</td><td>0.49</td><td>1.19</td></tr>
</table>

n.d. not detected (component could not be assigned to a specific peak

[a)] This peak consists of M222F005 (Carbimid) and characterised ($C_{12}H_{12}N_2O_3S$), value added to identified peaks.

[b)] This peak consists of M222F026 ($C_5H_8N_2OS_2$) and/or M222F025 ($C_4H_5N_3S_2$) and/or M222F007 (TDIT).

Table 5.1.1-9: Composition of radioactivity in faeces (12-24 hours and 24-48 hours) of male rats after oral administration of ^{14}C-metiram of Group 1, 2 and 3

<table>
<tr><th rowspan="4">Metabolite Identity</th><th colspan="18">Faeces of Male Rats</th></tr>
<tr><th colspan="18">Composition of radioactivity in % of the dose</th></tr>
<tr><th colspan="9">12-24 hours</th><th colspan="9">24-48 hours</th></tr>
<tr><th colspan="3">Methanol</th><th colspan="3">Water</th><th colspan="3">Sum</th><th colspan="3">Methanol</th><th colspan="3">Water</th><th colspan="3">Sum</th></tr>
<tr><th>Group</th><th>1</th><th>2</th><th>3</th><th>1</th><th>2</th><th>3</th><th>1</th><th>2</th><th>3</th><th>1</th><th>2</th><th>3</th><th>1</th><th>2</th><th>3</th><th>1</th><th>2</th><th>3</th></tr>
<tr><td colspan="19">Identified</td></tr>
<tr><td>M222F002 (ETU)
5.2-5.5 min</td><td>11.26</td><td>2.98</td><td>7.11</td><td>3.78</td><td>2.52</td><td>1.58</td><td>15.04</td><td>5.50</td><td>8.69</td><td>0.92</td><td>0.25</td><td>1.73</td><td>n.d.</td><td>n.d.</td><td>0.41</td><td>0.92</td><td>0.25</td><td>2.14</td></tr>
<tr><td>M222F003 (EU)
4.3-4.8 min</td><td>n.d.</td><td>0.12</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>0.25</td><td>n.d.</td><td>0.12</td><td>0.25</td><td>0.13</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>0.13</td><td>n.d.</td><td>n.d.</td></tr>
<tr><td>M222F005 (Carbimid)
15.7-16.2 min</td><td>2.10[a]</td><td>1.34[a]</td><td>1.07[a]</td><td>1.64[a]</td><td>n.d.</td><td>0.46[a]</td><td>3.74[a]</td><td>1.34</td><td>1.53[a]</td><td>0.27[a]</td><td>n.d.</td><td>n.d.</td><td>1.24[a]</td><td>n.d.</td><td>0.08[a]</td><td>1.51[a]</td><td>n.d.</td><td>0.08[a]</td></tr>
<tr><td>M222F026 ($C_5H_8N_2OS_2$)
16.1-17.0 min</td><td rowspan="3">2.26[b]</td><td>n.d.</td><td rowspan="4">1.44[b]</td><td rowspan="3">1.10[b]</td><td>n.d.</td><td>0.23</td><td rowspan="3">3.36[b]</td><td>n.d.</td><td rowspan="4">2.12</td><td>n.d.</td><td>n.d.</td><td rowspan="3">0.13[b]</td><td>n.d.</td><td>n.d.</td><td rowspan="4">0.14[c]</td><td>n.d.</td><td>n.d.</td><td rowspan="4">0.27[c]</td></tr>
<tr><td>M222F025 ($C_4H_5N_3S_2$)
16.1-17.0 min</td><td>1.37</td><td>n.d.</td><td></td><td>1.37</td><td>0.15</td><td rowspan="2">0.09</td><td>n.d.</td><td>n.d.</td><td>0.15</td><td rowspan="2">0.09</td></tr>
<tr><td>M222F007 (TDIT)
16.1-17.0 min</td><td></td><td>n.d.</td><td></td><td></td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td></tr>
<tr><td>M222F004 (EBIS)
17.0-17.4 min</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>2.13</td><td>0.45</td><td>n.d.</td><td>2.13</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td></tr>
<tr><td>M222F024 ($C_5H_8N_2S_3$)
17.6-18.1 min</td><td>0.48</td><td>0.58</td><td>0.88</td><td>0.50</td><td>n.d.</td><td>n.d.</td><td>0.98</td><td>0.58</td><td>0.88</td><td>0.18</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>n.d.</td><td>0.04</td><td>0.18</td><td>n.d.</td><td>0.04</td></tr>
<tr><td>Total Identified</td><td>16.10</td><td>6.39</td><td>10.50</td><td>7.02</td><td>4.65</td><td>2.97</td><td>23.12</td><td>11.04</td><td>13.47</td><td>1.65</td><td>0.34</td><td>1.86</td><td>1.24</td><td>0.00</td><td>0.67</td><td>2.89</td><td>0.34</td><td>2.53</td></tr>
<tr><td>Total Characterised</td><td>7.42</td><td>0.90</td><td>2.78</td><td>2.94</td><td>1.53</td><td>1.53</td><td>10.36</td><td>2.43</td><td>4.31</td><td>0.17</td><td>0.40</td><td>0.81</td><td>0.00</td><td>0.00</td><td>0.20</td><td>0.17</td><td>0.40</td><td>1.01</td></tr>
<tr><td>Total Identified and Characterised</td><td>23.52</td><td>7.29</td><td>13.28</td><td>9.96</td><td>6.18</td><td>4.50</td><td>32.90</td><td>13.47</td><td>17.78</td><td>1.82</td><td>0.74</td><td>2.67</td><td>1.24</td><td>0.00</td><td>0.87</td><td>3.06</td><td>0.74</td><td>3.54</td></tr>
</table>

n.d. not detected (component could not be assigned to a specific peak

[a] This peak consists of M222F005 (Carbimid) and characterised ($C_{12}H_{12}N_2O_3S$), value added to identified peaks.

[b] This peak consists of M222F026 ($C_5H_8N_2OS_2$) and/or M222F025 ($C_4H_5N_3S_2$) and/or M222F007 (TDIT).

[c] This peak consists of M222F026 ($C_5H_8N_2OS_2$), M222F025 ($C_4H_5N_3S_2$), M222F007 (TDIT) and M222F004 (EBIS).

Table 5.1.1-10: Summary of radioactive residues in faeces (early pool) of male rats of group 1, 2 and 3

Metabolite Identity	Faeces (early pool) of Male Rats Composition of radioactivity in % of the dose								
	Methanol			Water			Sum		
Group	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Identified									
M222F002 (ETU) 5.2-5.5 min	3.83	7.35	10.85	n.d.	8.85	3.80	3.83	16.20	14.65
M222F003 (EU) 4.3-4.8 min	0.27	0.71	n.d.	n.d.	n.d.	n.d.	0.27	0.71	n.d.
M222F005 (Carbimid) 15.7-16.2 min	0.21[a)]	0.87[a)]	0.53[a)]	n.d.	n.d.	1.37[a)]	0.21[a)]	0.87[a)]	1.90[a)]
M222F004 (EBIS) 17.0-17.4 min	0.84	1.64	1.68	n.d.	n.d.	1.68	0.84	1.64	3.36
M222F007 (TDIT) 16.1-17.0 min				n.d.	n.d.	n.d.			
M222F026 ($C_5H_8N_2OS_2$) 16.1-17.0 min	0.79[b)]	0.99[b)]	0.50[b)]	n.d.	n.d.	n.d.	0.79[b)]	0.99[b)]	0.50[b)]
M222F025 ($C_4H_5N_3S_2$) 16.1-17.0 min				n.d.	n.d.	n.d.			
M222F024 ($C_5H_8N_2S_3$) 17.6-18.1 min	0.43	0.57	0.48	n.d.	n.d.	n.d.	0.43	0.57	0.48
Total Identified	**6.37**	**12.13**	**14.04**	**0.00**	**8.85**	**6.85**	**6.37**	**20.98**	**20.89**
Total Characterised	**3.29**	**4.74**	**5.00**	**0.00**	**0.00**	**2.62**	**3.29**	**4.74**	**7.62**
Total Identified and Characterised	**9.66**	**16.87**	**19.04**	**0.00**	**8.85**	**9.47**	**9.66**	**25.72**	**28.51**

n.d. not detected (component could not be assigned to a specific peak

[a)] This peak consists of M222F005 (Carbimid) and characterised ($C_{12}H_{12}N_2O_3S$), value added to identified peaks.

[b)] This peak consists of M222F026 ($C_5H_8N_2OS_2$) and/or M222F025 ($C_4H_5N_3S_2$) and/or M222F007 (TDIT).

A summary of all identified metabolites in urine and faeces is shown in Table 5.1.1-11. A summary of all identified metabolites in bile is shown in Table 5.1.1-13. ETU and EU were also given as a percentage of the dose, when dose-normalised to molecular weight, see
Table 5.1.1-12 and Table 5.1.1-14.

Table 5.1.1-11: Identified metabolites in urine and faeces of male rats of group 1, 2 and 3

Designation	Group 1		Group 2		Group 3	
	Urine[a)] [% Dose]	Faeces[b)] [% Dose]	Urine [% Dose]	Faeces [% Dose]	Urine [% Dose]	Faeces [% Dose]
BAS 222 F	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M222F002 (ETU)	**17.76**	**5.95**	**9.63**	**24.20**	**12.12**	**23.76**
M222F003 (EU)	**5.80**	0.27	**3.54**	0.71	**3.87**	0.70
M222F005 (Carbimid)	n.d.	0.21	n.d.	0.87[d)]	n.d.	**1.90[d)]**
M222F004 (EBIS)	n.d.	0.84	n.d.	**1.64**	n.d.	**3.36**
M222F007 (TDIT)	n.d.	0.79[c)]	n.d.	0.99[c)]	n.d.	0.50[c)]
M222F026 ($C_5H_8N_2OS_2$)	n.d.		n.d.		n.d.	
M222F025 ($C_4H_5N_3S_2$)	n.d.		n.d.		n.d.	
M222F024 ($C_5H_8N_2S_3$)	**3.41**	0.43	0.79	0.57	0.90	0.48

Metabolites in order of retention time, components ≥ 1 % Dose in at least one dose group displayed in bold
n.d. not detected (component could not be assigned to a specific peak
a) Sum of radioactivity as detected in the early pool urine samples. As in the late pool samples no peaks were detected.
b) Sum of radioactivity as detected in the early pool faeces samples (methanol and water extract plus protease solubilizate). As in the late pool samples no peaks were detected.
c) This peak consists of M222F026, M222F025 and M222F007 (TDIT).
d) This peak consist of M222F005 (carbimid) and characterised ($C_{12}H_{12}N_2O_3S$), value added to the identified metabolites.

Table 5.1.1-12: Identified metabolites in urine and faeces of male rats of group 1, 2 and 3, values normalised to the molecular weight of EU and ETU

Designation	Group 1		Group 2		Group 3	
	Urine[a)] [% Dose]	Faeces[b)] [% Dose]	Urine[a)] [% Dose]	Faeces[b)] [% Dose]	Urine[a)] [% Dose]	Faeces[b)] [% Dose]
M222F002 (ETU)	6.67	2.23	3.62	9.09	4.55	8.92
M222F003 (EU)	1.83	0.09	1.12	0.22	1.22	0.22

a) Sum of radioactivity as detected in the early pool urine samples. As in the late pool samples no peaks were detected.
b) Sum of radioactivity as detected in the early pool faeces samples (methanol and water extract plus protease solubilizate). As in the late pool samples no peaks were detected.

Table 5.1.1-13: Identified metabolites in bile of male rats of group 4 and 5

Designation	Group 4	Group 5
	Bile[a)] [% Dose]	Bile[a)] [% Dose]
M222F002 (ETU) 5.2-5.5	2.16	0.57
M222F003 (EU) 4.3-4.8	n.d.	n.d.
M222F005 (Carbimid) 15.7-16.2	n.d.	n.d.
M222F004 (EBIS) 17.0-17.4	n.d.	n.d.
M222F007 (TDIT) 16.1-17.0	n.d.	n.d.
M222F024 ($C_5H_8N_2S_3$) 17.6-18.1	5.14	2.84
M222F025 ($C_4H_5N_3S_2$) 16.1-17.0	n.d.	n.d.
M222F026 ($C_5H_8N_2OS_2$) 16.0-16.3 min	0.26	0.31

n.d. not detected (component could not be assigned to a specific peak
a) Sum of radioactivity as detected in the early and mid pool bile samples. As in the late pool samples no peaks were detected.

Table 5.1.1-14: Identified metabolites in bile of male rats of group 4 and 5, values normalised to the molecular weight of EU and ETU

Designation	Group 4	Group 5
	Bile[a)] [% Dose]	Bile[a)] [% Dose]
M222F002 (ETU) 5.2-5.5	0.81	0.21
M222F003 (EU) 4.3-4.8	n.d.	n.d.

n.d. not detected (component could not be assigned to a specific peak

[a)] Sum of radioactivity as detected in the early and mid pool bile samples. As in the late pool samples no peaks were detected.

6. Metabolic Pathway

The proposed metabolic pathway of BAS 222 F (metiram) is depicted in Figure 5.1.1-1.

The major transformation steps in the metabolic pathway of BAS 222 F are:

(a) Conversion of metiram to ETU, which is further transformed into EU
(b) Conversion of metiram into TDIT and EBIS, which are further converted to ETU. EBIS is also transformed into M222F024 by methylation.
(c) Formation of smaller metabolites, like carbamid and M222F026 and M222F025.

Figure 5.1.1-1: Proposed Metabolic Pathway of BAS 222 F in Rats

Metiram, M222F000

Carbimid, M222F005

M222F026

M222F025

TDIT, M222F007

ETU, M222F002

EBIS, M222F004

M222F024

EU, M222F003

III. CONCLUSION

This study confirms that BAS 222 F (metiram) is extensively metabolised when applied to male rat in a single dose (both low dose and high dose). The excretion of radioactivity was similar for all groups. The majority of the radioactivity was excreted via faeces (> 55% of the dose) and smaller amounts via urine (< 43% of the dose). Bile accounted for up to 10% of dose. In general, excretion was rapid with the predominant part being excreted within 12-48 hours.

The metabolite patterns in urine, bile and faeces extracts were largely comparable for all groups.
Both in faeces and in urine, ETU (M222F002) was the most abundant component indicating a rapid excretion also for this transformation product.

In faeces, M222F002 (ETU) accounted for up to 11 % of applied dose. Further transformation products were found albeit at much lower level not exceeding 1.7% applied dose (namely M222F003 (EU), M222F005 (carbimid), M222F004 (EBIS), M222F007 (TDIT), M222F024, M222F025 and M222F026).

In urine, M222F002 (ETU) accounted for 9.6 – 17.8% Dose. When dose-normalised to molecular weight, see
Table 5.1.1-12 ETU accounted for 3.6% - 6.7% dose. Metabolites M222F003 (EU, 3.5 - 5.8% Dose, when dose-normalised values from 1.1 – 1.8% are calculated), and M222F024 (≤ 3.4% Dose) were present at significant concentrations.

In bile, metabolite M222F024 was the most abundant component in bile (2.8 – 5.1% dose). However, ETU (M222F002) was also identified, as was the metabolite M222F026, although both at lower concentrations (≤ 1.0% dose).

The metabolic pathway of BAS 222 F showed generation of a range of small molecular weight compounds from the parent compound: they can be considered dynamic intermediates (M222F026, M222F025, TDIT, ETU, EU, EBIS, M222F024, M222F005). Based on their molecular structure a plausible pathway is proposed, assuming a transformation of several intermediates (e.g. TDIT, EBIS, M222F024) into ETU which then is further transformed into EU.

Report:	*CA 5.1.1/2*
	[redacted] 1985a
	The biokinetics and metabolism of 14C-Metiram in the rat
	1985/0470
Guidelines:	*none*
GLP:	*yes (certified by Department of Health and Social Security, London, United Kingdom)*

Executive Summary

The distribution of [^{14}C]-BAS 222 F and [^{14}C]-ETU in Sprague-Dawley rats (adult, both sexes, dose levels 5 and 50 mg/kg bw ([^{14}C]-BAS 222 F) and 0.5 mg/kg bw ([^{14}C]-ETU) after single oral applications were investigated. The study evidenced that after low oral doses of ^{14}C-BAS 222 F approximately 37-45% of the radioactivity was excreted via the urine, whereas after higher doses the urinary excretion decreased to 21 to 26%. This indicates that the absorption at lower doses is considerably higher than at higher doses. In contrast, ^{14}C-ETU was almost quantitatively absorbed with more than 96% excreted in the urine. Small differences appeared in the disposition of BAS 222 F between males and females which could be related to differences in the rate of metabolism. The sex differences were more apparent in tissue residues where concentrations of radioactivity were significantly higher in females compared to males. Thin-layer chromatography in urine of rats dosed with BAS 222 F or ETU indicated the presence of about eight radioactive components (N-acetyl-ethylenediamine, ethylenediamine, ethanolamine, ethylene thiourea (ETU), ethyleneurea, one metabolite with glycine-like properties as well as other remaining metabolites). Similar results have been obtained from kidney solvent extracts as well as bile samples.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	BAS 222 F
Description:	^{14}C-Metiram (5 mCi), non-labeled BAS 222 F, ^{14}C-ETU (2 mCi), ^{15}N- Metiram (98% ^{15}N)
Batch/purity #:	^{14}C-Metiram (specific activity 37.71 µCi/mg) ^{14}C-BF222-ETU (specific activity 37.71 µCi/mg)
Development code:	Metiram: BAS 222 F ETU: M222F002
Stability of test compound:	not reported
2. Vehicle and/or positive control:	1% (w/v) sodium carboxymethylcellulose

3. Test animals:

Species:	CD rats (male and female)
Strain:	Sprague-Dawley origin
Age:	adult
Weight at dosing:	approx. 200g
Diet:	Standard Laboratory Diet Spratt's No.1)
Water:	tap water, *ad libitu*
Housing:	not reported
Husbandry:	not reported

4. Preparation of dosing solution

All dose suspensions were prepared by suspending ^{14}C-BAS 222 F in 1% aqueous sodium carboxymethylcellulose and the volume administered to each rat was 1 ml. Groups of rats were dosed with aliquots of dose solution using the same syringe for each animal. The administered radioactivity was determined by syringing identical aliquots of dose suspension into volumetric flasks, followed by measurement of radioactivity after dilution with pyridine.

B. STUDY DESIGN AND METHODS

1. Dates of work: 04-Oct-1983 - 01-Apr-1985

Studies on the kinetics of BAS 222 F (absorption, distribution, retention and elimination) and on the metabolism were carried out in male and female CD rats (approx. 200g). In order to follow the metabolic pathway the technical active ingredient was labeled with [^{14}C]. The animals were given oral doses of [^{14}C]-BAS 222 F (specific radioactivity: 37.71 µCi/mg) at nominal dose levels of 5 mg/kg and 50 mg/kg and [^{14}C]-ETU (specific radioactivity: 7.75 mCi/mmol) at a dose level of 0.5 mg/kg. All doses were administered by oral intubation, as suspensions in 1% (w/v) sodium carboxymethylcellulose aqueous solution at a level of 5 ml/kg.

2. Excretion and retention (Preliminary study):

Four rats (2 males/2 females) received single oral doses of [^{14}C]-BAS 222 F (dose level 50 mg/kg bw). Urine, faeces and expired air were samples during specific time intervals and five days after application animals were killed and tissues and carcasses were collected. All samples were stored at -20°C until taken for analysis.

3. Excretion and retention (low level doses):

Ten rats (5 males/5 females) received single oral doses of [^{14}C]-BAS 222 F (dose level 5 mg/kg bw). Urine, faeces and expired air were samples during specific time intervals and 168 hours after application animals were killed and tissues and carcasses were collected. All samples were stored at -20°C until taken for analysis.

4. Excretion and retention (high level doses):

Ten rats (5 males/5 females) received single oral doses of [^{14}C]-BAS 222 F (dose level 50 mg/kg bw). Urine, faeces and expired air were samples during specific time intervals and 168 hours after application animals were killed and tissues and carcasses were collected. All samples were stored at -20°C until taken for analysis.

5. Biliary excretion (low level doses):
The bile ducts of six rats (3 males/3 females) were cannulated. Rats received single oral doses of [^{14}C]-BAS 222 F (dose level 5 mg/kg bw). Bile was collected at 1.5 hours intervals; urine and faeces were collected every 24 hours for up to 48 hours. At 48 hours, animals were killed and gastrointestinal tract, liver and carcasses were collected. All samples were stored at -20°C until taken for analysis.

6. Biliary excretion (high level doses):
The bile ducts of six rats (3 males/3 females) were cannulated. Rats received single oral doses of [^{14}C]-BAS 222 F (dose level 50 mg/kg bw). Bile was collected at 1.5 hours intervals; urine and faeces were collected every 24 hours for up to 48 hours. At 48 hours, animals were killed and gastrointestinal tract, liver and carcasses were collected. All samples were stored at -20°C until taken for analysis.

7. Plasma concentrations (low level doses):
Ten rats (5 males/5 females) received single oral doses of [^{14}C]-BAS 222 F (dose level 5 mg/kg bw). Plasma samples were taken at specific time intervals for up to 216 hours after dosing. Plasma was measured for radioactivity immediately after centrifugation.

8. Plasma concentrations (high level doses):
Ten rats (5 males/5 females) received single oral doses of [^{14}C]-BAS 222 F (dose level 50 mg/kg bw). Plasma samples were taken at specific time intervals for up to 216 hours after dosing. Plasma was measured for radioactivity immediately after centrifugation.

9. Excretion and retention (ETU)
Three male rats received single oral doses of [^{14}C]-BF 222 F-ETU (dose level 0.5 mg/kg bw). Urine, faeces and expired air were samples during specific time intervals and 72 hours after application animals were killed and tissues and carcasses were collected. All samples were stored at -20°C until taken for analysis.

10. Measurement of radioactivity:
Tissues were finely minced. Faeces and carcasses were extracted by homogenisation with methanol (the high dose level faeces were also homogenised with dimethylformamide). After centrifugation, radioactivity was measured in both extracts and residues. Samples of urine, bile, plasma, solvent extracts, contents of expired air traps, cage washings, and other liquid samples were mixed with Special Scintillator M1 31. Samples of carcass and faecal residues, homogenized tissues and whole blood, were mixed with dry cellulose powder and burned in oxygen using an Automatic Sample Oxidiser.

The combustion products were absorbed into Carbosorb TM and mixed with Permafluor Scintillator system. Recoveries of radioactivity from carbon-14 standards for sample oxidisers burned in the oxidiser exceeded 95% Radioactivity was measured by liquid scintillation analysis using an Automatic Liquid Scintillation Analyzer. Radioactivity in amounts less than twice background levels was considered to be below the limit of accurate measurement. With equal counting times for both background and sample, the maximum statistical counting error at this limit was approximately 10% at the net count rate.

11. Thin-layer chromatography (TLC):
TLC was carried out using pre-layered glass plates coated with silica gel Chromatograms were developed using the following solvents:
1) Chloroform : methanol : water (6:4:1; by volume)
2) Acetonitrile : water (85:15; v/v)
3) Chloroform : methanol (1:1; v/v)
4) Chloroform : n-butanol : methanol : water (100:5:3:0,5; by volume)
5) Dichloromethane : methanol (9:1; v/v)
Radioactive components were detected by autoradiography and radio chromatogram scanning were done by a Berthold-TLC-analyzer.

II. RESULTS AND DISCUSSION

The results of the analytical measurements have been summarized in Table 5.1.1-15 and Table Table 5.1.1-16.

1. Excretion and retention (Preliminary study):
After a single dose of [^{14}C]-BAS 222 F (dose level 50 mg/kg bw) a total of 81 and 100% dose were excreted during 5 days. Urinary excretion accounted for 36 and 40% in males and females, respectively. About 44% and 58% of radioactivity was detected in faeces of males and females, respectively. Small quantities of radioactivity were eliminated via expired air.

Table 5.1.1-15: Detection of [^{14}C]-BAS 222 F and [^{14}C]-ETU after oral application of different duration and doses in percent of the initial radioactivity

Application (days after last application)	urine		faeces		expired air		carcass		bile	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
1 x 5 mg/kg bw BAS 222 F (7 days)	37	47	65	54	0.4	1.1	0.9	1.3	n/a	n/a
1 x 50 mg/kg bw BAS 222 F (7 days)	21	25	79	71	0.5	1.1	0.6	1.0	n/a	n/a
15 x 5 mg/kg bw BAS 222 F (7 days)	33	39	71	60	0.4	0.9	0.9	1.5	n/a	n/a
1 x 5 mg/kg bw BAS 222 F * (2 days)	46	55	34	29	n/a	n/a	3.7	6.8	14	7.1
1 x 50 mg/kg bw BAS 222 F * (2 days)	20	28	75	68	n/a	n/a	4.4	5.2	4.3	3.7
1 x 0.5 mg/kg bw ETU (2 days)	96	n/a	4	n/a	n/d	n/a	1	n/a	n/a	n/a

* = with bile duct cannulation
n/d = not detectable
m = male
f = female

2. Excretion and retention (low level doses):
After single oral administration of 5 mg/kg bw (nontoxic range), 37% (male) and 47% (female) of the initial radioactivity were detected in the urine within seven days. In the feces, this value was 65% (male) and 54% (female) respectively. The expired air contained 0.4% (male) and 1.1% (female) respectively. Seven days after test substance administration, 0.9% (male) and 1.3% (female) of the initial dose were still detected in the bodies of the five rats used per sex. Total recovery was reported as 104% and 103% for males and females, respectively.

3. Excretion and retention (high level doses):

After single oral doses of ^{14}C-metiram (50 mg/kg) to rats, the proportion of the dose that was absorbed appeared to be significantly less than that at the lower dose level. During 7 days after dosing, excretion of radioactivity in the urine (including cage wash) accounted for 21% and 25% dose in male and female rats respectively. Most of this radioactivity was excreted within 48 hours of dosing. Radioactivity excreted in the faeces up to 7 days after dosing accounted for 79% in males and 71% in females. A small proportion, 0.51% (male) and 1.13% (female) was excreted in the expired air. After sacrifice at 7 days, radioactivity remaining in the tissues and carcasses of the animals were between 0.9% in males and 1.0 in females. The high faecal excretion results may indicate that BAS 222 F is proportionately less well absorbed at the higher dose level. Total recovery was reported as 101% and 99% for males and females.

4. Biliary excretion (low and high level doses):

The measurement of biliary excretion carried out in three animals per sex showed 14% (male) and 7% (female) of the initial radioactivity used after a single administration in the low dose range. At the high dose level (50 mg/kg bw), only 4% was detected in both sexes. Together with the reduced values in the urine and the increased values in the feces, indications of a reduced absorption rate after repeated test substance administration were obvious.

Table 5.1.1-16: Mean concentrations of radioactivity in the plasma of rats after single oral doses of [^{14}C]-BAS 222 F

Time (hours)	Concentration (µg equivalents ^{14}C-BAS 222 F)			
	5 mg/kg		50 mg/kg	
	male	female	male	female
0.25	0.7	0.08	0.09	0.15
0.5	0.10	0.11	0.17	0.22
1	0.21	0.29	0.31	0.46
2	0.50	0.73	0.76	0.85
4	0.60	0.92	1.93	2.10
6	0.53	0.82	2.97	3.00
24	0.14	0.23	1.86	1.77
48	0.06	0.13	0.74	0.74
72	0.04	0.09	0.42	0.50
96	0.03	0.06	0.31	0.38
120	0.02	0.04	0.24	0.34
144	0.02	0.05	0.18	0.27
168	0.01	0.04	0.14	0.21
216	0.01	0.02	n/a	n/a

n/a = not applicable

5. Plasma concentrations (low and low level doses):

After a single administration of 5 mg/kg b.w of [^{14}C]-BAS 222 F, maximum radioactivity in the plasma was reached in males (0.60 µg µg/ml) and females (0.92 µg µg/ml) after four hours. These values declined gradually and reach concentrations ≤0.02 µg/ml. In the high dose range, the maximum plasma radioactivity was reached only six hours after administration. Seven days after test substance administration, the residual radioactivity in the plasma was still between 0.14 and 0.21 µg in both sexes. This again indicates that the absorption is reduced at higher doses.

6. Excretion and retention (ETU):
After oral administration of ETU, 96% of the initial radioactivity was excreted via the urine within three days. This shows that ETU was absorbed well. After this period, less than 1% of the radioactivity used was detected in the animal bodies. The remainder was found in the faeces (4.2%) and expired air (0.25%).

7. Thin-layer **chromatography (TLC):**
About eight radioactively labelled components were detected in the rat urine by thin-layer chromatography. No distinct sex difference was observed. Some metabolites differed quantitatively depending on the dose used (5 and 50 mg/kg bw). Among the polar components, N-acetyl-ethylene-diamine and ethylene-diamine were detected by mass spectroscopy. Whereas these two accounted for 20% of the radioactivity in the urine, the fraction of ethanolamine, which had also been identified, was low. A further polar substance (approx. 25%) chromatographically showed characteristics similar to those of glycine, a degradation product of ethylene diamine. Among the less polar components, ETU (18 to 28%) and ethylene urea (5 to 10%) were identified by mass spectroscopy. A similar distribution pattern of the metabolites was reproduced in the bile, but its excretion rate was reduced in rats due to the molecular weight of the test substance. The kidneys showed a similar distribution pattern, whereas in the liver an identifiable metabolite pattern was found only after treatment with β-glucuronidase/sulfatase. It consisted of 80% of polar metabolites and of only 5% of ETU and ethylene urea each.

III. CONCLUSION

The study evidenced that after low oral doses of ^{14}C-BAS 222 F approximately 37-45% of the radioactivity was excreted via the urine, whereas after higher doses the urinary excretion decreased to 21 to 26%. This indicates that the absorption at lower doses is considerably higher than at higher doses. In contrast, ^{14}C-ETU was almost quantitatively absorbed with more than 96% excreted in the urine. Small differences appeared in the disposition of BAS 222 F between males and females which could be related to differences in the rate of metabolism. The sex differences were more apparent in tissue residues where concentrations of radioactivity were significantly higher in females compared to males. Thin-layer chromatography in urine of rats dosed with BAS 222 F or ETU indicated the presence of about eight radioactive components (N-acetyl-ethylenediamine, Ethylenediamine, Ethanolamine, Ethylenethiourea (ETU), Ethyleneurea, one metabolite with glycine-like properties as well as other remaining metabolites). Similar results have been obtained from kidney solvent extracts as well as bile samples.

Report: *CA 5.1.1/3*
[redacted] 1989a
The bioconversion of Metiram to ETU in rats
1989/10732
Guidelines: *EPA 85-1*
GLP: *no*

Executive Summary

Since the molar mass of the formed ETU differs considerably from the mass of a monomeric BAS 222 F unit, the bioconversion was calculated on a mass bases. The mean conversion of BAS 222 F to ETU is 3%. That means a 10 mg dose of BAS 222 F would result in 0.30 mg of ETU, or the bioconversion factor is 0.030.

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 F, ETU
   Description: Metiram
   Batch/purity #: not applicable
   Development code: Metiram: BAS 222 F
   ETU: M222F002
   Stability of test compound: not applicable
2. **Vehicle and/or positive control:** not applicable

3. **Test animals:**
   Species: not applicable
   Strain: not applicable
   Age: not applicable
   Weight at dosing: not applicable
   Diet: not applicable
   Water: not applicable
   Housing: not applicable
   Husbandry: not applicable

4. **Preparation of dosing solution**
   not applicable

B STUDY DESIGN AND METHODS

1. Dates of work: 07-Dec-1989 - 07-Dec-1989

As a supplement to the study of (1985; see CA 5.1.1/3), in the rats, the bioconversion of BAS 222 F to ETU was calculated. For a toxicological risk assessment the calculation of bioconversion factors for the main toxic metabolite ETU is necessary. Since the molar mass of ETU differs considerably from the mass of a monomeric BAS 222 F unit, the bioconversion factor should rather be calculated on a mass than molar basis. This report addendum completes HRC Report BSF 410/85720 (see CA 5.1.1/3, this dossier) with the calculation of conversion factors using exclusively data contained in this report. In the Table 5.1.1-17 the results of a biliary excretion experiment with rats ((1985); see CA 5.1.1/3) are shown. Since the elimination was almost complete within the first 48 h, the sum of biliary and renally excreted radioactivity is a measure for the systemic bioavailability of BAS 222 F. The TLC analysis of urine and bile fluid showed that only a part of the radioactivity consisted of ETU (. (1985); see CA 5.1.1/3).

Table 5.1.1-17: Excretion of total radioactivity by rats with cannulated bile duct 0 - 48 h after single oral doses of 5 and 10 mg/kg ^{14}C-BAS 222 F

Sample	Percent of administered [^{14}C]- BAS 222 F dose			
	5 mg/kg		50 mg/kg	
	male	female	male	Female
Urine	45.9	54.8	20.3	27.8
Bile	14.3	7.1	4.3	3.7
Feces	34.1	28.5	74.7	68.2

II. RESULTS AND DISCUSSION

As can be seen in Table 5.1.1-18, the total amount of ETU was 5.3 to 10.5 mole percent of the administered dose, depending on sex and dose level. Assuming a 100% conversion, one BAS 222 F unit (x = 1) affords 4 molecules of ETU or, on a mass basis that 1089 mg of BAS 222 F give 408 mg of ETU. To get the amounts of formed ETU in weight percent, the mole percent values in Table 5.1/4 have to be multiplied with the molar mass ratio of 408/1089 = 0.38. The results are of this calculation is shown in Table 5.1.1-19; the values ranged from 2.0 to 4.0% with an average of 3.0%.

Table 5.1.1-18: Amounts of ETU in urine and bile of rats dosed with ^{14}C-BAS 222 F.

Sample	Mole percent of administered BAS 222 F			
	5 mg/kg		50 mg/kg	
	male	female	male	female
Urine (0-8 h)	1.6	3.9	1.4	1.1
Urine (8- 24 h)	3.1	5.5	3.6	4.6
Subtotal	4.7	9.4	5.0	5.7
Bile*	0.6	1.1	0.2	0.6
Total	5.3	10.5	5.2	6.3

* Bile was only analysed after dosage of 10 mg/kg ^{14}C-BAS 222 F. The composition of the bile of the 5 and 50 mg/kg dose groups was extrapolated from these values. Example: In the 10 mg/kg dose group 4.2% of the bile was ETU. Biliary excretion amounted to 14.3% of the dose in the 5 mg/kg dose group. Hence 14.3% × 0.042 = 0.60% of the biliary activity in the 5 mg/kg group were ETU.

Table 5.1.1-19: Amounts of ETU formed in rats dosed with [^{14}C]-BAS 222 F

Sample	Weight percent of administered BAS 222 F			
	5 mg/kg		50 mg/kg	
	male	female	male	female
Total ETU	2.0	4.0	2.0	2.4

III. CONCLUSION

The mean conversion of BAS 222 F to ETU is 3%. That means a 10 mg dose of BAS 222 F would result in 0.30 mg of ETU, or the bioconversion factor is 0.030.

CA 5.1.2 Absorption, distribution, metabolism and excretion by other routes

Report: CA 5.1.2/1
Funk D.,Possienke M., 2015b
Comparative in-vitro metabolism with 14C-BAS 222 F
2015/1000481
Guidelines: none
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

In order to investigate the possibility of formation of human-specific metabolites from metiram (BAS 222 F), a comparative in-vitro metabolism study was conducted using human hepatocytes as test system. As part of this investigations, in vitro metabolism of metiram in hepatocytes of animal species used in toxicological testing was compared to in vitro metabolism in human hepatocyte samples. The purpose was both determination of differences in the metabolic pattern as well as search for formation of unique human metabolite. Taken together, these comparisons show that the human hepatocytes did not form human-specific metabolites.

To address this question, the ^{14}C-labelled metiram was incubated with hepatocytes from human, rat, dog, rabbit and mouse (all mixed genders) at a final concentration of 5 µM. The concentration was chosen after a cell viability pre-test. The viability of the hepatocytes was determined after 180 min of incubation using a luminescent cell viability assay.
Positive controls were run in parallel to prove the metabolic activity of the hepatocytes (phase I and phase II metabolic reactions). Additionally, negative controls were conducted to verify the stability of the peak pattern of metiram in aqueous media over the test period (stability controls), to record the peak pattern at the beginning of the incubation (zero incubation controls) and to prove the absence of radiolabelled impurities in the cell media (blank controls). The control experiments yielded the expected results.
After incubation for 10, 30, 60 or 180 min, the reaction was terminated by addition of ethanol. The resulting supernatant was analysed by two different HPLC methods after concentration and the radioactive residues determined by LSC. The supernatant after incubation of 5 µM metiram with the hepatocytes contained less than 90% of the applied radioactivity (% AR). Therefore, the pellet was extracted with acetonitrile and subsequently with methanol. The radioactive residues in the resulting extracts and the final pellet were determined by LSC. The acetonitrile extracts were additionally analysed by HPLC. The radio-HPLC analyses of human and animal cell samples were compared in order to determine whether a unique human metabolite occurred or not.

After the incubation with human hepatocytes, seven ^{14}C peaks were detected that represented more than 5% AR on at least one time point (sum of supernatant and pellet extract).
Four peaks at 5.0 min, 17.1 min, 17.7 min and 18.1 min correspond to degradation products of metiram in aqueous liquids and are not a result of biotransformation in human hepatocytes as they were also detectable in stability controls (test item without cells).

Additionally, peaks at 16.6 min, 20.7 min and 22.2 min occurred in amounts of more than 5% AR in samples after incubation of metiram with human hepatocytes. These compounds were also found after incubation with animal hepatocytes and for the peak at 20.7 min additionally in zero incubation controls. In conclusion, these results confirm that metiram was not converted to any human-specific metabolite by the human hepatocytes.

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** ^{14}C-labelled metiram (BAS 222 F, Reg. No. 250284)
   Description: label: ethylene-C14
   Batch/purity #: 153-6001
   Stability of test compound:
   All analyses were accomplished within a period of less than six months after incubation with metiram. Therefore, no storage stability investigations were necessary.

2. **Vehicle and/or positive control:**
   vehicle: hepatocytes
   positive control: testosterone or 7-ethoxycoumarin instead of the test substances were incubated with hepatocytes from the different species to indicate the metabolic activity of the different hepatocytes

3. **Test animals:** Mammals
   Species: Rat, dog, rabbit and mouse
   Strain: Wistar, Beagle, New Zealand and B6C3F1

B. STUDY DESIGN AND METHODS

1. Dates of work: 03 Dec 2014 - 02 Jun 2015

This study was carried out at the Agricultural Centre of BASF in Limburgerhof, Germany.

2. Test substance preparation

For the preparation of the stock solution, the received amount of the radiolabelled test item ^{14}C-Metiram was dissolved in 1 mL DMSO. In order to determine the actual concentration, aliquots of diluted subsamples were analysed by LSC measurement. For this stock solution, a concentration of 14.737 mg/mL was determined.

The concentration of the test substance radiolabelled ^{14}C-testosterone in ethanol was determined by LSC measurement of aliquots of diluted subsamples (0.528 mg/mL).

For the preparation of the stock solution of the radiolabelled ^{14}C-7-ethoxycoumarin, 0.1235 mg substance were dissolved in 325 µL DMSO to yield a concentration of 0.350 mg/mL.

For the preparation of the application solution for experiments with 1 µM metiram, a specific amount of the stock solution of the radiolabelled metiram was concentrated to dryness in a stream of nitrogen and diluted with DMSO. Application solutions with 5 µM metiram and 10 µM metiram were prepared in a similar way. The actual concentrations of the radiolabelled metiram in the application solutions were determined by LSC of diluted aliquots. The specific radioactivity of the application solutions accounted for 458400 dpm/µg. A diluted aliquot of the application solution (5 µM metiram) was analysed using HPLC method LC04 and LC05.

For the preparation of the application for experiments with 10 µM testosterone, a specific amount of the stock solution of the radiolabelled testosterone was concentrated to dryness in a stream of nitrogen and diluted with DMSO. The actual concentration of testosterone in the application solution was determined by LSC of diluted aliquots. The specific radioactivity accounted for 402345 dpm/µg.

For the preparation of the application solution for experiments with 10 µM 7-ethoxycoumarin, a specific amount of the stock solution of the radiolabelled was used as the application solution. The actual concentration of 7-ethoxycoumarin in the application solution was determined by LSC of diluted aliquots. The specific radioactivity accounted for 898800 dpm/µg. The 7-ethoxycoumarin biotransformation products 7-hydroxycoumarin, 7-hydroxycoumarin glucuronide and 7-hydroxycoumarin sulphate were analysed using HPLC method LC02.

3. Preparation of hepatocytes

Cryopreserved hepatocytes from human, rat (variety: Wistar), dog (variety: Beagle), rabbit (variety: New Zealand) and mouse (variety: B6C3F1) were purchased from XenoTech. The hepatocytes were stored in liquid nitrogen. On each incubation day, the cells were thawed according to a protocol provided by the supplier using appropriate kits. The protocol was adapted for the incubation with mouse hepatocytes in order to generate a sufficient cell density. The number of viable cells in the resulting cell suspensions was measured using an automated cell counter. The cell suspensions were then adjusted to the desired cell density of 2×10^6 viable cells per mL with incubation medium. For the animal hepatocytes, male and female cells were combined in this final step in a ratio of 1:1. The human hepatocytes were purchased as a mixture of male and female cells.

4. *In vitro* assays

On each incubation day, the application solutions in DMSO were diluted with hepatocyte incubation medium by a factor of 100 to prepare the respective application medium. The final concentration of DMSO was 0.5%. Aliquots of the application media were analysed by LSC to calculate the amounts of applied radioactivity per well and of total applied metiram per assay (representing 100% AR). The application media were incubated at a final concentration of 5 µM metiram with human, rat, dog, rabbit or mouse hepatocytes. In case of testosterone and 7-ethoxycoumarin, the incubations were performed at 10 µM.

The purity of the application media for the relevant in-vitro assays were determined by HPLC analyses using HPLC methods LC04 and LC05, respectively.

The purity of the application media and the retention time of testosterone were determined by HPLC analyses using HPLC methods LC03 and LC04, respectively. The purity check using the HPLC method LC03 (optimised for testosterone) after preparation of the application media.

The purity of the application media and the retention time of 7-ethoxycoumarin were determined by HPLC analyses using HPLC methods LC02 and LC04, respectively. The purity check using the HPLC method LC03 (optimised for 7-ethoxycoumarin) after preparation of the application media.

Each sample (1.2 mL total incubation volume) comprised 0.6 mL of application medium and 0.6 mL of hepatocyte cell suspension in one of the wells of a 24-well plate. The final cell concentration was approximately 1 x 10^6 cells/mL (for mouse hepatocytes a cell density of approximately 0.6 x 10^6 viable cells per mL was used inadvertently). The reactions were performed for 10, 30, 60 or 180 min at 37°C and at 5% CO_2 in an incubator.

Two negative controls (stability and zero incubation control), two positive controls (testosterone and 7-ethoxycoumarin) and a medium with blank control (application DMSO instead of the test item) were performed for each analysed species and label.

In the negative controls no metabolisation should occur. For the “stability control”, the application medium was mixed with incubation medium instead of the cell suspension. Due to the fast degradation of metiram in aqueous liquids, the stability controls were performed at 0, 10, 30, 60 and 180 min. For the “zero incubation control” (t = 0 min), the reaction was stopped immediately after addition of the cell suspension.

In the positive controls, testosterone or 7-ethoxycoumarin instead of the test substances were incubated with hepatocytes from the different species to indicate the metabolic activity of the different hepatocytes. To prove the metabolic activity, the following amounts of testosterone should be metabolised at least: human 50%, rat 75%, dog 60%, rabbit 70% and mouse 50%. The following amounts of 7-ethoxycoumarin should be metabolised at least: human 30%, rat 70%, dog 70%, rabbit 70% and mouse 50%.

In each experimental setup, the incubation of the substrates as well as all control assays were performed in triplicates.

5. Viability test

The viability of human hepatocytes after incubation with 1 µM, 5 µM and 10 µM metiram was tested in order to select the appropriate concentration of the test item. Therefore, 250 µL of the respective application medium were incubated with 250 µL hepatocyte cell suspension at 37°C and 5% CO_2 for 180 min in a 24-well plate. The viability test for the stability controls was performed by addition of medium (without DMSO) without cells. Sextuplicates of 25 µL of the incubation mixtures or of the blank controls (cells without test item) as well as 50 µL of the stability controls (without hepatocytes) were transferred to a 96-well plate. These samples were diluted with incubation medium without DMSO to a volume of 100 µL. Additionally, 100 µL incubation medium (with DMSO) were also pipetted into the 96-well plate. 100 µL of the reagent of the luminescent cell viability assay were added. After shaking for 2 min, the plates were allowed to stand for 10 min at room temperature. Then, the luminescence was measured using a plate reader.

The viability of the hepatocytes after the exposure to 5 µM metiram was determined for each incubation date. After incubation and removal of 1 mL of the incubation mixtures for termination of the incubation and analysis, the wells still contained sufficient amounts of sample material for viability testing. Triplicates were taken from the incubation medium (with DMSO, 3 x 100 µL) and from the blank controls (cells without test item, 3 x 25 µL). Additionally, sextuplicates of 25 µL treated cells with 5 µM metiram and of 100 µL were taken from the stability controls. The samples were transferred to a 96-well plate and diluted with incubation medium (without DMSO) to a volume of 100 µL each. 100 µL of the reagent of the luminescent cell viability assay were added and the luminescence was determined as described above.
The luminescence measured (in Relative Luminescence Units, RLU) for the stability controls (without cells) was subtracted as background from the corresponding values of the samples with cells. The luminescence of the incubation medium was subtracted from the values of the blank samples (cells without test item). The viability of the cells incubated with metiram was compared to the viability of the cells incubated without the test item.

6. Sampling and sample storage

The incubation was terminated by pipetting 1 mL of the incubation mixture into a tube containing 2.33 mL cold ethanol to adjust the sample to an ethanol concentration of approximately 70% (v/v). Subsequently, cell lysis was performed by a 15 min ultrasonication step. The terminated incubation mixtures were concentrated at room temperature using a centrifugal evaporator (Genevac EZ 2.3 plus). The tubes were weighed after concentration, the supernatants were pipetted into HPLC vials and the tubes containing the pellets were weighed again. The volume of the supernatant collected from each tube was calculated from the difference of the two weights prior to and after removal of the supernatant and recorded in the raw data. The weight of each pellet was calculated by subtraction of the weight of the tube. The radioactive residues in the supernatants were determined by LSC analysis of aliquots. Additionally, the supernatants were analysed by HPLC method LC04 and LC05 (for controls with testosterone and 7-ethoxycoumarin using HPLC method LC03 and LC02, respectively). The pellets were stored in a freezer at -18°C.

7. Work-up of the residual pellet

In general, the supernatant of samples incubated with cells and the test item accounted for less than 90% AR. Therefore, the pellets after centrifugal evaporation (pellet 1) were additionally extracted with acetonitrile. They were resuspended in 50 µL water and mixed with 250 µL acetonitrile followed by ultrasonication for 5 min. After centrifugation, the radioactive residues in the acetonitrile extract were determined by LSC of aliquots. For the stability controls, the pellets were extracted for human and rat at all sampled time points, for dog, rabbit and mouse only for the first and last time point (0 and 180 min).

Additionally, the acetonitrile extracts were analysed using HPLC method LC04 (for stability controls after incubation with animal hepatocytes only time points 0 and 180 min). Furthermore, the remaining pellets were in some cases extracted with methanol. The methanol extraction was performed as described for the acetonitrile extraction and the radioactive residues in the supernatants were determined by LSC analysis. The final pellets were resuspended in 320 µL water, ultrasonicated for 10 min and the radioactive residues in the suspended samples were measured by LSC.

For the blank samples and the positive controls with testosterone and 7-ethoxycoumarin, no analysis of the pellets was performed.

A co-chromatography experiment was performed in order to check if peaks detected in samples incubated with human hepatocytes corresponded to peaks detected after incubation with rat hepatocytes. Therefore, the supernatant of 5 µM metiram incubated with human hepatocytes for 60 min (replicate 1) was co-injected with the supernatant of metiram incubated with rat hepatocytes for 60 min (replicate 1) using HPLC method LC04.

Additionally, a fractionation was performed with samples incubated with human hepatocytes for 10 min using HPLC method LC04.The fraction was cut from 15 to 19 min in three HPLC runs, pooled and concentrated. The sample was then analysed using HPLC method LC05 in order to support the assignment of the corresponding peaks in the second HPLC system. The expected peak region was detectable however two additional peaks at 15.3 min and in particular at 4.4 min were found. It must be assumed that both peaks represent compounds originating from the fractionated peak group and were formed after fractionation until reanalysis five days later.

II. RESULTS AND DISCUSSION

The present in-vitro study describes the biotransformation of metiram (BAS 222 F) after incubation with human, rat, dog, rabbit or mouse hepatocytes over a period of 180 min. ^{14}C-labelled metiram was used for the investigations. In cell viability pre-tests 5 µM metiram was found to represent an adequate concentration for the in-vitro assays.
Therefore, human hepatocytes were incubated with 5 µM metiram. The biotransformation products were compared to products from incubation of 5 µM metiram with the animal hepatocytes. Additionally, several negative and positive controls were conducted. In the supernatant the radioactive residues were quantified and in general an acetonitrile and subsequent methanol extraction of the remaining pellet was performed. All assays were performed in triplicates.

1. Control experiments

The blank controls performed for each species without the test item showed no significant amounts of radioactivity (LSC measurements of the supernatants after addition of ethanol to the incubation mixtures and concentration). Furthermore, no radioactive peaks were detected by HPLC analysis.

As negative controls, stability controls (test item without cells) and zero incubation controls (incubation stopped immediately) were performed in triplicates. Due to the fast degradation of metiram in aqueous liquids, the stability controls were performed at 0, 10, 30, 60 and 180 min.
The triplicates of the negative controls showed similar HPLC profiles. They all consisted of characteristic peaks found in aqueous samples of the active substance metiram.
Good recovery of radioactive residues after incubation of metiram with incubation medium (stability controls) was observed. The recoveries (sum of applied radioactivity, %) for human, rat, dog, rabbit and mouse range between 79 - 97%, 84 - 91%, 52 - 94%, 75 - 93% and 59 - 95%, respectively.

For the positive controls, the recovery of the radioactive residues in the concentrated supernatants accounting for 85% to 95% AR for testosterone and for 81% to 92% AR for 7-ethoxycoumarin, respectively. In the HPLC analyses of these samples, testosterone, 7-ethoxycoumarin and its biodegradation products were assigned to the corresponding peaks by retention time comparison.

The positive controls performed with testosterone showed that the metabolic activity of the hepatocytes was sufficiently high. Testosterone was metabolised completely (rat and dog) or almost completely (human, rabbit and mouse) after incubation with hepatocytes.
HPLC analyses of the positive controls performed with 7-ethoxycoumarin revealed mean portions of the metabolised 7-ethoxycoumarin reaching values above 70% AR after incubation with human hepatocytes and above 80% AR after incubation with animal hepatocytes. The phase I metabolite 7-hydroxycoumarin was found after the incubation with cells of all species except rat hepatocytes (accounting for 16.5% to 41.3% AR). The conjugated metabolite 7-hydroxycoumarin glucuronide was found only after the incubation with human and rabbit hepatocytes (equal or below 9.9% AR). The other phase II metabolite 7-hydroxycoumarin sulphate was detected after the incubation with all animal hepatocytes in high portions (accounting for 29.7% to 60.0% AR). Thus, the positive controls performed with 7-ethoxycoumarin indicate that the metabolic activity of the hepatocytes with respect to phase II metabolic reactions was sufficiently high for all species.

2. Viability of the hepatocytes

In order to select an appropriate concentration of the test item with a sufficiently high viability of the cells, concentrations of 1 µM, 5 µM and 10 µM metiram were tested on human hepatocytes. The viability of the human hepatocytes after the incubation with metiram at a final concentration of 1 µM accounted for 86% of the viability in comparison to the blank controls (cells incubated without the test item). For the final concentrations of 5 µM and 10 µM, the viability of the cells accounted still for 81% and 80%, respectively. Therefore, the final concentration of 5 µM metiram for the in-vitro biotransformation study was chosen.

The hepatocyte suspensions were adjusted to a cell density of 2 x 10^6 viable cells per mL to achieve a final cell density of approximately 1 x 10^6 cells per mL in the incubation assays. For mouse hepatocytes a cell density of approximately 0.6 x 10^6 viable cells per mL was used inadvertently. After the incubation for 180 min, the viability of the cells was determined using the luminescent cell viability assay.
The viability of the human hepatocytes incubated with 5 µM metiram was 78% of the viability in comparison to the blank controls. The viability of the animal hepatocytes incubated with 5 µM metiram was equal or above 80% of the viability of the blank controls. Therefore, it can be stated that the cell viability in the in-vitro assays was sufficient to generate valid results.

3. Workup procedures

After terminating the incubation of metiram with the hepatocytes the radioactive residues in the concentrated supernatants accounted from 74.6% to 87.6% AR (with one exception for dog cells at 0 min accounting for 65.0% AR). As the radioactive residues were below 90% AR, the residual pellets (pellet 1) were extracted with acetonitrile and subsequently with methanol. The radioactive residues in the acetonitrile extracts were below or equal to 5.5% AR in all experiments with metiram and hepatocytes, while the methanol extracts accounted for less than 2% AR. The radioactive residues in the final pellets of all experiments with metiram were equal or below 8.8% AR. Although for some samples the sum of radioactive residues was lower than 90% AR, no significant influence on the objective of this study is expected as the sum of recovery of radioactive residues in the majority of the assays accounted for more than 90% AR.

4. Comparison of metabolic profiles and designation of metabolite peaks

For quantitative evaluation of the biodegradation products of metiram, the chromatograms of analysis with HPLC methods LC04 and LC05 were used.

Biotransformation products of metiram were assigned by their retention time and the similar metabolite pattern in the HPLC chromatograms. Relevant peaks representing compounds accounting for more than 5% AR in the sum of supernatant and pellet extract on at least one analysed time point using HPLC method LC04 were named by their retention time (mean value of the replicates on first appearance). Thereby, seven relevant peaks P5.0, P16.6, P17.1, P17.7, P18.1, P20.7 and P22.2 were assigned.

In Table 5.1.2-1 (LC04) and Table 5.1.2-2 (LC05), the relevant peaks identified in human samples are compared with the peaks identified in the animal samples.

5. Peaks identified after incubation of metiram with hepatocytes

The active substance metiram is not detectable as a defined peak in HPLC analysis because of its chemical properties. Due to a fast degradation in aqueous liquids, a characteristic peak pattern corresponding to several degradation products could be observed. Radio-HPLC analyses of samples after incubation of human hepatocytes with 5 µM metiram allowed the assignment of seven relevant peaks.
Four of these relevant peaks with an abundance of more than 5% AR, namely **P5.0, P17.1, P17.7 and P18.1**, were detected in all species at all analysed time points. The compound corresponding to peak P5.0 accounted for up to 40.9% AR in samples obtained after incubation with human cells after 180 min (LC04) while P17.1 accounted for a maximum of 26.0% AR (10 min, LC04). For P17.7 and P18.1 amounts of up to 7.3% were detected (both 10 min) using HPLC method LC04. These results were confirmed by the second HPLC method LC05 whereby comparable amounts were detected.

Additionally, P5.0, P17.1, P17.7 and P18.1 were found in the stability controls (test item without cells). The comparison of the HPLC chromatograms of the stability controls and the incubation of metiram with human hepatocytes shows that the peak pattern was very similar for P5.0, P17.1, P17.7 and P18.1. Therefore, it can be concluded that these relevant peaks correspond to degradation products of metiram in aqueous liquids and are not a result of metiram biotransformation by human hepatocytes. They were also detected in largely comparable amounts after the incubation of metiram with animal hepatocytes at all analysed time points (Table 5.1.2-1 and Table 5.1.2-2 for LC04 and LC05, respectively). During the HPLC analysis using method LC04 the peaks P17.7 and P18.1 were not separated in samples from incubation with rat, dog and rabbit hepatocytes probably due to a decreasing separation performance of the HPLC column. This was confirmed by a co-chromatography experiment with the supernatant of samples from incubation with human and rat hepatocytes. When a new, equal column was used for the analysis of samples from mouse cells the separation of both peaks was re-established using HPLC method LC04. The existence of both peaks was additionally shown by using HPLC method LC05 for all analysed samples.

Besides these four peaks, P16.6, P20.7 and P22.2 occurred in amounts of more than 5% AR in samples after incubation with human hepatocytes.
The compound corresponding to peak **P16.6** was initially detected after 30 min in human hepatocytes (2.6% AR) and its amount increased up to 5.4% AR after 180 min (data obtained with HPLC method LC04). The metabolite corresponding to P16.6 was also found in samples after incubation with rat hepatocytes (30-180 min, up to 6.8% AR), dog hepatocytes (30-180 min, up to 3.8% AR) and mouse hepatocytes (all time points, up to 3.5% AR). These findings were confirmed by the second HPLC method LC05 whereby amounts of 2.6% AR (human), up to 5.9% AR (rat), 2.5% AR (dog), 1.7% AR (rabbit) and up to 1.9% AR (mouse) were detected.
It can be concluded, that the metabolite corresponding to peak P16.6 is no human-specific metabolite.

The peak at 20.7 min (**P20.7**) was found in human samples at all analysed time points accounting for up to 8.2% AR (data obtained with HPLC method LC04, maximum at 10 min). When using HPLC method LC05, the peak was detected in amounts of up to 4.0% AR (10-60 min). The compound was also found after incubation with rat hepatocytes (10 min, 2.8% AR). Additionally, the peak was detected in the zero incubation controls with human and rat hepatocytes (6.5% and 4.6% AR, respectively) and in the application media used for the incubation with human, rat and dog hepatocytes.
Therefore, it can be concluded, that the compound corresponding to peak P20.7 is no human-specific metabolite.

Likewise, the compound corresponding to peak **P22.2** was initially detected after 60 min in human hepatocytes (3.7% AR) and its amount increased up to 6.2% AR after 180 min (data obtained with HPLC method LC04). The metabolite was also found in samples after incubation with rat hepatocytes (60 min, 2.9% AR) and rabbit hepatocytes (10-60 min, up to 2.5% AR). The findings for incubation with human cells were confirmed by the second HPLC method LC05 whereby amounts of up to 5.0% AR were detected. Probably due to the low amounts in samples from metiram incubations with animal hepatocytes, the peak was not detectable with HPLC method LC05.
It can be concluded, that the metabolite corresponding to peak P22.2 is no human-specific metabolite.

In summary, this in-vitro metabolism study showed no human-specific metabolite formation from 5 µM metiram by the human hepatocyte cells within 180 min.

Table 5.1.2-1: Comparison of Relevant Metabolites of Metiram after Incubation with Different Hepatocytes Using HPLC Method LC04

Relevant Peak		P5.0	P16.6	P17.1	P17.7	P18.1	P20.7	P22.2
		[% AR][1, 2]						
0 min	Human	39.82	-	26.21	7.04	8.03	6.47	-
	Rat	44.90	-	15.48	10.65 [1]		4.63	-
	Dog	43.87	-	14.60	9.07		-	-
	Rabbit	52.54	-	11.86	10.02		-	0.30
	Mouse	56.05	1.43	15.98	2.81	6.25	-	-
10 min	Human	40.01	-	25.97	7.33	7.32	8.18	-
	Rat	49.62	-	19.39	11.89		2.84	-
	Dog	51.61	-	16.37	10.01		-	-
	Rabbit	52.17	-	11.48	11.08		-	2.27
	Mouse	58.70	2.51	16.24	2.43	6.39	-	-
30 min	Human	40.33	2.55	25.43	5.43	7.07	5.08	-
	Rat	47.23	2.95	19.17	10.31		-	-
	Dog	53.33	2.92	15.74	9.81		-	-
	Rabbit	55.13	-	13.65	8.09		-	2.50
	Mouse	57.30	2.72	14.50	2.91	6.11	-	-
60 min	Human	37.91	3.27	24.13	5.05	7.32	7.34	3.72
	Rat	50.38	5.43	17.18	8.52		-	2.85
	Dog	54.26	0.25	17.77	8.72		-	-
	Rabbit	56.73	-	13.74	7.26		-	0.31
	Mouse	59.05	0.16	15.98	2.65	6.49	-	-
180 min	Human	40.87	5.38	21.08	-	6.64	4.94	6.16
	Rat	50.35	6.83	10.36	8.27		-	-
	Dog	55.17	3.80	13.33	7.47		-	-
	Rabbit	59.37	-	11.34	6.23		-	-
	Mouse	56.70	3.47	12.33	3.51	5.64	-	-

1 Sum of supernatant and pellet extract

2 Peaks P17.7 and P18.1 were not split up in the analyses of samples after incubation with rat, dog and rabbit hepatocytes

Table 5.1.2-2: Comparison of Relevant Metabolites of Metiram after Incubation with Different Hepatocytes Using HPLC Method LC05

Relevant Peak		P5.0	P16.6	P17.1	P17.7	P18.1	P20.7	P22.2
		[% AR][1]						
0 min	Human	43.45	-	18.35	14.04	8.07	4.50	-
	Rat	49.56	-	14.82	8.48	6.14	-	-
	Dog	49.57	-	11.43	8.05	5.15	-	-
	Rabbit	52.80	-	10.12	6.17	5.69	-	-
	Mouse	56.06	-	10.26	5.97	5.51	-	2.18
10 min	Human	42.51	-	20.55	12.49	6.50	4.03	-
	Rat	51.63	-	16.44	8.52	6.42	-	-
	Dog	53.43	-	12.86	8.43	6.11	-	-
	Rabbit	56.91	-	10.15	5.81	5.73	-	-
	Mouse	58.67	-	11.03	6.36	6.00	-	-
30 min	Human	47.37	-	19.32	9.07	6.66	2.79	2.54
	Rat	50.65	2.27	14.85	8.74	5.77	-	-
	Dog	55.83	-	12.60	6.62	7.29	-	-
	Rabbit	56.87	-	10.94	4.95	5.51	-	-
	Mouse	58.69	1.66	9.85	6.04	6.65	-	-
60 min	Human	45.02	2.60	19.61	10.69	6.83	3.30	-
	Rat	51.84	4.34	15.81	6.77	5.77	-	-
	Dog	56.56	-	12.66	5.98	5.02	-	-
	Rabbit	56.87	1.55	10.35	3.57	5.28	-	-
	Mouse	58.51	1.68	11.63	5.07	6.12	-	-
180 min	Human	49.08	-	20.80	4.05	6.82	-	4.98
	Rat	54.33	5.86	13.03	6.92	5.71	-	-
	Dog	56.14	2.50	13.42	4.33	5.98	-	-
	Rabbit	56.65	1.65	8.39	4.68	5.57	-	-
	Mouse	57.79	1.85	9.26	5.03	6.98	-	-

1 Sum of supernatant and pellet extract

III. CONCLUSION

In this comparative in-vitro metabolism study with metiram (BAS 222 F), the biotransformation products after incubation of 5 µM metiram with human hepatocytes were compared to products from incubation with animal hepatocytes (rat, dog, rabbit or mouse) over a period of 180 min.

After the incubation of human hepatocytes with the active substance, seven ^{14}C peaks were detected that represented more than 5% AR on at least one time point (sum of supernatant and pellet extract). Four peaks namely P5.0, P17.1, P17.7 and P18.1 correspond to degradation products of metiram in aqueous liquids and are not a result of biotransformation by human hepatocytes as these peaks were also detectable in stability controls (test item without cells).
Besides these peaks, P16.6, P20.7 and P22.2 occurred in amounts of more than 5% AR in samples after incubation with human hepatocytes.
These compounds were also found after incubation with animal hepatocytes and for P20.7 additionally in zero incubation controls. Therefore, it can be concluded, that these compounds corresponding to peak P16.6, P20.7 and P22.2 are no human-specific metabolites.

Therefore, it was shown in this in-vitro metabolism study that no human-specific metabolites were formed from metiram by the human hepatocytes.

CA 5.2 Acute Toxicity

Studies evaluated in the draft monograph of Rapporteur Member State Italy of July 2000:

Metiram has been tested in various species and via different routes of administration. All studies are scientifically valid, however partially the studies have been conducted before the release of study guidelines and are without GLP according to the usual practice in those days. These studies listed in Table 5.2-1 have been evaluated and peer reviewed during the previous Annex I inclusion process. Summaries of the respective studies are presented under the respective chapters.

Table 5.2-1: Summary of already peer-reviewed acute toxicity studies with metiram

Route/species/sex	Dose range	Vehicle	Result	Reference (BASF DocID)
Oral (metiram) Rat, SD, m/f	2150; 3160; 4640; 6810 mg/kg bw	water	LD_{50} > 6810 mg/kg bw	1992/10669
Oral (metiram + 2% ETU) Rat, SD, m/f	5620; 6810; 8250; 10000; 12100 mg/kg bw	0.8% aqueous hydroxypropylmethylcellulose-gel	LD_{50} = 8900 mg/kg bw	1979/0161
Dermal (metiram) Rat, SD, m/f	2000 mg/kg bw	water	LD_{50} > 2000 mg/kg bw	1979/032
Intra peritoneal metiram + 2% ETU) Mouse, NMRI, m/f	68.1; 100; 147; 215; 316; 464 mg/kg bw	0.8% aqueous hydroxypropylmethylcellulose-gel	LD_{50} = 212 mg/kg bw	1979/0162
Intra peritoneal (metiram) rat, SD, m/f	215; 237; 261; 287; 316; 348; 383; 424; 464 mg/kg bw	0.5% aqueous CMC	LD_{50} = 318 mg/kg bw	1975/006
Inhalation (metiram) rat, SD, m/f	5.7; 20.3 mg/L	None	LC_{50} > 5.7 mg/L air (4h)	1983/064
Skin irritation (metiram) Rabbit – New Zealand White	0.5 g	Water	Not irritating	2002/1005292
Eye irritation (metiram) Rabbit- New Zealand White	40 mg	water	Not irritating	2002/1005291, 2002/1006988
Skin sensitisation, Maximisation Test (metiram) Guinea pig, Dunkin/Hartley, f	Intradermal: in mixture Freund's adjuvant /parafine oil Epidermal: 50% in water	water	sensitising	1982/068

Studies submitted in this AIR 3 dossier (not yet peer-reviewed):

In vivo studies characterizing the acute irritation profile were performed with metiram, which were not submitted during the previous Annex I inclusion process. The respective studies are listed in Table 5.2-2.

Table 5.2-2: Summary of not yet peer-reviewed acute toxicity studies with metiram

Type of study	Test substance	Result Classification	Reference (BASF DocID)
In vitro NRU Phototoxicity Test in Balb/c 3T3 cells	Metiram Solvent: DMSO	Phototoxic	2013/1358979

Metiram has very low acute toxicity by the oral, dermal and inhalation route of administration. Metiram is neither a skin nor eye irritant.

In a Maximisation Test metiram was sensitizing to the skin. Therefore, a classification with R43 according to EU and Skin Sensitisation 1B according to GHS classification criteria is warranted.

A phototoxic potential was observed in an in vitro NRU Phototoxicity Test in Balb/c 3T3 cells.

Based on the studies previously peer-reviewed, the following EU agreed endpoints are given in the Review Report of metiram (SANCO/4059/2001-Final, 3. June 2005):

Rat LD_{50} oral:	> 5000 mg/kg bw
Rat LD_{50} dermal:	> 2000 mg/kg bw
Rat LC_{50} inhalation:	> 5.7 mg/L
Skin irritation:	Not irritating
Eye irritation:	Not irritating
Skin sensitization (test method used and result):	sensitising (M & K maximisation test)

The proposed endpoints based on all available studies are shown below in Table 5.2-3.

Table 5.2-3: Proposed acute toxicity endpoints of metiram*

Study type/species	Results	Classification	
		EU Dir. 67/548/EEC 2001/59 EC	**Reg. EC 1272/2008 (CLP)**
Acute oral toxicity, rat	LD_{50} > 5000 mg/kg bw		
Acute dermal toxicity, rat	LD_{50} > 2000 mg/kg bw		
Acute inhalation toxicity, rat	LC_{50} > 5.7 mg/L		
Dermal irritation, rabbit	Not irritating		
Eye irritation, rabbit	Not irritating		
Maximisation test, guinea pig	sensitising (M & K maximisation test)	R43	H317
In vitro NRU Phototoxicity Test, Balb/c 3T3 cells	**phototoxic**		

* new endpoints or values differing from the current agreed EU endpoints are marked in bold

CA 5.2.1 Oral

The acute oral toxicity study of metiram was evaluated and peer reviewed during the previous Annex I inclusion process. The studies are scientifically valid, however the studies have been conducted before the release of study guidelines and are without GLP according to the usual practice in those days. Metiram has a very low acute oral toxicity.

Report:	*CA 5.2.1/1*
	[redacted] 1981a
	Report on the study of the acute oral toxicity of Metiram technical grade in the rat
	1992/10669
Guidelines:	*none*
GLP:	*no*

Executive Summary

In an acute oral toxicity study 10 Sprague-Dawley rats (5/sex) were orally exposed to a single dose of metiram (batch and purity not indicated) as an aqueous suspension at dose levels of 2150, 3160, 4640 and 6810 mg/kg bw.

Mortality rates (males/females) were as follows: 2/1 (6810 mg/kg bw), 1/0 (4640 mg/kg bw). All other animals survived the 14-day observation period. Accordingly, the oral LD50 was found to be greater than 6810 mg/kg bw for male and female rats.

Rat, oral: LD50 > 6810 mg/kg bw

Signs of toxicity noted in the 2150 mg/kg bw to 6810 mg/kg bw dose groups comprised dyspnoea, apathy, staggering, spastic gait, piloerection, alopecia, exsiccosis and poor general state.
Necropsy findings in animals that died were sporadically bloody ulcerations in the stomach, widespread hemorrhages in the fore stomach and muscles sporadically somewhat exsiccotic.
No abnormalities were noted at necropsy of animals sacrificed at the end of the study.
According to the EU and GHS classification criteria, no classification is warranted as to acute oral toxicity for metiram.

(BASF DocID 1992/10669)

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** metiram technical grade
   Description: no data
   Test substance #: 80/340
   Purity: no given in the report
   Stability of test compound: no data given in the report

2. Vehicle: distilled water

3. Test animals:

Species: Rat
Strain: Sprague-Dawley / WIGA
Sex: males and females
Age: about 12 weeks
Weight at dosing (mean): mean: female: 185 g; male: 192.5 g
Source: [redacted]
Acclimation period: at least one week
Diet: Herilan (MRH-Haltung, H. Eggersmann KG, Rinteln, Germany) ad libitum
Water: demineralized water each workday, tap water on holidays ad libitum
Housing: 5 per in stainless steel wire mesh cages, type DK-III (Becker&Co, Castrop-Rausxel, Germany)

Environmental conditions:

Temperature: 20 – 26°C
Humidity: 45 – 75%
Air changes: no data
Photo period: 12-hour light-dark cycle (06:00 – 18:00, 18:00 – 06:00)

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 02-April-1981 (day of first administration)

2. Animal assignment and treatment:

Sixteen hours before treatment the animals received no food but water ad libitum. Ten rats (5 per sex per group) received doses of 2150, 3160, 4640 and 6810 mg/kg bw of test substance suspended in water by gavage. The observation period lasted 14 days. Individual body weights were recorded shortly before application (day 0), on the 2nd, 3rd or 4th day, on the 7th and on the 13th day after administration. Clinical signs and symptoms were recorded several times on the day of administration and the once each workday for the individual animals. Check for moribund and dead animals were carried out twice each workday and once on holidays. After the observation period a necropsy of all animals with gross pathology followed.

II. RESULTS AND DISCUSSION

A. MORTALITY

Mortalities observed in the single dose groups are described in the following table.

Table 5.2.2-1: Mortality rates in rats dosed with metiram

Dose [mg/kg bw]	Toxicological results*	Time of death	Mortality [%]
Males			
2150	0/5	-	0
3160	0/5	-	0
4640	1/5	Day 7	20
6810	2/5	Day 7	40
Females			
2150	0/5	-	0
3160	0/4	-	0
4640	0/45	-	0
6810	1/5	Day 7	20

* x/x = number of dead animals / number of animals used

At the highest dosage 3/10 animals died which corresponds to 30% mortality. At 3160 mg/kg bw only 1 of 10 animals died which corresponds to 10% mortality.

B. CLINICAL OBSERVATIONS

All animals showed dyspnea, apathy, staggering, spastic gait, piloerection, alopecia, exsiccosis and poor general state. Only the animals from the lowest dose group did not show spastic gait.

C. BODY WEIGHT

In the two highest dose group of male rats and in all dose groups of female rats, animals lost weight in the beginning. Then the animals gained weight until the end of the observation period.

D. NECROPSY

Necropsy findings in animals that died were sporadically bloody ulcerations and widespread hemorrhages in the fore stomach and muscles sporadically somewhat exsiccotic.
No abnormalities were noted at necropsy of animals sacrificed at the end of the study.

III. CONCLUSION

Under the experimental conditions of this study the dermal LD50 of metiram technical in rats was determined to be greater than 6810 mg/kg bw for males and females. Based on the results of this study, metiram does not warrant classification as to acute oral toxicity according to EU criteria (28th ATP of Council Directive 67/548/EEC by Commission Directive 2001/59/EC / Council Regulation 1272/2008/EC) and according to the OECD Globally Harmonized System (GHS, revision 2, 2007)

Report: *CA 5.2.1/2*
[redacted] 1979a
The acute oral toxicity of the preparation Metiram, techn. agent with 2% ETU in rats
1979/0161
Guidelines: *none*
GLP: *no*

Executive Summary

In an acute oral toxicity study 20 Sprague-Dawley rats (10/sex) were orally exposed to a single dose of metiram (batch and purity not indicated) with 2% ethylenethiourea (ETU) as suspension in 0.8% aqueous hydroxypropylmethyl-cellulose-gel at dose levels of 5620, 6810, 8250, 10000 and 12100 mg/kg bw.

Mortality (males/females) occurred in the following doses: 10/10 at 12100 mg/kg bw, 4/5 at 10000 mg/kg bw, 2/3 at 8250 mg/kg bw, 2/2 at 6810 mg/kg bw. All other animals survived the 14-day observation. Accordingly, the oral LD_{50} was found to be 9000 mg/kg bw for male and female rats, 9100 mg/kg bw for male rats and 8900 mg/kg bw for female rats. Since female LD50 is the lowest value it is taken as final LD_{50}.

Rat, oral: LD_{50} = 8900 mg/kg bw

Signs of toxicity noted in the 6810 mg/kg bw to 12100 mg/kg bw dose groups comprised comatose condition, slight decreased motility and slight ataxia. Animals of the 5620 mg/kg bw dose group did not show any symptoms. Necropsy findings in animals that died were pale parenchymatous organs, bloody masses in the intestines and single hemorrhagic erosions. No abnormalities were noted at necropsy of animals sacrificed at the end of the study.
According to the EU and GHS classification criteria, no classification is warranted as to acute oral toxicity for metiram.

(BASF DocID 1979/0161)

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** metiram technical agent with 2% ETU
   Description: solid, yellow
   Test substance #: no data given in the report
   Purity: no given in the report
   Stability of test compound: no data given in the report

2. **Vehicle:** 0.8% aqueous hydroxypropyl-methylcellulose-gel (type E4M, w/v)

3. Test animals:

Species:	Rat
Strain:	Sprague-Dawley
Sex:	males and females
Age:	male: 52 days, females: 70 days
Weight at dosing (mean):	between 165 and 185 g
Source:	
Acclimation period:	at least one week
Diet:	Altromin 1323 (Atromin GmbH, POB 285, Lage/Lippe, Germany) ad libitum
Water:	tap water ad libitum
Housing:	single housing in Makrolon cages, type III

Environmental conditions:

Temperature:	18 – 24°C
Humidity:	57 – 63%
Air changes:	no data
Photo period:	12-hour light-dark cycle (06:00 – 18:00, 18:00 – 06:00)

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: December 1977 until January 1978

2. Animal assignment and treatment:

Sixteen hours before treatment the animals received no food but water ad libitum. 20 rats (10 per sex per group) received doses of 5620, 6810, 8250, 10000 and 12100 mg/kg bw of test substance suspended in 0.8% aqueous hydroxypropyl-methylcellulose-gel by gavage. The observation period lasted 14 days. Body weights and food intake were recorded on the first, second and 7th day after administration. Clinical signs and symptoms were recorded several times on the day of administration and the once each workday for the individual animals. After the observation period a necropsy of all animals with gross pathology followed.
Mortality rate was evaluated according to Litchfeld and Wilcoxon (LD_{50}).

II. RESULTS AND DISCUSSION

A. MORTALITY

Mortalities observed in the single dose groups are described in the following table.

Table 5.2.2-2: Mortality rates in rats dosed with metiram

Dose [mg/kg bw]	Toxicological results*	Time of death	Mortality [%]
Males			
5620	0/10	-	0
6810	2/10	72 hours	20
8250	2/10	72 hours	20
10000	4/10	72 hours	40
12100	10/10	72 hours	100
Females			
5620	0/10	-	0
6810	2/10	72 hours	20
8250	3/10	72 hours	30
10000	5/10	72 hours	50
12100	10/10	72 hours	100

* x/x = number of dead animals / number of animals used

The LD_{50} of rats after single oral administration of metiram is amounted to:
Male animals: 9100 mg/kg bw (8350 – 9920)
Female animals: 8900 mg/kg bw (8170 – 9700)
Male and female animals: 9000 mg/kg bw (8490 – 9540)

B. CLINICAL OBSERVATIONS

Signs of toxicity noted in the 6,810 mg/kg bw to 12,100 mg/kg bw dose groups.
For the dose groups with 8250, 10000 and 12100 mg/kg bw approximately 60 minutes after administration in all animals slight decreased motility and slight ataxia for 1 to 3 hours occurred. Approximately 48 hours after administration in 2 males and 3 females for the 8250 mg/kg dose group, 4 males and 5 females for the 10000 mg/kg dose group and all males and females for the 12100 mg/kg dose group comatose condition, in which exitus occurred within 2 to 4 days after administration were observed. In all animals in the 6810 mg/kg dose group on the 1st and 2nd day no signs of intolerance reactions were seen. Approximately 48 to 72 hours after administration in 2 males and 2 females comatose condition, in which exitus occurred within 60 to 120 minutes after administration were observed. Animals of the 5620 mg/kg bw dose group did not show any symptoms.

C. BODY WEIGHT

Body weights per dose group decreased between 5 to 8% until 7th day after administration.

Table 5.2.2-3: Decrease of body weight and food intake (mean values per group)

	1st day	2nd day	7th day
5620 mg/kg bw			
Body weight [%]	0	4	6
Food intake [%]	4	0	2
6810 mg/kg bw			
Body weight [%]	0	4	5
Food intake [%]	18	10	12
8250 mg/kg bw			
Body weight [%]	4	8	8
Food intake [%]	31	14	11
10000 mg/kg bw			
Body weight [%]	6	4	8
Food intake [%]	54	10	12
12100 mg/kg bw			
Body weight [%]	4	10	-
Food intake [%]	100	100	-

* x/x = number of dead animals / number of animals used

D. NECROPSY

Necropsy findings in animals that died were pale parenchymatous organs, bloody masses in the intestines and single hemorrhagic erosions.
No abnormalities were noted at necropsy of animals sacrificed at the end of the study.

III. CONCLUSION

Under the experimental conditions of this study the dermal LD_{50} of metiram in rats was determined to be 8900 mg/kg bw. Based on the results of this study, metiram does not warrant classification as to acute oral toxicity according to EU criteria (28th ATP of Council Directive 67/548/EEC by Commission Directive 2001/59/EC / Council Regulation 1272/2008/EC) and according to the OECD Globally Harmonized System (GHS, revision 2, 2007)

CA 5.2.2 Dermal

The acute dermal toxicity study of metiram was evaluated and peer reviewed during the previous Annex I inclusion process. The study is scientifically valid, however the studies have been conducted before the release of study guidelines and are without GLP according to the usual practice in those days. Metiram has a very low acute dermal toxicity.

Report:	*CA 5.2.2/1*
	[redacted] 1979a
	Study of the acute dermal toxicity of Metiram tech. with 2% ETU on the rat
	1979/032
Guidelines:	*none*
GLP:	*no*

Executive Summary

In an acute dermal toxicity study 10 Sprague-Dawley rats (5/sex) were dermally exposed to a single dose of metiram (compound no.: 79/443) with 2% ethylene thiourea (ETU) as 50% aqueous suspension at a dose level of 2000 mg/kg bw. The treated skin was clipped and exposed under occlusive dressing for 24 hours.

No mortality occurred. Accordingly, the dermal LD_{50} was found to be greater than 2000 mg/kg bw for rats.

Rat, dermal: LD_{50} > 2,000 mg/kg bw

No signs of systemic toxicity were observed in the animals during the 14 day observation period. Initially slight dermal irritation was observed. The expected body weight gain was observed in the course of the study. No abnormalities were noted at necropsy of animals sacrificed at the end of the study.
According to the EU and GHS classification criteria, no classification is warranted as to acute dermal toxicity for metiram.

(BASF DocID 1979/032)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	metiram technical agent with 2% ETU
Description:	not reported
Compound #:	79/443
Purity:	not indicated in the report
Stability of test compound:	not indicated in the report
2. Vehicle:	water

3. **Test animals:**

Species:	Rat
Strain:	Sprague-Dawley
Sex:	males and females
Age:	not reported
Weight at dosing (mean):	female: 160 g; male: 220 g
Source:	[redacted]
Acclimation period:	not reported
Diet:	ad libitum Herilan MRH-Kraftfutter (H. Eggersmann, Rinteln/Weser, Germany)
Water:	tap water ad libitum
Housing:	not reported

Environmental conditions:

Temperature:	not reported
Humidity:	not reported
Air changes:	not reported
Photo period:	not reported

B. STUDY DESIGN AND METHODS

1. **Dates of experimental work:** September 1979

2. **Animal assignment and treatment:**

Fifteen to twenty four hours before treatment the fur was clipped from the dorsal and lateral parts of the trunk. Ten rats (5 per sex per group) received a single dose of 2000 mg/kg bw of test substance in 50% aqueous suspension to the clipped epidermis. Test substance was covered with an occlusive dressing (aluminum foil secured in positions with adhesive tape) for 24 hours. Afterwards the occlusive dressing was removed and possible residual substance was removed with warm water or a mixture of water and Lutrol and the skin was dried with cellulose. The application area was about 42 cm² which corresponds to at least 10% of the body surface. The observation period lasted 14 days. Clinical signs and symptoms and local irritations were recorded. After the observation period a necropsy of all animals with gross pathology followed.

II. RESULTS AND DISCUSSION

A. MORTALITY

No deaths occurred after dermal administration of 2000 mg/kg bw of the test substance.

B. CLINICAL OBSERVATIONS

No systemic clinical signs were observed during clinical examination. Initially slight dermal irritation was found which was fully reversible until the end of the observation period.

C. BODY WEIGHT
No body weight was recorded during the study.

D. NECROPSY

No macroscopic anomalies were noted in the organs examined after dermal treatment.

III. CONCLUSION

Under the experimental conditions of this study the dermal LD_{50} of metiram in rats was determined to be greater than 2000 mg/kg bw for males and females. Based on the results of this study, metiram does not warrant classification as to acute dermal toxicity according to EU criteria (28th ATP of Council Directive 67/548/EEC by Commission Directive 2001/59/EC / Council Regulation 1272/2008/EC) and according to the OECD Globally Harmonized System (GHS, revision 2, 2007)

CA 5.2.3 Inhalation

The acute inhalation toxicity study of metiram was evaluated and peer reviewed during the previous Annex I inclusion process. The study is scientifically valid, however the studies have been conducted before the release of study guidelines and are without GLP according to the usual practice in those days. Metiram has a very low acute inhalation toxicity.

Report:	*CA 5.2.3/1*
	[redacted] 1980a
	Report on the determination of the acute inhalation toxicity LC50 of Metiram techn. with 2% ETU as a dust aerosol after 4-hour exposure in Sprague-Dawley rats
	1983/064
Guidelines:	*none*
GLP:	*no*

Executive Summary

In an acute inhalation toxicity study, groups of 10 male and 10 female Sprague-Dawley rats were exposed to metiram as a dust aerosol (compound number: 79/443) at a maximum achievable concentration of 5.7 mg/L for 4 hours. The animals were observed for 14 days after exposure.

No mortality occurred at the tested concentration. Accordingly, the acute inhalation LC_{50} for aerosol dust inhalation exposure of metriam was determined to be

LC_{50} (male and female rats) > 5.7 mg/L

Clinical signs of toxicity consisted of accelerated respiration during the test. After treatment no abnormalities were detected. The body weight gain of the test group was lower than of the control animals but increased throughout the study period. No gross pathological abnormalities were noted during the necropsy at termination of the post exposure observation period.
Cascade impactor measurements resulted in particle size distributions with mass median aerodynamic diameters (MMADs) of 3.44 µm.

According to the EU and GHS classification criteria, no classification is warranted as to acute inhalation toxicity for metiram.

(DocID 1983/064)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Metiram technical with 2% ethylene thiourea (ETU)
Description:	not reported
compound #:	79/443
Purity/content:	not reported
Stability of test compound:	not reported

2. Vehicle: none, the test substance was doses unchanged

3. Test animals:

Species:	Rat
Strain:	Sprague-Dawley (Caw-Ico-Wiga)
Sex:	male and female
Age:	not reported
Weight at dosing (mean):	185 ± 15 g
Source:	
Acclimation period:	not reported
Diet:	Herilan MRH feed (H. Eggersmann KG, Rinteln/Weser, Germany), ad libitum
Water:	Tap water, ad libitum
Housing:	no reported

Environmental conditions:

Temperature:	20 - 24°C
Humidity:	50 - 60%
Air changes:	Fully air-conditioned rooms; number of air changes not indicated in the report
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: September 1980

2. Animal assignment and treatment:
For determination of the acute inhalation toxicity (single head-nose inhalation, 4-hour-exposure) groups of ten male and ten female rats were exposed to 5.7 mg/L of the test substance metiram that was dosed unchanged as a dust aerosol. After exposure, animals were observed for at least 14 days.
Individual body weights were recorded shortly before exposure (day 0), after 7 days and at the end of the observation period.
Clinical observations and mortalities were recorded each day.
At the end of the observation period the surviving animals were sacrificed by CO_2-inhalation and were subjected to gross-pathological examination.

3. Statistics/calculations:
For results of the type "LC_{50} greater than", "LC_{50} approx.", or "LC_{50} smaller than", the binomial test was used for statistical evaluation. [Witting, H.: Mathematische Statistik 1974, pp. 32-35]

4. Generation of the test atmosphere and exposure:
The dust aerosol was generated with a vibration dust metering equipment. By means of a dust generator the substance to be tested was generated into a dust aerosol, which was passed into the inhalation system.
A head-nose inhalation system was used. The animals were restrained in glass tubes and their snouts projected into the inhalation system.
By means of an exhaust air system the pressure ratios in the inhalation chamber were adjusted in such a way that the amount of fresh air was about 10% higher (excess pressure). This ensured that no fresh air from outside reached the animals noses.
The animals were exposed to the inhalation atmosphere for 4 hours.

5. Analytical investigation:
The nominal concentration was calculated from the amount of substance dosed and the supply air flow.

The concentration of the test substance in the inhalation atmosphere was determined via gravimetrical measurement. Preweighed filters were placed into the filtration equipment. By means of a vacuum pump (Millipore) metered volumes of the dust were drawn through the filter. For each sample the dust concentration in mg/L was calculated from the difference between the preweight of the filter and the weight of the filter after sampling with reference to the sample volume of the inhalation atmospheres.

- Sampling devices: Millipore vacuum pump
- Sampling position: immediately adjacent to the animals' noses
- Sampling flow: 3 L/min
- Sampling velocity: 1.25 m/s
- Sample volume: up to 1 L

6. Particle Size Analysis:
The particle size analysis was done using a Stack Sampler Mark III (Andersen) and a vacuum pump (Millipore). The sampling probe had an internal diameter of 6.9 mm.
Before sampling, the impactor stages were assembled with pre-weighed glass-fiber and equipped with a backup particle filter. The impactor was connected to the vacuum pump and a sample (9 L) was removed.
After sampling the impactor was taken apart. The collecting discs and the backup particle filter were re-weighed. The amount of material adsorbed to the walls of the impactor and in the sampling probe (wall losses) was also determined quantitatively.

II. RESULTS AND DISCUSSION

A. MORTALITY

No lethality occurred at the tested concentration of 5.7 mg/L during the study period of 14 days. Therefore, the study satisfies the criteria of a limit test.
Based on the absence of mortality the following LC_{50} value was determined:

LC_{50} (both sexes combined):	> 5.7 mg/L
LC_{50} (male rats)	> 5.7 mg/L
LC_{50} (female rats)	> 5.7 mg/L

The statistically reliability is 99%.

B. CLINICAL OBSERVATIONS

Clinical signs of toxicity comprised accelerated respiration during the test (heads conglutinated by the test substance after the test). Otherwise no abnormalities were observed. After exposure no abnormalities were detected in any of the animals.

C. BODY WEIGHT

The mean body weights of the animals increased throughout the study period.

Table 5.2.3-1: Relative body weight gain [g]

Mean body weight gain [g]	Before the study		After 7 days		After 14 days	
	male	female	male	female	male	female
Test group	0	0	+31	+9	+79	+28
Control group	0	0	+47	+18	+90	+32

The body weight gain of the test group was lower than that of the control group.

D. NECROPSY

No gross pathological abnormalities were detected in the animals that underwent necropsy at termination of the study.

E. ANALYTICAL MEASUREMENTS

The result of the analytical concentration measurements are presented in Table 5.2.3-2.

Table 5.2.3-2: Measurement of the analytical concentration

Mean concentration (mg/L)	Standard deviation	Nominal concentration (mg/L)
5.7	0.17	20.3

The measurements of particle-size distribution revealed mass median aerodynamic diameters (MMAD) in the respirable range of 3.44 µm with geometric standard deviations of 2.4 µm.

III. CONCLUSION

Under the conditions of this study the 4 hour inhalation LC_{50} of metiram for male and female rats was estimated to be > 5.7 mg/L. Based on the results of this study, metiram does not warrant classification as to acute inhalation toxicity according to EU criteria (28th ATP of Council Directive 67/548/EEC by Commission Directive 2001/59/EC / Council Regulation 1272/2008/EC) and according to the OECD Globally Harmonized System (GHS, revision 2, 2007).

CA 5.2.4 Skin irritation

The skin irritation study of metiram was evaluated and peer reviewed during the previous Annex I inclusion process. Metiram has no skin irritation potential.

Report:	*CA 5.2.4/1* ███████ *2002c* *Metiram technical (synonym: Metiram TC or BAS 222 29 F) - Acute dermal irritation / corrosion in rabbits* *2002/1005292*
Guidelines:	*OECD 404, EEC 92/69 B 4, EPA 870.2500, JMAFF No 12 Nosan No 8147*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a primary dermal irritation study the skin irritation/corrosion potential of metiram (batch: 2001-1, purity: 88.6%) was tested. The clipped intact skin of three White New Zealand rabbits was exposed to 0.5 g of the test-substance for four hours under semiocclusive dressing. Observation of local reactions was performed 1, 24, 48 and 72 hours after removal of the patch. No erythema or edema of intact skin was noted after 24 to 72 hours in the 3 rabbits treated with metiram.
Based on the findings of this study metiram does not show a skin irritation potential and thus has not to be classified for skin irritation potential according to EU criteria (28th ATP of Council Directive 67/548/EEC by Commission Directive 2001/59/EC / Council Regulation 1272/2008/EC) and according to the OECD Globally Harmonized System (GHS, revision 2, 2007).

(BASF DocID 2002/1005292)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Metiram technical (synonym: Metiram TC or BAS 222 29 F)
Description:	powder, light yellow
Lot/Batch #:	2001-1
Purity:	88.6%
Stability of test compound:	The stability of the test substance was guaranteed for the duration of the study. The homogeneity of the test substance was confirmed by analysis.
2. Vehicle:	test substance was moistened with water

3. Test animals:

Species:	Rabbit
Strain:	New Zealand White A1077 (SPF)
Sex:	1 male and 2 females
Age:	about 8 months
Weight at dosing (mean):	3.67 - 3.93 kg
Source:	[redacted]
Acclimation period:	at least 5 days
Diet:	Kliba-Labordiaet, Provimi Kliba SA, Kaiseraugst, Switzerland, about 130 g/animal per day
Water:	tap water ad libitum
Housing:	Single housing in stainless steel wire mesh cages with grating, floor area: 3000 cm²)

Environmental conditions:

Temperature:	20 – 24°C
Humidity:	30 – 70%
Air changes:	no data available (fully air-conditioned)
Photo period:	12-hour light-dark cycle (06:00 – 18:00, 18:00 – 06:00)

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 25-Feb-2002 (day of application)

2. In-vitro pre-test: No *in vitro* pre-test was conducted.

3. Animal assignment and treatment:

The potential of metiram to cause acute dermal irritation or corrosion was assessed by a single topical application of the unchanged test substance (moistened with water) to the clipped intact dorsal skin of three New Zealand White rabbits. A dose of 500 mg Metiram, moistened with doubly-distilled water, was applied to the test site (dorsolateral part of the trunk). The test substance was applied in a single dose to the intact untreated skin. The test patch was secured in position with a semiocclusive dressing. The patch was held in place for the duration of 4 hours. The test substance was removed at the end of the exposure period with Lutrol® and Lutrol® / water (1:1). Observation of local reactions was performed 1, 24, 48 and 72 hours after removal of the patch.

II. RESULTS AND DISCUSSION

No cutaneous reactions were observed during the study. Mean scores over 24, 48 and 72 hours for each animal were 0.0, 0.0 and 0.0 for erythema and 0.0, 0.0 and 0.0 for edema.

III. CONCLUSION

Based on the findings of this study metiram does not show a skin irritation potential under the test conditions chosen. No classification of BAS 222 29 F for skin irritation potential according to EU criteria (28th ATP of Council Directive 67/548/EEC by Commission Directive 2001/59/EC / Council Regulation 1272/2008/EC) and according to the OECD Globally Harmonized System (GHS, revision 2, 2007) is needed.

CA 5.2.5 Eye irritation

The eye irritation study of metiram was evaluated and peer reviewed during the previous Annex I inclusion process. Metiram has no eye irritation potential.

Report:	*CA 5.2.5/1* *[redacted] 2002d* *Metiram technical (synonym: Metiram TC or BAS 222 29 F): Acute eye irritation in rabbits* *2002/1005291*
Guidelines:	*OECD 405, EPA 870.2400, EEC 92/69 B 5, JMAFF No 12 Nosan No 8147*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Report:	*CA 5.2.5/2* *[redacted] 2002b* *Amendment No. 1: Metiram technical (synonym: Metiram TC or BAS 222 29 F) - Acute eye irritation in rabbits* *2002/1006988*
Guidelines:	*OECD 405, EEC 92/69 B 5, EPA 870.2400, JMAFF No 12 Nosan No 8147*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Note: The report amendment (CA 5.2.5/2) corrects some clerical errors, which do not affect the outcome or interpretation of the study.

Executive Summary

In an eye irritation study, the eye irritation/corrosion potential of metiram (batch: 2001-1, purity: 88.6%) was determined by instillation of about 40 mg of the test substance into the conjunctival sac of the right eye of three New Zealand White rabbits. After about 24 hours after application the eye was rinsed with tap water.

The ocular reactions were assessed approximately 1, 24, 48 and 72 hours after the administration of the test substance.

Slight to moderated conjunctival redness, slight or marked conjunctival chemosis and slight discharge were observed during the course of the study. In addition injected scleral vessels in a circumscribed area were observed. The ocular reactions were reversible in all animals within 72 hours after application. Eye irritation average scores (24 to 72 hours) were 0.0 for corneal opacity and iris, 0.9 for conjunctival redness and 0.4 for chemosis.

Considering the described ocular reactions as well as the average score for irritation, metiram does not give indication of an irritant property to the eye under the test conditions chosen. Therefore no classification as to eye irritation is warranted according to the EU (28th ATP of Council Directive 67/548/EEC by Commission Directive 2001/59/EC / Council Regulation 1272/2008/EC) and according to the OECD Globally Harmonized System (GHS, revision 2, 2007).

(BASF DocID 2002/1005291)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: Metiram technical (synonym: Metiram TC or BAS 222 29 F)

Description: powder, light yellow
Lot/Batch #: 2001-1
Purity: 88.6%
Stability of test compound: The stability of the test substance was guaranteed for the duration of the study. The homogeneity of the test substance was confirmed by analysis.

2. Vehicle: test substance was moistened with water

3. Test animals:

Species: Rabbit
Strain: New Zealand White A1077 INRA (SPF)
Sex: 1 male and 2 females
Age: about 8 months
Weight at dosing (mean): 3.22 - 3.35 kg
Source: [redacted]
Acclimation period: at least 5 days
Diet: Kliba-Labordiaet, Provimi Kliba SA, Kaiseraugst, Switzerland, about 130 g/animal per day
Water: tap water ad libitum
Housing: Single housing in stainless steel wire mesh cages with grating, floor area: 3000 cm²)

Environmental conditions:

Temperature: 20 – 24°C
Humidity: 30 – 70%
Air changes: no data available (fully air-conditioned)
Photo period: 12-hour light-dark cycle (06:00 – 18:00, 18:00 – 06:00)

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 25-Feb-2002 (day of application)

2. In-vitro pre-test: No *in vitro* pre-test was conducted

3. Animal assignment and treatment:
The potential of Metiram to cause acute eye irritation/corrosion was assessed by instillation of 0.1 mL bulk volume which corresponds to about 40 mg of the comminuted test substance into the conjunctival sac of the right eye. The left eye, which remained untreated, served as the negative control. The substance was washed out with tap water about 24 hours after application (before 24 hour reading). As the irritant potential of the test substance was unknown one animal has been tested in a first step. As no severe findings have been noted in the animal, another two animals were tested. The results of the three animals were pooled for evaluation.

The ocular reactions were assessed approximately 1, 24, 48 and 72 hours after the administration of the test substance.

II. RESULTS AND DISCUSSION

Slight to moderate conjuctival redness (grade 1-2), slight conjunctival chemosis (grade 1) and slight discharge (grade 1) were observed in almost all animals 1 hour after application. 24 hours after application conjunctival redness and chemosis partly incrased but were decreased again after 48 hours. Moreover, infected scleral vessels in a circumscribed area were observed in two animals 24 and 48 hours after application. The ocular reactions were reversible in all animals within 72 hours after application (see Table 5.2.5-1). Mean scores calculated for each animal over 24, 48 and 72 hours were 0.0 in each animal for corneal opacity and for iris lesions, 1.0, 0.7 and 1.0 for redness of the conjunctiva and 1.3, 0.0 and 0.0 for chemosis.

Table 5.2.5-1: Individual and mean eye irritation scores after ocular application of metiram

Readings	Animal	Cornea	Iris	Conjunctiva			Additional finding
		Opacity		Redness	Chemosis	Discharge	
1 h	01 (♂)	0	0	1	0	1	
	02 (♀)	0	0	2	1	1	
	03 (♀)	0	0	1	1	1	
24 h	01	0	0	2	3	0	48
	02	0	0	1	0	0	48
	03	0	0	2	0	0	
48 h	01	0	0	1	1	0	48
	02	0	0	1	0	0	48
	03	0	0	1	0	0	
72 h	01	0	0	0	0	0	
	02	0	0	0	0	0	
	03	0	0	0	0	0	
Mean 24 – 72 h	01	0.0	0.0	1.0	1.3		
	02	0.0	0.0	0.7	0.0		
	03	0.0	0.0	1.0	0.0		
Mean		**0.0**	**0.0**	**0.9**	**0.4**		

48: Scleral vessels incjected, circumscribed area

III. CONCLUSION

Based on the findings of this study metiram does not have an eye irritation potential under the test conditions chosen. Consequently, no classification as to eye irritation is required according to the EU (28th ATP of Council Directive 67/548/EEC by Commission Directive 2001/59/EC / Council Regulation 1272/2008/EC) and according to the OECD Globally Harmonized System (GHS, revision 2, 2007).

CA 5.2.6 Skin sensitisation

The skin sensitisation study (Maximisation Test) of metiram was evaluated and peer reviewed during the previous Annex I inclusion process. The study is scientifically valid although in the study report no guideline is reported. But the study is performed similar to OECD guideline 406. Metiram is sensitizing to the skin.

Report:	*CA 5.2.6/1*
	[redacted] 1981b
	Report on the study of the sensitizing effect of Metiram techn. in the guinea pig - Maximization test
	1982/068
Guidelines:	*none*
GLP:	*yes*

Executive Summary

For the determination of potential sensitizing properties of the test substance metiram (test substance #: 81/171) a Maximization test based on the method of Magnusson and Kligman was conducted using a control and a treated group of 6 and 12 female Dunkin Hartley guinea pigs, respectively.

The test-substance concentrations for the main test were selected based on the results of the pretests. The intradermal induction was performed with a 10% test substance preparation in paraffin oil or a 5% test substance preparation in Freund's complete adjuvant / water (1:1). The epicutaneous induction was conducted with a 50% aqueous test substance preparation. For the challenge a 50% test substance preparation in distilled water was chosen.

The intradermal induction caused distinct erythema and definite edema in all test group animals. After the epicutaneous induction, crusts could be observed in all test group animals. After the challenge, slight to severe erythema and edema was noted in all 12 test group animals 24 hours after removal of the patch which in only one animal had completely subsided after 48 and 72 hours. Three of six control animals displayed slight erythema and one of them additionally showed slight edema 24 hours after removal of the patch. After 48 hours only one of these animals had slight erythema. In two of six animals of the control group the second challenge, which was also carried out with a 50% aqueous suspension, led to slight erythema which was not reversible within 72 hours. Only one of these two animals was identical with one of the three control animals in which a finding was obtained after the first challenge. The second control animal even showed rather distinct erythema and slight edema 24 hours after the second challenge, but after 48 hours only slight erythema was still observed.
Eight of 11 animals of the test group exhibited slight to distinct erythema 48 hours after the second challenge, and six of 11 animals additionally had slight edema. After 72 hours five of 11 animals showed scaling.

Based on the results of the Maximization Test and applying the evaluation criteria, it was concluded that metiram does have a skin sensitizing effect in guinea pigs. Thus, classification of metiram as a skin sensitizer with R43 and Skin Sensitizing Category 1 (H317) is required according to EU criteria (28th ATP of Council Directive 67/548/EEC by Commission Directive 2001/59/EC /Council Regulation 1272/2008/EC) as well as according to the OECD Globally Harmonized System (GHS, revision 2, 2007).

(DocID1982/068)

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** Metiram techn.

Description:	Not indicated in the report
Lot/Batch #:	81/171
Purity/content:	not reported
Stability of test compound:	according to the manufacturer at least until June 1983.

2. **Vehicle and/or positive control:** water

3. **Test animals:**

Species:	Guinea pigs
Strain:	Pirbright White, Dunkin Hartley, HOE DHPK [SPF-LAC] BÖ
Sex:	female
Age:	not reported
Weight on day 0:	235 - 295 g
Source:	[redacted]
Acclimation period:	at least 7 days before application
Diet:	Ssniff GK 4 mm, standard diet for rabbits and guinea pigs (Ssaniff-Versuchstierdiäten GmbH, Soest, Germany)
Water:	water ad libitum
Housing:	groups of 6 animals housed in Makrolon cages (type IV)

Environmental conditions:

Temperature:	20 - 24°C
Humidity:	30 - 70%
Air changes:	not indicated in the report
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 15-July-1981 (intradermal induction) to 10-August-1981 (second challenge)

2. Animal assignment and treatment:
The skin sensitizing potential of metiram was assessed using the Guinea Pig Maximization Test. This test consists of a pretest for the determination of suitable induction and challenge concentrations and the Maximization test itself.
For this, female Guinea pigs were distributed to groups according to the randomization instructions of „Salfi, R.: A Long-Period Random Number Generator with Application to Permutation, Compstat 1974, pp. 28 – 35".
For the intradermal and epicutaneous occlusive route of application, animal fur was clipped about 3 to 5 hours before each test-substance application at the appropriate treatment sites.
Individual body weights were determined on study day 0 and on the last day of observation. Mortality was checked on each working days. Evaluations of the skin reactions were performed according to the grading scale of Magnusson and Kligman (The Identification of Contact Allergens by Animal Assay. The Guinea Pig Maximization Test. J. Invest. Dermatol. 52, 268 - 276 (1969)).

3. Preliminary tests
Four guinea pigs were treated percutaneously under occlusive conditions with 5, 10, 25, and 50% aqueous formulations of the test substance. The evaluation of the skin reactions was performed 24 h after application.

4. Main study - Induction
The intradermal induction consisted of 6 intradermal injections in groups of two into the shoulder region of the animals according to the following scheme:

Intradermal injections:
A) front row: 2 injections each of 0.1 mL Freund's complete adjuvant without the test substance emulsified with water in a ratio of 1:1 (w/w)
B) middle row: 2 injections each of 0.1 mL of a test substance preparation in an appropriate vehicle at the selected concentration
C) back row: 2 injections each of 0.1 mL Freund's complete adjuvant / water (1:1 w/w) with test substance at the selected concentration.

Control animals received the same injections as described above but without test substance.
Skin reactions were assessed 24 hours after injection. The test and control groups consisted of 12 and 6 females, respectively.

Epidermal induction was carried out one week after the intradermal induction. For this 0.3 g of the test substance preparation by means of a 2x4 cm filter paper strip was applied to each animal under occlusive conditions to the shoulder region previously used for intradermal induction. The exposure duration lasted 48 hours. The readings were performed directly after removal of the patch. Control animals were not treated since the formulating agent used, water, was not expected to influence the result of the study.

5. Challenge
The test concentration should be a nonirritant dose. The first challenge was carried out 14 days after epidermal induction. The second challenge about one week later. For this 0.15 g of the test substance preparation was applied on a 2x2 cm filter paper strip under occlusive conditions to the intact flank of the animals for 24 hours. Skin reactions were determined 24, 48 and 72 hours after removal of the patches.

6. Positive controls:
A positive control (reliability check) with a known sensitizer was not performed in this study.

7. Evaluation of results:
The number of animals with skin findings at 48 hours after removal of the patch was taken into account for the determination of the sensitization rate.
The evaluation "sensitizing" results if at least 30% of the test animals exhibit skin reactions in this adjuvant test.
The evaluation is based on the criteria of the Commission Directive 67/548/EEC and the OECD Harmonized Integrated Classification System that were in place on the date of report signature.

8. Statistics:
Not performed in this study

II. RESULTS AND DISCUSSION

A. PRE-TEST

In the pretests with four guinea pigs 5, 10, 25 and 50% aqueous formulations of the test substance were applied percutaneously. No skin irritation was observed at any of the concentrations tested.

As no skin findings were observed after dermal administration of a 50% test substance preparation in water this concentration was chosen for the challenge.

Solubility: 10% formulation in paraffin oil and 5% formulations in adjuvant/water (1:1) could be injected with a syringe.

B. INDUCTION REACTIONS

Intradermal injection of

- front row: 2x0.1 mL of Freund's complete adjuvant / water (1:1) in control and treated animals,
- middle row: 2x0.1 mL of 10% metiram preparation in paraffin oil
- back row: 2x0.1 mL of 5% test substance formulation in Freund's complete adjuvant / water

caused moderate erythema and swelling (grade 2).

Table 5.2.6-1: Skin irritation scores 24 hours after intradermal injection - Main test

Control Animals (1st and 2nd group)				
Skin irritation score	Application site	A) Freund's adjuvant / water (1 : 1)	B) paraffin oil	C) Freund's complete adjuvant / water (1 : 1)
Erythema score 2	left	6/6	6/6	6/6
	right	6/6	6/6	6/6
Edema score 2	left	6/6	0/6	6/6
	right	6/6	0/6	6/6
Treated Animals				
Skin irritation score	Application site	A) Freund's adjuvant / water (1 : 1)	B) 10% metiram in paraffin oil	C) 5% metiram in A)
Erythema and edema score 2	left	12/12	12/12	12/12
	right	12/12	12/12	12/12

x/y = number of positive reactions / number of animals in the test
Grading: 2: well defined erythema or slight edema (edges of area well defined by definite raising)

The epicutaneous induction with a 50% aqueous test substance led to incrustation, partially open (caused by the intradermal induction) in addition to moderate and confluent erythema and swelling in all test group animals.

C. CHALLENGE

24 hours after the first challenge with a 50% aqueous suspension slight erythema was observed in three of six animals of the control group, and one of these three animals additionally showed slight edema. After 48 hours only one of these animals had slight erythema. All 12 animals of the test group exhibited slight to severe erythema and edema, which in only one animal had completely subsided after 48 and 72 hours.
In two of six animals of the control group the second challenge, which was also carried out with a 50% aqueous suspension, led to slight erythema which was not reversible within 72 hours. Only one of these two animals was identical with one of the three control animals in which a finding was obtained after the first challenge. The second control animal even showed rather distinct erythema and slight edema 24 hours after the second challenge, but after 48 hours only slight erythema was still observed.
Eight of 11 animals of the test group exhibited slight to distinct erythema 48 hours after the second challenge, and six of 11 animals additionally had slight edema. After 72 hours five of 11 animals showed scaling.

Table 5.2.6-2: 1st Challenge skin reaction scores 24, 48 and 72 hours after intradermal injection - Main test

Skin findings	1st Control group			Test group		
	24 h	48 h	72 h	24 h	48 h	72 h
Grade 0	3/6	5/6	5/6	0/12	1/12	1/12
Grade 1	3/6	1/6	1/6	6/12	3/12	7/12
Grade 2	-	-	-	5/12	7/12	3/12
Grade 3	-	-	-	1/12	1/12	1/12

x/y: number of animals with findings / number of animals tested

For the 2nd challenge additionally a 2nd control group was treated. This control group showed no animals with skin reactions. The skin reaction observed for the 1st control group and the test group are listed in Table 5.2.6-3.

Table 5.2.6-3: 2nd Challenge skin reaction scores 24, 48 and 72 hours after intradermal injection - Main test

Skin findings	1st Control group			Test group		
	24 h	48 h	72 h	24 h	48 h	72 h
Grade 0	4/6	4/6	4/6	1/11	3/11	3/11
Grade 1	1/6	2/6	2/6	2/11	4/11	6/11
Grade 2	1/6	0/6	0/6	8/11	4/11	2/11
Grade 3	-	-	-	-	-	-

x/y: number of animals with findings / number of animals tested

Summarizing these results metiram induces 92% positive reaction after 1st challenge and 73% positive reaction after second challenge at 48 hour reading point.

D. OBSERVATIONS

No clinical signs of systemic toxicity was observed. One animal died during the second challenge.

E. BODY WEIGHTS

The expected body weight gain was generally observed in the course of the study.

III. CONCLUSION

Based on the results of this study and applying the evaluation criteria, it was concluded that metiram does have clear skin sensitizing properties. Thus, classification of metiram as a skin sensitizer (R43, Skin Sens. Cat 1, H317) is required according to EU criteria (28th ATP of Council Directive 67/548/EEC by Commission Directive 2001/59/EC / Council Regulation 1272/2008/EC) as well as according to the OECD Globally Harmonized System (GHS, revision 2, 2007).

CA 5.2.7 Phototoxicity

Report: CA 5.2.7/1
Cetto V.,Landsiedel R., 2013a
BAS 222 29 F (Metiram TK) - In vitro 3T3 NRU phototoxicity test
2013/1358979

Guidelines: OECD 432 (2004) In vitro 3T3 NRU Phototoxicity test, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.41 No. L 142

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

Metiram (batch 300015; purity 91.5%) was tested for its ability to induce phototoxic effects in Balb/c 3T3 cells in vitro. The photo-cytotoxicity was estimated by the means of the Neutral Red Uptake (NRU) method. A single experiment was carried out with and without irradiation with an UVA source. Vehicle and positive controls were included and clearly fulfilled the acceptance criteria.

Based on an initial range-finding phototoxicity test for the determination of the experimental concentrations, the following concentrations were tested in this study with and without UVA irradiation: 0, 0.5, 1.0, 2.2, 4.6, 10.0, 21.5, 46.4 and 100.0 µg/mL and 0, 2.2, 4.6, 10.0, 21.5, 46.4, 100.0, 215.4 and 464.2 µg/mL, respectively.

Precipitation was only seen at 464.2 µg/mL without irradiation. In the absence and the presence of UVA irradiation in the highest concentrations applied cytotoxicity was noted and EC_{50} values were calculated. Based on the results of the present study, the test substance was predicted to have a phototoxic potential as indicated by Photo-Irritancy-Factor (PIF) values of 6.2. The threshold for a negative response is ≤ 2. The positive control chlorpromazine led to the expected increased cytotoxicity with UVA irradiation as indicated by PIF values of 41.3.
Thus, under the experimental conditions of this study, Metiram is considered to be a phototoxic substance in the in vitro 3T3 NRU Phototoxicity Test using Balb/c 3T3 cells.

(DocID 2013/1358979)

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material** BAS 222 29 F (Metiram TK)
   Description: Solid, beige
   Lot/Batch #: 300015
   Purity: 91.5%
   Stability of test compound: Expiry date: 30 Oct 2013
   Solvent used: Dimethylsulfoxide (DMSO)

2. Control Materials:

Vehicle control: DMSO 1% (v/v) in PBS

Positive control compounds: Chlorpromazine (CPU) was dissolved in DMSO; 8 concentrations tested - 1.9 to 180 µg/mL without radiation, 0.03 to 3.2 µg/mL with radiation

3. Test organisms: BaIb/c 3T3, clone A31: fibroblast cell line isolated from the muscle tissue of a mouse embryo. The Balb/c 3T3 cell line which was used in this experiment was obtained from the "European Collection of Cell Cultures" Salisbury, Wiltshire SP4 OJG, UK on 09 Aug 2006 and is stored at -196°C (liquid nitrogen).

4. Culture media and reagents:

Culture medium: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with
- 10% (v/v) newborn calf serum (NCBS)
- 4 mM L-glutamine
- 100 IU penicillin
- 100 µg/mL streptomycin

Neutral Red solution:
- 0.4 g Neutral Red powder (NR; Sigma N4638)
- 100 mL deionized water

Neutral Red medium:
- 1 mL Neutral Red solution
- 79 mL culture medium (DMEM incl. supplements) (incubated overnight at 37°C with 5% CO_2 and filtered with a 0.22 µm filter prior to use)

Other solutions and reagents:
- phosphate buffered saline (PBS) without Ca/Mg
- trypsin/EDTA solution (0.05%; 0.02%)
- Neutral Red desorb solution (1 mL acetic acid, 50 mL ethanol, 49 mL deionized water)

5. Irradiation source: The Sol 500 solar simulator (Dr. Hönle AG, 82166 Gräfelfing, Germany) used with filter H1 produced wavelength > 320 nm. The exposure rates were determined with UV-meter RM-21 (Dr. Gröbel GmbH, 76275 Ettlingen, Germany).

6. Test concentrations:

Pretest:	Up to 1000 µg/mL with and without irradiation. The EC_{50} values determined were 34.1 µg/mL without and 6.8 µg/mL with UVA irradiation (no detailed data provided in the report).
Main NRU test:	Based on the results of the pretest the following concentrations were used in the main study: Without UVA: 0, 2.2, 4.6, 10.0, 21.5, 46.4, 100.0, 215.4 and 464.2 µg/mL With UVA: 0, 0.5, 1.0, 2.2, 4.6, 10.0, 21.5, 46.4 and 100.0 µg/mL

B. TEST PERFORMANCE:

1. Dates of experimental work: 17-Sep-2013 to 19-Sep-2013

2. Treatment and NRU Phototoxicity Test:
Two 96 well-plates per substance (test substance or positive control) were used for cultivation of cells (1.5 x 10^5 cells/well). After an attachment period of about 24 hours the cells were washed once with 100 µL PBS and subsequently treated with the respective substance (8 concentrations each with 6 replicates of the test substance or the positive control) and the vehicle control. After pre-incubation for 1 hour in the dark (5% (v/v) CO_2, ≥90% humidity; 37°C) one 96 well-plate per substance was irradiated for 50 minutes with UVA (UV intensity underneath the lid 1.5 - 2.1 mW/cm^2 = 5 J/cm^2) whereas the respective reference plate was kept in the dark for the same period. Thereafter the test- respectively control-substance was removed and the cells washed at least once with 100 µL PBS. After replenishing the wells with culture medium the cells were incubated overnight under the conditions indicated above. The medium was removed 24 hours after the start of treatment and after washing with 100 µL PBS the wells were filled with 100 µL medium containing 50 µg/mL Neutral Red. Subsequently the plates were incubated for another 3 hours. Each step was performed under light protected conditions in the lab to prevent uncontrolled photo activation. Finally, the cells were washed again with 100 µL and the dye was extracted by 100 µL Neutral Red desorb solution. Cytotoxicity was determined by measuring the Neutral Red Uptake by means of a microplate reader (Perkin Elmer, Waltham, Massachusetts, US; Wallac 1420 multilabel counter) equipped with a 550 nm filter to read the absorption of the extracted dye. The absorption shows a linear relationship with the number of surviving cells.

3. Evaluation/Assessment

3.1 Cytotoxicity

The mean absorbance values obtained for each test group of every plate were used to calculate the percentage of cell viability relative to the respective vehicle control, which is arbitrarily set at 100%.

$$Viability^{\S}\ [\%] = \frac{\text{Absorbance}_{\text{mean}} \text{ of the test group}}{\text{Absorbance}_{\text{mean}} \text{ of the vehicle control}} \times 100$$

§ The authors of the study denominate the above quotient as 'cytotoxicity', which is strictly speaking not correct. Thus, in this summary the appropriate term 'viability' is used. This applies also to Table 5.2.7-1 to Table 5.2.7-2.

In case of cytotoxicity, an EC_{50} value (concentration at which the viability is reduced by 50% relative to the respective vehicle control) was calculated by a linear interpolation method (linear dose-response curve). Therefore two viability values were needed: one between 100% and 50% and one between 50% and 0%. From these two points the concentration that inhibits the Neutral Red uptake by 50% of the respective control was calculated.

For the assessment of the phototoxic potential of a compound two prediction models are currently available:

- The Photo-Irritancy-Factor (PIF) prediction model for substances which allow the comparison of two equi-effective concentrations (EC_{50}) in the concurrently performed experiments in the presence and absence of light. This model includes two special cases: Case 1 accounts for situations in which an EC_{50} can only be calculated in the presence of UVA irradiation. Case 2 accounts for situations where an EC_{50} cannot be calculated in absence and presence of irradiation. These special cases do not apply to this study. Even though described in the report these cases are not described in this summary.
- The Mean Photo Effect prediction model which is used if no EC_{50} was obtained in the absence and presence of UV light. This is not the case in this study. Even though described in the report this prediction model is not described in this summary.

3.2 Photo-Irritancy-Factor (PIF)

For substances which induce a 50% cytotoxicity (EC_{50}) in the presence and absence of light the Photo-Irritancy-Factor (PIF) is calculated based on comparison of the EC_{50} values in the absence (-UVA) and presence (+UVA) of UVA irradiation.

$$PIF = \frac{EC50\ (-UVA)}{EC50\ (+UVA)}$$

resulting in the following classification rules:

PIF ≥5	phototoxic potential predicted
2 < PIF < 5:	probable phototoxic potential predicted
PIF ≤ 2:	no phototoxic potential predicted

3.3 Other parameters
pH:
The pH was measured at least for the two top doses and for the vehicle controls with and without irradiation.

Osmolarity:
Osmolarity was measured at least for the two top doses and for the vehicle controls with and without irradiation.

Solubility:
Test substance precipitation was checked immediately after treatment and at the end of treatment.

Cell morphology:
Test cultures of all test groups were examined microscopically before staining with NRU, which allows conclusions to be drawn about attachment of the cells.

4. Statistics:
No special statistical tests were performed.

5. Acceptance criteria:
The assay has to be considered valid if the following criteria are met:

- The vehicle control needs to fulfill the following criteria:
    - The mean OD_{550} value (with and without UVA irradiation) should be > 0.3.
    - Cell viability after irradiation should be at least 80% of the concurrent non-irradiated vehicle control.
    - The standard deviation of the mean values of both vehicle control rows should not exceed ± 15%.
- The positive control chlorpromazine needs to fulfill the following criteria:
    - the EC50 value should be in the ranges:
      With irradiation (+UVA): 0.1 - 2.0 µg/mL
      Without irradiation (-UVA): 7.0 - 90.0 µg/mL
    - and the PIF ≥ 6.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Osmolarity and pH values were not influenced by test substance treatment. In this study, in the absence of UVA irradiation precipitation in culture medium was observed at test substance concentration of 464.2.4 µg/mL.

B. CYTOTOXICITY OF THE TEST SUBSTANCE

After treatment with the test substance, clear cytotoxic effects indicated by Neutral Red absorbance values of below 50% of control were observed in the main experiments in the absence and the presence of UVA irradiation in at least the highest concentration (see Table 5.2.7-1).
Without UVA irradiation, there was a decrease in the cell number at concentrations ≥ 46.4 µg/mL (EC_{50}: 49.0 µg/mL). With UVA irradiation, there was a decrease in the cell number at concentrations ≥ 10.0 µg/mL (EC_{50}: 7.9 µg/mL). Cell morphology changes were observed at the end of exposure period at 46.4 µg/mL and above without irradiation and from about 10.0 µg/mL onward with irradiation.

Based on the EC_{50} values a PIF of 6.2 was calculated, indicating a phototoxic potential for Metiram.

Table 5.2.7-1: Mean relative viability of metiram with (+) and without (-) UVA irradiation in Balb 3T3 cells

Test group	UVA irradiation*	Precipitation**	Mean $OD_{corr.}$ ***	Relative viability [% of control]	
				Mean	SD
Vehicle control 1	-		0.425	-	10.0
Vehicle control 2	-		0.438	-	1.6
Vehicle mean	-		0.432	100.0	6.9
Metiram					
2.2 µg/mL	-	-	0.400	92.5	8.8
4.6 µg/mL	-	-	0.362	83.8	20.7
10.0 µg/mL	-	-	0.368	85.2	18.6
21.5 µg/mL	-	-	0.424	98.2	6.2
46.4 µg/mL	-	-	0.227	52.5	12.4
100.0 µg/mL	-	-	0.000	0.0	0.9
215.4 µg/mL	-	-	0.003	0.8	0.9
464.2 µg/mL	-	+	0.001	0.3	0.7
Vehicle control 1	+		0.334	-	8.9
Vehicle control 2	+		0.372	-	12.0
Vehicle mean	+		0.353	100.0	11.7
Metiram					
0.5 µg/mL	+	-	0.318	90.1	5.2
1.0 µg/mL	+	-	0.357	101.2	4.8
2.2 µg/mL	+	-	0.367	104.0	8.3
4.6 µg/mL	+	-	0.348	98.6	13.3
10.0 µg/mL	+	-	0.082	23.2	10.9
21.5 µg/mL	+	-	-0.002	-0.7	0.5
46.4 µg/mL	+	-	0.000	0.1	1.0
100.0 µg/mL	+	-	0.001	0.3	0.9

*: Irradiation with Sol 500 solar simulator for 50 minutes (approx. 5 J/cm²)
**: Precipitation in PBS at the end of exposure period
***: Mean OD corrected: mean absorbance (test group) minus mean absorbance (blank)

C. CYTOTOXICITY OF THE POSITIVE CONTROL

After treatment with the positive control chlorpromazine clear cytotoxic effects indicated by Neutral Red absorbance values of below 50% of control were observed in the absence and the presence of UVA irradiation (see Table 5.2.7-2).
In the experiment without UVA irradiation, there was a decrease in viability at ≥ 30.0 µg/mL (EC_{50}: 23.6 µg/mL). With UVA irradiation, there was a decrease in viability at ≥ 0.8 µg/mL (EC_{50}: 0.6 µg/mL). Cell morphology was distinctively changed in both experiments at ≥ 30 µg/mL and ≥ 0.8 µg/mL without and with UVA irradiation.

Based on the EC_{50} values a PIF of 41.3 was obtained, indicating a strong phototoxic potential, thus confirming the sensitivity of the test system.

Table 5.2.7-2: Mean relative viability of Chlorpromazine with (+) and without (-) UVA irradiation in Balb/c 3T3 cells

Test group	UVA irradiation	Mean OD *	Mean $OD_{corr.}$ **	Relative viability [% of control]	
				Mean	SD
Blank	-	0.037	-	-	-
Vehicle control 1	-	0.367	0.330	-	6.8
Vehicle control 2	-	0.428	0.391	-	6.9
Vehicle mean	-	0.398	0.361	100.0	11.0
Chlorpromazine					
1.9 µg/mL	-	0.393	0.356	98.6	5.1
3.8 µg/mL	-	0.401	0.364	101.0	5.5
7.5 µg/mL	-	0.388	0.350	97.2	4.7
15.0 µg/mL	-	0.339	0.302	83.7	4.5
30.0 µg/mL	-	0.127	0.090	25.0	7.3
60.0 µg/mL	-	0.038	0.001	0.2	0.3
90.0 µg/mL	-	0.041	0.004	1.0	1.2
180.0 µg/mL	-	0.036	-0.001	-0.2	0.2
Blank	+	0.039	-	-	-
Vehicle control 1	+	0.383	0.344	-	11.1
Vehicle control 2	+	0.374	0.335	-	8.7
Vehicle mean	+	0.378	0.339	100.0	9.6
Chlorpromazine					
0.03 µg/mL	+	0.382	0.343	101.0	7.9
0.05 µg/mL	+	0.351	0.312	91.9	2.5
0.10 µg/mL	+	0.348	0.309	91.1	6.3
0.20 µg/mL	+	0.348	0.309	91.0	6.2
0.40 µg/mL	+	0.319	0.280	82.5	15.6
0.80 µg/mL	+	0.059	0.020	5.8	3.6
1.60 µg/mL	+	0.037	-0.002	-0.5	0.8
3.20 µg/mL	+	0.042	0.003	1.0	1.7

*: Mean absorbance at 550 nm of 6 wells, in general
**: Mean absorbance (test group) minus mean absorbance (blank)

III. CONCLUSION

According to the results of the present study, Metiram is considered to be a phototoxic substance in the in vitro 3T3 NRU Phototoxicity Test.

Influence of the phototoxic potential of metiram for hazard assessment:

According to the data requirements a positive result should be taken into account when considering potential human exposure, however there is no clear guidance available, which further studies are required in case of a positive results, seen in an in vitro phototoxicity study. From the ICH Guidance S10 on photosafety testing it can be deduced, that four different effects have been discussed in connection with photosafety testing: phototoxicity, photoallergy, photogenotoxicity and photocarcinogenicity. It is further written, that photogenotoxicity and photocarcinogenicity is not currently considered useful [for human pharmceuticals], because the experiences with photogenotoxicity tests indicated, that these tests are substantially oversensitive. The expert panel on photosafety testing concluded in an international workshop on Genotoxicity, that photogenotoxicity testing should no longer be recommended as part of the standard photosafety testing strategy (Lynch et al., Mutation Research 723, 91 – 100, 2011)

There are a number of considerations indicating that phototoxicity at concentrations around the EC50 values are not relevant for the human exposure situation.

Metiram is rapidly degrading in DMSO solution. The degradation products are in quantity and quality not identical to a hydrolysis in aqueous system (which occurs very slowly, as metiram is basically unsoluble in aqueous systems), however some of the degradation products are seen in the hydrolysis study as well as in the photolysis study (e.g. ETU, EU). For further details please refer to Chapter 7.2 of the dossier. The Ames assay of metiram using DMSO as a solvent, as well as genotoxicity studies conducted with ETU, EU, EBIS, EDA (metabolites and degradation products of metiram) were negative. According to in vivo studies e.g. ethylene diamine (EDA) – which is a metabolite/degradation product of metiram occurring in residues - is severely irritating to rabbit´s skin and also a skin sensitizer (see Chapter 5.8). Metiram itself is likewise classified as a skin sensitizer (R43/Skin Sens Cat 1B). Respective recommendations for safe handling and use are therefore in place. There is no evidence for skin sensitization in humans in > 50 years practical use in the field in Europe and in the US.

From these considerations it is concluded:

- No further a photo-allergy test is deemed necessary.
- No further photo-mutagenicity test is deemed necessary.

Based on the overall evidence BASF does consider the phototoxicity to be of no relevance under human exposure conditions, and no further testing is proposed at this stage.

CA 5.3 Short-Term Toxicity

Studies evaluated in the draft monograph of Rapporteur Member State Italy of July 2000:

Studies evaluated in the metiram draft monograph consisted of: short-term toxicity studies (28 - 90 days) with oral administration in three different species (rats, mice, dogs). Further the 1-year dog study is included in this chapter. In addition a 21-day dermal toxicity study in rabbits and a 13-week inhalation toxicity study in rats were conducted. These studies have been evaluated by European authorities and Italy as RMS and were considered to be acceptable. For the convenience of the reviewer, the relevant studies are summarized below in tabular form and under the respective chapters as extracted from the monograph (see Table 5.3-1).

In the old subchronic toxicity studies performed in rats, mice and dogs metiram was intentionally spiked with 2.2% ETU. *This was considered to represent a worst-case approach with regard to thyroid activity. However in the more recent studies (1990ies), a representative metiram tox batch has been used, which is equivalent to the tox batches used for the most recently conducted toxicological studies (immunotoxicity, 2-generation toxicity, acute and subchronic neurotoxicity studies). An overview over the representativeness of the different tox batches is given in Doc J of this dossier.* In all the three species investigated the signs of toxicity observed were similar, with thyroid as target organ.
It is important that the findings in the examinations of the thyroid were reversible and almost exclusively of functional nature. It is known from literature that thiourea and its derivatives reduce the synthesis of hormone precursors in the follicular cells of the thyroid, probably by inhibiting the enzymatic iodine oxidation to elementary iodine by intercellular peroxidase (the available toxicological data package of ETU is summarized in chapter 5.8.1 of this dossier). Kinetics and metabolism studies have shown that ETU is formed endogenously in rats as a metabolite – and this is the reason why the studies were performed spiking metiram with ETU – which can be detected in the thyroid. Therefore a reduced uptake of ^{131}I can be explained as a consequence of a reduced enzymatic synthesis of thyroid hormones. Consequently this reduction leads to the observed drop of T4. As the peripheral concentration of T4 is much higher than that of T3, a reduction in T4 concentration does not necessarily result in reduction of T3. These findings indicate a drop of the hormone production in the thyroid with compensatory release of TSH from the pituitary. The latter is manifest indirectly in form of slight, yet detectable growth stimulation of the hormone-producing tissue. These effects are assessed to be the results of ETU – mediated reduction in thyroid synthesis.
Pareses of the hindlimbs with muscular atrophy occurred in rat subacute and subchronic studies. This finding was not observed in dogs or B6C3F1 mice, although the doses used were comparable. No evidence for neurotoxicity could be observed in the existing data package, as well as in the newly conducted subchronic neurotoxicity study. For further details, please refer to chapter 5.7 of this dossier.

The overall subchronic NOAELs from oral studies (as derived during the European peer-review) were:

Rats:	6	mg/kg bw/day
Mice:	100	mg/kg bw/day
Dogs:	2.6	mg/kg bw/day

Taking into account more recently conducted studies or re-evaluation (e.g. subchronic neurotoxicity study; thyroid hormone levels determined in the 2-Generation toxicity study), the following NOAELs are proposed to be included in the list of endpoints and for reference dose derivation:

Rats: 17 mg/kg bw (corresponding to a dietary dose of 200 ppm observed in the 90-day neurotoxicity study (see Chapter 5.7), as no effects were seen for thyroid hormone levels, as well as for thyroid weights or histopathology, or skeletal muscle findings at this dose in males and females. This value is further supported by the absence of thyroid hormone changes at 100 ppm in the 2-generation toxicity study (changes were observed at the two next higher dose levels of that study)
Mice: 100 mg/kg bw
Dogs: 5.2 mg/kg bw (using $BMDL_{20}$ calculations based on the thyroid weights)

The 21-day dermal study performed in rat with the formulation Polyram DF showed no signs of systemic toxicity. For further information on comparability of Polyram DF and metiram, please refer to DocJ of this dossier. The clinicochemical, haematological and pathological examinations did not reveal any abnormalities. Slight oedema connected with erythema and scaling were observed in the highest dose group. The NOAEL for the systemic toxicity is >250 mg/kg bw, the NOAEL for local irritation effect is 50 mg/kg bw.

A subchronic inhalation study, 13 weeks was performed on rats, to clarify if particles of the AI and of its transformation products reaching the alveoli might induce functional and anatomical thyroid changes via the lungs. It can be stated that neither the hormone parameter (T3, T4, TSH) nor the thyroids showed any thyroid weight, gross-pathological or histopathological changes indicating an effect of the test substance. With the exception of an increased relative lung weight in the highest dose females group, no organ weight changes were observed compared to control. The deposit of brown pigment in the kidneys at 101 mg/m³ was the only histopathological finding, whose toxicological relevance is unknown. The alveolitis observed histopathologically is regarded as a nonspecific dust reaction of the polymeric active ingredient artificially ground to particles reaching the alveoli, which has no relevance to risk assessment in practice.

Table 5.3-1: Summary of reviewed metiram short-term toxicity studies

Study	Dosages (mg/kg bw/ day)	NOAEL (mg/kg bw/day)	Main adverse effect	Reference (BASF DocID)
4-week **feeding** with 2- to 4 week reversibility periods (range finding) SD rat 0, 100, 300, 1000, 3000 ppm metiram + 2.2% ETU*	M: 10, 30, 110 and 296 F: 11, 33, 113 and 292	10 (100 ppm)	Target organs: thyroid (hyperplasia), liver (increased weight) and skeletal muscle (atrophy and hind limb paresis)	1976/024
4-week **feeding** (range finding) CFLP mouse 0, 100, 300, 1000, 3000 ppm metiram + 2.2% ETU*	M: 16, 40, 120 and 399; F: 15, 44, 142 and 433	40 (M); 44 (F) (300 ppm)	Liver weight increased from 1000 ppm onward; heart weight increase at all doses	1976/014

Table 5.3-1: Summary of reviewed metiram short-term toxicity studies

Study	Dosages (mg/kg bw/ day)	NOAEL (mg/kg bw/day)	Main adverse effect	Reference (BASF DocID)
4-week **feeding** (range finding) Beagle dog 0, 100, 30, 600, 900 ppm metiram + 2.2% ETU*	M&F: 5, 14, 28 and 42	5 (100 ppm)	Increased liver weights, minimal hyperplasia in thyroid	1978/0154
13-week **feeding** with 6-week reversibility SD rat 0, 5, 100, 300, 900 ppm metiram + 2.2% ETU*	M: 3, 6, 20 and 61 F: 4, 8, 24 and 76	6 (M) 8 (F) (100 ppm)	Thyroid (hyperplasia and functionality), hindlimb paresis, skeletal muscle atrophy. Most findings reversible.	1977/043 1987/0205
3-month **feeding** and neurofunctional observations Wistar rats 0, 5, 80,320, 960 ppm metiram	M&F: 0.4, 6.3, 25.4 and 81.4	No NOAEL; *a NOAEL of 320 ppm can be derived taking into account the new subchronic toxicity study (Chapter 5.7)*	960 ppm: reduced bw, altered clinic-chemical parameters, increased thyroid weight, reduced T4 values, neuromuscular weakness without pathological changes; 320 ppm: decreased red blood cell parameters TSH increase at all doses	1992/11224 *2015/1171917*[#]
3-month **feeding** B6C3F1 mouse 0, 300, 1000, 3000, 7500 ppm metiram	M&F: 100, 400, 1200 and 3000	~100 (300 ppm)	3000 ppm: increased liver and adrenal weights (including adrenal fatty degeneration). Thyroid hypertrophy and vacuolization ; 1000 ppm: reduced T4 values	1992/11223
19-week **feeding** Beagle dog 0, 100, 400-6000, 1600, 6000 ppm metiram	M&F: 3.8, 16 – 167, 55, 184	No NOAEL	Thyroid toxicity	1989/5128
1-year **feeding** Beagle dog 0, 30, 80, 1000, 3000 ppm metiram	M&F: 0.98, 2.59, 29.9 and 84.8	~2.6 (80 ppm) *Using $BMDL_{20}$ a value of 5.2 (162 ppm) mg/kg bw is derived*	1000 and 3000 ppm: slightly reduced bw gain and red blood cell parameters, decreased T4 values, thyroid weight increase with enlargement and hyperplasia.	1991/10786 1992/12594 *2015/1174483** *2017/1134476*[#]
21-day **dermal** NZW rabbit 0, 25, 50 ,250 mg/kg bw (Polyram DF®)	M&F: 25, 50 and 250	>250 (systemic) 50 (dermal)	No systemic toxicity, marginal skin irritation	1987/0260
13-week **inhalation** SD rat 0, 2.1, 20, 101 mg/m³ air metiram + 0.2% ETU	M&F: 2.1, 20 and 101 mg/m³ air	20	Body weight gain impaired at 101 mg/m³ air; alveolitis at 20 and 101 mg/m³ not related to metiram but to artificially generated dust.	1986/407, 1987/0414

*The studies conducted with metiram + 2.2% spiked ETU are considered to represent a worst-case assessment (esp. with regard to thyroid-related endpoints). The results of these studies are only used as supporting information.
[#]References (BASF DocIDs) in italics are submitted the first time in this supplemental dossier. They are listed in this table for the sake of completeness of information.

Based on the available studies, the following endpoints were determined in the Annex I listing of metiram.

Short term toxicity	
Target / critical effect:	Thyroid (inhibition of thyroid peroxidase, hyperplasia/hypertrophy), liver (increased weight), atrophy of hindlimb muscles in rats.
Lowest relevant oral NOAEL:	1-year dog: 80 ppm = 2.6 mg/kg bw/d
Lowest relevant dermal NOAEL:	21-day rabbit: > 250 mg/kg bw/d
Lowest relevant inhalation NOAEC:	90-day rat: 20 mg/m³ air

Submission of not yet per-reviewed studies/amendments in this dossier:

In this dossier a bench mark dose calculation for left and right thyroid weights to the already submitted one-year dog feeding study is submitted. The NOAEL was found to be at 80 ppm. Since between 80 ppm and 1000 ppm (which is the next higher dose level), there is a factor of > 10, the real NOAEL is considered to be between 80 and 1000 ppm. Therefore a benchmark dose calculation was conducted, based on the thyroid weight changes.
With regard to rat studies, a new 90-day neurotoxicity study in rats has been conducted, which included also the endpoint thyroid hormone measurements. This new – not yet peer-reviewed - study is included in chapter 5.7 of this dossier. Based on this study the questionable findings on female grip strengths (without histopathological correlate in none of the doses) and on thyroid hormone changes of the existing 90-day rat study are assessed to be not treatment-related. This is further supported by recently collected historical control data, showing that the slight effects in female grip strengths are fully within the historical controls and the confirmation, that there is no histopathological findings seen in none of the relevant tissues additionally investigated. These new data are included in the first amendment to the study (BASF DocID 2015/1171917).

Thus, the conclusion for relevant endpoints for the current re-registration remains as follows:

Target / critical effect:	Thyroid (inhibition of thyroid peroxidase, hyperplasia/hypertrophy), liver (increased weight), atrophy of hindlimb muscles in rats.
Lowest relevant oral NOAEL:	1-year dog: 80 ppm = 2.6 mg/kg bw/d 1-year dog: 167 ppm = 5.7 mg/kg bw/d ($BMDL_{10}$)
Lowest relevant dermal NOAEL:	21-day rabbit: > 250 mg/kg bw/d
Lowest relevant inhalation NOAEC:	90-day rat: 20 mg/m³ air

Note: The one-year dog feeding study report with its amendment (CA 5.3.2/1 and CA 5.3.2/2) were already submitted and reviewed in the course of the initial registration of metiram. These studies are submitted for completeness as the basis for the benchmark dose calculation (CA 5.3.2/3) and because, they were the basis for reference dose setting of metiram.

CA 5.3.1 Oral 28-day study

28-day study in rats (BASF DocID 1976/024)

Groups of 20 male and 20 female Spraque Dawley rats per dose group received metiram with 2.2% ETU (purity about 96%) at dietary doses of 0, 100, 300, 1000 and 3000 ppm for 28 consecutive days. The nominal test substance intake was 10; 30; 100 and 296 mg/kg bw for males and 11; 33; 113 and 292 mg/kg bw for females. After 4-week treatment the group size was reduced to ten animals, i.e. ten animals per sex and dose group would be sacrificed provided no mortalities occurred. After a first withdrawal period of two weeks and a second withdrawal of two weeks each time five animals per sex and dose group were sacrificed.

The following observations were made:
At 3000 ppm 19/20 females and 7/20 males exhibited signs of hindlimb paralysis accompanied by muscular atrophy. At this dose level food consumption and body weight gain were severely reduced, especially in the females. Increased absolute and relative thyroid weights with a varying degree of minor thyroid hyperplasia was noted in some of the male and female animals. Kidney weights were also increased (absolute and relative) with tubular lesions in two females. For the increased relative liver weights no histopathological changes at cellular level were found. The same is true for the increased absolute and relative gonad weights in males and females.
At 1000 ppm reduced body weight gain, renal tubular lesions in two females and minor thyroid hyperplasia in some males and females were noted at this dose level.
At 300 ppm effects noted at this dose level were limited to lower body weight gain in females and minor thyroid hyperplasia in some males and females.
No changes were noted at 100 ppm.

After two weeks of withdrawal reversibility was indicated for all of the above-mentioned findings, however, this process was not yet complete. After another two weeks full reversibility was observed for rats treated with 100; 300 and 1000 ppm. Rats treated with 3000 ppm showed after the 4 week recovery period only vacuoles and or areas of fat cells within muscle.

Conclusion
Based on the described findings at 300 ppm and above, the NOAEL in this study was identified at 100 ppm, which is equivalent to mean daily intakes of 10 and 11 mg/kg bw/day in male and females rats, respectively.

28-day study in mouse (BASF DocID 1976/014)

Eight mice (CFLP) per sex and dose group received the metiram (purity: 96.82% metiram complex) with 2.2% ETU via the diet at concentrations of 0; 100; 300; 1,000 and 3,000 ppm. The nominal test substance intake was 16, 40, 120 and 399 mg/kg bw for males and 15, 44, 142 and 433 mg/kg bw for females.

The following observations were made:
A lower body weight gain was noted for males during week 1 at 1000 and 3000 ppm and for females at 3000 ppm. Increased liver weights were also noted for both sexes at these dose levels. The absolute and relative heart weights were significantly increased in males and reduced in females starting from 300 ppm.
It should be noted that there were no information on the thyroid, which is the target organ for rat.

Conclusion
Based on the described findings at 100 ppm and above, the NOAEL in this study was identified at 300 ppm, which is equivalent to mean daily intakes of 40 and 44 mg/kg bw/day in male and females mice, respectively.

28-day study in dogs (BASF DocID 1978/0154)

In a subacute study four Beagle dogs per sex and dose group were administered the test substance metiram (purity: 96.82%) with 2.2% ETU in doses of 0; 100; 300; 600 and 900 ppm via the diet for a period of four weeks. The test substance intake corresponds to 5, 14, 28, 42 mg/kg bw/day.

The following observations were made:
No mortalities and no clinical signs were observed. Apart from one dog receiving 300 ppm and one dog receiving 600 ppm which lost weight, there was no adverse effect on body weight. Food consumption remained unaffected whereas water consumption was increased for all groups receiving the test compound with the exception of dogs receiving 300 ppm. Ophthalmoscopy revealed no abnormalities of the eyes. After three weeks dosing the mean values relating to blood cells (PCV, Hb and RBC) although well within normal limits, were significantly lower for animals receiving 900 ppm than for the controls. Gross-pathological examinations revealed no treatment-related changes. The mean relative liver weights for dogs receiving 600 ppm and 900 ppm were significantly greater than the control value. Relative and absolute thyroid weights were increased at all the doses considered. However, no morphological abnormalities were detected to account for the relative weight increase noted at post mortem examination in dogs treated at these doses. The possibility that the minimal thyroid hyperplasia encountered in four dogs that had received 900 ppm was treatment-related cannot be excluded. Spleen absolute and relative weights were increased at 100, 300 and 600 ppm, but were in normal range at 900 ppm. No other morphological abnormality or variation from normal was encountered in any other tissues examined that would be attributable to treatment with metiram. The results of a 4 week intake of metiram by beagle dogs revealed significant increases in liver weight at dietary concentrations of 600 and 900 ppm without morphological correlates. Histopathological, however, there were indications of minimal hyperplasia of the thyroid in 50% of the animals of either sex at 900 ppm.

Conclusion
Based on the described findings, the NOAEL in this study was identified at 100 ppm, which is equivalent to mean daily intakes of 5 mg/kg bw/day in male and females dogs.

New information from the open literature presented in the AIR process:

Report: CA 5.3.1/1
Sakr S.A. et al., 2009a
Protective effect of licorice on Metiram fungicide induced liver injury in mice
2009/1131682
Guidelines: none
GLP: no

Healthy adult male albino mice (Mus musculus) approximately three months old and weighing 15 to 25 g were treated orally with 1240 mg/kg bw metiram (purity: 80%; 20% inert ingredients) for 10 days dissolved in water. After one, two or three weeks the treated animals and their controls were killed by cervical dislocation.

Histological results: Examination of liver of control animals showed normal architecture. Examination of liver specimens treated with metiram fungicide for one week showed impaired structural organization of the hepatic lobules, the characteristic cord-like arrangement of the normal liver cells was lost. In addition, central and portal veins were enlarged, congested with blood and surrounded by leucocytic infiltration. The histopathological changes of the liver were more pronounced after two weeks of treatment with metiram. In these specimens, the intrahepatic central and portal veins were congested with blood. The sinusoidal spaces were widen and filled with activated Kupffer cells and there were masses of leucocytic infiltrations. After 3 weeks of metiram treatment, the inflammatory leucocytic infiltration was increased and cytoplasmic vacuolization appeared in most of the hepatocytes and their nuclei were pyknotic. In addition, severe necrosis around central veins was observed.

Immunohistochemical results: Some of hepatocytes displayed faint stain of PCNA in controls. Animals treated with metiram for 1 and 2 weeks showing stimulation of DNA synthesis and increased PCNA expression in most of hepatocytes when compared with control animals. After 3 weeks the expression of PCNA in all hepatocytes was strong. PCNA –labeling index is significantly higher ($p<0.05$) in animals given metiram compared with control animals.

Biochemical results: As shown in Table 5.3.1-1, animals treated with metiram showed significant increase in serum AST and ALT activity in all periods of treatment.

Table 5.3.1-1: Effect of metiram on AST and ALT

	AST [U/L]		ALT [U/L]	
Treatment period	control	metiram	control	metiram
1st week	32.4 ± 1.64	77.4 ± 9.2*	37.8 ± 1.89	100.4 ± 7.6*
2nd week	32.4 ± 1.66	80.4 ± 9.4*	37.8 ± 1.91	105.2 ± 8.4*
3rd week	32.5 ± 1.69	67.4 ± 8.6*	37.8 ± 1.9	113.6 ± 8.3*

n=5 animals for each group
*: significant increase ($p<0.5$) in comparison with control animals

Conclusion: Mice treated orally with 1240 mg/kg bw metiram showed hepatotoxicity in mice after one to three week treatment.

Comment from the applicant:
Abstract and material and methods are referring to 10-day oral treatment of metiram whereas the result concerning histology, immunohistochemistry and biochemistry show results of mice treated for one, two or three weeks with metiram. Further it is not described whether the animals receive metiram by gavage or by feed.
Since the intention of this publication was to show a protective effect of licorice on metiram induced liver injury in mice only one dose was tested. For the toxicological evaluation of metiram the results of this publication show that an oral treatment of mice with 1240 mg/kg bw metiram for one to three weeks results in liver injury. The lowest NOAEL for mice in other studies was found to be 100 mg/kg bw which is far below the here tested dose. Therefore, the study was not considered relevant to be taken into account in the overall conclusion.

Classification of study: not further considered

Report: CA 5.3.1/2
Sakr S. et al., 2011a
Metiram-induced nephrotoxicity in albino mice: Effect of licorice aqueous extract
2013/1419081
Guidelines: none
GLP: no

Healthy adult male swiss albino mice (15 to 25 g) were treated daily orally with 124 mg/kg bw metiram (purity: 80%; 20% inert ingredients) for 3 weeks dissolved in water. Control animals received water additionally to their food. Animals of the different groups were sacrificed after 1, 2, and 3 weeks post-treatment.

Biochemical results: In the sera of mice treated with metiram compared with that of control creatinine was significantly ($p<0.05$) increased after two and three weeks of treatment. Blood urea exhibited a significant increase only after 3 weeks of treatment with metiram.

Histological results: Histological examination of the kidney of control mice revealed entirely normal histological features. Examination of the kidney sections of animals after treatment with metiram for 1 week, revealed intertubular hemorrhage. Some tubules showed cloudy degeneration and the interstitium contained few inflammatory cells. After 2 weeks, Malpighian corpuscles lost their characteristic configuration. The Bowman's capsules revealed marked alteration where some of them lacked most of the parietal and visceral epithelial cells. Some glomeruli seemed to have lost their attachments and mesangial stroma and others were atrophied. Congestion of intertubular veins and leukocytic infiltrations were observed. After 3 weeks, most of the renal tubules rendered highly damaged that they have almost lost their characteristic appearance and their lining epithelial cells became undistinguished, lost its brush border and the cells appeared with pyknotic nuclei. The lumen of the tubules was filled with degenerated and desquamated epithelial cells, as well as apoptotic cells.

Immunohistochemical results: Examination of kidney sections of control animals show that few nuclei displayed faint stain of PCNA. PCNA served as valuable marker for cell proliferation. Animals treated with metiram for 1 and 2 weeks show stimulation of DNA synthesis and increased PCNA expression in some epithelial cells of renal tubules. After 3 weeks the expression of PCNA in most of renal epithelial cells was strong. The PCNA-labeling index is significantly higher ($p<0.05$) in animals given metiram for 3 weeks compared to the controls.

Conclusion: Mice treated orally with 124 mg/kg bw metiram showed clear renal toxicity in mice after three week treatment.

Comment from the applicant:
In this publication it is not described whether the animals receive metiram by gavage or by feed. Furthermore, the figures showed in the publication do not match with the description in the results. All figures show significant differences between the treatment with metiram and control at all time points but in the text it is described for instance only for the 3 week interval.
Since the intention of this publication was to show a protective effect of licorice on metiram induced nephrotoxicity in mice only one dose was tested. For the toxicological evaluation of metiram the results of this publication show that an oral treatment of mice with 124 mg/kg bw metiram for one to three weeks results in kidney injury. The lowest NOAEL for mice in other studies was found to be 100 mg/kg bw which is still below the here tested dose. Therefore, the study was not considered relevant to be taken into account in the overall conclusion.

Classification of study: not further considered

CA 5.3.2 Oral 90-day study

13-week study in rats (BASF DocID 1977/043 and 1987/0205)

Metiram (purity: 96.82%) with 2.2% ETU was administered to 35 Sprague-Dawley rats per sex and dose group via the diet in doses of 0; 50; 100; 300 and 900 ppm over a period of 13 weeks. The nominal mean test substance intake was 3, 6, 20, 61 mg/kg bw for males and 4, 8, 24, 76 mg/kg bw for females. The test substance administration was followed by a 6-week post dosing observation period for ten male and ten female animals per group. At a later date (addendum report 1987/0205) a histopathological examination of the sciatic nerve sections of 10 male and 10 female control animals as well as of 15 males and 15 females treated with 900 ppm was performed.

The following observations were made:
As in the range-finding study [1976/024], especially the female rats showed pareses of the hind limbs. In the main study, about 90% of the females were affected after 2-week test substance administration of 900 ppm. Abnormal movements, unphysiological reflexes and muscular atrophies of the hind limbs were observed as clinically evident signs. In the further course of the feeding period, the number of rats affected was reduced indicating adaptation. At the end of the 13-week test substance administration, only 20% of the females of this dose group showed the findings described above. After six weeks none of the 15 animals used for observation showed clinical signs in the form of paresis. Atrophy of muscle fibers, accompanied by occasional fatty cell infiltration and nuclear proliferation in the sarcolemma, was probably the histopathological correlate of the clinical findings. Generally, such findings are typical of neurogenic muscular atrophy, which is consistent with the clinical findings obtained in this study (unphysiological reflexes). The histopathological findings were dose-dependent and were observed from 300 ppm onward in the females and only at 900 ppm in the males. The histopathological examination of the animals sacrificed at the end of the observation period indicated reversibility of muscular atrophy. Additional examination of the sciatic nerve, which innervates the muscles of the hindlimbs, did not indicate any damage to the nerves at the highest dose level examined (900 ppm) as compared with the control. Therefore, the low and intermediate doses were not investigated.

In the examinations of the thyroid merely functional parameters (hormones and iodine uptake) were changed with the exception of a marginal hyperplasia in about 13% of the males of the highest dose (900 ppm). In this connection, it should be pointed out that the results of isolated investigations on the effects of substances which, as in this case, interfere with a feedback mechanism (hypothalamus - pituitary - thyroid) should be considered with caution since it is difficult to exactly define the dose-response-time relationship in organs involved in a feedback mechanism. In the ^{131}I thyroid function tests, the plasma clearance and the ^{131}I binding capacity of the thyroid proteins were unaffected compared with the control. The total uptake of ^{131}I in the thyroid, however, was reduced at all doses of metiram tested. Whereas the T3 values were unchanged in the peripheral blood compared with the control, reduced T4 values were observed towards the end of the administration period at the 900 ppm dose level (11th and 12th test weeks) and at the 300 ppm dose level (12th test week). At the end of the 6-week post dosing observation period, the parameters of "^{131}I total uptake in the thyroid" and "T4 in the peripheral blood" which were changed compared with the control were again in the normal range of the untreated animals.

Conclusion

Based on the described findings, the NOAEL under this test conditions is established at 100 ppm, corresponding to 6 mg/kg bw for males and 8 mg/kg bw for females, respectively.

Report:	CA 5.3.2/1
	[redacted] 1992 a
	Study of the oral toxicity of Metiram Premix 95% in Wistar rats - Administration in the diet for 3 months including the examination of neurotoxicology (neurofunctional observational battery)
	1992/11224
Guidelines:	EEC 87/302 B, OECD 408, EPA 82-1, EPA 798.6050, EPA 798.6400
GLP:	yes
	(certified by Ministerium fuer Umwelt und Gesundheit, Postfach 3160, 6500 Mainz)

Report:	CA 5.3.2/2
	[redacted] 2015 a
	Amendment No. 1 to the report: Study of the oral toxicity of Metiram Premix 95% in Wistar rats - Administration in the diet for 3 months including the examination of neurotoxicology (neurofunctional observational battery)
	2015/1171917
Guidelines:	EEC 87/302 B, OECD 408, EPA 82-1, EPA 798.6050, EPA 798.6400
GLP:	yes
	(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Report:	CA 5.3.2/3
	[redacted] 2016 a
	Amendment No. 2 to the report: Study of the oral toxicity of Metiram Premix 95% in Wistar rats - Administration in the diet for 3 months including the examination of neurotoxicology (neurofunctional observational battery)
	2016/1306727
Guidelines:	EEC 87/302 B, OECD 408, EPA 82-1, EPA 798.6050, EPA 798.6400
GLP:	yes
	(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

3-month study in rats (BASF DocID 1992/11224, BASF DocID 2015/1171917)

Metiram (purity: 94,8%) was administered to 13 male and 13 female Wistar rats per group in dose levels of 5; 80; 320 and 960 ppm corresponding to 0.4/0.4, 5.8/6.7, 23.5/27.3, and 73.9/88.8 mg/kg bw (male/female animals) over a period of three months. As a control 13 male and 13 female animals were used. The study was conducted according to the OECD Guideline 408, under GLP. It included the investigation of the neurofunction ("neurofunctional observational battery") and additional neuropathology. Further thyroid hormones (T4, T3, TSH) were determined in serum collected at the terminal sacrifice of the animals.

The purpose of the amendment to this study was to evaluate the reason for the findings on hindlimb weakness, ataxia and reduced grip strengths seen in the study. Further histopathological examinations were conducted, as well as a collection of historical control data for grip strength values of fore- and hindlimbs had been performed.

The following observations were made:
Thus, the 3-month administration of metiram led to reduced body weight in both sexes at a dose of 960 ppm (81 mg/kg bw), the females being more affected than the males. At 960 ppm decreased body weight of the male (about -4 to -6%) and female (about -12 to -15%) rats when compared to the control group was noted (see table below).

Table 5.3.2-1: Mean body weight before and at end of treatment

Test group Concentration in diet	Body weight (g)			
	Males day 0	**Males day 91**	**Females day 0**	**Females day 91**
Controls	190 (SD 6.2)	455.7 (SD 41.7)	145.4 (SD 6.6)	269.1 (SD 22.9)
5 ppm	191.7 (SD 5.9)	464.6 (SD 28.1)	145.7 (SD 5.5)	274 (SD 18.5
80 ppm	192.6 (SD 6.2)	458.6 (SD 25.5)	145.9 (SD 5.6)	265.2 (SD 24.3)
320 ppm	191.3 (SD 6.0)	445.5 (SD 21.8)	144.9 (SD 5.2)	255.6 (SD 23.3)
960 ppm	190.9 (SD 6.6)	429 (SD 25.6)	146.1 (SD 5.9)	234.1 (SD 23.3)**

**p<0.01

In both sexes a decrease in red blood cells, creatinine, inorganic phosphate, calcium, potassium and magnesium was noted. Hemoglobin, hematocrit, alanine aminotransferase, alkaline phosphatase and sodium were decreased in females, Urea was decreased in males. As clinical observations, three female rats showed reduced general state, two of these females showed additionally ataxia, another female rats showed in addition ataxia (hind limb weakness). Both observations (reduced general state of health as well as the ataxia) were only observed during the weeks 3 – 7 of the study, indicating the transient nature of this finding. A respective table of the individual females in given below:

Table 5.3.2-2: Individual clinical observations of the female rats of the 960 ppm group

Animal number	Observations	Week of study
118	No remarkable clinical observations	Week 1 -13
119	No remarkable clinical observations	Week 1 -13
120	No remarkable clinical observations	Week 1 -13
121	No remarkable clinical observations	Week 1 -13
122	No remarkable clinical observations	Week 1 -13
123	No remarkable clinical observations	Week 1 -13
124*	Ataxia	Week 4 – 7
125*	Reduced general state	Week 3 – 7
126*	Ataxia Reduced general state	Week 4 – 7 Week 3 – 7
127	No remarkable clinical observations	Week 1 -13
128	No remarkable clinical observations	Week 1 -13
129	No remarkable clinical observations	Week 1 -13
130*	Ataxia Reduced general state	Week 4 – 7 Week 3 - 7

*During the other weeks no remarkable clinical observations were recorded

From day 15 onwards reduced grip strength of the fore limbs of the female animals (between -18% and -25%) and from day 7 onwards reduced grip strength of the hind limbs (between -12% and -52%) were observed at doses of 80, 320 and 960 ppm. Male animals exhibited reduced grip strength of the hindlimbs (-12%) at the end of the study. Table 5.3.2-3 shows the grip strength findings in females at the different time points.

Table 5.3.2-3: Grip strength in females (in Nm) at different time points

Doses	Hind limbs (day 15)	Hind limbs (day 27	Hind limbs (day 56	Hind limbs (day 90)
0	2.3 SD 0.3	2.3 SD 0.5	4.1 SD 0.3	5.0 SD 0.4
5 ppm	2.3 SD 0.3	2.1 SD 0.5	3.9 SD 0.6	5.0 SD 0.4
80 ppm	2.4 SD 0.5	2.2 SD 0.4	3.9 SD 0.5	4.9 SD 0.6
320 ppm	2.2 SD 0.5	2.1 SD 0.4	3.8 SD 0.6	4.7 SD 0.5
960 ppm	1.1 SD 0.5*	1.8 SD 0.4	3.3 SD 0.8*	4.4* SD 0.5
	Fore limbs (day 15)	**Fore limbs (day 27)**	**Fore limbs (day 56)**	**Fore limbs (day 90)**
0	4.9 SD 0.4	5.0 SD 0.7	5.3 SD 0.4	6.9 SD 0.6 (HCD: 3.1 – 6.9)
5 ppm	5.1 SD 0.4	5.2 SD 0.4	5.4 SD 0.5	*6.3 SD 0.9*
80 ppm	4.9 SD 0.5	5.2 SD 0.7	5.1 SD 0.5	*6.2 SD 0.7***
320 ppm	4.6 SD 0.4	5.0 SD 0.5	5.0 SD 0.6	*6.0 SD 0.5***
960 ppm	3.7 SD 0.8**	4.1 SD 0.7**	4.2 SD 0.5**	5.1 SD 0.7**

The statistically significant increases in the fore limb grip strengths at 80 and 320 ppm are not considered to be treatment-related, as they do not correlate with the clinical observations on ataxia (which is only transiently observed and not evident in week 13 of the study). Further there is only a very small effect seen in the mid doses, compared to the much stronger effect seen at the top dose, which is considered to be treatment-related, although the fore limb grip strength of 5.1 is also within the historical control values. A retrospective collection of historical control data shows that the value of 6.9 for female fore limb grip strengths – determined in controls of this study - is unusually high, when compared to the control values determined in 5 other studies conducted in the same time frame in the same lab (DocID 2015/1171917 and 2016/1306727). The control values for female fore limb grip strengths are between 3.1 – 5.6 measured at either day 85 or day 87 of week 13 (see Table 5.3.2-3).

This assessment is further supported by the new 90-day neurotoxicity study (BASF DocID 2014/1315300), which showed only very slight effects on female grip strengths at top dose of 900 ppm at study days 22 and 85 (see Chapter MCA 5.7). The lower doses in this study (40 and 200 ppm were without any effects.) For further details please refer to Chapter 5.7 of this dossier. Moreover, no morphological changes in the peripheral or central nervous system were observed in this study, although thorough neuropathological examinations similar to the OECD TG 424 requirements were performed using perfusion fixed tissues of the animals. Additional histopathological examinations were performed on the sciatic nerve transversal for perfusion-fixed and sciatic nerve of formalin-fixed female animals of control and all test groups had been conducted. During the histopathological examinations of the sciatic nerve no treatment-related findings were recorded, confirming the original assessment, that there is no evidence for a specific neurotoxicity after dietary metiram exposure to rats. Regarding pathology, no morphological findings could be detected to explain the hindleg weakness in females observed clinically (BASF DocID 2015/1171917).

Histopathologically, there were no substance-induced findings detected.
All findings noted were considered to be of incidental or spontaneous character, since they consisted either of single observations, or were of low incidence, or they occurred in control animals only, or at comparable incidence and graded severity in control and treated males and/or females.

This also includes the minimal axonal lesion in the tibial nerve of one male animal in the 320 ppm dose group, as no further indications of any peripheral neuropathy or axonopathy were found in the nerve sections examined.

Table 5.3.2-4: Selected microscopic findings in Wistar rats administered metiram for 91 days

Sex		Males					Females				
Dose	**[ppm]**	**0**	**5**	**80**	**320**	**960**	**0**	**5**	**80**	**320**	**960**
Animals in selected groups	**[n]**	**13**	**13**	**13**	**13**	**13**	**13**	**13**	**13**	**13**	**13**
Organ	**Grade**										
Liver [animals examined]		10	10	10	10	10	10	10	10	10	10
- Granuloma(s), Kupff. cells	1	-	3	1	1	3	5	2	6	6	1
	2	10	6	8	9	6	5	8	4	4	9
	3	-	1	1	-	1	-	-	-	-	-
	*mean**	*[2.0]*	*[1.8]*	*[2.0]*	*[1.9]*	*[1.8]*	*[1.5]*	*[1.8]*	*[1.4]*	*[1.4]*	*[1.9]*
- Cholangitis, focal		0	0	0	0	1	0	0	0	0	0
- Fatty change, focal		0	0	0	0	1	0	0	0	0	0
- Fatty change, diffuse		1	0	0	0	0	0	0	0	0	0
- Fatty change, peripher	1	2	3	-	2	1	6	4	4	4	5
	2	4	6	6	3	3	3	2	1	2	2
	3	2	1	3	4	-	-	2	-	1	-
	*mean**	*[2.0]*	*[1.8]*	*[2.3]*	*[2.2]*	*[1.8]*	*[1.3]*	*[1.8]*	*[1.2]*	*[1.6]*	*[1.3]*
Kidney [animals examined]		10	10	10	10	10	10	10	10	10	10
- Mononuclear cell infiltrates	2	0	0	0	1	0	0	0	0	0	0
- Lithiasis, intratub.	1	2	-	1	-	-	2	-	1	1	-
	2	-	-	-	-	-	8	9	8	8	9
	3	-	-	-	-	-	-	1	1	1	1
	*mean**	*[1.0]*	*[0.0]*	*[1.0]*	*[0.0]*	*[0.0]*	*[1.8]*	*[2.1]*	*[2.0]*	*[2.0]*	*[2.1]*
- Hyperplasia, urothel.	1	-	-	-	-	1	1	-	-	-	-
	2	-	-	-	1	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-
	*mean**	*[0.0]*	*[0.0]*	*[0.0]*	*[2.0]*	*[1.0]*	*[1.0]*	*[0.0]*	*[0.0]*	*[0.0]*	*[0.0]*
Lung [animals examined]		10	10	10	10	10	10	10	10	10	10
- Foreign body (no inflammation)		0	0	1	1	0	1	0	0	0	0
- Mineralisation (artery)		4	4	5	4	4	3	3	2	4	4
- Pneumonitis, focal	1	2	3	5	4	-	2	2	5	2	5
	2	3	2	1	1	4	-	2	2	1	1
	3	-	-	-	1	1	-	-	1	1	1
	*mean**	*[1.6]*	*[1.4]*	*[1.2]*	*[1.5]*	*[2.2]*	*[1.0]*	*[1.5]*	*[1.5]*	*[1.8]*	*[1.4]*
Mesenteric lymph node [animals exam.]		10	-	-	-	10	10	-	-	-	10
- Hyperplasia, reactive	2	10	-	-	-	10	10	-	-	-	10
Thyroid gland [animals examined]		10	10	10	10	10	10	10	10	10	10
- Cyst(s)		0	0	1	1	1	1	0	0	0	0
Skin [animals examined]		-	-	-	-	-	-	-	1	-	-
- Atrophy of hair follicles	3	-	-	-	-	-	-	-	1	-	-
Sciatic nerve [animals examined]		13	13	13	13	13	13	13	13	13	13
- Findings		0	0	0	0	0	0	0	0	0	0
Tibial nerve [animals examined]		3	3	3	3	3	3	3	3	3	3
- Axonal degeneration	1	0	0	0	1	0	0	0	0	0	0

* The mean severity is the sum of the gradings divided by the incidence
Gradings : 1 = minimal, 2 = slight, 3 = moderate, 4 = marked/severe, 5 = massive

Within the framework of the histopathologic examinations of the test animals subjected to perfusion fixation, sciatic, tibial and sural nerves were investigated additionally morphometrically in the control and top dose animals in 2015. The measurements of myelinated axons did not reveal any significant difference in area [see **Table 5.3.2-5**] which allowed a conclusion to be drawn with regard to an axonal reaction in the form of an axonal atrophy or decrease of the nerve fiber diameter.
Signs of any neuropathological changes due to the substance administration were not found in the examined material of the CNS either.

Table 5.3.2-5: Morphometrical evaluation in perfusion fixed Wistar rats administered metiram for 91 days

Parameter Unit	Sex	Dose level	Sciatic nerve		Sural nerve		Tibial nerve	
		ppm	Mean	SD	Mean	SD	Mean	SD
Areas of myelinated axons [µm²]	♂	0	53.71	5.43	45.01	13.04	41.42	0.94
	n =3	960	48.85	3.25	47.71	16.45	41.91	1.37
	♀	0	59.19	9.65	32.56	4.97	46.84	6.98
	n =3	960	46.57	5.22	30.30	5.90	39.03	7.35

* $p \leq 0.05$; ** $p \leq 0.01$ Student's t-tes

Serum thyroxine (T4) was decreased in both sexes, while T3 was decreased in males at the two top doses (see table below). The serum TSH concentrations were increased at all doses, however the biological relevance of this finding is unclear, as there was no dose-dependency observed. Further the experimentally complex RIA method used to determine TSH values at that time, is no longer used. A more accurate and better established method has been used in the new rat neurotoxicity study, which is described in chapter 5.7 of this dossier, and clear NOAELs for thyroid hormone changes (including TSH) has been determined at 200 ppm. The values are shown in Table 5.3.2-6).

Table 5.3.2-6: Serum T4, T3 and TSH concentrations in male and female rats determined at terminal blood collections

Test group	T3 [ng/dl]		T4 [µg/dl]		TSH [µU/ml]	
	Males	females	Males	Females	Males	females
0 ppm	64 (4)[1]	82 (5)	5.1 (0.2)	3.8 (0.5)	134 (7)	90 (4)
5 ppm	72 (3)	79 (4)	5.9 (0.3)	3.5 (0.3)	229 (28)*	129 (9)*
80 ppm	76 (5)	94 (5)	5.2 (0.3)	4.1 (0.4)	287 (37)**	146 (8)**
320 ppm	89 (4)**	93 (3)	5.2 (0.3)	3.0 (0.2)	260 (24)**	136 (13)**
960 ppm	80 (4)*	82 (5)	3.3 (0.2)**	1.9 (0.3)**	232 (32)*	147 (9)**

[1]Standard deviation is given in +/-S.E.M., *p<0.05, **p<0.01

At the top dose of 960 ppm, increased absolute organ weights of the thyroid was observed in males and relatively increased organ weights of thyroid glands and liver was seen in both sexes (see Table 5.3.2-7).

Table 5.3.2-7: Mean liver and thyroid gland weights (absolute and relative)

Test group	Liver weight [g]				Thyroid weight [mg]			
	Males		Females		Males		females	
	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.
Control	13.287 (1.846)	3.056 (0.222)	7.365 (0.788)	2.929 (0.185)	27 (4.028)	0.006 (0.001)	20.5 (5.701)	0.008 (0.002)
5 ppm	13.66 (1.844)	3.07 (0.282)	7.146 (0.713)	2.802 (0.131)	29.1 (6.471)	0.007 (0.001)	20.9 (7.475)	0.008 (0.003)
80 ppm	13.788 (1.045)	3.152 (0.21)	6.967 (0.771)	2.905 (0.133)	26.222 (4.549)	0.006 (0.001)	20.6 (3.596)	0.009 (0.002)
320 ppm	13.103 (0.97)	3.115 (0.217)	6.799 (0.763)	2.948 (0.185)	26.9 (4.122)	0.006 (0.001)	23.6 (7.806)	0.01 (0.003)
960 ppm	13.87 (1.429)	3.4** (0.197)	7.85 (0.842)	3.51** (0.201)	34 (6.245)	0.008** (0.001)	24.3 (4.572)	0.011* (0.002)

$^{*}p<0.05$, $^{**}p<0.01$

The liver and the thyroids did not show histopathological changes deviating from controls. There were no histopathological changes observed in any of the examined organs.

Conclusions

The clinical and the neurofunctional observations revealed in the highest dose signs of muscle weakness (hind limb weakness, ataxia, reduced grip strength), female animals being more affected than males. However, at this dose also body weight was impaired in the animals. No evidence for neurotoxicity was found in extensive neuropathological testing. A CS_2-related neurotoxicity can be excluded with certainty.

Hormone determinations revealed the following statistically significant changes: at 960 ppm serum T3 was increased (males) and T4 was decreased (both sexes); at 320 ppm T3 was increased in males. TSH was increased in all treated animals when compared to the controls, even if there was no dose-response relationship. Based on the described findings, no NOAEL under this test conditions was established.

The relevance of the measured TSH and T3 levels is unclear, as the analytical method used at the time of the study is no longer used and also no biological background data are available. In the new study (BASF DocID 2014/1315300; see chapter 5.7) clear NOAELs for thyroid hormone (only T4 was changed) changes were derived at 200 ppm in males and females. This observation is further supported by the thyroid hormone measurements conducted in the 2-generation toxicity study, where serum T3, T4 and TSH levels were measured in parental F0 and F1 animals and in offspring and changes were seen at 350 (= 31 mg/kg bw) and 1000 (= 92 mg/kg bw) ppm, but not at 100 ppm (BASF DocID 2011/1264813, see Chapter MCA 5.6).

Taking into account the more recently generated study results (subchronic neurotoxicity and 2-generation toxicity study) a **NOAEL of 320 ppm** (corresponding to **23.5/27.3 mg/kg bw**) based on findings on body and organ weight and on the skeletal muscle is derived for this study.

3-month study in mice (BASF Doc ID 1992/11223)

Metiram (purity: 94.8%) was administered to ten male and ten female B6C3F1 mice per group, via the diet for three months at doses of 300; 1,000; 3,000 and 7,500 ppm corresponding to about 100; 400; 1,200; 3,000 mg/kg bw/day. For comparison, groups of untreated (ten males and ten females) mice were used as controls.

The following observations were made:
There were no changes during the clinical examinations that were test substance-related. Moreover, clinical chemistry and hematology also revealed no test substance-related effects.
Total serum T4 concentrations were found to be reduced in both sexes at dose levels ≥1000 ppm. In addition, an increase in T3 was found in high dose males. A minimal or slight hypertrophy and vacuolization of the thyroid follicular epithelium in both sexes were found at dose levels ≥3000 ppm.
Absolute and relative adrenal weights were increased in females, accompanied by an increase in the severity of fatty degeneration of the "X" zone in the adrenal glands of the females at ≥3000 ppm. Absolute liver weight was significantly increased in males at 7500 ppm, relative liver weights at 7500 ppm for both sexes and at 3000 ppm also for males.
The decrease in total serum T4 concentrations in both male and female mice fed 1000; 3000 and 7500 ppm of metiram suggests that metiram may act as a mild anti-thyroidal agent resulting in an inhibition of thyroid hormone production. The statistically significant increase in total serum T3 in males may be explained by an interaction of several factors that are involved in a compensatory response by the thyroid; however, the biological relevance of this finding is rather uncertain.

Conclusion
Based on the described findings, the NOAEL under this test conditions is established at 300 ppm, corresponding to about 100 mg/kg bw.

19-week study in dogs (BASF DocID 1989/5128)

Metiram (purity: 95.2%) was administered to two pure-bred Beagle dogs per sex and dose group via the diet at doses as shown in the table below:

Table 5.3.2-8: Dosing of metiram in the 19-week feeding study in Beagle dogs

Group	Test substance concentration in the feed [ppm]	Average test substance intake [mg/kg bw/day]
1	0	0
2	100 (days 1-56)	3.8
3	400 (days 57-94) 3200 (days 95-102) 3200* (days 95-102) 6000 (days 103-130)	16 105 97 167
4	1600	55
5	6000 (days 95-102)	184

* One male and one female were temporarily made to group 5 between day 95 and 102 of the study

The following observations were made:
It was concluded that the administration of the test substance was associated with thyroid toxicity at doses of 1600 ppm and above.
There was no clear evidence of morphological changes at the lowest dose of 100 ppm, the presence of slight basophil hypertrophy in the adenohypophesis of two animals of this group and minimal basophil hypertrophy in the third may suggest an association with treatment. In the absence of an obvious relationship to dose, however, the significance of this finding has been considered to be questionable and felt that this should be further investigated in the 12 month dog feeding study described next [1989/10786].

Conclusion
Based on the described findings, no NOAEL under this test conditions is established.

Report:	*CA 5.3.2/4*
	1991a
	52-week oral toxicity (feeding) study with Metiram Premix 95% in the dog
	1991/10786
Guidelines:	*EPA 83-1*
GLP:	*yes*
	(certified by Eidgenoessisches Departement des Innern, Bern, Schweiz)

Report:	*CA 5.3.2/5*
	1992a
	Amendment 1: 52-week oral toxicity (feeding) study with Metiram Premix 95% in the dog
	1992/12594
Guidelines:	*EPA 83-1*
GLP:	*yes*
	(certified by Eidgenoessisches Departement des Inneren, Bern, Schweiz)

Report:	CA 5.3.2/6
	Dammann M., 2015a
	BMD-1-year dog thyroid weights – left and right weights combined
	2015/1174483
Guidelines:	none
GLP:	no

Report:	CA 5.3.2/7
	Melching-Kollmuss S.,Dammann M., 2017 a
	Explanations on Benchmark Dose Calculations conducted on thyroid parameters determined in the 1-year dog study (DocID 1991/10786)
	2017/1142341
Guidelines:	none
GLP:	no

Material and Methods:
The chronic toxicity of metiram (batch no.: WF 5103; purity: 94%) was investigated in a 52 week dog feeding study. The study consisted of five groups each containing five male and five female Beagle dogs ([redacted]). The animals were treated with 0; 30; 80; 1,000 or 3,000 ppm in the diet. These concentrations resulted in a test substance intake of 0; 0.98; 2.59; 29.9 or 84.8 mg/kg bw.
Ophthalmological examinations were carried out before initiation of treatment and on study weeks 13, 26 and 52. Neurological examinations were carried out before initiation of treatment and on study weeks 14, 26, 40, 46 and 51. The neurological examinations investigated a number of parameters with respect to 1) postural reactions, 2) spinal reflexes and 3) cranial nerves. Haematological and clinicochemical examinations as well as urinalysis were carried out before initiation of treatment and on study weeks 13, 26 and 52.

For the determination of the benchmark dose the software 'Benchmark dose Software' (BMDS, released by EPA) was used. The Exponential (M2) model was used for the calculations.

A dose with 5% and 10% increase of the thyroid weight in comparison to the control group mean and its 95% lower confidence limit was calculated. As in this study the left and the right thyroid was weighed separately, the values determined for this paired organ are combined and the BMD calculations was done using the combined value of male and female thyroid weights.
In addition benchmark dose calculations had been conducted using the PROAST model for absolute and relative thyroid weights and for the T_4 changes (2017/1142341, 2017/1143423, 2017/1143425). A benchmark dose response (BMR = %effect size) was derived for the absolute and relative thyroid weight changes based on the median coefficient of variation (CV), calculated by dividing the standard deviations by the mean values of controls and the treated groups (2017/1142341). As the CVs using the control groups showed a high variation, also the CVs of the treatment groups had been calculated. No difference between the CVs of the controls and the treated groups were found, thus the median value of roughly 20% had been used as the BMR. For the T_4 changes BMRs (%effect sizes) of 10% have been calculated using the values from the control groups of males and females.

At the end of the study gross and histopathology were performed. In addition to the standard pathological techniques and examinations, one of the hindlimbs of each dog was perfused with glutaraldehyde and embedded in epoxy resin blocks for subsequent histopathological investigation of the peripheral nervous system.

Findings:
Results of the dietary analysis indicated that metiram was homogeneously mixed in the diets at all levels and was stable under the conditions of this test. The mean concentrations of metiram were within 10% of the nominal concentrations.

No animal died during the treatment period. A slight to markedly increased incidence of diarrhea was evident in individual dogs treated at 80 (males only); 1000 or 3000 ppm. There were no other clinical signs that could be related to treatment with metiram. A slight to marked decrease in group mean food intake was recorded in the females at 1000 ppm and the males and females at 3000 ppm in the first five to ten weeks of treatment when compared with the control and pretest values. Thereafter, food intake improved although it remained slightly, but consistently, lower than that of the controls throughout the treatment period. Food intake in the dogs at 30 ppm or 80 ppm was unaffected by treatment. Slight to moderate weight loss was recorded in dogs at 1000 ppm or 3000 ppm during the first weeks of treatment. Thereafter, slight weight loss was recorded over the treatment period in one male at 3000 ppm and one female at 1000 ppm. Group mean body weight gain at 3000 ppm was statistically significantly reduced during the first half of the study and remained lower than that of the controls during the second half of the study. Group mean bodyweight gain over the treatment period in the dogs at 30; 80 or 1000 ppm metiram was unaffected by treatment.

There was no indication that treatment with the test substance had any adverse effect upon the eyes. Examination of the haematology data revealed the following changes in comparison with the controls: A slight decrease in erythrocyte count, haemoglobin concentration and, on occasions, haematocrit was noted with increased mean cell volume and decreased mean cell haemoglobin concentration in the males and females at 1000 ppm or 3000 ppm at each investigation (see Table 5.3.2-9 and Table 5.3.2-10).

Table 5.3.2-9: Summary of substance-related haematology data (males)

Weeks	Concentration in diet	RBC [T/L]	HB [mmol/L]	MCV [fL]	MCHC [mmol/L]	Retic. [L]
13	0 ppm	6.26	9.0	66.1	21.8	0.002
	30 ppm	6.12	8.7	65.6	21.7	0.002
	80 ppm	5.84	8.3	65.5	21.8	0.001
	1000 ppm	5.46*	8.2	70.8**	21.1**	0.003
	3000 ppm	5.58	8.2	70.8**	20.9**	0.006**
26	0 ppm	6.86	9.8	66.9	21.5	0.014
	30 ppm	6.36	8.9	66.0	21.3	0.009
	80 ppm	6.31	9.0	66.2	21.7	0.009
	1000 ppm	5.87**	8.8*	70.7**	21.1	0.009
	3000 ppm	5.96*	8.6*	70.3*	20.6**	0.017
52	0 ppm	6.82	9.8	69.0	20.8	0.005
	30 ppm	6.53	9.3	67.9	20.9	0.005
	80 ppm	6.64	9.4	67.9	21.0	0.008
	1000 ppm	5.95*	9.0	72.6*	20.9	0.010
	3000 ppm	6.10	8.9	72.6*	20.1**	0.016*

* $p < 0.05$; ** $p < 0.01$ Dunnett-test based on pooled variance
RBC = red blood cells
HB = hemoglobin
MCV = mean corpuscular volume
MCHC = mean corpuscular hemoglobin concentration
Retic = reticulocyte count

Table 5.3.2-10: Summary of substance-related haematology data (females)

Weeks	Concentration in diet	RBC [T/L]	HB [mmol/L]	MCV [fL]	MCHC [mmol/L]	Retic. [L]
13	0 ppm	6.91	9.8	66.0	21.6	0.001
	30 ppm	6.83	9.6	65.7	21.5	0.003
	80 ppm	6.60	9.4	65.6	21.7	0.001
	1000 ppm	6.19	9.2	71.0**	21.1	0.004
	3000 ppm	5.77*	8.4*	70.2*	20.5**	0.005*
26	0 ppm	7.05	10.0	66.6	21.4	0.010
	30 ppm	7.03	10.0	66.5	21.4	0.009
	80 ppm	6.71	9.6	66.4	21.5	0.005
	1000 ppm	6.33	9.3	71.0**	20.7	0.020**
	3000 ppm	6.00	8.8	70.2*	20.8	0.016
52	0 ppm	7.23	10.3	69.6	20.4	0.008
	30 ppm	6.84	9.7	69.0	20.7	0.006
	80 ppm	6.71	9.6	68.1	21.0*	0.003
	1000 ppm	6.34	9.3	72.6	20.2	0.014**
	3000 ppm	5.80*	8.5	73.5*	19.8*	0.018*

* $p < 0.05$; ** $p < 0.01$ Dunnett-test based on pooled variance
RBC = red blood cells
HB = hemoglobin
MCV = mean corpuscular volume
MCHC = mean corpuscular hemoglobin concentration
Retic. = reticulocyte count

Slight reticulocytosis was observed in males at 3000 ppm and in females at 1000 ppm or 3000 ppm in weeks 26 and 52. This was associated with an increased incidence of slight polychromasia in these groups at the latter investigation. Examination of the clinical biochemistry data revealed the following changes in comparison with the controls: In males and females, at 3,000 ppm group mean glucose concentration was slightly decreased (see Table 5.3.2-11 and Table 5.3.2-12). In males, at 1000 ppm and in males and females, at 3000 ppm total lipids, cholesterol, phospholipids and, particularly in males, triglyceride values were slightly increased. Group mean thyroxine (T_4) concentration in males at 1,000 ppm and in males and females at 3000 ppm was significantly decreased starting from the 13th week. At 3000 ppm group mean alkaline phosphatase activity in the dogs, particularly in females was slightly increased. At this dose level group mean total protein levels and a disturbance of the protein-electrophoretic pattern was increased in males. The latter was characterized by decreased albumin, increased alpha-1, beta and gamma globulins and a decreased albumin/globulin ratio. The differences from the controls of these parameters were generally statistically significant. These effects were dose-dependent in severity and present at each investigation.

Table 5.3.2-11: Clinical biochemistry summary (males) of metiram affected parameters

							Thyroid function	Prot. electroph. (rel.)	
weeks	Concen-tration in diet	Glucose [mmol/L]	Lipids T. [g/L]	Cholest. T [mmol/L]	Trigl. [mmol/L]	Phos. Lipid [mmol/L]	T4 [nmol/L]	Albumin [L]	A1-Glob. [L]
13	0 ppm	6.06	2.9	2.92	0.45	3.36	41.3	0.495	0.127
	30 ppm	5.54*	3.0	3.50	0.41	3.88	36.3	0.489	0.131
	80 ppm	5.67	3.2	3.87	0.40	3.92	36.7	0.502	0.138
	1000 ppm	5.58*	3.9	4.41	0.55	4.33	28.3*	0.488	0.142*
	3000 ppm	5.52*	6.4*	6.29**	0.84*	5.23**	17.0**	0.460	0.142*
26	0 ppm	5.62	2.6	2.72	0.43	2.92	43.3	0.515	0.123
	30 ppm	5.46	3.1	3.37	0.49	3.43	40.2	0.500	0.128
	80 ppm	5.26	3.2	3.73	0.48	3.65	44.4	0.501	0.128
	1000 ppm	5.53	3.7	4.43*	0.55	4.09*	36.6	0.483	0.135
	3000 ppm	5.25	4.9**	5.85**	0.62	4.94**	31.6**	0.454*	0.144*
52	0 ppm	6.26	2.6	3.13	0.45	3.32	53.9	0.488	0.103
	30 ppm	5.67	3.3	3.86	0.55	4.02	48.7	0.481	0.111
	80 ppm	5.66	3.3	3.55	0.60	3.75	50.5	0.466	0.110
	1000 ppm	5.96	4.0*	4.63*	0.68*	4.33	39.9*	0.464	0.121*
	3000 ppm	5.37**	5.4**	6.41**	0.71*	5.36**	31.3**	0.428	0.133**

* p < 0.05; ** p < 0.01 Dunnett-test based on pooled variance

Table 5.3.2-12: Clinical biochemistry summary (females) of metiram affected parameters

						Thyroid function
Weeks	concen-tration in diet	Glucose [mmol/L]	Lipids T. [g/L]	Cholest. T. [mmol/L]	Phos. Lipid [mmol/L]	T4 [nmol/L]
13	0 ppm	6.17	3.1	3.55	3.74	39.0
	30 ppm	5.77	2.8	3.20	3.52	39.9
	80 ppm	5.79	3.6	4.42	4.46	41.4
	1000 ppm	5.71	3.8	4.50	4.31	35.0
	3000 ppm	5.67	5.2**	5.79**	5.02**	20.3**
26	0 ppm	5.81	3.3	3.84	3.90	44.6
	30 ppm	5.37	2.9	3.24	3.38	41.9
	80 ppm	5.33	4.9	5.19	4.82	44.9
	1000 ppm	5.28	4.6	5.09	4.61	39.2
	3000 ppm	5.08**	5.0	5.52	4.77	31.1*
52	0 ppm	6.61	3.8	3.86	4.05	37.9
	30 ppm	5.55**	3.1	4.07	4.19	45.3
	80 ppm	5.78*	5.8	6.38*	5.57*	55.5**
	1000 ppm	5.74**	5.2	5.69	5.07	38.2
	3000 ppm	5.56**	6.8	6.88*	5.63*	29.4

* p < 0.05; ** p < 0.01 Dunnett-test based on pooled variance

Benchmark dose calculations had been done for the T4 hormone changes. %effect sizes of 10% had been calculated for relevant changes in T4, based on median variation coefficient of controls (including pretest I and II). An analysis of the dose response curves revealed that the difference between males and females is marginal, thus it has been decided to use the combined calculations of male and female T_4 hormone changes and using sex as co-variable in the models (see DocID 2017/1142341) The $BMDL_{10}$ for combined T_4 changes in males and females at the time points 13, 26 and 52 weeks was 97, 110 and 204 ppm. For week 13 and week 26 the ratio of BMDL to BMD was above 4. This means that the 95% confidence limit is much lower than the estimated BMD itself, and can thus be considered to represent very conservative values.

Urinalysis parameters were unaffected by treatment with metiram.
Examination of the organ weight data revealed a slight to marked increase in group mean thyroid gland weight in males and females at 1000 ppm or 3000 ppm, respectively, in comparison with the controls. The differences from the controls for the absolute weight and ratios were statistically significant in the high dose group. Thyroid enlargement and thickening were consistently seen macroscopically at 3000 ppm (see Table 5.3.2-13). Thyroid thickening alone was observed in the other treated groups, with a dose-related incidence, but as it was also seen in a control animal and is of uncertain significance. Further the animals only showing thyroid thickening were indistinguishable between control animals at histopathological examination. There were no other treatment-related necropsy findings identified.

Table 5.3.2-13: Thyroid weights after 52 weeks (absolute, relative)

Concen-tration in diet (ppm)	Thyroid weight after 52 weeks							
	absolute (g) (SD)				relative (%) (SD)			
	males		females		males		females	
	L	R	L	R	L	R	L	R
0	0.394 (0.118)	0.323 (0.120)	0.403 (0.083)	0.397 (0.087)	0.0042 (0.0012)	0.0035 (0.0014)	0.0049 (0.0011)	0.0048 (0.0007)
30	0.380 (0.050)	0.361 (0.065)	0.334 (0.045)	0.347 (0.057)	0.0039 (0.0006)	0.0037 (0.0005)	0.0045 (0.0011)	0.0046 (0.0013)
80	0.448 (0.129)	0.295 (0.101)	0.409 (0.046)	0.448 (0.076)	0.0051 (0.0014)	0.0033 (0.0009)	0.0052 (0.0004)	0.0057 (0.0009)
1000	0.543 (0.081)	0.615 ((0.148)	0.488 (0.155)	0.490 (0.143)	0.0058 (0.0011)	0.0065 (0.0016)	0.0065 (0.0018)	0.0066 (0.0020)
3000	1.553** (0.527)	1.345** (0.558)	1.296** (0.311)	1.245** (0.276)	0.0171** (0.0046)	0.0144** (0.0040)	0.0179** (0.0038)	0.0173** (0.0036)

* $p < 0.05$; ** $p < 0.01$ Dunnett-test based on pooled variance

For the purpose of benchmark modelling the left and right thyroid weights were combined for females and males and these values were used for modeling. A benchmark dose with 5% and 10% increase of the absolute thyroid weights in comparison to the control group mean and its 95% lower confidence limit in males and females was calculated. The model with the best fit was used (see Table 5.3.2-14). In case of thyroid weights a 10% relative risk value is considered appropriate, thus the $BMDL_{10}$ for male thyroid weights is calculated to be 176 ppm.

Table 5.3.2-14: Benchmark Dose Computation for absolute thyroid weights in males and females

Endpoint	Model	BMD05	BMDL05	BMD10	BMDL10
Thyroid weight, males	Exponential (M2), inhomogeneous variance modeled	105 ppm	90.2 ppm	205 ppm	**176 ppm**
Thyroid weight, females	Exponential (M2), inhomogeneous variance modeled	125 ppm	107 ppm	245 ppm	210 ppm

Using PROAST, similar model calculations were provided by the Software (2017/1142341, 2017/1143425). The relative thyroid weight changes lead to a $BMDL_{20}$ value of 162 ppm for male and female dogs using the most conservative value of the model with the best fit. All BMDLs were derived by using a BMR of 20% to be relevant. Comparing the two values of 176 ppm coming from the EPA model and the 162 from PROAST, they are all in the same order of magnitude, indicating a certain robustness of the approach.

Thyroid follicular hyperplasia, dose-related in severity, was seen histologically in all 1000 ppm and 3000 ppm animals. At 1000 ppm males and females showed thyroid follicle hyperplasia of grade 1 (each one animal) and grade 2 (each 4 animals) and at 3000 ppm among the females the grading was between 3 (one animal), 4 and 5 (each two animals) and between grade 4 (2 animals) and 5 (3 animals) in the males. No meaningful BMD calculation could be conducted for thyroid histopathological changes, as all animals were affected at 1000 ppm and no animal was affected at the next lower dose level of 80 ppm.

There was an increase, dose-related in severity, in hepatic pigment deposition seen in many 1000 ppm and 3000 ppm animals. The pigment is thought to be lipofuscin and the significance of this very non-specific response remains uncertain. There were no other treatment-related histological findings identified.

There was no effect on the neurological parameters considered to be attributable to the administration of metiram. Also the extensive specific microscopic examination (longitudinal and transverse 1 micrometer sections of sciatic nerve were cut from all animals and stained with toluidine blue) of the hindlimb did not reveal any abnormalities in the sciatic nerve.

Conclusion:
A dose of 3000 ppm had a clear effect on body weight development and absolute and relative thyroid weight. The thyroids were identified as a target organ by clinical chemical examination (decrease of thyroxine concentration) and confirmed by histological examination (follicular hyperplasia) at doses of ≥ 1000 ppm. In males, protein levels were increased and a disturbance of the protein-electrophoretic pattern was observed. Also, effects on hematology and clinical biochemistry data were observed at 1000 and 3000 ppm.
Based on the findings in this study, the NOAEL is 80 ppm equivalent to 2.59 mg/kg bw for male and female animals. As the next higher dose level was 1000 ppm (factor between NOAEL and LOAEL > 10), it was considered appropriate to conduct benchmark doses in order to derive more realistic points of departure from this study. Benchmark dose calculations (according to EPA and PROAST model) had been conducted for relative thyroid weight and T_4 hormone changes. No benchmark dose calculations could be done for the histopathological findings. When a **BMDL** is calculated, using a specified effect of 0.10 (appropriate for thyroid weights) and the model, which is delivering the best fit for the input data (Exponential (M2), a value of 176 ppm is derived. This corresponds to a dose of 5.7 mg/kg bw. Using the PROAST model a value of 162 ppm was found using a BMR (% effect size) of 20%, corresponding to 5.2 mg/kg bw. For T_4 a $BMDL_{10}$ of 97, 110 and 204 ppm was identified for the 13, 26 and 52 weeks timepoint, where the value for the 52 weeks time point seems to be more realistic.

Following a WoE assessment, a point of departure of 162 ppm (corresponding to a dose of 5.2 mg/kg bw) – which is equivalent to the $BMDL_{20}$ calculated for relative thyroid weight increases by the PROAST model – is considered to represent the appropriate endpoint for this study and shall be used for risk assessment.
~~**Overall the more appropriate point of departure for this study seems to be 176 ppm, based on $BMDL_{10}$ calculations. The dietary concentration of 176 ppm corresponds to a dose of 5.7 mg/kg bw.**~~

CA 5.3.3 Other routes

Regarding other routes of exposure, the applicant performed a 21-day study by the dermal route and a 13-week study by inhalation route.

21-day dermal toxicity in rabbits (BASF DocID 1987/0260)

Toxicological data from a 21-day dermal study were obtained using the formulated product Polyram DF® (BAS 222 28 F). This study was done with the formulated product because for risk assessment purposes for the user, toxicological data from the formulated product, provide the most relevant data. The Polyram DF® formulation, which contains metiram as the only active ingredient at a concentration of 70%, was applied dermally to five rabbits (New Zealand White) per sex and test group (0; 25; 50 and 250 mg/kg bw). Altogether, the animals were treated on 21 consecutive days, the test substance remaining under a semiocclusive dressing for six hours in each case.

The following observations were made:
No death occurred during the study. No systemic symptoms were observed. Local findings on the treated skin were only observed in male and female animals of the 250 mg/kg bw dose group. They mainly consisted of erythema, edema, scales and hyperemia. All findings were described as minimal to slight. No differences in food consumption and body weight gain were observed between the animals of the test article treated and control groups. No treatment-related ophthalmoscopic changes were observed in any rabbit of the test article-treated or control groups. The assessment of hematology, clinical biochemistry and urinalysis data indicated no changes of toxicological significance at termination of the treatment. Organ weights and organ to bw ratios were comparable between the rabbits of the control and test article-treated groups. No obvious differences were observed. Treatment-related findings were recorded only in the skin of rabbits from the highest dose group and consisted of exfoliation and ulcerative dermatitis. The latter finding is regarded as an indication of toxicity at the highest dose level, whereas the two intermediate dose groups were free of adverse skin findings.

Conclusion
Based on the described findings, the local NOAEL under this test conditions is established at 50 mg/kg bw and the systemic NOAEL at >250 mg/kg bw.

13-week inhalation toxicity in rats (BASF DocID 1986/407 and 1987/0414)

28 male and 28 female Sprague-Dawley rats per dose group were exposed, nose only, six hours per day, five days per week for 13 consecutive weeks to 0; 2.1; 20 or 101 mg/m³ metiram (purity: 94%) 0.2% ETU. These gravimetrically determined exposure concentrations were found to be within +11% to -5% of samples analyzed chemically for metiram. Concentrations of ETU in the exposure chambers ranged from 0.02, 0.033 and 1.8 mg/m³ in the low, the mid and the high metiram exposure groups, respectively. Half of the animals were observed for further 13 weeks after the exposure period to examine the reversibility of possible findings.

The following observations were made:
Apart from nonspecific signs, which are probably related to the exposure to dust per se, no specific findings were obtained which were observed, for example, after oral administration of the technical active ingredient. Retarded body weight gain, which was more pronounced in the males, was determined in the 101 mg/m³ test group. This finding was completely reversible during the 13-week post-exposure observation period. The clinicochemical, haematological, urinalytical and ophthalmological examinations did not reveal any abnormalities in any test group. The study concentrated mainly on the question whether particles of the technical active ingredient and of its transformation products reaching the alveoli might induce functional and anatomical thyroid changes via the lungs, as was known from subchronic feeding studies in high doses. It can be stated that neither the functional parameters (T3, T4, TSH) nor the thyroid weights showed any gross-pathological or histopathological changes indicating an effect of the test substance.

Since, however, the sensitive thyroid hormone levels were unaffected, the conclusion may be drawn that, under the given test conditions, metiram reaching the lungs did not lead to any impairment of the thyroid up to a concentration of 101 mg/m³ after subchronic exposure.
With the exception of an increased relative lung weight in the females of the highest test group, no organ weight changes were observed as compared with the control. The deposit of brown pigment in the kidneys at 101 mg/m³ was the only histopathological finding, the toxicological significance of which is unknown. The alveolitis observed histopathologically cannot be regarded as substance-related. This is rather a nonspecific dust reaction of the polymeric active ingredient artificially ground to particles reaching the alveoli, which has no relevance to a risk assessment in practice. Overall, metiram produced a low order of toxicity with no specific effects on the thyroid and no significant morphological changes.

Conclusion
Although the thyroid hormone levels were not significantly affected, a sensible reduction was observed at 100 mg/m³, as well as an increase in thyroid weights. For this reason the NOAEC established at 20 mg/m³.

CA 5.4 Genotoxicity Testing

Studies evaluated in the draft monograph of Rapporteur Member State Italy of July 2000:

A sufficient data-package of in vitro genotoxicity studies in bacterial and mammalian cell systems and of in vivo genotoxicity studies conducted with metiram is available. These studies as listed in Table 5.4.1-1, Table 5.4.2-1 and Table 5.4.3-1 below have been evaluated by European authorities and Italy as Rapporteur Member State (European Commission Peer Review Program) and were considered to be acceptable. For the convenience of the reviewer, these studies are summarized below in table form as extracted from the monograph.

The summary of genotoxicity, as given in the monograph of metiram.

"The technical active ingredient metiram (as different batches containing metiram in a range 80-89%, with the intentional addition of 2% ETU in the majority of the studies, as described in the table) was investigated in a series of *in vitro* and *in vivo* tests which cover the various end points of possible genetic damage (point or gene mutation, chromosome mutation, DNA damage and repair), using bacterial and mammalian somatic cells as well as mammalian germ cells. Furthermore, the technical active ingredient was tested for cell-transforming properties.
Two *in vitro* mutagenicity tests considering different genetic endpoints (CHO/HGPRT test without S-9 mix and CHO/SCE test without activation and with S-9 mix of mice) shown an equivocal or a weakly positive result. These results were not confirmed by further *in vitro* or *in vivo* experiments. Moreover, a dominant-lethal test was conducted to examine the mutagenic potential in germ cells and did not give any indications of a positive mutagenic response. Also, the *ex vivo* host mediated assay shows no mutagenic response in bacterial cells.
The overall evidence provided by the available studies is that metiram is not significant genotoxic.

Thus, the conclusion for relevant endpoints for the current re-registration remains as follows:

In vitro studies	No genotoxic concern
In vivo studies	No genotoxic concern
In vivo studies in germ cells	No genotoxic concern

Submission of not yet per-reviewed studies in this dossier:

The database has been extended (see Table 5.4-1) with an Ames test (with spiked impurities) and an in vitro and in vivo micronucleus test to fulfill the new data requirement in order to demonstrate toxicological equivalence of the proposed specification. Robust study summaries of these new studies are provided in detail below. The Ames test is summarized in document J of this dossier. By weight of evidence it was concluded that metiram is not genotoxic.

Table 5.4-1: Not-yet peer-reviewed *in vitro* and *in vivo* mutagenicity study with metiram

Study type	Test System	With S9-mix	Result	Reference
In vitro Mutagenicity in bacterial cells (Ames test) OECD 471*	Salmonella typhimurium (TA 98, 100, 1535, 1537, E. coli) Concentration up to 333 µg/plate metiram	No Yes (rat)	Negative Negative	see DocJ
In vitro MNT	V79 Chinese hamster cells 4 h treatment, 24 h harvest time 3.13 – 100 µg/mL (±S9) 10 – 120 µg/mL (-S9)	No Yes (rat)	Positive Negative	2014/1315333
In vivo MNT	NMRI mice (erythrocytes) 24/48 h sacrifice 500, 1000, 2000 mg/kg bw (bioavailability was confirmed)	-	Negative	2014/1315334 2015/1112085

*The summary of the study is provided in DocJ of this dossier.

CA 5.4.1 In vitro studies

A summary of the in vitro genotoxicity studies evaluated for Annex I inclusion of metiram is summarized in Table 5.4.1-1 below.

Table 5.4.1-1: In vitro mutagenicity studies with metiram

Study type	Test System	With S-9 mix	Result	Reference
In vitro Mutagenicity in bacterial cells (Ames test)	*Salmonella thyphimurium* (TA 98, 100, 1537) Concentration up to 2000 µg/plate metiram (purity: 98%)	No Yes (rat)	Negative Negative	1977/027
In vitro Mutagenicity in bacterial cells (Ames test) OECD 471	*Salmonella thyphimurium* (TA 98, 100, 1535, 1537) Concentration up to 5000 µg/plate metiram with 2.2% ETU (purity: 98%)	No Yes (rat) Yes (mouse)	Negative Negative Negative	1985/020
ex vivo Host-mediated assay	*Salmonella thyphimurium* (TA 1530) Dose: 500, 1670, 5000 mg/kg bw metiram with 2.2% ETU (purity: 98%)	In mice (male)	negative	1985/210
In vitro Mutagenicity in mammalian cells CHO/HGPRT Based on OECD 476	Chinese Hamster ovary cells; Concentrations up to 100 µg/mL metiram (purity: 99%)	No Yes (rat)	Weakly positive (borderline significance Negative	1985/238
In vitro Mutagenicity in mammalian cells CHO/HGPRT OECD 476	Chinese Hamster ovary cells; Concentrations up to 500 µg/mL metiram (purity: 93%)	No Yes (mouse)	Negative Negative	1990/0285
In vitro Mutagenicity in mammalian cells SCE	Chinese Hamster ovary cells; Concentrations up to 200 µg/mL metiram (purity: 99%)	No Yes (rat) Yes (mouse)	Weakly positive Negative Weakly positive	1986/082
In vitro DNA damage & repair UDS test	Rat hepatocytes; Concentrations up to 49.2 µg/mL metiram with 2.2% ETU (purity 99%)	Primary cells	negative	1984/209

Report: CA 5.4.1/1
Schulz M.,Landsiedel R., 2015a
BAS 222 29 F (Metiram TK) - In vitro micronucleus assay in V79 cells (cytokinesis block method)
2014/1315333

Guidelines: OECD 487 (2010), Commission Regulation EU No. 640/2012 of 06 July 2012 - B.49: In vitro Mammalian Cell Micronucleus Test

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

Metiram (Batch: 300015; Purity: 91.5%) was tested for its potential to induce micronuclei in V79 cells *in vitro* in the absence and presence of metabolic activation by S9 mix. Two independent experiments were carried out, incubating the cells for 4 h (±S9 mix; 24 h harvest time) with the test substance at concentrations in the range of 3.13 to 120 µg/mL. The vehicle DMSO served as negative control, ethyl methanesulfonate (EMS) as positive controls in the absence of metabolic activation and cyclophosphamide (CCP) as positive control in the presence of metabolic activation. Thereafter cytochalasin B was added and the cultures were fixed and stained finally after another 20 hours. Cytokinesis-block proliferation index and cell count were determined as cytotoxicity parameters and number of micronucleated cells were determined in 2000 binucleated cells per test group for evaluation of mutagenicity.

The vehicle controls gave frequencies of micronucleated cells within our historical negative control data range for V79 cells. Both positive control substances, EMS and CCP, led to the expected increase in the number of cells containing micronuclei.

Cytotoxicity indicated by clearly reduced cell count and/or low cell quality was observed at least at the highest applied test substance concentrations in all experimental parts. In the absence of S9 mix, after 4 hours exposure period a statistically significant increased micronucleus rate was found at the highest scorable concentration either in the first or the second experiment. Both values exceeded our laboratory's historical negative control data range. In contrast, in a single experiment in the presence of S9 mix no relevant increase of micronucleated cells was obtained after 4 hours exposure period.

On the basis of the results of the present study, the test substance caused a statistically significant and biologically relevant increase in the number of cells containing micronuclei in the absence of a metabolizing system.

Thus, under the experimental conditions described, Metiram TK is considered to have a chromosome-damaging (clastogenic) effect or to induce numerical chromosomal aberrations (aneugenic activity) under in vitro conditions in V79 cells in the absence of metabolic activation.

(BASF DocID 2014/1315333)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 222 29 F (Metiram TK)
Description: solid / beige
Lot/Batch #: 300015
Purity/content: 91.5%
Stability of test compound: The stability of the test item under storage conditions over the study period was guaranteed by the sponsor (expiry date January, 2016).
Vehicle used: DMSO (1% final concentration)

2. Control Materials:
Negative: No negative control was employed in this study.
Vehicle control: Culture medium with 1% DMSO
Positive control: Without metabolic activation:
Ethyl methanesulfonate (EMS, 400 and 500 µg/mL) dissolved in MEM without FCS;
With metabolic activation:
Cyclophosphamide (CCP, 0.5 and 1 µg/mL) dissolved in MEM without FCS

3. Activation: S9 was produced from the livers of rats pretreated with β-naphthoflavone/phenobarbital. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction is thawed at room temperature mixed with an appropriate volume of S9-supplement (cofactors). This preparation, the so-called S9-mix, was kept on ice until used.
The S9-mix was prepared immediately before use and had the following composition:

Component	Concentration
Phosphate buffer (pH 7.4)	15 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM

One part S9-fraction and 9 parts S9-supplement were mixed, yielding the S9-mix.

4. Test organism: V79 Chinese hamster cells (permanent cell line with a high proliferation rate, high plating efficiency, and stable karyotype)
Each batch of cells were checked for mycoplasma contamination, karyotype stability and plating efficiency.

5. Culture media:

Culture medium: MEM containing a L-glutamine source supplemented with 10% (v/v) fetal calf serum (FCS), Pen/Strep (10000 IU/10000 µg/mL), and 1% amphotericine B (250 µg/mL)

6. Test concentrations:

Micronucleus assay

Experiment I
(4-h exposure, ±S9): 3.13, 6.25, 12.5, 25, 50, 100 µg/mL

Experiment II
(4-h exposure, -S9): 10, 20, 40, 60, 80, 100, 120 µg/mL

B. TEST PERFORMANCE

1. Dates of experimental work: 20-Oct-2014 to 28-Apr-2015

2. Dose selection:

Dose selection was performed according to the current OECD Guideline for the *in vitro* micronucleus test. The highest test item concentration should be 5000 µg/mL, 5 µL/mL or 10 mM, whichever is the lowest. At least three test item concentrations should be evaluated for cytogenetic damage.

A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. Cytotoxicity is characterized by the percentages of reduction in the CBPI in comparison with the controls (% cytostasis) by counting 1000 cells per culture in duplicate. The experimental conditions in this pre-experimental phase were identical to those required and described below for the mutagenicity assay. The pre-test was performed with 8 concentrations of the test item separated by no more than a factor of √10 and a solvent control. All cell cultures were set up in duplicate. Exposure time was 4 hrs (with and without S9 mix). The preparation interval was 24 hrs after start of the exposure.

Test item concentrations ranging from 21.5 to 2750 µg/mL (with and without S9 mix) were chosen for the evaluation of cytotoxicity. In the pre-test for toxicity, precipitation of the test item in the treatment medium was observed at 1375 µg/mL and above in the absence of S9 mix. In the presence of S9 mix precipitation of the test substance in culture medium occurred at 171.9 µg/mL and above at the end of exposure period. Cytotoxicity indicated by reduced cell numbers of about or below 40-50% was observed at 43 µg/mL and above after 4 hours treatment in the absence and presence of S9 mix and after 24 hours continuous treatment in the absence of S9 mix.

3. Micronucleus test:

Seeding of the cells:

A single cell suspension with the required cell count (3 - $5x10^5$ cells per culture, depending on the schedule) was prepared in MEM incl. 10% (v/v) FCS. 5 mL cell suspension was transferred into 25 cm² cell culture flasks using a dispenser. Subsequently, the test cultures were incubated at 37°C, 5% (v/v) CO2 and ≥ 90% relative humidity. The cultures were visually checked for attachment and viability before treatment of the test cultures.

Treatment of test cultures
A test culture consists of two cytospin preparations on glass slides. A test group consists of two separately treated test culture flasks, means four preparations and thus of four slides. After the attachment period, about 20 - 24 hours after seeding, the medium was removed from the flasks and the treatment medium was added. The cultures were incubated for the respective exposure period at 37°C, 5% (v/v) CO2 and ≥ 90% relative humidity. At the end of the exposure period, the medium was removed and the cultures were rinsed twice with 5 mL HBSS (Hanks Balanced Salt Solution). Subsequently, 5 mL MEM (incl. 10% [v/v] FCS) supplemented with CytB (final concentration: 3 µg/mL; stock: 0.6 mg/mL in DMSO) was added and incubated at 37°C, 5% (v/v) CO2 and ≥ 90% relative humidity for the respective recovery time.

Preparation of test cultures
Just before preparation the culture medium was completely removed. Single cell suspensions were prepared from each test group by trypsination. Then, the cell numbers per flask of each single cell suspension were determined using a cell counter. Subsequently, $5x10^4$ cells per slide were centrifuged at 1400 rpm for 7 minutes onto labelled slides using a Cytospin centrifuge. After drying, the slides were fixed in 90% (v/v) methanol for 10 minutes.

Staining
Before scoring, the slides were stained with a mixture of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; stock: 5 mg/mL) and propidium iodide (stock: 5 mg/mL) in Fluoroshield™ at a concentration of 0.25 µg/mL each. By the use of the combination of both fluorescence dyes it can be differentiated between DNA (DAPI; excitation: 350 nm, emission: 460 nm) and cytoplasm (PI; excitation: 488 nm, emission: 590 nm).

4. Statistics:
The statistical evaluation of the data was carried out using the MUVIKE program system (BASF SE). The proportion of cells containing micronuclei was calculated for each group. A comparison of each dose group with the concurrent vehicle control group was carried out using Fisher's exact test for the hypothesis of equal proportions. This test is Bonferroni-Holm corrected versus the dose groups separately for each time and was performed one-sided. If the results of this test were statistically significant compared with the respective vehicle control, labels (* p ≤ 0.05, ** p ≤ 0.01) have been printed in the tables.

5. Proliferation index:
The cytokinesis-block proliferation index (CBPI) is a direct measure of the proliferative activity of the cells and it was determined in at least 1000 cells per culture (at least 2000 cells per test group). This value indicates the average number of cell cycles per cell during the period of exposure to the actin polymerisation inhibitor cytochalasin B. The number of mononucleated, binucleated and multinucleated cells was recorded and the CBPI was calculated using the following formula:

$$CBPI = \frac{(MONC \text{ x } 1) + (BINC \text{ x } 2) + (MUNC \text{ x } 3)}{n}$$

CBPI: Cytokinesis-block proliferation index
n: Total number of cells
MONC: Mononucleate cells
BINC: Binucleate cells
MUNC:Multinucleate cells

Cytostasis % = 100 – 100 [($CBPI_T$ – 1) / ($CBPI_C$ – 1)]
T: Test item; C: Solvent control

6. Micronucleus evaluation:

At least 1000 cells per culture, means at least 2000 cells per test group, were evaluated and the number of micronuclei-containing binucleated cells was recorded.
The analysis of micronuclei was carried out following the criteria:

- The diameter of the micronucleus is less than 1/3 of the main nucleus.
- The micronucleus and main nucleus retain the same color.
- The micronucleus is not linked to the main nucleus and is located within the cytoplasm of the cell.
- Only cells clearly surrounded by a nuclear membrane were scored.

Slides were coded before microscopic analysis by MUVIKE software. Cultures with few isolated cells were not analysed for micronuclei. Since the absolute values shown were rounded but the calculations were made using the unedited values, there may be deviations in the given relative values.

7. Evaluation criteria:

Acceptability criteria:

- The rate of micronuclei in the solvent controls falls within the historical laboratory control data range.
- The rate of micronuclei in the positive controls is statistically significant increased.
- The quality of the slides must allow the evaluation of a sufficient number of analyzable cells.

Assessment criteria:

A test item can be classified as non-clastogenic and non-aneugenic if:

- The number of micronucleated cells in the test groups is not distinctly increased above the concurrent vehicle control and is within our laboratory's historical negative control data range

A test item can be classified as clastogenic and aneugenic if:

- the number of micronucleated cells is not in the range of the historical laboratory control data and not within the current vehicle control and
- a significant, dose-related and reproducible increase in the number of cells containing micronuclei was observed

II. RESULTS AND DISCUSSION

A. CYTOTOXICITY

In both experiments in the absence of S9 mix cytotoxicity indicated by clearly reduced cell counts was observed at the highest applied test substance concentrations at 100 µg/mL or at 120 µg/mL (54.7% or 47.0% of control, respectively). Besides, in the presence of S9 mix the cell numbers were not clearly reduced after 4 hours exposure up to the highest applied test substance concentration. However, in all experimental parts at least the slides of the highest applied test substance concentrations were not scorable for cytogenetic damage.

No reduced proliferative activity was observed after 4 hours exposure interval in the absence and presence of S9 mix in the test groups scored for cytogenetic damage. However, in both experiments in the absence of S9 mix a dose-related decrease in cell growth was found. At least the slides of the highest applied test substance concentrations were not scorable for cytogenetic damage due to strong cytotoxicity (e.g. low cell proliferation).

Cell morphology/attachment was not adversely influenced (grade > 2) at any concentration evaluated for micronuclei. In both experiments under all conditions cell morphology/attachment was not adversely influenced from 100 µg/mL onward. The slides of these test groups were not scorable for cytogenetic damage due to strong cytotoxicity.

Osmolarity and pH values were not influenced by test substance treatment. Test substance precipitation in culture medium at the end of exposure period was only observed at 100 µg/mL in the 1st Experiment with metabolic activation (macroscopical assessment).

B. MICRONUCLEUS ANALYSIS

A statistically significant and biologically relevant increase in the number of micronucleated cells was observed after 4 hours test substance treatment in the absence S9 mix in two independently performed experiments.
In the 1st Experiment in the absence of metabolic activation after 4 hours treatment with the test substance at 50 µg/mL a statistically significant increased micronucleus rate (2.0% micronucleated cells) was obtained. This value was corroborated by increasing the sample of evaluation up to 4000 cells. The micronucleus rate exceeded either the concurrent vehicle control value (0.6% micronucleated cells) or our historical negative control data range (0.1 - 1.8% micronucleated cells). This observation was reproduced in the 2nd Experiment following the same test procedure. In this repeat experiment a dose-related increase in micronucleated cells was observed from 40 to 80 µg/mL (0.7, 1.6 and 3.9%). The value at the highest scored concentration of 80 µg/mL was statistically significantly increased. It exceeded either the concurrent vehicle control value (1.3% micronucleated cells) or our historical negative control data range (0.1 - 1.8% micronucleated cells).
Contrary, in the 1st Experiment in the presence of S9 mix after 4 hours exposure no biologically relevant increased micronucleus rates compared to the respective vehicle control values were obtained. At all evaluated test groups the values (0.7% micronucleated cells, all) were close to the concurrent vehicle control value (0.3% micronucleated cells) and clearly within our historical negative control data range (0.1 - 1.8% micronucleated cells).

The positive control substances EMS (without S9 mix; 400 µg/mL) and CPP (with S9 mix; 0.5 µg/mL) induced statistically significant increased micronucleus frequencies in both independently performed experiments. In this study, in the absence and presence of metabolic activation the frequency of micronucleated cells (2.4 – 2.9% micronucleated cells) was clearly above the range of our historical negative control data range (0.1 - 1.8% micronucleated cells) and within our historical positive control data range (2.3 – 26.6% micronucleated cells).

Table 5.4.1-2: Summary of results of the in vitro micronucleus test in V79 cells with metiram

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cell count in %	Micronucleated cells in %[a]
Exposure period 4 h without S9 mix					
I	24 h	Solvent control[1]	0.0	100.0	0.6
		Positive control[2]	12.3	109.0	2.8**
		12.5	2.8	107.9	0.6
		25	11.9	107.0	0.4
		50	22.1	110.6	2.0**
		100	n.s.	54.7	n.s.
Exposure period 4 h without S9 mix					
II	24 h	Solvent control[1]	0.0	100.0	1.3
		Positive control[2]	4.9	97.6	2.9**
		40	3.4	91.7	0.7
		60	6.7	99.0	1.6
		80	15.9	100.8	3.9**
		100	n.s.	75.3	n.s.
Exposure period 4 h with S9 mix					
I	24 h	Solvent control[1]	0.0	100.0	0.3
		Positive control[3]	35.5	89.3	2.4**
		6.25	2.7	99.3	0.7
		12.5	5.7	86.3	0.7
		25.0	5.1	97.8	0.7
		50.0	n.s.	62.7	n.s.

a: The number of micronucleated cells was determined in a sample of 2000 binucleated cells
**: p≤0.01; n.s.: not scorable due to strong cytotoxicity
1 DMSO 1 % (v/v)
2 EMS 400 µg/mL
3 CCP 0.5 µg/mL

III. CONCLUSION

Thus, under the experimental conditions chosen here, the conclusion is drawn that metiram has the potential to induce micronuclei (clastogenic and/or aneugenic activity) under in vitro conditions in V79 cells in the absence of metabolic activation.

This positive result in the absence of metabolic activation is considered to be a false-positive artificial result, as metiram (in order to test it in an in vitro system) was dissolved in DMSO, which is leading to a direct degradation of the product and thus not corresponding to the physiological situation. Therefore an in vivo micronucleus test in mice was conducted to confirm, that metiram is not genotoxic.

CA 5.4.2 In vivo studies in somatic cells

A summary of the in vivo genotoxicity studies evaluated for Annex I inclusion of metiram is summarized in Table 5.4.2-1 below.

Table 5.4.2-1: In vivo mutagenicity studies with metiram

Study type	Test System	Test material	Result	Reference
In vivo Cytogenicity assay Chromosome analysis	Rat Study on bone marrow cells; single and multiple administration Dose (acute): 0, 240, 1200, 2400 mg/kg bw Dose (5 consecutive days): 0, 20, 100, 200 mg/kg bw/d	Metiram with 2.2% ETU (purity: 99%)	Negative	1986/265
In vivo Mutagenicity test SCE OECD	Chinese hamster bone marrow Dose: 0, 1000, 3330, 10000 mg/kg bw	Metiram (purity: 93%)	Negative	1990/0569

Report:	CA 5.4.2/1 [redacted] 2015a BAS 222 29 F (Metiram TK) - Micronucleus assay in bone marrow cells of the mouse 2014/1315334
Guidelines:	OECD 474 (2014), (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.12, EPA 870.5395
GLP:	yes (certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Report:	CA 5.4.2/2 [redacted], 2015 a Metiram: Analysis of ETU and EU in mouse plasma samples originating from an in vivo micronucleus study (26M0734/01X071) with BAS 222 29 F (Metiram formulation) 2015/1112085
Guidelines:	none
GLP:	yes (certified by Landesamt fuer Umwelt, Messungen und Naturschutz Baden Wuerttemberg, Karlsruhe, Germany)

Executive Summary

Metiram (Batch: 300015; Purity: 91.5%) was tested for chromosomal damage (clastogenicity) in NMRI mice using the micronucleus test method. For this purpose, the test substance, suspended in methyl cellulose was administered once orally to groups of 5 to 7 male mice at dose levels of 0, 500, 1000, and 2000 mg/kg body weight in a volume of 10 mL/kg body weight. The vehicle served as negative and cyclophosphamide as positive control. The animals were sacrificed 24 or 48 (additional high dose group) hours after administration; the bone marrow of the two femora was prepared from each animal. After staining of the preparations, 4000 polychromatic erythrocytes were evaluated per animal and investigated for micronuclei. The normochromatic erythrocytes occurring per 500 polychromatic erythrocytes were also recorded. In addition, blood samples taken from satellite group (vehicle and high dose group) animals at both time points were analyzed to verify the bioavailability of the test substance.

The oral administration of metiram did not lead to any biologically relevant increase in the number of polychromatic erythrocytes containing micronuclei. The rate of micronuclei was mostly close to the concurrent negative control and was within the range of the historical control data. No effect on erythropoiesis induced by the treatment of mice with metiram was observed in any dose group.
The bioavailability of the test substance in blood after oral administration was clearly confirmed by determining ETU in the mouse plasma samples. No signs of systemic toxicity were observed in any of the animals treated with the positive control substances, the vehicle or the test substance. The positive control chemical cyclophosphamide led to the expected increase in the rate of polychromatic erythrocytes containing micronuclei, thus demonstrating the sensitivity of the test system. Thus, under the experimental conditions of this study, metiram did not induce cytogenetic damage in bone marrow cells of NMRI mice in vivo.

(BASF DocID 2014/1315334)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material	BAS 222 29 F (Metiram TK)
Description:	solid / beige
Lot/Batch #:	300015
Purity/content:	91.5%
Stability of test compound:	The stability of the test item under storage conditions over the study period was guaranteed by the sponsor (expiry date January, 2016).
Vehicle used:	Methyl Cellulose (1%)
2. Control Materials:	
Negative control:	A negative control was not employed in this study
Solvent control:	Methyl Cellulose (1%)
Positive control:	Cyclophosphamide (CPP) 40 mg/kg bw (dissolved in sterile water)
3. Test animals:	
Species:	Mouse
Strain:	Crl:NMRI
Sex:	Male for the main study; male and female for the range finding study
Age:	8-9 weeks (pre-test); 7-8 weeks (main experiment)
Mean body weight:	37±2.4 g
Source:	
Number of animals per dose:	
Range finding study:	2 per sex and dose
Micronucleus assay:	5 males per vehicle/positive control groups; 7 males in treatment groups
Blood sampling:	3 males per vehicle and high dose group per sampling time point (additional satellite group)
Acclimation period:	At least 5 days
Diet:	Standardized pelleted feed, ad libitum
Water:	Tap water, ad libitum
Housing:	Single housing in Makrolon cages, type II/III with wire-mesh top
4. Environmental conditions:	
Temperature:	20-24 °C
Humidity:	45-65%
Air changes:	not indicated (central air-conditioning)
Photo period:	12-hour light-dark cycle (06:00 - 18:00, 18:00 - 06:00)
5. Test compound doses:	
Range finding test:	2000 mg/kg bw
Micronucleus assay:	500, 1000 and 2000 mg/kg bw The test substance was administered once by oral gavage using an application volume of 10 mL/kg bw.

B. TEST PERFORMANCE:

1. Dates of experimental work: 06-Feb-2015 to 09-Mar-2015

2. Preliminary range finding test

In a pretest for the determination of the acute oral toxicity, 2 male and 2 female animals were treated once by oral gavage with a test substance dose of 2000 mg/kg bw. The animals were treated once orally with the test item and examined for acute toxic symptoms at intervals of approx. 0-1 h, 2-4 h, 5-6 h, 24 h, 30 h, and 48 h after administration of the test item.

3. Micronucleus test

Treatment and sampling:

Groups of 5 (controls) or 7 (test item) male mice were treated once with either the vehicle or 500, 1000, and 2000 mg test substance per kg bodyweight by oral gavage (24 h sacrifice). An additional test group was used for the 48 h sacrifice period, were 5 animals were treated with vehicle and 7 animals were treated with 2000 mg/kg bw of the test substance. The application volume was 10 mL/kg bw and the volume to be applied was calculated based on actual weight on the day of administration. The positive control substance CPP was administered once by oral gavage and the mice sacrificed after 24 h. The animals were observed for evident clinical signs of toxicity throughout the study.

Blood sampling

For further analysis blood samples (~0.5 mL each) of additional satellite animals were taken. For this purpose, blood sampling was performed 1 h and 4 h after a single oral application of the highest test item dose. 3 animals for each sampling time were used, resulting in 6 samples. Additionally, 3 animals were dosed with the negative control item once and their blood was withdrawn 1 h after application.

The blood of the animals was collected in tubes containing K_3-EDTA. The blood samples were centrifuged at 10000 rpm for about 5 minutes to obtain plasma samples (approximately 0.2 mL).

The plasma samples were stored at ≤ - 80°C at Harlan CCR and were send to the sponsor for further analysis. The analysis was performed as a separate study under the responsibility of the sponsor, and the results are reported in a separate report (BASF DocID 2015/1112085).

Slide preparation

Finally, 24 or 48 hours after the administration the animals were sacrificed using CO_2 followed by bleeding. The femora were removed, the epiphyses were cut off and the marrow was flushed out with foetal calf serum using a syringe. The cell suspension was centrifuged at 1500 rpm (390 x g) for 10 minutes and the supernatant was discarded. A small drop of the re-suspended cell pellet was spread on a slide. The smear was air-dried and then stained with May-Grünwald/Giemsa. Cover slips were mounted with EUKITT. At least one slide was made from each bone marrow sample.

Slide evaluation

Evaluation of the slides was performed using NIKON microscopes with 100x oil immersion objectives. Per animal 4000 polychromatic erythrocytes (PCE) were analysed for micronuclei. To describe a cytotoxic effect the ratio between polychromatic and normochromatic erythrocytes was determined in the same sample and expressed in polychromatic erythrocytes per total erythrocytes. The analysis was performed with coded slides.

4. Statistics

The number of polychromatic erythrocytes with micronuclei was analyzed by comparing the dose groups with the vehicle control using the nonparametric Mann-Whitney test.

5. Acceptance and evaluation criteria

The study is considered valid as the following criteria are met:
- at least 5 animals per group could be evaluated.
- PCE to total erythrocyte ratio was not less than 20 % of the negative control.
- the positive control shows a statistically significant and biological relevant increase of micronucleated PCEs compared to the vehicle control.
- the negative/vehicle control data are consistent with the historical negative control data.

A test item is classified as mutagenic if it induces either a dose-related increase or a clear increase in the number of micronucleated polychromatic erythrocytes in a single dose group above the laboratory's historical solvent control data range. Statistical methods (nonparametric Mann-Whitney test) were used as an aid in evaluating the results, if necessary. However, the primary point of consideration is the biological relevance of the results.
A test item that fails to produce a biological relevant increase in the number of micronucleated polychromatic erythrocytes is considered non-mutagenic in this system.

II. RESULTS AND DISCUSSION

A. PRE-EXPERIMENT FOR TOXICITY

The animals treated in the pre-experiments received the test item metiram suspended in 1% MC once orally. The volume administered was 10 mL/kg bw. The animals treated with 2000 mg/kg bw metiram showed no clinical signs. On the basis of these data 2000 mg/kg bw were estimated to be suitable. No differences between sexes in toxicity were observed, so that only male animals were used in the main experiment.

B. MICRONUCLEUS ASSAY

Clinical examinations
In the main experiment for each test item dose group 7 males received the test item metiram dissolved in 1% MC once orally. The volume administered was 10 mL/kg bw. At the dose groups of 500, 1000, and 2000 mg/kg bw the animals did not show any sings. The animals treated with the vehicle control (1% MC) did not express any clinical symptoms.

Micronucleus test results
The mean number of polychromatic erythrocytes was not decreased after treatment with the test item as compared to the mean value of PCEs of the vehicle control indicating that Metiram did not have any cytotoxic properties in the bone marrow.
In comparison to the corresponding vehicle controls there was no statistically significant or biologically relevant enhancement in the frequency of the detected micronuclei at any preparation interval and dose level after administration of the test item. The mean values of micronuclei observed after treatment with metiram were below or near to the value of the vehicle control group.

The bone marrow exposure was confirmed by bioanalytical detection of metabolites ETU and EU formed by the test item in the plasma (BASF DocID 2015/1112085). All mouse EDTA plasma originating from the animals treated with metiram (at 2000 mg/kg body weight) and sampled 1 hour after treatment showed ETU levels in a range from about 1.5 mg/L to about 1.8 mg/L. All mouse EDTA plasma originating from the animals treated with metiram (at 2000 mg/kg body weight) and sampled 4 hours after treatment showed ETU levels in a range from about 3.9 mg/L to about 5.4 mg/L. All plasma samples originating from treated animals had detectable concentrations of EU, however, these levels were always below the LOQ of 50 µg/L (but always higher than the LOD of 10 µg/L). The analytical method used for ETU and EU dermination has been validated.

Further, studies on the kinetics of [^{14}C]-metiram (absorption, distribution, retention and elimination) and on the metabolism following oral administration of 5 and 50 mg/kg bw, respectively, were carried out in rats of either sex. Summing up, the test item was found to be excreted in feces, urine, and bile, and a small proportion of the radioactivity was found in the expired air. After single oral doses of [^{14}C]-metiram at dose levels of 5 and 50 mg/kg bw, peak mean plasma levels occurred at 4 h and 6 h after dosing, respectively. Furthermore, whole-body autoradiographs of rats which received [^{14}C]-metiram confirmed the distribution pattern observed in above mentioned studies. 40 mg/kg bw cyclophosphamide administered orally was used as positive control which showed a statistically significant increase of induced micronucleus frequency.

Table 5.4.2-2: Summary of Micronucleus Test Results

Sampling: 24 h post-dosing		MN/4000 PCE [Mean±SD]	Mean % MN	Range	Ratio PCE/total Ery.	% ratio vehicle
Metiram:	0 mg/kg bw	4.6 ± 2.7	0.1	2-9	0.682	100
Metiram:	500 mg/kg bw	5.0 ± 2.4	0.1	1-7	0.683	100.15
Metiram:	1000 mg/kg bw	6.4 ± 3.0	0.2	2-10	0.720	105.57
Metiram:	2000 mg/kg bw	5.9 ± 2.1	0.2	3-8	0.685	100.44
CPA:	40 mg/kg bw	70.8 ± 14.8**	1.8	54-91	0.634	92.96
Sampling: 48 h post-dosing						
Metiram:	0 mg/kg bw	4.8 ± 2.3	0.1	2-8	0.654	100
Metiram:	2000 mg/kg bw	8.1 ± 3.5	0.2	4-13	0.737	112.69

**: $p<0.01$

III. CONCLUSION

Based on the result of this study metiram did not induce micronuclei in mouse polychromatic erythrocytes under the conditions of the study, i.e. is devoid of clastogenic activity in vivo. Bioavailability of metiram in this study has been confirmed via detection of metiram-derived metabolites ETU and EU in mouse plasma according to a validated method.

CA 5.4.3 In vivo studies in germ cells

A summary of the in vivo genotoxicity studies in germ cells evaluated for Annex I inclusion of metiram is summarized in Table 5.4.3-1 below.

Table 5.4.3-1: In vivo studies in germ cells with metiram

Study type	Test System	Test material	Result	Reference
In vivo Dominant lethal test	mouse multiple oral administration dose: 0, 600, 1200, 2400 mg/kg bw/day (5 consecutive days)	Metiram with 2.2% ETU (purity: 98%)	Negative	1979/069

CA 5.5 Long-Term Toxicity and Carcinogenicity

Studies evaluated in the draft monograph of Rapporteur Member State Italy of July 2000:

Studies evaluated in the metiram draft monograph consisted of: one chronic toxicity and carcinogenicity study in rats performed with metiram and one carcinogenicity study in mice performed with metiram. These studies have been evaluated by European authorities and Italy as the rapporteur member state and were considered to be acceptable. For the convenience of the reviewer, these studies are summarized below as extracted from the monograph. The results are summarized in Table 5.5-1.

Table 5.5-1: Summary of already peer-reviewed long-term toxicity and carcinogenicity studies with metiram

Study	Dosages (mg/kg bw/day)	NOAEL (mg/kg bw/day)	LOAEL (mg/kg bw/day)	Main adverse effect	Reference and year
24 month chronic toxicity and carcinogenicity study in rats (diet administration of 0, 5, 20, 80, 320 ppm)	M: 0.2, 0.8, 3.1, 12.3 F: 0.2, 1.0, 3.8, 15.5	M: ~3.1 F: ~3.8 [80 ppm]	M: 12.3 F: 15.5 [320 ppm]	Systemic toxicity: Slight muscular atrophy at 320 ppm Oncogenicity: no carcinogenic effects	1981/280 1989/0001 2002/1007052 2002/1006228
18-month carcinogenicity study in CFLP mice (diet administration of 0, 100, 300, 1000 ppm)	M: 8, 24, 79 F: 9, 29, 95	M: ~24 [300 ppm] F: ~29 [300 ppm]	M: 79 [1000 ppm] F: 95 [1000 ppm]	Systemic toxicity: Reduced body weight gain Oncogenicity: no carcinogenic effects	1979/033

In the chronic toxicity study with rats, Sprague-Dawley rats were fed metiram (+ 2% ETU) at doses ranging from 5 to 320 ppm, according to the results obtained in the subchronic feeding study (1977/043). Histopathologically, an atrophy of the skeletal muscles, as was detected in the subchronic rat study before, was observed as the only finding in the highest dose group (320 ppm). In comparison with the subchronic study, it should be noted that no functional or morphological changes occurred in the thyroid at similar dose levels indicating that the effects on the thyroid do not aggravate with chronic administration but rather show signs of adaptation. An increased sensitivity of the CFY rat strain used in the subchronic study to metiram would be a possible explanation of this. On the basis of the above findings, the NOAEL in rats is 80 ppm. This corresponds to a daily intake of test substance of 3.1 mg/kg bw (males) and 3.8 mg/kg bw (females).
The study in mouse was intended to examine only a possible carcinogenicity effect of metiram. It will be used for the carcinogenicity risk assessment. Thus, the lowest NOAEL for long-term toxicity can be derived from the two year rat study: 80 ppm (3.1 mg/kg bw/day).

Two studies on carcinogenicity were conducted using Sprague-Dawley rats and CFLP mice. In both studies, metiram with 2% intentionally added ETU at toxic dose levels were used (320 and 1000 ppm, respectively). Toxicity was expressed by induction of atrophy of the skeletal muscles in the rat study (see above) and by retarded body weight gain and reduced food consumption in the mouse study.

Neither in rats nor in mice were the spontaneous tumour rates of the strains used increased by the administration of metiram. Thus, metiram and its degradation product ETU induced no carcinogenic effect.

Based on the available data, the following EU agreed endpoint was determined during the last Annex I listing of metiram (SANCO/4059/2001-Final, 3. June 2005):

Long term toxicity and carcinogenicity	
Target/critical effect	Thyroid (inhibition of thyroid peroxidase, hyperplasia/hypertrophy), atrophy of hind limb muscles in rats
Lowest relevant NOAEL	2-year, rat: 3.1 mg/kg bw/day
Carcinogenicity	No evidence of carcinogenicity in rats and mice

Submission of not yet peer reviewed studies in this dossier:

In this dossier one amendment to the already submitted long-term and carcinogenicity study in rats is submitted. In addition two new reports containing historical control data for thyroid effects were submitted (BASF DocIDs 2008/1102756 and 2016/1296090).

This amendment to the chronic and carcinogenicity study in rats further evaluates thyroid parameters by additional statistical methods. These additional statistical analyses confirmed the absence of effects on thyroid parameters (T3, T4 and thyroid weight). Therefore, these additional findings did not affect the original interpretation of the rat chronic and carcinogenicity study.

Thus, the conclusion for relevant endpoints for the current re-registration remains as follows:

Long term toxicity and carcinogenicity	
Target/critical effect	Thyroid (inhibition of thyroid peroxidase, hyperplasia/hypertrophy), atrophy of hind limb muscles in rats No classification required
Lowest relevant NOAEL	2-year, rat: 3.1 mg/kg bw/day
Carcinogenicity	No evidence of carcinogenicity in rats and mice No classification required

Note: The rat chronic toxicity and carcinogenicity study report CA 5.5/1, CA 5.5/2, CA 5.5/4 and CA 5.5/5 were already submitted and reviewed in the course of the initial registration of metiram. This study with its amendments is submitted for completeness as the basis for the assessment of report amendments submitted under CA 5.5/3. This amendment provides the results of additional statistical evaluation of the thyroid parameters. Taking into account that the majority of the data were already reviewed, the following study summary is extracted from the monograph including the additional statistical evaluation.

Report:	*CA 5.5/1*
	1981a
	Metiram - Toxicity and tumorigenicity in prolonged dietary administration to the rat
	1981/280
Guidelines:	*none*
GLP:	*yes*

Report:	*CA 5.5/2*
	1988a
	Metiram - Toxicity and tumorigenicity in prolonged dietary administration to the rat
	1989/0001
Guidelines:	*none*
GLP:	*yes*

Report:	*CA 5.5/3*
	2002a
	Metiram - Toxicity and tumorigenicity in prolonged dietary administration to the rat - WNT No. 77/951 - Additional statistical evaluation of the parameters Triodothyronine (T3), Thyroxine (T4) and thyroid weights via the Wilcoxon-test
	2002/1007052
Guidelines:	*none*
GLP:	*no*

Report:	*CA 5.5/4*
	Iversen B.S.,Vassallo E., 2002a
	Metiram - Position paper - Long term toxicity and carcinogenicity in the rat (Annex IIA 5.5)
	2002/1006228
Guidelines:	*none*
GLP:	*no*

Report:	*CA 5.5/5*
	, 2008a
	Historical histopathology data - Long term studies - CD rats - Liver tumors, thyroid tumors
	2008/1102756
Guidelines:	*none*
GLP:	*no*

Report:	CA 5.5/6
	[redacted] 2016 a
	Historical histopathology data - CD rats - Long term studies starting between 1978 and 1982 - Selected neoplastic findings
	2016/1296090
Guidelines:	none
GLP:	no

Deviations from the current guideline (OECD TG 453 – Combined chronic toxicity /carcinogenicity studies):

- Several biochemical parameters were evaluated only for the control and high dose group during the study.
  According to the OECD guideline 453 "Chronic Toxicity Studies" it is recommended that blood samples should be collected for at least 10 animals/sex of all groups 3 months, 6 months, and at approximately 6 month intervals thereafter. This was done throughout the entire study except that it was performed only for the control groups and the 320 ppm groups (male/female). As there were no significant differences seen between the high dose and the control groups, this deviation is considered to be of minor relevance and this part of the study still considered acceptable. For the urine analysis only 5 sample for the groups were analysed and not 10, but as no difference between the control groups and the high dose groups (male/female) were reported at any time during the two years, the lower sample number could be justified. In addition an extra control of biochemical parameters for all groups was done on males after 119 weeks, because they survived by months the target of 2 years. Also in this case 10 animals for each group were analyzed as recommended by OECD TG 452
- All dose groups were evaluated at the end of the study, when the percent survival of animals is very low (at maximum 38%), even if not related to treatment; in a satellite group at 104 weeks the % survival was 6% (1/15 animals) in the control group, not allowing statistical evaluation.
  The number of female animals in the control group of the satellite study was too low only for the "after 104 weeks" comparison. For this reason the female animals from the main study control group were used for the "after 104 weeks" comparison with treated animals in the satellite study. This is due to assumption of randomness fully acceptable. In the OECD guideline it is also stated that the blood samples should be collected from the same animals only if possible. The survival rates for the control groups in the main study were respectively 50% and 52% after 104 weeks. For carcinogenicity a termination is acceptable according to the OECD TG 451 when the number of survivors of the lower doses or control groups reaches 25%. This study was terminated with survival rates for the groups between 22-38%. It should be noted that, in order to comprise the majority of the normal life span of the animals, the study was continued until approximately 20% survival was attained in any group. This happened after 111 and 119 weeks for females and males respectively.
- So the study can be considered as an additional source of information for risk assessment.
  According to the notifier's opinion the study is also suitable to assess the long-term risk beside the hematological and urinary endpoints. Clinical and pathological data relating to individual main group rats and satellite group rats, dying during the course of the study or killed at termination, are included in the study, making it possible to compare the data not only for the terminally sacrificed animals.

Although there are some deviations from the OECD guidelines evident in this chronic/carcinogenicity study, a new study was not conducted, because the results on thyroid toxicity and skeletal muscle effects (which are the major target organs in rats) were thoroughly evaluated (including a 60 animal/dose satellite group to conduct mechanistic thyroid studies), as well as a robust enough picture on chronically toxic and the carcinogenic potential of metiram can be derived from the study. Also it was the intention, not to unnecessarily use a huge number of laboratory animals.

Material and Methods:
The chronic toxicity and possible carcinogenicity of metiram (batch no.: BAS 945 F 791 and BAS 946 H1 59; purity: not indicated in the original report, however a retrieval from archive gave a content as CS_2 cleavable of 41.2%, corresponding to 96.8% Metiram) with 2/2.2% ETU was investigated in Sprague-Dawley rats (CD) ([redacted] [redacted]) in diet concentrations of 0; 5; 20; 80 and 320 ppm corresponding to 0.2; 0.8; 3.1; and 12.3 mg/kg bw/day for males and 0.2; 1.0; 3.8 and 15.5 mg/kg bw/day for females. 50 animals per sex and dose group were used in the main groups and 30 animals/sex/dose groups in the satellite groups. The satellite groups were used for blood sampling and thyroid function tests. Additionally T3 and T4 was investigated in each 10 male and female animals (controls and high dose) from the main study in weeks 5, 12, 24, 51, 77 and 103. The test substance was administered for 111 weeks (females) and 119 weeks (males) corresponding to an age-related survival rate of about 20% of the rat strain used.
All animals were checked at least twice per working day for dead and moribund animals. Food consumption and body weight change were recorded weekly, and water consumption daily (working days only) in control groups and treatment groups 5 (320 ppm) during weeks 12 and 24. Ophthalmological examinations were carried out before dosing commenced and during weeks 7, 13, 26, 52, 78, 104, and 119 (for males only). Haematological and clinicochemical examinations, including hormones, as well as urinalysis were carried out from the satellite groups for doses 0 and 320 ppm in the interim observations (6, 12, and roughly 6 months thereafter) and for all dose groups at the end of the study. After 4, 12, 26, and 52 weeks of treatment and during week 104, 3 males and females from each satellite group were used for the assessment of thyroid function, assessed by measuring the clearance of an intravenous dose of ^{131}I (5.3 µCi) from the plasma and by measuring the incorporation of radioiodine into the thyroid and protein of the thyroid. Rats from the satellite group interim sacrifices were not subjected to macroscopic examination. However, the tissues listed under “Terminal studies” were preserved from each animal. Longitudinal and transverse sections (stained with haematoxylin and eosin) of skeletal muscle samples were examined microscopically from rats killed at weeks 12, 26, 52 and 104.

On completion of the laboratory investigations in week 102, all surviving rats in the satellite groups, and at the end of the study, all surviving rats of the main groups were killed and subjected to detailed macroscopic examination and organ weight analysis (Brain, pituitary, thyroid, heart, liver, spleen, kidney, adrenals, testes, ovaries, uterus). A full set of tissues and organs were preserved for microscopic examination.

Findings:
No substance-induced clinical findings were noted in the animals. Mortality, food and water consumption, body weight and food utilisation efficiency remained unaffected by the treatment. Figure 5.5-1 shows the growth of male and female over the entire study duration:

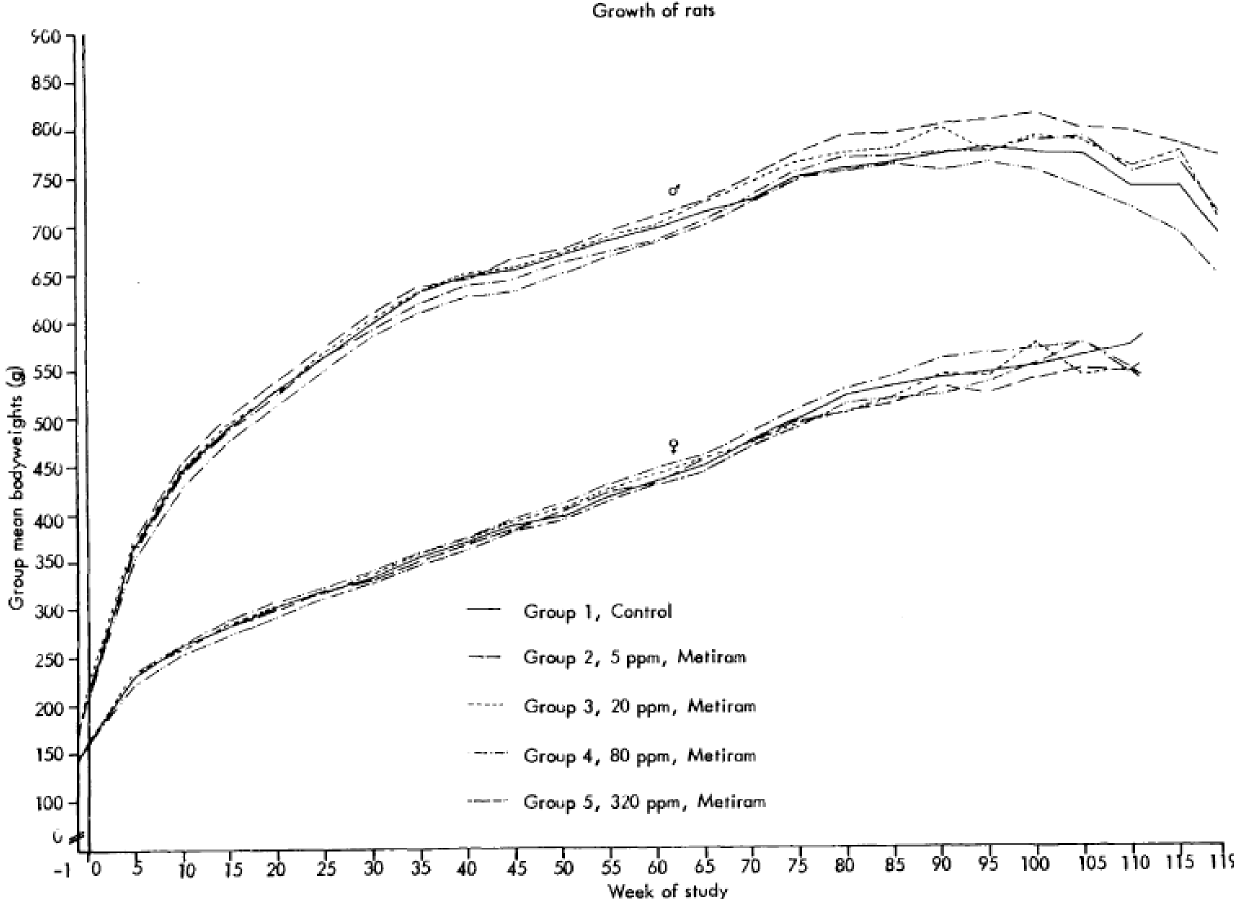

Figure 5.5-1: Group mean body weights [g] in rats over the entire study duration

Ophthalmoscopy revealed no treatment-related lesions. Haematological and clinicochemical examinations did not show any adverse effect of the test substance on any of the parameters. This also applies to the functional parameters of the thyroid (T3 and T4 determinations, thyroid function test). Variations in these parameters that occasionally occurred in the study are due to physiological variation, as was also described by [redacted] (1975, cited from the monograph). No consistent trend or any dose-response-time relationship was observed in this study. A more detailed summary of the thyroid parameter is presented in Table 5.5-2 below:

Table 5.5-2: T3 and T4 hormones in blood collected at different time point (each 10 male and 10 female animals used)

	T3 males [ng/100 ml]		T3 females [ng/100 ml]		T4 males [µg/100 ml]		T4 females [µg/100 ml]	
Week	**Control**	**320 ppm**	**Control**	**320 ppm**	**Control**	**320 ppm**	**Control**	**320 ppm**
5	97 (SD 12.0)	87 (SD 23.5)	100 (21.2)	119 (21.1)	3.9 (SD 0.22)	3.8 (SD 0.36)	3.3 (SD 0.71)	2.8* (SD 0.35)[1)]
12	75 (SD 16.1)	83 (SD 23.3)	102 (21.6)	115 (SD 22.5)	3.2 (SD 0.60)	2.8 (SD 0.60)	2.6 (SD 0.55)	2.3 (SD 0.31)
24	84 (SD 12.5)	75 (SD 20.9)	115 (SD 12.8)	125 (17.8)	4.1 (SD 0.59)	3.7 (SD 0.60)	2.8 (SD 0.61)	2.8 (SD 0.36)
51	92 (SD 14.5)	101 (SD 22.9)	95 (SD 18.1)	99 (SD 11.3)	4.4 (SD 0.71)	4.3 (SD 0.75)	3.1 (SD 0.52)	2.7* (SD 0.32)
77	98 (SD 24.3)	105 (SD 22.5)	89 (SD 15.6)	108* (SD 18.8)	4.1 (SD 0.66)	3.9 (SD 0.87)	2.8 (SD 0.46)	2.7 (SD 0.57)
103	80 (SD 9.2)	96 (SD 19.2)	84 (SD 12.7)	84 (SD 8.8)	2.9 (SD 0.85)	3.0 (SD 0.90)	2.1 (SD 0.55)	2.3 (SD 0.80)

1) In week 7, blood of 10 female animals of all dose groups was collected and T3/T4 was determined (Values for T3 were in dose groups 0, 5, 20, 80, 320 ppm 104 (16.6), 103 (21.3), 115 (23.6), 98 (22.7), 122 (23.4), and for T4 3.2 (0.53), 2.6* (0.50), 3.4 (0.63), 2.6* (0.68), 2.6* (0.28))
2) In week 119, blood of 10 male animals of all dose groups was collected and T3/T4 was determined (Values for T3 were in dose groups 0, 5, 20, 80, 320 ppm 70 (14.2), 81 (17.0), 78 (14.9), 82 (16.2), 117** (59.1), and for T4 2.4 (0.72), 2.5 (0.31), 2.8 (0.44), 2.5 (0.47), 2.9 (0.70)
*p<0.05
**p<0.01

Further thyroid functional tests were investigated in the satellite groups. For these investigations, each 3 males and females from each satellite group were investigated after 4, 12, 26 and 52 weeks of treatment and during week 104. Thyroid function was assessed by measuring the clearance of an intravenous dose of ^{131}I from the plasma and by measuring the incorporation of radioiodine into the thyroid. Radioiodine incorporated into the protein fraction of the thyroid was also measured. Also the thyroid weights were determined. The results of these investigation is presented in the table below.

Table 5.5-3: Thyroid function tests (group mean values of each 3 male and female animals)

Group	Week	Body weight [g]	Thyroid weight [mg]	% injected ^{131}I in thyroid (after 24 h)	% ^{131}I in thyroid bound to protein m/f	Plasma clearance %131I in plasma	
						After 4 h m/f	After 24 h m/f
Control m	4	330 (26.2)	17.1 (0.76)	5.56 (1.467)	82.1 (5.38	5.84 (1.036	1.12 (0.174)
5 ppm m		360 (39.1)	22.3 (4.57)	12.03** (4.251)	86.7 (2.34)	7.40 (1.870)	1.27 (0.116)
20 ppm m		367 (25.2)	24.2* (2.42)	17.02*** (2.273)	86.5 (23.42)	9.01** (0.785)	1.96** (0.400)
80 ppm m		348 (8.7)	18.7 (1.80)	8.48 (1.955)	77.8 (13.45)	6.86 (1.093)	1.73* (0.397)
320 ppm m		344 (7.8)	25.2** (4.12)	7.71 (0.780)	86.4 (3.66)	7.18 (0.607)	1.58 (0.108)
Control, f	4	243 (7.0)	21.7 (1.30)	8.75 (2.471)	89.8 (2.05)	7.10 (0.991)	0.72 (0.156)
5 ppm, f		230 (4.2)	18.8 (1.14)	10.89 (2.095)	83.7 (22.86)	6.59 (0.523)	1.02* (0.136)
20 ppm, f		233 (20.4)	17.5 (2.91)	11.18 (0.177)	97.9 (17.77)	7.28 (1.048)	1.00* (0.074)
80 ppm, f		218* (16.0)	18.4 (5.40)	12.84 (1.950)	86.5 (7.84)	7.01 (0.537)	1.06** (0.092)
320 ppm, f		211* (8.3)	18.3 (1.15)	10.83 (4.242)	75.3 (3.75	7.93 (2.456)	1.10* (0.117)
Control m	12	452 (40.0)	22.0 (9.45)	8.33 (0.336)	85.7 (4.90)	8.12 (0.315)	1.13 (0.336)
5 ppm m		481 (108.5)	26.0 (3.31)	8.66 (2.462)	87.3 (3.13)	6.79* (0.341)	1.20 (0.105)
20 ppm m		460 (150.6)	27.3 (5.49)	7.73 (2.848)	86.7 (1.30)	7.43 (1.81)	1.55 (0.707)
80 ppm m		439 (95.0)	25.1 (5.11)	8.72 (2.772)	86.8 (3.47)	7.75 (0.695)	1.76 (0.411)
320 ppm m		418 (3.1)	26.0 (1.76)	8.08 (3.245)	87.9 (3.20)	7.69 (0.680)	1.49 (0.178)
Control, f	12	277 (34.8)	19.8 (3.77)	9.11 (2.390)	88.6 (3.04)	6.59 (0.498)	0.63 (0.157)
5 ppm, f		272 (14.2)	23.2 (4.64)	11.02 (2.708	89.4 (4.50)	6.64 (0.197)	0.85 (0.341)
20 ppm, f		263 (11.5)	20.4 (2.81)	12.04 (4.1029)	89.5 (1.44)	7.62 (1.463)	0.97 (0.219)
80 ppm, f		249 (22.1)	17.1 (2.37)	8.93 (2.531)	88.9 (4.65)	7.91 (0.871)	0.83 (0.027)
320 ppm, f		250 (36.5)	22.3 (6.69	10.61 (5.342)	89.7 (5.62)	8.59* (1.557)	0.99 (0.308)
Control m	26	606 (23.1)	30.5 (10.77)	9.97 (2.742	87.6 (3.72)	8.44 (1.292)	1.83 (0.565)
5 ppm m		587 (35.9)	27.0 (8.28)	7.65 (1.274)	88.1 (6.10)	8.37 (0.859)	1.76 (0.408)
20 ppm m		576 (113.6)	24.7 (1.40)	10.04 (1.506)	87.8 (2.16)	9.68 (1.212)	2.05 (0.577)
80 ppm m		616 (35.9)	33.5 (2.64)	9.01 (1.930)	93.5 (4.97)	8.33 (0.216)	2.17 (0.869)
320 ppm m		570 (76.3)	30.1 (5.27)	8.56 (3.792)	90.3 (1.85)	8.20 (0.337)	2.27 (0.242)
Control, f	26	343 (87.2)	17.9 (2.61)	6.66 (1.614)	85.8 (2.597)	9.20 (1.410)	1.24 (0.191)
5 ppm, f		309 (42.2)	19.1 (0.87)	10.59 (5.121)	91.1 (1.905)	8.94 (0.207)	1.44 (0.100)
20 ppm, f		326 (18.9)	22.9 (4.05)	12.07* (1.180)	90.9 (3.251)	8.47 (0.480)	1.55 (0.554)
80 ppm, f		353 (98.6)	17.5 (3.21)	8.10 (0.989)	89.5 (4.372)	10.49 (3.372)	2.80* (1.445)
320 ppm, f		299 (36.2)	23.9* (4.08)	10.69 (0.906)	91.4 (8.080	8.11 (0.347)	1.11 (0.059)
Control m	52	726 (84.5)	20.7 (2.49)	9.06 (1.976)	87.1 (1.83)	8.47 (2.702)	1.94 (0.377)
5 ppm m		738 (132.2)	28.3 (5.20)	11.42 (3,873)	90.5 (1.60)	8.36 (1.218)	1.87 (0.316)
20 ppm m		690 (89.0)	33.6** (5.47)	12.07 (3.551)	91.1 (3.03)	8.02 (1.362)	2.28 (0.877)
80 ppm m		757 (90.8)	26.9 (3.55)	9.44 (1.453)	88.2 (3.18)	9.59 (0.433)	2.27 (0.137)
320 ppm m		583 (35.8	29.7* (3.90)	12.77 (3.519)	91.6* (1.56)	9.16 (0.984)	2.71 (0.558)
Control, f	52	399 (49.0)	17.3 (2.97)	8.58 (2.058)	89.2 (0.87)	9.17 (1.517)	1.20 (0.339)
5 ppm, f		405 (91.8)	24.6** (2.12)	11.33 (3.227)	90.04 (5.00)	8.25 (2.087)	1.54 (0.586)
20 ppm, f		418 (102.3)	22.8* (2.34)	13.62 (7.197)	94.6 (1.93)	10.40 (1.427)	1.20 (0.204)
80 ppm, f		389 (70.0)	20.6 (2.10)	11.09 (2.361)	92.6 (1.86)	10.00 (1.695)	2.18* (0.790)
320 ppm, f		352 (30.0)	23.4** (1.18)	8.70 (1.048)	88.8 (4.00)	9.36 (1.360)	1.44 (0.370)
Control m	104	780 (87.5)	31.5 (4.95)	9.88 (5.058)	89.7 (4.63)	8.75 (1.506)	2.67 (0.795)
5 ppm m		899 (46.9)	32.1 (6.09)	9.08 (4.770)	91.7 (4.49)	13.87** (1.818)	6.06** (1.247)
20 ppm m		783 (84.3)	32.3 (6.86)	11.59 (1.446)	91.4 (2.16)	10.14 (1.875)	2.57 (0.380)
80 ppm m		752 (83.3)	44.7 (19.14)	13.75 (1.932)	93.1 (1.19)	10.42 (1.512)	3.28 (1.644)
320 ppm m		758 (69.6)	42.8 (11.95)	13.69 (7.204)	93.9 (0.36)	10.12 (0.553)	4.38 (0.791)
Control, f	104	587 (154.7)	38.1 (16.72)	13.45 (3.222)	91.8 (3.03)	11.76 (3.829)	2.86 (1.510)
5 ppm, f		579 (113.2)	24.0 (3.15)	13.45 (3.996)	85.7 (13.27)	11.46 (1.413)	3.74 (1.672)
20 ppm, f		611 (108.0)	29.5 (3.76)	14.16 (1.975)	94.2 (3.08)	14.05 (4.161)	4.72 (3.790)
80 ppm, f		562 (70.5)	30.9 (0.89)	9.99 (1.850)	93.4 (3.80)	9.68 (0.269)	1.91 (0.153)
320 ppm, f		559 (72.4)	35.4 (8.52)	8.52* (0.286)	92.8 (2.47)	12.39 (0.535)	2.93 (0.420)

The thyroid weights of the remaining 15 animals (each male and female) in the satellite groups are shown in the Table below:

Table 5.5-4: Thyroid weights of satellite group rats killed after 104 weeks of treatment (each 15 male and female animals)

Male groups	Body weight [g] males	Thyroid weight [mg] males	Body weight [g], females	Thyroid weight [mg], females
Controls	719 (114.2)	40 (17.9)	490*	29*
5 ppm	821 (156.2)	43 (5.5)	486 (116.8)	33 (4.5)
20 ppm	807 (122.9)	34 (13.3)	534 (69.4)	36 (15.9)
80 ppm	726 (99.4)	36 (7.3)	502 (130.8)	32 (8.0)
320 ppm	691 (151.6)	39 (9.0)	467 (103.8)	33 (12.6)

*only one surviving animal

The thyroid parameters T3, T4 and thyroid weights have been additionally statistically evaluated via the Wilcoxon test (DocID: 2002/1007052). For the parameters T3, T4 and thyroid weight a pairwise comparison of the dose groups with the control group was carried out using the Wilcoxon-test for the hypothesis of equal medians (one-sided). This evaluation was performed to supplement the Student's t-test which was already performed in the original report. For the parameter T4 the results of the Wilcoxon-test are similar to the Student's t-test. For the parameter T3 there were no statistical significant results for the one-sided Wilcoxon-test because physiologically a substance related decrease of the values of T3 would be the assumed effect.

Conclusion on thyroid effects, investigated in the satellite group

With regard to thyroid-related parameters, there were no statistically significant changes seen in the thyroid weights, as well as no consistent effects on the blood thyroid hormones (T3 and T4). The isolated statistically significant increase in T3 at week 77 only in females is considered to be incidental as physiologically a decrease in T3 hormones would be expected. At week 5 and week 51, statistically significant decreases of T4 were observed (Table 5.5-2). However as the changes in week 5 occurred not together with increased thyroid weights or consistent changes radioiodine incorporation into the thyroid or into the protein fraction of the thyroid, the treatment-relationship is questionable. The decreased T4 value at week 51 is accompanied by statistically increased thyroid weights seen at week 52 (interim sacrifice). However, as for the terminal thyroid weight there was no statistical significant difference, this is also of questionable relevance and may well reflect the occasional occurrence of physiological variations as assessed by ████, 1976.

Macroscopic pathology at interim sacrifice after 104 weeks of treatment (satellite groups) revealed no evidence of a reaction to treatment in any organ or tissue of the treated rats. Organ weights for treated rats were essentially similar to those of the controls.

At terminal sacrifice (main groups) macroscopic pathology revealed no treatment-related change in any organ or tissue of the treated rats. Organ weights for treated rats were essentially similar to those of the controls. Myelograms showed that bone marrow smears for treated and control rats were essentially similar. Non-neoplastic histopathology revealed an increased incidence of skeletal muscular atrophy among rats treated with 320 ppm (see Table 5.5-10). No other significant changes were noted. Neoplastic histopathology revealed no treatment-related effect on the spontaneous tumor profile of the CD rat strain.

Table 5.5-5: Mortality rates in the satellite group – interim kill after 104 weeks –

Number of survivor at week 104	Group									
	1 males Control	2 males 5 ppm	3 males 20 ppm	4 males 80 ppm	5 males 320 ppm	1 females control	2 females 5 ppm	3 females 20 ppm	4 females 80 ppm	5 females 320 ppm
	7/15	7/15	4/15	9/15	9/15	1/15	8/15	5/15	5/15	8/15
% survival at week 104	47	47	27	60	60	6	53	33	33	53

Table 5.5-6: Mortality rates at terminal sacrifice (111 weeks males and 119 weeks females)

Number of survivor at terminal sacrifice	Group									
	1 males Control	2 males 5 ppm	3 males 20 ppm	4 males 80 ppm	5 males 320 ppm	1 females control	2 females 5 ppm	3 females 20 ppm	4 females 80 ppm	5 females 320 ppm
	11/50	12/50	13/50	13/50	15/50	19/50	13/50	13/50	19/50	11/50
% survival at terminal sacrifice	22	24	26	26	30	38	26	26	38	22

A summary of the neoplastic microscopic changes occurring in main group male and female rats dying during the study or sacrificed at termination are given in the tables below.

Table 5.5-7: Summary of neoplastic microscopic changes occurring in main group rats dying during the study or killed at termination - males

Group	**1**	**2**	**3**	**4**	**5**
Compound	Metiram spiked with ETU				
Level (ppm)	**Control**	**5**	**20**	**80**	**320**
Number of male rats examined	**50**	**50**	**50**	**50**	**50**
Endocrine					
Pituitary					
Adenoma	17	19	27	22	20
Adenocarcinoma	0	0	0	0	1
Pancreas					
Islet cell adenoma	2	7	2	2	4
Islet cell adenocarcinoma	1	0	0	0	0
Thyroid					
Parafollicular cell adenoma	3	5	6	1	5
Parafollicular cell carcinoma	1	0	0	0	0
Follicular adenoma HCD: 0.0 – 13.3%*	1 (2%)	1 (2%)	0	1 (2%)	3 (6%)
Follicular adenocarcinoma HCD: 0.0 – 3.3%*	0	0	0	1 (2%)	1 (2%)
Adrenal					
Cortical cell adenoma	2	1	0	1	0
Cortical cell carcinoma	0	1	1	0	0
Phaeochromocytoma	6	4	1	3	4
Malignant phaeochromocytoma	2	0	0	0	1
Malignant medullary tumour	0	1	0	0	0
Ganglioneuroma	0	0	0	0	1

Group	1	2	3	4	5
Compound	Metiram spiked with ETU				
Level (ppm)	**Control**	**5**	**20**	**80**	**320**
Number of male rats examined	**50**	**50**	**50**	**50**	**50**
Parathyroid					
Adenoma	0	0	0	1	0
Testes					
Interstitial cell adenoma	1	0	0	1	1
Cutaneous					
Kerato-acanthoma	2	4	6	5	7
Squamous cell papilloma	3	1	0	1	2
Sebaceous adenoma	0	0	0	0	1
Squamous cell carcinoma	0	0	1	1	2
Basal cell carcinoma	2	0	0	0	1
Dermal fibroma	0	3	1	2	1
Zymbals gland					
Squamous cell papilloma	0	0	0	1	0
Squamous cell carcinoma	0	2	2	2	1
Subcutaneous					
Fibroma	10	7	6	4	5
Fibrosarcoma	3	3	2	7	5
Lipoma	4	3	3	5	1
Haemangiosarcoma	0	1	0	0	0
Mammary					
Fibro-adenoma	2	5	0	2	3
Adenoma	0	0	1	1	0
Preputial gland					
Squamous cell papilloma	0	0	0	1	0
Brain					
Astrocytoma	1	0	0	0	1
Glioma	0	0	0	0	1
Meningioma	1	0	0	0	0
Tongue					
Squamous cell papilloma	0	0	0	0	1
Stomach					
Squamous cell papilloma	1	0	0	0	0
Jejunum					
Leimyosarcoma	0	0	0	1	0
Intestinal adenocarcinoma	0	0	1	0	0
Liver					
Benign liver cell tumour	2	0	0	2	0
Malignant liver cell tumour	1	0	0	3	0
Haemangiosarcoma	1	0	0	0	0
Spleen					
Haemangioma	0	1	0	0	1
Haemangiosarcoma	0	0	0	0	1
Thymus					
Adenocarcinoma	0	0	1	0	0
Lymphoreticular system					
Lymphosarcoma	0	0	0	1	0
Lymphocyte/lymphoblastic leukaemia	2	0	1	0	0
Myeloid leukaemia	0	1	1	0	0
Reticulum cell sarcoma	1	2	0	2	0
Lung					
Alveolar mucinous carcinoma	0	0	0	1	0
Kidney					
Renal lipoma	0	1	2	0	0

Group	1	2	3	4	5
Compound	Metiram spiked with ETU				
Level (ppm)	**Control**	**5**	**20**	**80**	**320**
Number of male rats examined	**50**	**50**	**50**	**50**	**50**
Bone					
Osteosarcoma	1	0	0	0	0
Abdominal cavity					
Haemangiosarcoma	1	0	0	0	0
Liposarcoma	0	1	0	0	0
Fibrosarcoma	0	0	1	1	0
Leimyosarcoma	0	0	0	1	0
Mesothelioma	0	0	1	0	0

*Historical control data were collected by [redacted] for 20 long term CD rat studies performed at [redacted] between 1978 and 1982 (see DocID 2016/1296090).

Focusing on the thyroid adenoma and adenocarcinoma incidences in males, the following incidences were reported: The incidences for parafollicular cell adenoma were 3, 5, 6, 1 and 5 and 1, 0, 0, 0, 0 for the parafollicular cell carcinoma in each the controls, 5, 20, 80, and 320 ppm dose groups. The incidences for follicular adenoma were 1, 1, 0, 1 and 3 and 0, 0, 0, 1, 1 for follicular carcinoma. The Rapporteur Member State concluded, that there was a treatment-related increase in thyroid follicular adenoma seen at the top dose males, however, when historical control data, derived at [redacted] in chronic studies in the same rat strain between the years 1978 and 1984, incidences between 0/50 and 8/50 (for thyroid follicular adenoma) were seen in males. The incidences for follicular cell carcinoma were between 0/50 and 4/50 in the same time frame. The historical control data are summarized in document DocID 2008/1102756. More specifically historical control data collected by [redacted], who conducted the study) for 20 long term CD rat studies performed at [redacted] between 1978 and 1982 also revealed historical control incidences of up to 13.3% for thyroid follicular adenoma and up to 3.3% for adenocarcinoma in males (see Table; DocID: 2016/1296090). Based on these additional information the increased incidences of thyroid follicular adenoma seen in the 320 ppm dose group are considered to be not treatment-related and incidental.

Table 5.5-8: Summary of neoplastic microscopic changes occuring in main group rats dying during the study or killed at termination - Females

Group	1	2	3	4	5
Compound	Metiram spiked with ETU				
Level (ppm)	**Control**	**5**	**20**	**80**	**320**
Number of male rats examined	**50**	**50**	**50**	**50**	**50**
Endocrine					
Pituitary					
Adenoma	29	32	31	39	37
Adenocarcinoma	2	2	2	0	1
Pancreas					
Islet cell adenoma	0	1	0	1	0
Thyroid					
Parafollicular cell adenoma	2	0	2	2	2
Follicular adenoma	0	0	1	0	1
Follicular adenocarcinoma	0	0	0	0	0
Adrenal					
Cortical cell adenoma	0	3	2	0	0
Phaeochromocytoma	0	1	1	0	1
Cutaneous					
Squamous cell papilloma	0	0	0	0	1
Squamous cell carcinoma	0	0	0	0	1
Tricho-epithelioma	1	0	0	0	0
Dermal fibroma	0	1	0	0	0
Zymbals gland					
Squamous cell carcinoma	0	0	1	0	0
Subcutaneous					
Fibroma	3	4	1	3	4
Fibrosarcoma	2	1	0	1	1
Lipoma	1	0	1	1	0
Liposarcoma	0	1	0	0	0
Myosarcoma	0	0	0	0	1
Mammary gland					
Fibroma	0	0	0	0	1
Fibro-adenoma	30	36	38	34	31
Adenoma	2	6	5	6	6
Adenocarcinoma	9	9	6	4	7
Ovary					
Tubular adenoma	6	2	1	1	1
Malignant granulosa cell tumour	0	0	0	1	1
Uterus					
Haemangioma	0	1	0	0	0
Brain					
Astrocytoma	1	0	1	0	0
Liver					
Benign liver cell tumour	3	1	1	0	1
Malignant liver cell tumour	0	1	0	0	1
Lymphoreticulum					
Lymphosarcoma	0	1	0	1	0
Myeloid leukaemia	0	0	1	0	0
Reticulum cell sarcoma	0	0	1	2	0
Lymphatic/lymphocytic leukaemia	0	0	1	1	0
Abdominal					
Fibrosarcoma	2	0	0	1	1
Haemangioma	0	0	1	0	0

Group	1	2	3	4	5
Compound	Metiram spiked with ETU				
Level (ppm)	**Control**	**5**	**20**	**80**	**320**
Number of male rats examined	**50**	**50**	**50**	**50**	**50**
Miscellaneous					
Malignant neurilemmoma	0	0	1	0	0

In the following table a summary of the factors contributing to death in the main group rats during the study.

Table 5.5-9: Summary of factors contributing to death in the main group rats during the study

Group	**1**		**2**		**3**		**4**		**5**	
Compound	Metiram									
Level (ppm)	**Control**		**5**		**20**		**80**		**320**	
Group	M	F	M	F	M	F	M	F	M	F
N. of rats examined	39	31	38	37	37	37	37	31	35	39
N.of rats with:										
Neoplasia	18	29	21	35	18	36	24	27	19	35
Nephropathy	6	1	9	0	5	0	4	0	6	0
Fractured skull	1	0	0	0	0	0	0	0	0	0
Broken upper jaw	0	0	0	0	0	0	0	1	0	0
Broken snout/damaged muzzle	0	0	0	1	0	0	1	0	0	0
Probable nasal haemorrhage	0	0	0	0	0	0	0	0	0	1
Retroperitoneal/abdominal haemorrhage	1	0	0	0	0	0	1	0	0	0
Possible haemorrhage of unknown origin	0	0	1	0	0	0	0	0	0	0
Possible uterine haemorrhage/polyps	0	0	0	0	0	0	0	1	0	0
Probable prolapse	0	0	0	0	0	0	0	0	0	1
Probable ruptured oesophagus	1	0	0	0	0	0	0	0	0	0
Gastric ulceration	1	0	1	0	0	0	1	0	0	0
Diaphragmatic hernia	0	0	0	0	0	0	1	0	0	0
Hepatic necrosis	0	0	0	0	1	0	0	0	0	0
Prostatitis	1	0	0	0	0	0	0	0	0	0
Subcutaneous abscesses	0	1	0	0	0	0	0	0	0	0
Ulcerated feet	0	0	1	0	0	0	0	0	0	0
Inflamed hind limb	0	0	0	0	0	0	0	0	0	1
Muscular atrophy/paralysis	0	0	0	0	0	0	0	0	1	0
Suspected/probable middle ear disease	0	0	0	0	1	1	1	0	0	0
Unknown	8	2	4	0	10	0	4	1	7	2
Killed on humane grounds	4	0	2	1	2	0	1	1	2	0

The skeletal muscle findings are shown in the below table:

Table 5.5-10: Results of principal microscopic findings pertaining to skeletal muscle and occurring in main group rats dying during the study (D) or killed at termination (T)

Group (mg/kg diet)	Number of rats with atrophic changes															
	Males								Females							
	No. of rats examined		Minimal		Mode-rate		marked		No. of rats examined		minimal		Mode-rate		Marked	
	D	T	D	T	D	T	D	T	D	T	D	T	D	T	D	T
Control	39	11	7	4	3	3	1	1	31	19	4	5	1	1	0	0
5	38	12	9	8	7	0	0	0	37	13	2	3	0	0	0	0
20	37	13	7	8	1	5	0	0	37	13	3	2	0	1	0	0
80	37	13	8	6	1	4	0	0	31	19	0	5	0	2	0	0
320	35	15	10	12	13	3	0	0	39	11	14	7	16	2	0	0

Conclusion:
Histopathologically, an atrophy of the skeletal muscles, as was detected in the subchronic rat study before, was observed as the only finding in the highest dose group (320 ppm).
On the basis of the above findings, the NOAEL in rats is 80 ppm. This corresponds to a daily intake of test substance of 3.1 mg/kg bw (males) and 3.8 mg/kg bw (females).
There was no consistent trend for thyroid changes seen in this chronic study, neither in the blood thyroid hormones T3 and T4, nor for thyroid weights or the ^{131}I intake experiments or the % plasma clearance of ^{131}I. In comparison with thyroid-related results observed in rat subchronic and the reproduction toxicity studies, it should be noted that no functional or morphological changes occurred in the thyroid at least at similar dose levels indicating that the effects on the thyroid do not aggravate with chronic administration but rather show signs of adaption.
No increase in the malignant tumor incidence compared with the spontaneous rate of the animal strain used was observed, however the high dose animal groups had a higher incidence of benign follicular adenomas compared to controls. Based on newly submitted historical control data, these findings are considered to be not treatment-related, as they were well within the historical control data.

Report:	*CA 5.5/7*
	[redacted] 1979a
	Metiram - Tumorigenicity to mice in long term dietary administration
	1979/033
Guidelines:	*none*
GLP:	*no*

Comments on the validity of the study and deviations from the guideline:
The mouse carcinogenicity study was conducted prior to the adoption of the OECD TG 451 guideline, however it broadly meets the OECD TG in terms of number of animals, administration period, and examined organs.
In the final EU commission document (No. 283/2013) on data requirement for active substances in accordance with Regulation (EC) No. 1107/2009, it is stated, that a "second carcinogenicity study of the active substance shall be conducted using mouse as test species, unless it can be scientifically justified that this is not necessary." As the available study is only to be used for carcinogenicity assessment in the second species, it is considered acceptable. Further, the mouse has been found to be the least sensitive species after administration of metiram (especially what concerns the effects on the thyroid) in all available subacute and subchronic studies. A new mouse carcinogenicity study was also not required after the US evaluation has been finalized and a DCI (Data-call-in) was issued in 2007. Also it was the intention, not to unnecessarily use a huge number of laboratory animals.

Material and Methods:
The carcinogenicity of metiram (batch no.: 945 F 791; purity: not indicated in the original report, however a retrieval from archive gave a content as CS_2 cleavable of 41.2%, corresponding to 96.8% metiram, see BASF Reg. DocID 1986/1000164) with 2.2% ETU was studied in mice of the CFLP strain. Four groups of each 52 male and 52 female animals were fed with a diet containing metiram at dose levels of 0; 100; 300 and 1,000 ppm for a treatment period, depending on the survival rate (25%), of 88 weeks (females of the 1,000 ppm group), 94 weeks (remaining females) and 96 weeks (male animals). Samples of the diet were analysed to verify the concentration, homogeneity and stability of metiram in the diets. Food consumption data was recorded weekly. Body weight was recorded weekly for the first twelve weeks and fortnightly thereafter. The group mean intake of metiram (mg/kg/day) was calculated from the body weight and food consumption data, and was 8; 24; 79 mg/kg bw/day for males and 9; 29; 95 mg/kg bw/day for females. Clinical signs of toxicity were examined daily. Blood smears were prepared where possible.
A full set of organs and tissues (aorta*, trachea*, heart*, lung*, thymus*, lymph nodes (cervical and mesenteric), liver, gall bladder*, spleen, pancreas*, kidneys*, urinary bladder*, uterus*, prostate*, thyroid, adrenals, salivary gland*, oesophagus*, stomach (glandular and non-glandular)*, duodenum*, jejunum*, ileum*, colon*, caecum*, ovaries, testes*, skin*, skeletal muscle*, mammary gland*, tongue*, eye*, second eye*, Brain*: cerebral cortex/thalamic nuclei/mid-brain/cerebellum/medulla, pituitary, sciative nerve*, bone marrow (sternum)*, femur*, seminal vesicles*) was examined macroscopically and microscopic evaluation consisted of the following step:

- All macroscopically abnormal tissues from decedents were examined microscopically in an attempt to confirm the cause of deaths.

- Adrenals, thyroids, ovaries, liver, spleen, lymph nodes and pituitary gland, plus all macroscopically observed lesions suggestive of neoplasia were routinely examined for every animal, along with blood smears. Abnormalities seen on the blood smear were confirmed by examination of a bone marrow section.

*tissues marked with a star were preserved but not process further in the first instance

Findings:
At the highest dose level of 1,000 ppm, retarded body weight gain was observed during the first 14 weeks of treatment (see figure below).

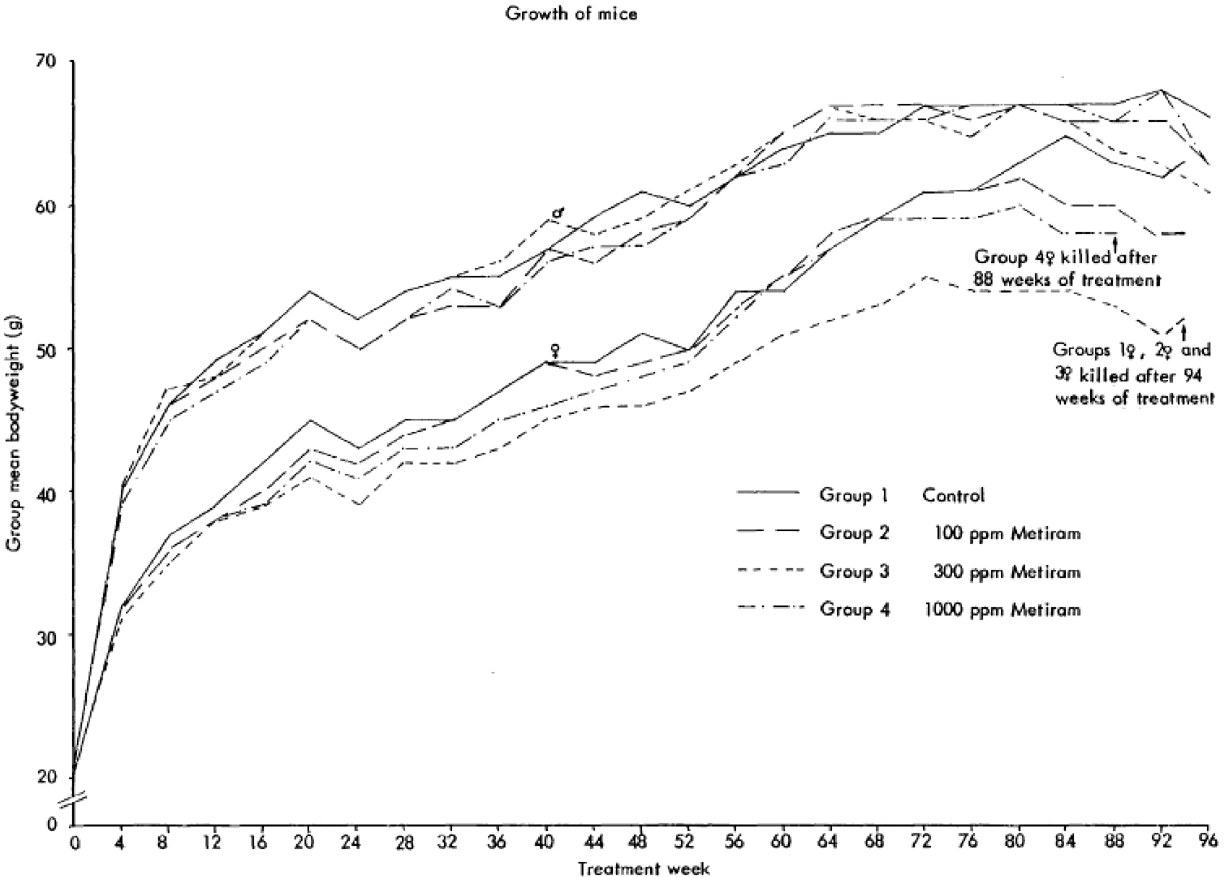

Figure 5.5-2: Group mean body weights [g] over the treatment weeks in male and female mice

At 1000 ppm the food consumption of the males was reduced by about 5% during the first 52 test weeks and that of the females was increased by about 10% from the 53rd test week onward. 300 ppm and 100 ppm of metiram did not lead to any substance-induced effects.
The spontaneous tumor rate of the animal strain used was not increased by the administration of metiram. Further substance-induced pathological changes were not detected either. The following tables give summaries on the tumor incidences for male and female animals respectively.

Table 5.5-11: Histopathology – summary of tumor incidences – male animals

Group	1	2	3	4
Compound	**Metiram spiked with 2.2% ETU**			
Level (ppm)	**control**	**100**	**300**	**1000**
Number of male mice examined	**51**	**52**	**51**	**52**
Alimentary system and associated organs				
Intestinal adenocarcinoma	0	1	0	0
Squamous cell papilloma of stomach	0	0	0	2
Liver				
Benign liver cell tumor (single)	2	4	4	6
Benign liver cell tumors (multiple)	1	0	0	5
Malignant liver cell tumor (single)	14	15	14	10
Malignant liver cell tumors (multiple)	7	14	9	15
Malignant liver cell tumor(s) with metastases	1	0	0	1
Unclassified liver cell tumors (autolysis)	1	0	0	0
Number of mice with liver cell tumor(s)	23	30	24	28
Haemangioma	1	1	1	0
Endocrine system				
Adrenals				
Adenoma	0	1	0	0
Bilateral phaeochromocytoma	1	0	0	0
Pituitary				
Adenoma	2	0	0	0
LYMPHORETICULAR SYSTEM				
Lymphosarcoma	10	8	6	11
Reticulum cell sarcoma	2	3	4	0
Lymphoblastic lymphoma	0	0	1	0
Myeloid leukaemia	0	0	1	0
Lymphocytic leukaemia	1	0	0	0
Total number of mice with a lymphoreticular tumour	13	11	12	11
URINARY SYSTEM				
Kidney				
Adenoma	0	0	0	1
Adenocarcinoma	0	1	1	0
Urinary bladder:				
Haemangioma	0	0	0	1
REPRODUCTIVE SYSTEM				
Testes				
Interstitial cell adenoma	3	1	3	5
Sertolis cell tumour	0	0	1	0
RESPIRATORY SYSTEM				
Pulmonary adenomas and carcinomas	24	31	28	27
No. of mice with lung tumour(s)	21	25	27	22
CUTANEOUS TISSUES				
Squamous papilloma	0	0	1	0
SUBCUTANEOUS TISSUES				
Haemangiosarcoma	0	0	0	1
Anaplastic sarcoma	0	0	1	0
MISCELLANEOUS TUMOURS				
Osteosarcoma	0	1	0	0
Harderian gland carcinoma	0	0	1	0

Table 5.5-12: Histopathology – summary of tumor incidence – female animals

Group	**1**	**2**	**3**	**4**
Compound	**Metiram**			
Level (ppm)	**control**	**100**	**300**	**1000**
Number of female mice examined	**51**	**52**	**51**	**52**
Alimentary system and associated organs				
Fibrosarcoma of stomach	0	0	1	0
Squamous carcinoma of stomach	0	0	0	1
Liver				
Benign liver cell tumor (single)	0	0	1	2
Malignant liver cell tumor (single)	2	4	3	1
Malignant liver cell tumors (multiple)	0	2	2	3
Number of mice with liver cell tumor(s)	2	6	6	6
Endocrine system				
Pituitary				
Adenoma	2	4	1	3
Thyroid				
Adenoma	0	0	1	0
LYMPHORETICULAR SYSTEM				
Lymphosarcoma	13	12	18	14
Reticulum cell sarcoma	5	3	1	1
Myeloid leukaemia	0	0	0	1
Lymphocytic leukaemia	2	0	0	1
Total number of mice with a lymphoreticular tumour	20	15	19	17
REPRODUCTIVE SYSTEM				
Ovarian/paraovarian tissue				
Tubular adenoma	2	0	0	0
Cystadenoma	1	0	1	0
Granulosa cell tumour	0	0	1	0
Uterus/vagina				
Leiomyosarcoma	0	0	0	1
RESPIRATORY SYSTEM				
Pulmonary adenomas and carcinomas	11	13	20	13
No. of mice with lung tumour(s)	10	11	18	11
CUTANEOUS TISSUES				
Basal cell carcinoma	0	1	0	0
Trichoepithelioma	0	1	0	0
Squamous cell papilloma	1	0	0	0
SUBCUTANEOUS TISSUES				
Fibrosarcoma	1	1	1	1
Haemangiosarcoma	0	1	0	1
MISCELLANEOUS TUMOURS				
Haemangiosarcoma of tail	1	0	0	0
Abdominal haemangiosarcoma	0	1	0	0
Mammary adenocarcinoma	0	0	0	1

Conclusions:

No carcinogenic effect was induced by metiram (spiked with 2.2% ETU) under the given test conditions. Based on decreased body weights/body weight gains and decreased food consumption (in males), the NOAEL is 300 ppm equivalent to about 24 and 29 mg/kg bw for males and females.

CA 5.6 Reproductive Toxicity

Studies evaluated in the draft monograph of Rapporteur Member State Italy of July 2000:

The metiram studies – evaluated in the draft monograph of the rapporteur member state Italy of July 2000 – consisted of a three generation reproductive toxicity study in rats and developmental toxicity studies in rats and rabbits. These studies have been evaluated by European authorities and Italy as Rapporteur member state and were considered to be acceptable. For the convenience of the reviewer, these are summarized below as extracted from the monograph. For a better overview the endpoints parental toxicity, reproductive toxicity and developmental toxicity were addressed separately.

Table 5.6-1: Summary of already peer-reviewed reproduction toxicity studies as available in the monograph (2000)

Study	Dosages (mg/kg bw/day)	NOAEL (mg/kg bw/day)	LOAEL (mg/kg bw/day)	Main adverse effect	Reference and year
3-Generation study including investigation for teratogenic changes, oral, feed, Sprague Dawley rats (diet concentrations: 0, 5, 40, 320 ppm)	ca. 0, 0.5, 4 and 32	Parental toxicity: ~4 (40 ppm) Offspring toxicity: 32 (320 ppm) Reproductive toxicity: 32 (320 ppm) No teratogenic properties	Parental toxicity: 32 Reproductive toxicity: > 32	Parental toxicity: slight toxicity (food consumption, body weight gain) in F0 and F1 males and reduced body weight gain during gestation at 320 ppm. No effects on offspring. Reproductive toxicity: no effects	1981/132
Prenatal toxicity, oral, gavage (days 7-19), Himalayan rabbits	0, 10, 40 and 120	Parental toxicity: 10 Developmental toxicity: 40 No teratogenic effects	Parental toxicity: 40 Developmental toxicity: 120	Parental toxicity: Severe maternal toxicity (body weight loss, reduced fecal output and food consumption) and abortions at 120 mg/kg bw and to a lesser extent at 40 mg/kg bw Developmental toxicity: no effects	1988/0154 1988/0262
Developmental toxicity, gavage (days 6-15), Sprague-Dawley rat	0, 40, 80, and 160	Maternal toxicity: 80 Developmental toxicity: 160 No teratogenic effects	Maternal toxicity: 80 Developmental toxicity: 160	Maternal toxicity: Slightly reduced food consumption at ≥ 80 mg/kg bw and body weight gain of dams at 160 mg/kg bw, reduced litter size and weight due to pre-/post-implantation* loss at 160 mg/kg bw. Effects on pre-implantation losses are not considered treatment-related. Developmental toxicity: no effects	1979/065

*primarily preimplantation loss (see below)

The reproduction toxicity of metiram was investigated in a multigeneration study in rats as well as in prenatal toxicity studies in rats and rabbits. In the multigeneration study rats were fed with metiram (+ 2% ETU). There were no dose-dependent effects in animals treated with metiram (+ 2% ETU) in any of the three subsequent generation investigated. The male rats of the highest dose showed slightly reduced food consumption that might explain the minimally retarded weight gains of the males of the same group in the F0 and F1 generations. Also the littering behaviour was not influenced by the treatment. The reduced litter size and the slightly increased pup mortality observed in the 2nd mating of the F1 generation were within the range of historical data of the laboratory. The F3b generation investigated on day 20 of gestation did not show any influence on the embryonic or foetal development related to the treatment. The NOAEL for parental toxicity was 40 ppm, or 4 mg/kg bw; the NOAEL for offspring development and reproduction parameters was 320 ppm or 32 mg/kg bw (these values were calculated using a factor of 10).
The teratogenic potential of metiram (+ 2% ETU) was investigated in rats and rabbits. In the prenatal toxicity study in rats, there was only a marginal reduction of food consumption in the dams belonging to the two upper dose groups. Compared to control, the body weight gain was slightly retarded in animals from the two upper dose groups. The slight and statistical non – significant increase of pre- and post-implantation losses, led to a statistically significant decline in the litter size and total litter weight in the highest group. No other findings were observed which could be related to the treatment regarding parental and developmental toxicity. Under the condition of this study the NOAEL for maternal toxicity is 40 mg/kg bw and the NOAEL for embryo/fetotoxicity is 80 mg/kg bw.
Developmental toxicity in presence of evident maternal toxicity (reduced food consumption and pronounced loss of body weight) was also observed at the highest dose tested in the rabbit prenatal toxicity study. Eight dams aborted and one animal died during the observation period. The only effect determined in fetuses was the slightly reduced weight. Also two of the dams treated with 40 mg/kg bw had abortions. Teratogenic changes were not detected at any dose. The NOAEL for maternal toxicity was 10 mg/kg bw, the NOAEL for embryo/fetotoxicity was 40 mg/kg bw, due to the reduced fetuses weight.
In conclusion: The lowest NOAEL for maternal/parental is 4 mg/kg bw (multigeneration study) (when using the conversion factor of 10 from the 40 ppm dose group), the lowest NOAEL for developmental toxicity is 40 mg/kg bw (developmental study in rabbits).

Based on the available data, the following endpoints were determined during the last Annex I listing of metiram:

Reproductive toxicity (SANCO/4059/2001)

Target / critical effect - Reproduction:	No reproductive toxic effects at parentally toxic dose levels.
Lowest relevant reproductive NOAEL:	40 ppm, ca 2.7 mg/kg bw/d (systemic toxicity). 320 ppm, ca. 21 mg/kg bw/d (reproductive toxicity).
Target / critical effect - Developmental toxicity:	Decreased litter size and foetal weights at maternally toxic dose levels.
Lowest relevant developmental NOAEL:	Rabbit: 40 mg/kg bw/d.

Submission of not yet peer reviewed studies in this dossier:

A new two-generation study in Wistar rats was conducted according to OECD 416 (2001). This study was required by the US-EPA in a Data-call-in process. Following that study a benchmark dose calculation was performed concerning follicular hyperthrophy/hyperplasia in male thyroid glands, as no NOAEL was achieved in parental males.

Table 5.6-2: Summary of not yet peer-reviewed reproduction toxicity studies

Study	Dosages (mg/kg bw/day)	NOAEL (mg/kg bw/day)	LOAEL (mg/kg bw/day)	Main adverse effect	Reference and year
2-Generation study oral, feed, Wistar rats (diet concentrations: 0, 100, 350, 1000 ppm)*	ca. 0, 9, 31, 92	Parental toxicity: M:<9 (100 ppm) $BMDL_{10}$ = 5.5 (61 ppm) F: 9 (100 ppm) Reproductive toxicity: 92 (1000 ppm) Developmental toxicity: 92 (1000 ppm) No teratogenic properties	Parental toxicity: M: 9 F: 31 Reproductive and Developmental toxicity: not applicable since NOAEL was at highest dose tested	Parental toxicity: Decreased food consumption and body weight/b.w.gain, changed thyroid hormone levels and thyroid morphology Reproductive toxicity: no effects Developmental toxicity: no adverse effects	2011/1264813 2015/1094108

*Study was enhanced by the addition of thyroid hormone and thyroid histopathological investigations in adults and offspring

The reproduction toxicity of metiram was further investigated in a two-generation study in rats. In this study rats were fed with metiram. The food contained either no, 100 ppm, 350 ppm or 1000 ppm of metiram. At the mid and high dose the main systemic effects for the F0 and F1 parental female rats were decreased food consumption and lower body weight/body weight gain as well as changed thyroid hormone levels (at 1000 ppm only) and thyroid morphology. For the F0 and F1 parental male rats the NOAEL is below 100 ppm because, in addition to the described mid- and high-dose effects, changed thyroid morphology is still present at this dose. Therefore, the parental NOAEL for females is at 100 ppm (= 9 mg/kg bw/day) and for males below 100 ppm. Since a NOAEL for parental toxicity for males could not be determined a benchmark dose calculation was performed for F0 and F1 males concerning the follicular hypertrophy/hyperplasia of the thyroid gland. The lowest $BMDL_{10}$ was 61 ppm (corresponding to appr. 5.5 mg/kg bw).
Fertility and reproductive performance for the F0 and F1 parental rats is not effected up to the highest dose; leading to a NOAEL for fertility and reproductive performance of 1000 ppm (= 92 mg/kg bw/day).

At the mid dose for the F1 and F2 progeny slightly decreased pre-weaning pup body weights/pup weight gain, as well as slightly changed thyroid hormone levels in female F1 pups on PND 4 were observed. These effects did not affect any other developmental parameters such as postnatal survival as well as post-weaning development of the offspring until sexual maturity. Thus, they are not regarded as independent adverse or biologically relevant developmental toxicity which leads to a NOAEL for developmental toxicity of 1000 ppm (=92 mg/kg bw/day).

The results of the new 2-generation study (including additional thyroid parameters) support the existing overall evaluation for reproduction and developmental toxicity. However the NOAEL doses can be adjusted according to the results of the new study. Thus, the conclusion for relevant endpoints would be as follows:

Target / critical effect - Reproduction:	No reproductive toxic effects at parentally toxic dose levels.
Lowest relevant reproductive NOAEL:	61 ppm, ca 5.5 mg/kg bw/d (systemic toxicity). 1000 ppm, ca. 92 mg/kg bw/d (reproductive and developmental toxicity).
Target / critical effect - Developmental toxicity:	Decreased litter size and foetal weights at corresponding maternally toxic dose levels.
Lowest relevant developmental NOAEL:	Rabbit: 40 mg/kg bw/d.

No classification for reproduction or developmental toxicity is considered necessary.

The available studies as provided in the monograph and the new 2-generation study in Wistar rat including the benchmark dose calculation are summarized below.

CA5.6.1 Generational studies

3-generation study, rat (BASF DocID 1981/132)

Material and Methods: The possible influence of metiram (batch no.: 946 H 159; purity: not indicated in the original report, however a retrieval from archive gave a content as CS_2 cleavable of 41.2%, corresponding to 96.8% Metiram, see BASF Reg. DocID 1986/1000164) with an ETU content of 2% on the reproductivity of Sprague Dawley rats (Crl: COBS CD BR strain) was investigated in a multi-generation study (F0, F1a, F1b, F2a, F2b, F3a and F3b) in doses of 0; 5; 40 and 320 ppm via the diet.
The three treatment groups and one control group each consisted of 12 male and 24 female animals per generation. Fresh batches of diet were mixed weekly. Treatment continued throughout the study for all generations. Feeding and housing of the animals was performed under standard conditions.

For parent animals, signs of toxicity, mortalities, food consumption, body weight changes and the pregnancy rate were recorded. Parent animals of the F0 and F1B generation were sacrificed after the second litters had been weaned and examined macroscopically. The following litter data were recorded: litter size, weight, external and internal abnormalities (after sacrifice), sex of pups. Teratology data were recorded using the F2B generation, (second mating). Rats of the F3A generation were sacrificed at 3 weeks and subjected to gross examination and histopathology.

Findings:
Throughout the three generations, there were no consistent dosage-related effects on adult animals with respect to clinical symptoms, mortalities, food conversion ratios, mating performance, pregnancy rate, duration of gestation or findings at terminal autopsy. There was a slight decrease in food consumption for males receiving 320 ppm over the three generations, females were unaffected. Body weight gain was slightly retarded for males and females receiving 320 ppm during the F0 and F1 generations and was essentially comparable with controls during the F2 generation. At 5 and 40 ppm, no effects on body weight gain could be observed. Weight gain during gestation was slightly but consistently reduced at 320 ppm only.
Over the three generations, mean litter parameters, as assessed by the incidence of total litter loss, litter size, cumulative pup mortality and findings at terminal autopsy provided no consistent evidence of adverse treatment related effects. At the second mating of the F1 generation, mean litter sizes were lower and pup mortality slightly higher than that of controls but were within the historical data of this rat strain in the test laboratory, which is in the range of 10.7 to 14.8, with a mean of 12.4 pups. As these effects were not clearly dose-related and could not be observed at the F2 and F3 generations - although these animals were exposed to metiram in utero and for a longer time period than the F1 – the observed effects are considered to be not substance-related.
Mean litter and pup weights were generally unaffected by treatment; changes at the second mate of the F1 generation (reduced mean litter size and mean litter weights from day 4 at 40 ppm and from day 8 at 320 ppm) reflected the above mentioned lower litter sizes.
The F3B generation sacrificed on day 21 post-partum did not show dosage-related adverse effects upon: mean litter parameters, as assessed by pre- and post-implantation loss, litter size, litter and mean fetal weights and embryonic and fetal development, as assessed by the incidence of major malformations, minor anomalies and skeletal variants.

The F3A offspring sacrificed on day 21 p.p. did not show dosage-related adverse effects following an analysis of the weights of major organs from ten male and ten female pups from each group and following macroscopic pathological examination of ten male and ten female pups from each group and microscopic pathological examination of ten male and ten female pups from the control group and from the 320 ppm treated group.

Conclusions:
Under the conditions of this study the NOAEL for parental toxicity is 40 ppm or approximately 4 mg/kg bw using a conversion factor of ten. The NOAEL for offspring development and reproduction parameters is 320 ppm or approximately 32 mg/kg bw. The study was considered acceptable.

Report:	CA 5.6.1/1
	2011a
	BAS 222 29 F (Metiram TK) - Two-generation reproduction toxicity study in Wistar rats - Administration via the diet
	2011/1264813
Guidelines:	(EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. L 142, EPA 870.3800, OECD 416
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Report:	CA 5.6.1/2
	2015b
	Metiram - Benchmark dose calculation of the reproduction toxicity (DocID 2011/1264813) study conducted with Metiram
	2015/1094108
Guidelines:	none
GLP:	no

Report:	CA 5.6.1/3
	2016 a
	Historical control data - Thyroid gland weights and histopathology
	2016/1295144
Guidelines:	none
GLP:	no

Report:	CA 5.6.1/4
	2016
	BAS 222 29 F - Histrorical control data - Liver weights
	2016/1345367
Guidelines:	none
GLP:	no

Executive Summary

In a 2-generation reproduction toxicity study, metiram (batch: 300015; purity 91.5%) was administered in the diet to groups of 25 male and 25 female Crl:WI(Han) Wistar rats at nominal dose levels of 0, 100, 350 and 1000 ppm throughout 2 generations. The dietary concentrations of metiram were adjusted to 0, 50, 175, 500 ppm during lactation, thereby maintaining constant dose-levels during this period of increased food intake. The overall mean dose of metiram administered to the male and female Wistar rats during the entire study period was approx. 9 mg/kg body weight/day (mg/kg bw/d) in the 100 ppm group, approx. 31 mg/kg bw/d in the 350 ppm group and approx. 92 mg/kg bw/d in the 1000 ppm group. This study was enhanced by the addition of thyroid hormone measurements and histopathological examination of thyroids in parental animals and in pups.
No treatment-related mortality was observed throughout the study. No clinical signs or changes of general behaviour, which may be attributed to the test substance, were detected in any of the male and female parental animals in any of the generations. Food consumption was impaired in mid and high dose F0 and F1 parental animals. The high-dose F0 and F1 parental animals had statistically significantly lower body weights/body weight gain during several study segments, which led to a statistically significant reduction of the mean terminal body weight resulting in secondary weight changes of a number of organs. In both generations also body weight/body weight gain of the mid dose males and females was affected which resulted in a lower terminal weight of these animals as well.

Clinical pathological examinations revealed that changes in thyroid hormone levels were evident primarily in the adult F0 and F1 generation parental animals. In the adult F0 and F1 generation males of the mid- and high-dose groups (350 ppm and 1000 ppm) decreased T4 and/or increased TSH levels were measured, whereas in females T4 levels were slightly decreased only in the high dose group (1000 ppm) of the F0 generation. Thyroid hormone levels of dosed pups in the F1 and F2 generations were not affected on PND 4 as well as 21, the only exception were female high-dose F1 pups (1000 ppm) with lower T4 and higher TSH levels on PND 4. In addition, higher cholesterol levels were measured in adult male F0 and F1 rats of the high dose group (1000 ppm), which is a well described phenomenon in hypothyroid rats. Hypercholesterolemic males of the F1 generation also had higher levels of cholesterol transport globulins in the blood.
In the thyroid glands, an increased incidence and severity of follicular cell hypertrophy/hyperplasia was noted in male animals in all treated groups of both F0 and F1 generations as well as in mid- and high-dose F1 parental females correlating with increased weights. In addition, in one high-dose F0 parental male (1000 ppm) and in one high-dose F1 parental male a single unilateral follicular adenoma was observed. As the adenomas were thought to have arisen in the hypertrophic/hyperplastic epithelium, they were considered to be probably treatment-related. The described morphological changes in the adult thyroid glands correlate well with the thyroid hormone effects.
A treatment related effect on liver weights of test group 03 and 13 (1000 ppm) animals cannot be ruled out, although a histological correlate was not detected.
There were no indications from clinical examinations as well as gross and histopathology, that metiram adversely affected the fertility or reproductive performance of the F0 or F1 parental animals up to and including a dose of 1000 ppm. Estrous cycle data, mating behavior, conception, gestation, parturition, lactation and weaning as well as sperm parameters, sexual organ weights and gross and histopathological findings of these organs (including differential ovarian follicle counts in the F1 females) were comparable between the rats of all test groups and ranged within the historical control data of the test facility.

For all liveborn pups of the F0 and F1 parents, no test substance-induced signs of developmental toxicity were noted at dose levels as high as 1000 ppm. Postnatal survival as well as post-weaning development of the offspring of these test groups until sexual maturity remained unaffected by the test substance. Furthermore, clinical and/or gross necropsy examinations of the F1 and F2 pups revealed no adverse findings. The F1 and F2 offspring in the high-dose group 1000 ppm had significantly reduced body weights and gained also less body weight than the control offspring prior to weaning. Because it is present in the progeny of both generations, this reduced weight gain is regarded as a treatment-related slight delay of postnatal development. However, the more consistent reduction of weight gain was observed towards the end of lactation, i.e. from PND 14 onwards, when the offspring usually starts to consume solid food. Thus, it may be assumed that the reduced weight gain could at least be partly attributed to the direct exposure of offspring with the test compound via the diet as it was observed for their parents. At any rate the effect did not affect any other developmental parameters such as postnatal survival as well as post-weaning development of the offspring of the high-dose group until sexual maturity. Secondary to the reduced pup body weight gain, lower weights of thymus and/or higher relative brain weights were noted in the high-dose offspring, these effects were not regarded as independent adverse or toxicologically relevant findings. Furthermore, all values were well within the historical control range.

Based on the effects on body weight development, food consumption as well as changed thyroid hormone levels and thyroid morphology for the F0 and F1 parental female rats the parental NOAEL in this study was identified at 100 ppm (9 mg/kg bw/d). For the F0 and F1 parental male rats the NOAEL is below 100 ppm because, in addition to the described mid- and high-dose effects, changed thyroid morphology is still present at this dose. Since a NOAEL for parental toxicity for males could not be determined a benchmark dose calculation was performed for F0 and F1 males concerning the follicular hypertrophy/hyperplasia of the thyroid gland. The lowest $BMDL_{10}$ was 61 ppm (5.5 mg/kg bw/d).

In absence of any effects on fertility and reproductive performance the reproductive NOAEL was ≥ 1000 ppm (92 mg/kg bw/d). The NOAEL for developmental toxicity in the F1 and F2 progeny is 1000 ppm, the highest dose tested. The NOEL is 350 ppm for the F1 and F2 progeny based on slightly decreased pre-weaning pup body weights/pup weight gain, as well as slightly changed thyroid hormone levels in female F1 pups on PND 4, observed at the LOEL of 1000 ppm. These effects did not affect any other developmental parameters such as postnatal survival as well as post-weaning development of the offspring until sexual maturity. Thus, they are not regarded as independent adverse or biologically relevant developmental toxicity.
Developmental effects did not occur in the absence of parental effects.

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I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 222 29 F (Metiram TK)

Description:	solid / beige
Lot/Batch #:	300015
Purity:	91.5%
Stability of test compound:	The test substance was stable over the study period (Expiry date 30.06.2011).

2. Vehicle and/or positive control: Vehicle: diet (see below)

3. Test animals:

Species:	Rat, Wistar
Strain:	Crl:WI(Han)
Sex:	Male and female
Age:	28 ± 1 days at delivery; 37 ± 1 days at commencement of treatment
Weight at dosing:	♂: 110.1 – 145.0 g, ♀ 103.9 – 139.6 g
Source:	
Acclimation period:	about 9 days
Diet:	Kliba maintanance diet rat/mouse Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water ad libitum
Housing:	individual in type Makrolon type MIII cages supplied by Becker & Co., Castrop-Rauxel, Germany (floor area of about 800 cm²), with the following exceptions:
•	male and female mating pairs were housed together in type MIII cages overnight
•	pregnant animals and their litters housed in Makrolon type M III cages (Becker & Co., Castrop-Rauxel, Germany) until PND 21. Pregnant females were provided with nesting material (cellulose wadding) toward the end of gestation.
Environmental conditions:	
Temperature:	20 - 24°C
Humidity:	30 - 70%
Air changes:	15 per hour
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 23-March-2010 - 11-Nov-2010 (Inlife dates: 01-Apr-2010 (start of administration of F_0 parental animals) to 13-Dec-2010 (sacrifice of selected F_1 females)

2. Animal assignment and treatment:

Metiram was administered to groups of 25 male and 50 female rats at nominal dose levels of 0, 100, 350 and 1000 ppm. During the lactation period the metiram concentrations in the diet of the F0 and F1 females were reduced to 50%. This dietary adjustment, derived from historical body weight and food consumption data, maintained the dams at the desired target doses of metiram during this period of increased food intake. The animals used as F_0 parental animals were derived from different litters according to a written statement from the breeder. By this sibling mating was avoided. The animals were randomly assigned to the treatment groups based on the body weight at day -3.

After the acclimatization period F_0 parental animals continuously received the test-substance throughout the entire study. About 16 hours prior to sacrifice food was withdrawn.

At least 73 day after commencement of treatment, male and female rats from the same dose group were mated, (details see below).

Females were allowed to litter and rear their pups (F_1 pups) until day 4 (standardisation; see below) or day 21 parturition. After weaning of F_1 pups the F_0 parental animals were sacrificed.

After weaning, 25 male and 25 female F_1 pups of each treatment group were randomly selected as F_1 generation parental animals. It was attempted to take each litter into account. If fewer than 25 litters in these groups were available for selection or if one sex was missing in a litter, more animals were taken from different litters from the relevant test group to make up the required number of animals.

All selected animals were treated with the test substance at the same dose level as their parents, from post-weaning through adulthood. At least seventy-three days after assignment of the F1 generation parental animals, the males and females were mated. The partners were randomly assigned, however matings between siblings were avoided.

Like F_0 females, F_1 females were allowed to litter and rear their pups (F_2 pups) until day 4 or 21 after parturition. Shortly after weaning of F_2 pups F_1 parental animals were sacrificed.

F_1 and F_2 weanlings not selected as parental animals or reared to sexual maturity were sacrificed after weaning.

Mating procedure: Males and females were mated overnight at a 1 : 1 ratio for a maximum of 2 weeks. Throughout the mating period, each female was paired with a predetermined male form the same dose groups. The animals were paired by placing the female in the cage of the male mating partner from about 4.00 p.m. until 7.00 - 9.00 a.m. of the following morning. Deviations from the specified times were possible on weekends and public holidays.
A vaginal smear was prepared after each mating and examined for sperm. If sperm was detected, pairing of the animals was discontinued. The day on which sperm were detected was denoted "gestation day (GD) 0" and the following day "gestation day (GD) 1".

Standardization of litters: On day 4 p.p. (post-partum), the individual litters were standardized in such a way that, where possible, each litter contained 4 male and 4 female pups (always the first 4 pups/sex and litter were taken for further rearing). If it was not possible in single litters to have 4 pups/sex, it was proceeded in such a way that 8 pups per litter were present for further rearing (e.g., 5 male and 3 female pups). Standardization of litters was not performed in litters with ≤ 8 pups.

3. Test substance preparation and analysis:
For diet preparation the test substance was weighed out and thoroughly mixed with a small amount of food. Then corresponding amounts of food, depending on the dose group, were added to this premix in order to obtain the desired concentration. Mixing was carried out for about 10 minutes in a laboratory mixer.

The following nominal dose levels were selected for the study:

100 ppm as low dose
350 ppm as intermediate dose
1000 ppm as high dose

Metiram concentrations in the diet of the F_0 and F_1 females were reduced to 50% during the lactation period. This dietary adjustment, derived from historical body weight and food consumption data, maintained the dams at constant dose-levels of metiram during this period of increased food intake.

Analytical verifications of the stability of the test substance in the diet for a period of 42 days at room temperature were carried out before the study was initiated. Homogeneity and concentration control analyses were carried out at the beginning and toward the end of the premating periods, as well as during the gestation (except in the F_1 females) and lactation periods.

Table 5.6.1-1: Analysis of diet preparations for homogeneity and test-item content

Expected value [mg/kg]	Sampling	Analytical values [mg/kg]			Recoveries in %
		Arithmetic mean			
100	01.04.2010	103	±	6.2	103
100	01.04.2010	98	±	5.0	98
100	01.04.2010	98	±	1.7	98
350	01.04.2010	348	±	13.3	99
1000	01.04.2010	980	±	40.5	98
1000	01.04.2010	1077	±	89.8	108
1000	01.04.2010	944	±	145.2	94
100	02.06.2010	90	±	3.2	90
100	02.06.2010	91	±	2.5	91
100	02.06.2010	81	±	2.0	81
350	02.06.2010	359	±	6.2	103
1000	02.06.2010	1027	±	72.9	103
1000	02.06.2010	938	±	75.2	94
1000	02.06.2010	985	±	10.5	99
50	29.06.2010	46	±	2.0	92
50	29.06.2010	49	±	0.6	98
50	29.06.2010	41	±	2.6	82
175	29.06.2010	160	±	8.9	107
500	29.06.2010	550	±	142.7	110
500	29.06.2010	518	±	27.3	104
500	29.06.2010	579	±	54.2	116
100	25.07.2010	100	±	9.6	100
100	25.07.2010	102	±	7.0	102
100	25.07.2010	94	±	8.2	94
350	25.07.2010	373	±	32.0	107
1000	25.07.2010	1065	±	79.8	107
1000	25.07.2010	1072	±	103.8	107
1000	25.07.2010	1109	±	13.9	111
100	19.09.2010	94	±	10.8	94
100	19.09.2010	86	±	9.1	86
100	19.09.2010	102	±	10.3	102
350	19.09.2010	388	±	11.8	111
1000	19.09.2010	1006	±	42.8	101
1000	19.09.2010	1062	±	56.5	106
1000	19.09.2010	1032	±	11.4	103
50	07.11.2010	57	±	1.0	114
50	07.11.2010	55	±	1.2	110
50	07.11.2010	51	±	2.5	102
175	07.11.2010	189	±	11.4	108
500	07.11.2010	591	±	25.4	118
500	07.11.2010	493	±	17.6	99
500	07.11.2010	484	±	29.0	97

Most measured values for metiram were in the intended range of the target concentrations (90 - 110%), demonstrating the correctness of the diet preparations. Some individual samples were outside the target range (minimum 81%, maximum 118%), but however, still within the admissible tolerance of the measurement method of 80 – 120% (see Table 5.6.1-1). Thus, these individual departures from the target range are not considered to compromise the overall validity of the study.

4. Statistics and Benchmark Dose calculation:

Means and standard deviations of each test group were calculated for several parameters. Further statistical analyses were performed according to following tables:

Parameter	Statistical test
Food consumption[3] (parental animals), body weight and body weight change (parental animals and pups; for the pup weights, the litter means were used), estrous cycle duration, number of mating days, duration of gestation, number of implantation sites, postimplantation loss and % postimplantation loss, number of pups delivered per litter, duration of sexual maturation (days to vaginal opening, days to preputial separation)	Simultaneous comparison of all dose groups with the control group using the DUNNETT-test (two-sided) for the hypothesis of equal means
Male and female mating indices, male and female fertility indices, gestation index, females with liveborn pups, females with stillborn pups, females with all stillborn pups, live birth index, pups stillborn, pups died, pups cannibalized, pups sacrificed moribund, viability index, lactation index, number of litters with affected pups at necropsy, sexual maturation data (vaginal opening, preputial separation), males with a certain amount of abnormal sperm (cutoff value: 0.9-quantile [90%] of control groups	Pairwise comparison of each dose group with the control group using FISHER'S EXACT test for the hypothesis of equal proportions
Proportions of affected pups per litter with necropsy observations Total spermatids/g testis, total sperm/g cauda epididymides,	Pairwise comparison of the dose group with the control group using the WILCOXON-test (onesided) for the hypothesis of equal medians
Sperm motility (%)	Pairwise comparison of the dose group with the control group using the WILCOXON-test (one-sided) with Bonferoni-Holm-Adjustment for the hypothesis of equal medians
Pup organ weights (absolute and relative)	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided).If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using the WILCOXON-test (two-sided) for the equal medians
Clinical pathology parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided).If the resulting p-value was equal or less than 0.05, a pair-wise comparison of each dose group with the control group was performed using Wilcoxon-test (two-sided) for the equal medians
Weigh parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians
DOFC (differential ovarian follicular count)	Pair-wise comparison of the dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal medians

Benchmark Dose Calculation:
For the determination of the benchmark dose the software ‘Benchmark dose Software’ (BMDS, released by EPA) was used. The Multistage model was used.

P[Response] = background + (1 – background) * [1 – EXP(-beta1 * dose^1-beta2 * dose^2)] with the parameters background, beta1 and beta2, which have to be estimated.

A dose with an extra risk of follicular hypertrophy/hyperplasia in thyroid glands of male rats of 5% and 10% and its 95% lower confidence limit was calculated.

C. Methods

1. Observations:

The animals, i.e. parental animals and pups, were examined for mortality twice daily on working days and once daily on weekends and public holidays. Observation for evident signs of toxicity was performed daily. The littering and lactation behavior of the dams was generally evaluated in the mornings in connection with the daily clinical inspection of the dams. Only special findings (e.g., animal could not litter, umbilical cord not cut) were documented on an individual dam basis. Except on public holidays littering behavior was also checked in the afternoons.

2. Body weight:

Body weight of **parental animals** was determined on the first day of the administration period and weekly thereafter. The following exceptions are notable for female parental animals:

a. During the mating period body weight was not determined for F0 and F1 females.

b. During pregnancy, body weight of the F0 and F1 females with evidence of sperm was determined weekly for GD 0-7, 7-14 and 14-20.

c. Body weight was not determined for F0 and F1 females without positive evidence of sperm and females without litter.

d. During lactation, body weight of the F0 and F1 females, which gave birth to a litter was determined for PND 1-4, 4-7, 7-14 and 14-21.

e. Females without litter were not weighed during the lactation phase.

Pup body weights were determined on the day after birth (PND 1) as well as on PND 4, 7 14 and 21.

3. Food consumption, food efficiency and compound intake:

Generally, food consumption was determined once a week for male and female F0 and F1 **parental** animals, with the following exceptions:

a. Food consumption was not determined after the 10th premating week (male F0 and F1 animals) and during the mating period (female F0 and F1 animals).

b. During pregnancy, food consumption of the F0 and F1 females with evidence of sperm was determined weekly for GD 0-7, 7-14 and 14-20.

c. During lactation, food consumption of the F0 and F1 females, which gave birth to a litter was determined for PND 1-4, 4-7, 7-14, and 14-21.

Food consumption was not determined for F0 and F1 females without positive evidence of sperm and females without litter.

The mean daily intake of test substance (group means) was calculated based upon individual values for body weight and food consumption.

$$\text{Substance intake for day } x = \frac{FC_x \times C}{BW_y}$$

FC_x = daily food consumption of day x [g]
C = concentration on ppm;
BW_y = body weight on day y [g] (last weighing before day x)

4. Ophtalmoscopy:
Not performed in this study

5. Hematology and clinical chemistry:
Parental animals:
Blood was withdrawn from the retroorbital venous plexus following Isoflurane anesthesia. Blood was withdrawn from 12 animals per test group and sex of the F_0 and F_1 parental animals shortly before scheduled sacrifice (study days 106/110 in F0/F1 males and 123 in F0 & F1 females).
Pups:
On PND 4, as a result of standardization, the surplus pups were sacrificed by decapitation (following isoflurane anesthesia) and blood were sampled from vena cava cranialis. On PND 21 blood was sampled from one F1 and F2 male and female pup per litter of all surviving blood-sampled dams per group.

The following haematological and clinical chemistry parameters were determined:

Haematology:					
	Red blood cells		*White blood cells*		*Clotting Potential*
✓	Erythrocyte count (RBC)	✓	Total leukocyte count	✓	Platelet count (PLT)
✓	Haemoglobin (Hb)	✓	Neutrophils (differential)		
✓	Haematocrit (Hct)	✓	Eosinophils (differential)		
✓	Mean corp. volume (MCV)	✓	Basophils (differential)		
✓	Mean corp. haemoglobin (MCH)	✓	Lymphocytes (differential)		
✓	Mean corp. Hb. conc. (MCHC)	✓	Monocytes (differential)		
✓	Reticulocytes	✓	Large unstained cells		
Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Phosphorus (inorganic)	✓	Bilirubin (total)	✓	Aspartate aminotransferase (AST)
		✓	Cholesterol	✓	Alkaline phosphatase (ALP)
		✓	Creatinine	✓	-glutamyl transpeptidase (-GT)
		✓	Globulin		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		
Hormones:					
	Total triiodothyronine (T3)				
	Total thyroxine (T4)				
	Thyroid stimulating hormone (TSH)				

6. Estrous cycle determination:

Estrous cycle length was evaluated by daily analysis of vaginal smear for all F0 and F1 female parental rats for a minimum of 3 weeks prior to mating. Determination was continued throughout the pairing period until the female exhibited evidence of copulation. At necropsy, an additional vaginal smear was examined to determine the stage of estrous cycle for each F0 and F1 female with scheduled sacrifice.

7. Male reproduction data

The pairing partners, the number of mating days until vaginal sperm was detected in the female animals, and the gestational status of the females were recorded for F0 and F1 breeding pairs. For the males, mating and fertility indices were calculated for F1 and F2 litters according to the following equations:

$$\text{Male mating index [\%]} = \frac{\text{number of males with confirmed mating *}}{\text{number of males placed with females}} \times 100$$

* defined by a female with vaginal sperm or with implants in utero

$$\text{Male fertility index [\%]} = \frac{\text{number of males proving their fertility *}}{\text{number of males placed with females}} \times 100$$

* defined by a female with implants in utero

8. Sperm parameters

Immediately after necropsy and organ weight determination the right testis and cauda epididymis were taken from the F0 and F1 males of all dose groups. The following parameters were determined:

- sperm motility
- sperm morphology
- sperm head count (cauda epididymis)
- sperm head count (testis)

Sperm motility examinations were carried out in a randomized sequence. Sperm morphology and sperm head count (cauda epididymis and testis) were evaluated for the control and highest dose group, only. Sperm motility was investigated by microscopic evaluation. Sperm morphology was evaluated microscopically after vital staining with eosin. Sperm head counts in cauda epididymis and testes were determined microscopically after homogenization using a MAKLER chamber.

9. Female reproduction and delivery data

For the females, mating, fertility and gestation indices were calculated for F1 and F2 litters according to the following equations:

$$\text{Female mating index [\%]} = \frac{\text{number of females mated *}}{\text{number of females placed with males}} \times 100$$

* defined as the number of female with vaginal sperm or with implants in utero

$$\text{Female fertility index [\%]} = \frac{\text{number of females pregnant *}}{\text{number of females mated **}} \times 100$$

* defined as number of female with implants in utero
** defined as the number of females with vaginal sperm or with implants in utero

$$\text{Female gestation index [\%]} = \frac{\text{number of females with live pups on the day of birth}}{\text{number of females pregnant *}} \times 100$$

* defined as number of female with implants in utero

The total number of pups delivered and the number of liveborn and stillborn pups were noted, and the live birth index was calculated:

$$\text{Live birth index [\%]} = \frac{\text{number of liveborn pups at birth}}{\text{total number of pups born}} \times 100$$

The implantations were counted* and the postimplantation loss (in %) was calculated:

$$\text{Postimplantation loss [\%]} = \frac{\text{number of implantations} - \text{number of pups delivered}}{\text{number of implantations}} \times 100$$

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* To determine the number of implantation sites, the apparently non-pregnant uteri were stained for about 5 minutes in 10% ammonium sulfide solution according to the method of SALEWSKI. (Salewski, E.; 1964). Then the uteri were rinsed carefully under running water. Thereafter the implantation sites were recorded for calculation of the postimplantation loss.

10. Litter data

All F_1 and F_2 pups were examined as soon as possible on the day of birth to determine the total number of pups and the number of liveborn and stillborn members of each litter. Pups, which died before the first determination of their status on the day of birth, were designated as stillborn pups.

The number of live pups/litter was calculated on the day of birth, and on lactation days 4, 7, 14, and 21. Furthermore, viability and lactation indices were calculated as follows:

$$\text{Viability index [\%]} = \frac{\text{number of live pups on day 4* after birth}}{\text{number of live pups on the day of birth}} \times 100$$

* before standardization of litters (i.e. before culling)

$$\text{Lactation index [\%]} = \frac{\text{number of live pups on day 21 after birth}}{\text{number of live pups on day 4* after birth}} \times 100$$

* after standardization of litters (i.e. after culling)

On the day of birth the sex of the pups was determined by determination of the anogenital distance. Subsequently the sex of the pups was assessed by the external appearance of the anogenital region and/or the mammary line of the animals and was finally confirmed at necropsy. The sex ratio was calculated at day 0 and 21 after birth using the following equation:

$$Sex\ ratio\ [\%] = \frac{number\ of\ live\ male\ or\ female\ pups\ on\ day\ 0\ and\ 21}{number\ of\ live\ male\ and\ female\ pups\ on\ day\ 0\ and\ 21} \times 100$$

Sexual maturation was determined in F_1 pups selected as parental animals. **Females** were evaluated daily for **vaginal opening** with examinations initiating on PND 27. At the day of vaginal opening the body weights of the respective animals were additionally determined. **Males** were evaluated daily for **preputial separation** with examinations initiating on PND 38. At the day of preputial separation the body weights of the respective animals were additionally determined.

11. Sacrifice and pathology:

All F0 and F1 **parental animals** were sacrificed by decapitation under CO_2-anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology; special attention was given to the reproductive organs. A quantitative assessment of primordial and growing follicles in the ovaries was performed for all control and high-dose F1 parental females.

Pathology:

The following organs were collected (column C), weighed (W) and examined histopathologically (H, ✓: all groups, #: All animals of control and high dose groups and mating pairs suspected of reduced fertility of the low and mid dose groups.

C	W	H		C	W	H		C	W	H	
✓	✓	#	adrenals	✓	✓	#	Ovaries	✓	✓	✓*	Thyroid w. parathyroid gland
	✓		brain	✓		#	Oviducts	✓	✓	#	uterus
	✓		cauda epididymides	✓	✓	#	Pituitary gland	✓		#	cervix uteri
✓	✓	#	epididymides§	✓	✓	#	prostate	✓		#	vagina
✓		✓	gross lesions	✓	✓	#	seminal vesicles[a]		✓		body (anesthetized)
	✓		kidneys		✓		spleen				
✓	✓	#	liver	✓	✓	#	testes§				

§ left; [a] with coagulation glands; * all male F0 animals/test group and all F1 animals/test group and each 5 m&f PND4 and each 12 m&f PND21 F1 and F2 pups

For F_1 maternal animals differential ovarian follicle count (DOFC) five ovarian sections each were taken from the proximal and distal part from both ovaries at least 100 µm apart from the inner third of the ovary. In order to prevent multiple counting - especially of the growing follicles - only follicles with an oocyte with visible chromatin on the slide were counted.

All **pups** were examined macroscopically (including weight determinations of brain, spleen and thymus in one pup/sex/litter). On PND 4, the thyroid glands/parathyroid glands were prepared for histopathological examinations from five selected culled F1 and F2 male and female pups per group. On PND 21 glands/parathyroid glands were prepared for histopathological examinations from one F1 and F2 male and female pup per litter of all surviving blood-sampled dams per group (each 12 m&f pups examined). All surplus pups, (with the exception of those F1 generation pups which were chosen as F1 generation parental animals) were sacrificed under isoflurane anesthesia with CO_2. After sacrifice, all pups were examined externally, eviscerated and their organs were assessed macroscopically. All pups without any notable findings or abnormalities were discarded after their macroscopic evaluation. Animals with notable findings or abnormalities were further evaluated on a case-by-case basis (e.g., histopathological evaluation or special staining), depending on the findings noted.

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

See section B3 above.

B. OBSERVATIONS

1. Clinical signs of toxicity

No clinical signs or changes of general behavior, which may be attributed to the test substance, were detected in any of the male and female F0 parental animals in any of the groups.

There were no test substance-related clinical findings in any females of all dose groups during the gestation period for F1 litter.
One female of dose group 01 (100 ppm) had vaginal haemorrhage and blood in bedding on GD 25.
There were no test substance-related clinical findings in all F0 females of all dose groups during the lactation period.

No treatment-related or spontaneous mortalities were observed in any of the groups throughout the study.

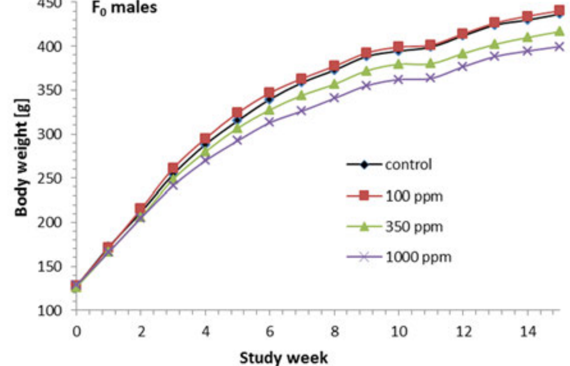
C. PARENTAL BODY WEIGHT AND BODY WEIGHT GAIN

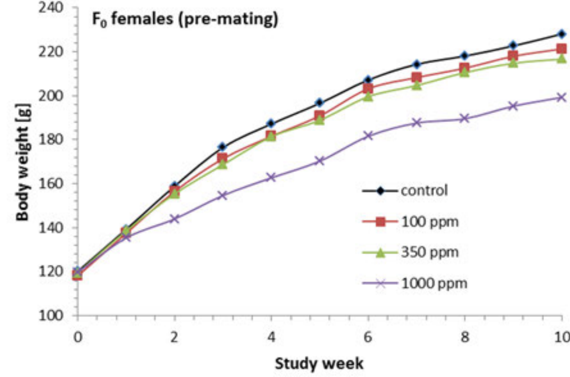
Body weight development was impaired in parental males and females at the top dose level.

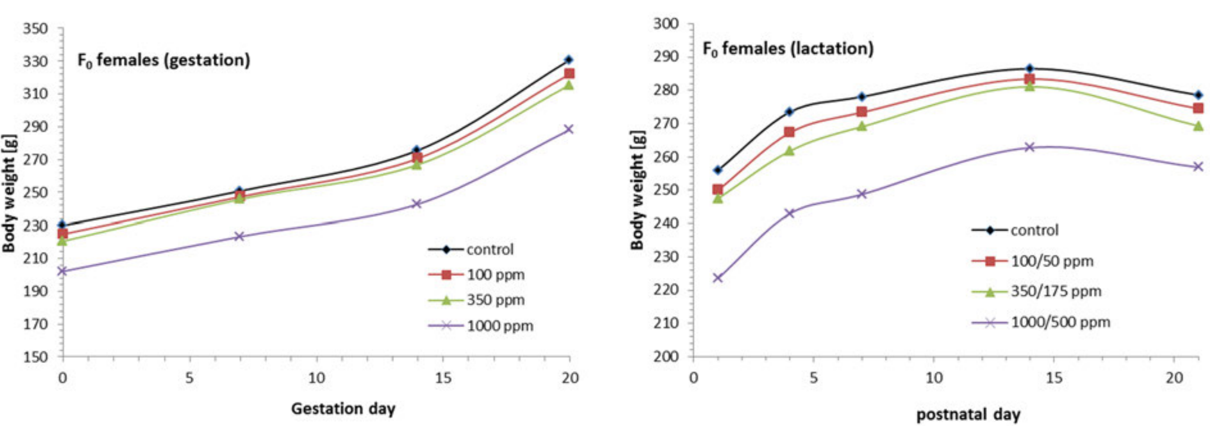
F_0 parental animals:
Body weights of high dose males were significantly reduced from week 3 until the end of treatment [see Figure 5.6.1-1]. The body weight difference to controls amounted to 8%. Their body weight gain was statistically significantly decreased when calculated as average for the entire study (-12%), showing maximal decreases during weeks 2 -5 (-17%) and 6 - 7 (-31%). A slight but noticeable effect on body weights/body weight gain was still present in the mid dose males (350 ppm, average weight gain -6%, final weight -5%) although the difference to control was only statistically significant during week 13 (body weight, -5%) and 0 – 1 (body weight gain, -10%).
The body weights and the body weight gain of the low-dose parental males (100 ppm) were comparable to the concurrent control group throughout the entire study. The statistically significantly increased body weight gain in the low-dose males during study weeks 1 - 2 was considered as spontaneous in nature.

High-dose parental females had statistically significantly lower body weights from test week 2 until the end of premating (final weight -13%), their body weights remained below control also during gestation period (maximum -13%). Their body weight gain was statistically significantly decreased during premating weeks 0 - 3 (up to 56%) and when calculated for whole premating (about -26%), as well as during gestation (GD 7 - 14 about -22%, average gestation -15%). Average body weight gain of high-dose F0 females was higher than control during lactation (+48%) however, body weights did not fully recover (final weight 8% below control).
Occasionally, the mid-dose parental F0 females also had slightly but statistically significantly lower body weights in premating, gestation and lactation, such as in premating weeks 3 (about 4%), 7 (about 4%) and 10 (about 5%), as well as on GD 0 (about 4%) and PND 4 (about 4%). Their body weight gain was decreased during premating weeks 2 - 3 (about -24%) and when calculated for whole premating (about -10%) and gestation (about -6%).
Neither mean body weights nor mean body weight gain of the F0 parental females in the low dose group were influenced by the test substance during premating, gestation and lactation periods.

Figure 5.6.1-1: Body weight development of parental F_0 rats



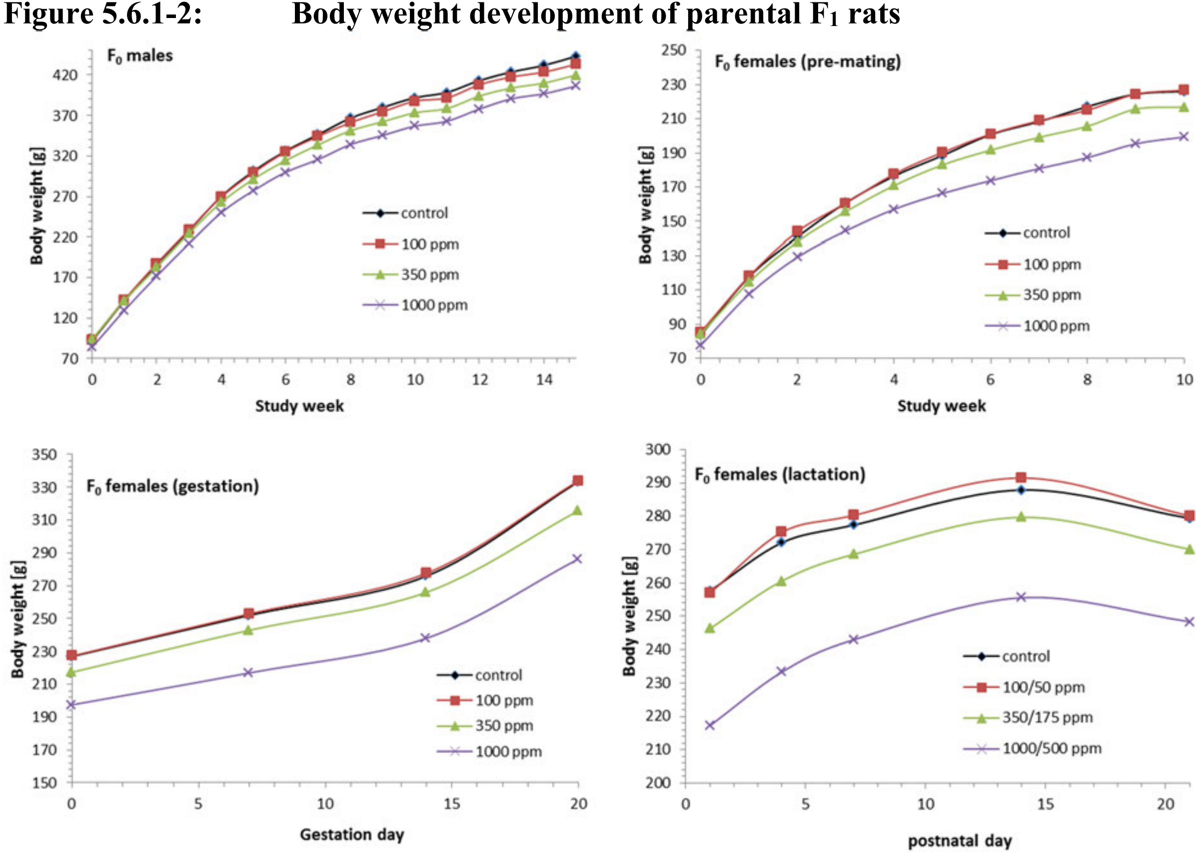




F1 parental animals:

Body weights of high dose males were significantly reduced throughout treatment (final weight -8%) [see Figure 5.6.1-2]. Their body weight gain was statistically significantly decreased when calculated as average for the entire study (-8%), showing maximal decreases during weeks 0 -1 (-8%), 3 - 5 (-13%) and 6 - 7 (-22%). A noticeable effect on body weights/body weight gain was still present in the mid dose males (350 ppm, average weight gain -7%, final weight -5%) although the difference to control in weight gain was only statistically significant during weeks 3 – 4 (-9%). The body weights and the body weight gain of the low-dose parental males (100 ppm) were comparable to the concurrent control group throughout the entire study.
High-dose F1 parental females had statistically significantly lower body weights during the entire premating period (final weight -12%), their body weights remained below control also during gestation (final weight -14%) and lactation periods (final weight -11%). Their body weight gain was statistically significantly decreased during premating weeks 0 - 1 (-12%), during weeks 2 - 3 (-23%), during weeks 5 - 6 (-39%) and when calculated for whole premating (-15%), as well as during gestation (GD 0 - 7 about -22%, GD 14 - 20 about -16%, average gestation -17%). Average body weight gain of high-dose F1 females was higher than control during lactation (+43%), however, body weights did not recover (final weight 11% below control). Occasionally, the mid-dose parental F1 females also had slightly but statistically significantly lower body weights in premating and gestation, such as in premating week 8 (about -5%), as well as on GD 20 (about -5%). Their body weight gain was decreased during premating weeks 0 - 1 (about -11%), 5 - 6 (about -28%), calculated for whole premating (about -7%) and on GD 14 - 20 (about -14%). No changes in body weight gain of the mid-dose females were noted during lactation. Neither mean body weights nor mean body weight gain of the F1 parental females in the low dose group were influenced by the test substance during premating, gestation and lactation periods. The statistically significantly lower body weight gain during premating weeks 2 – 3 (about -16%) was considered as incidental finding since there was no dose response.

Figure 5.6.1-2: Body weight development of parental F_1 rats



D. PARENTAL FOOD CONSUMPTION AND COMPOUND INTAKE

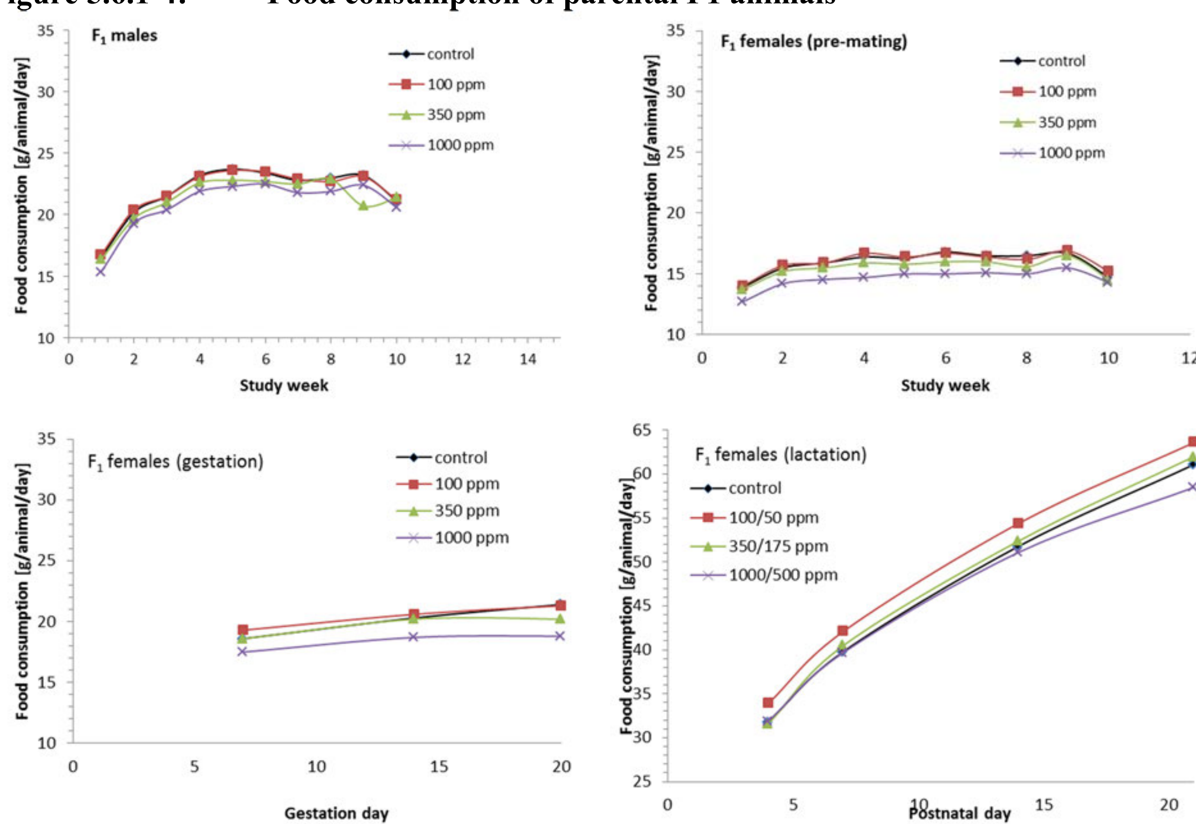
Food consumption was impaired in both parental generations at the high dose level. The observed effects on food consumption are in line with the observed body weight effects.

F_0 parental animals:
Food consumption of the high-dose F0 males was below control during the entire treatment period (maximum -8%, average -6%). The difference was statistically significant during weeks 0 - 1, weeks 2 - 7 and during weeks 8 – 10. Food consumption of the male F0 rats in the mid and low dose groups (350 and 100 ppm) was comparable to the concurrent control throughout the entire study.
Food consumption of the high-dose F0 females was statistically significantly below control during major parts of premating (weeks 0 - 5 up to -11%, weeks 6 - 7 about -5%, during weeks 9 - 10 about -5%) and it was below control during gestation, being statistically significant on GD 14 - 20 (about -12%). Occasionally, the mid-dose females also showed slightly, but statistically significantly reduced food consumption, like during premating weeks 4 - 5 (about 5% below control) and 9 - 10 (about 5% below control).
Food consumption of the high-dose F0 females was comparable to the concurrent control during lactation and food consumption of the mid-dose F0 females was comparable to the concurrent control during gestation and lactation. The low-dose females did not show any test substance-related changes of food consumption during the whole treatment period.

Figure 5.6.1-3: Food consumption of parental F0 animals

F₁ parental animals:
Food consumption of the high-dose F1 males was below control during the entire treatment period (maximum -7%, average -5%). The difference was statistically significant during weeks 0 - 1 and weeks 3 – 5.
Food consumption of the high-dose F1 females was statistically significantly below control during major parts of premating (weeks 0 - 9 up to -11%, average -8%), and it was below control during gestation, being statistically significant in each interval during GD 0 and GD 20 (maximum -12%). No changes in food consumption of the high-dose females were noted during lactation.
Food consumption of the male and female F1 rats in the mid- and low-dose group was comparable to the concurrent control throughout the entire study.

Figure 5.6.1-4: Food consumption of parental F1 animals



The intake of metiram (in mg/kg bw/d) was calculated on the basis of most recent individual body weights in each test group. The overall approximate values are shown in Table 5.6.1-2.

Table 5.6.1-2: Average metiram intake in parental rats

Dose level	**100/50 ppm (9 mg/kg bw/d)**		**350/175 ppm (31 mg/kg bw/d)**		**1000/500 ppm (92 mg/kg bw/d)**	
Group & sex	mean intake	min/max intake	average intake	min/max intake	average intake	min/max intake
F0 males	8.6	5.4 / 14.2	30.3	19.5 / 49.4	84.5	55.8 / 134.6
F1 males	9.6	5.7 / 18.0	33.6	20.1 / 61..5	99.5	59.8 / 184.2
F0 females (premating)	8.9	6.7 / 11.6	31.1	23.2 / 41.1	92.2	73.3 / 112.2
F0 females (gestation)	8.1	7.7 / 8.3	28.6	27.0 / 30.4	84.7	78.6 / 90.2
F0 females (lactation*)	8.8	6.5 / 11.3	31.2	22.4 / 39.6	96.5	74.0 / 119.4
F1 females (premating)	10.0	6.8 / 16.5	35.0	23.6 / 56.8	102.3	73.4 / 164.5
F1 females (gestation)	8.1	7.7 / 8.5	28.6	26.7 / 30.0	84.6	79.0 / 88.7
F1 females (lactation*)	8.7	6.6 / 10.9	30.7	22.5 / 38.8	94.6	73.7 / 114.2

* Days 0 - 14 only

E. ESTROUS CYCLE DETERMINATIONS

Estrous cycles as determined during 3 week prior to mating were very regular in all dose groups in **F_0** and **F_1** females. The mean cycle length was 4.3, 4.0, 4.1 and 4.3 days in F_0 and 4.0, 4.2, 4.4 and 4.6 days in F_1 females at 0, 100, 350, and 1000 ppm, respectively.

F. MATING AND GESTATION DATA

1. Male reproductive performance

Male reproductive performance was not affected by treatment. The effects on mating and fertility displayed no dose-response relationship and were within the normal variation of this strain of rats used.

Copulation was confirmed for all F0 parental males, which were paired with females to generate F1 pups. Thus, the male mating index was 100% in all test groups.
Fertility was proven for almost all F0 parental males with confirmed copulation. One low-dose male did not generate F1 pups. Thus, the male fertility index ranged between 96% and 100% without showing any effect of dosing (see Table 5.6.1-3). For nearly all F1 parental males, which were placed with females to generate F2 pups, copulation was confirmed. Copulation was not confirmed for one high-dose male paired with a high-dose female. Thus, the male mating index was 100% in the control, low and mid-dose groups and 96% in the high-dose group. Fertility was proven for most of the F1 parental males within the scheduled mating interval for F2 litter. Two control males and one high-dose male did not generate F2 pups. Thus, the male fertility index ranged between 92% and 100% without showing any relation to dosing.
These values reflect the normal range of biological variation inherent in the strain of rats used for this study. All respective values are within the range of the historical control data of the test facility.

The apparently infertile male rats did not show histopathological findings that could explain infertility.

Table 5.6.1-3: Reproduction parameters of male rats treated with metiram

Parental generation	F_0				F_1			
Dose [ppm]	0	100	350	1000	0	100	350	1000
Animals per dose	25	25	25	25	25	25	25	25
Male fertility								
- placed with females [n]	25	25	25	25	25	25	25	25
- mated [n]	25	25	25	25	25	25	25	24
- mating index [%]	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**96**
- with females pregnant [n]	25	24	25	25	23	25	25	24
- fertility index [%]	**100**	**96**	**100**	**100**	**92**	**100**	**100**	**96**

2. Sperm analysis

Sperm analysis (number of homogenisation resistand sperm heads in testes and cauda eypidymidis, % abnormal sperm and motility) did not indicate any effects of treatment in F_0 and F_1 males [see Table 5.6.1-4].

Table 5.6.1-4: Sperm parameters of males administered metiram

Parental generation	F0				F1			
Dose [ppm]	0	100	350	1000	0	100	350	1000
Sperm count [10^6 / g								
Testis	114			124	103			98
Cauda epididymis	606			673	513			541
Normal sperm [%]	97.3			97.2	96.5			92.5
Abnormal sperm [%]	2.7			2.8	3.5			7.5
Abnormal sperm > 5%# [n]	1			1	3			3
Sperm motility [%]	88	89	89	88	86	89	88	84

corresponds to the 90%-quantile of the control group

3. Female reproductive performance

Female reproductive performance was not affected by treatment (see Table 5.6.1-5).

The female mating index calculated after the mating period for F1 litter was 100% in all test groups whereas for F2 litter it varied between 100% (control, low and mid dose group) and 96% (high dose group).
The mean duration - until sperm was detected (GD 0) - varied between 1.8 and 2.5 or 2.6 and 3.5 days for the F0 or the F1 parental animals, respectively, without any relation to dosing.
All female F0 rats delivered pups or had implants in utero except one female in the low dose group. All female F1 rats delivered pups or had implants in utero except two females from the control group and one female from the high dose group. There were no corroborative histopathological findings in the sexual organs of the non-pregnant females.
The fertility index for the F0 parental females varied between 96% in the low dose group and 100% in all other groups whereas for the F1 females it varied between 92% (control) and 100% (low-, mid- and high dose group). These values reflect the normal range of biological variation inherent in the strain of rats used for this study. All respective values are within the range of the historical control data of the test facility and do not show any relation to dosing.

The mean duration of gestation was similar in all test groups (i.e. between 21.9 and 22.0 days for F0 and 22.0 and 22.1 days for F1 females). For the F0 generation females the gestation index varied between 92 and 100% and the gestation index for the F1 generation females was 100% for all test groups (0, 100, 350 and 1000 ppm). All values are within the historical control range of the test facility.
Implantation was not affected by the treatment since the mean number of implantation sites was comparable between all test substance-treated groups and the controls, taking normal biological variation into account. Furthermore, there were no indications for test substance-induced intrauterine embryo- /fetolethality since the postimplantation loss did not show any statistically significant differences between the groups, and the mean number of F1 or F2 pups delivered per dam remained unaffected.
The rate of liveborn pups was also not affected by the test substance, as indicated by live birth indices of 98% to 100%. Moreover, the number of stillborn pups was comparable between the groups.

Table 5.6.1-5: Reproduction and gestational parameters of female rats treated with metiram

Parental generation			F_0			F_1		
Dose [ppm]	0	100	350	1000	0	100	350	1000
Animals per dose	25	25	25	25	25	25	25	25
Female fertility								
- placed with males	25	25	25	25	25	25	25	25
- mated [n]	25	25	25	25	25	25	25	24
- mating index [%]	100	100	100	100	100	100	100	96
- pregnant [n]	25	24	25	25	23	25	25	24
- **fertility index** [%]	100	96	100	100	92	100	100	100
Pre coital interval [days]	2.4	2.2	1.8	2.5	3.5	2.6	2.8	3.0
Duration of gestation [days]	22.0	21.9	22.0	22.0	22.1	22.0	22.1	22.1
Implantation sites, total [n]	302	287	279	308	286	330	310	307
- dto per dam [n]	12.1	12.0	11.2	12.3	12.4	13.2	12.4	12.8
Post implantation loss [n]	18	17	11	22	24	19	22	19
- dto per dam [n]	0.7	0.7	0.4	0.9	1.0	0.8	0.9	0.8
- dto per litter [mean %]	5.9	12.5	10.4	7.4	9.7	5.9	7.7	6.1
Females with liveborn	25	22	23	24	23	25	25	24
- **Gestation index** [%]	100	92	92	96	100	100	100	100
- with stillborn pups [n]	1	3	1	1	2	5	0	1
- with all stillborn [n]	0	0	0	0	0	0	0	0
Pups delivered [n]	284	270	268	286	262	311	288	288
- per dam [mean n]	11.4	12.3	11.7	11.9	11.4	12.4	11.5	12.0
- liveborn [n]	282	266	267	285	260	304	288	287
- stillborn [n]	2	4	1	1	2	7	0	1
- **Live Birth index** [%]	99	99	100	100	99	98	100	100

G. PUP DATA

1. Survival / Clinical observations

Survival of pups was not affected in either generation. The viability index (survival days 0 to 4 pre cull) was 99% and 100% in F_1 pups and between 98 and 100% in F_2 pups. The lactation index (survival day 4 post cull to 21) was 100% in F_1 and F_2 litters [see Table 5.6.1-6].

For one female pup (mid-dose group 100 ppm) an anophthalmia (left eye) was recorded on PND 21. This observation is not considered to be treatment-related. The F2 generation pups did not display any clinical signs until weaning.

2. Sex ratio

The sex ratios at day 0 and 21 were not affected by treatment in both generations. All differences were within the historical control range and not indicative of a treatment-related effect [see Table 5.6.1-6].

Table 5.6.1-6: Pup viability and sex-ratio

Parental generation	F0				F1			
Dose [ppm]	0	100	350	1000	0	100	350	1000
Number of litters	25	22	23	24	23	25	25	24
- with live born pups	25	22	23	24	23	25	25	24
Pups live born [n]	282	266	267	285	260	304	288	287
Pups died [n]	2	0	1	0	1	0	1	0
Pups cannibalized [n]	1	0	1	3	1	1	1	5
Pups culled day 4 [n]	79	90	83	93	81	103	92	95
Pups day 4 - pre-cull [n]	279	266	265	282	258	303	286	282
- **Viability index** [%]	99	100	99	99	99	100	99	98
Pups day 4 - post cull [n]	200	176	182	189	177	200	194	187
Pups day 21 [n]	200	176	182	189	177	199	194	187
- **Lactation index** [%	100	100	1001	100	100	100	100	100
Sex ratio [% live males]								
- Day 0	48.2	58.3	49.4	50.5	45.4	46.4	45.1	47.7
- Day 21	51.0	54.5	51.1	51.9	49.2	47.7	47.4	48.7
Male pup weight [g]								
- day 1 [g]	6.8	**6.3****	6.5	**6.3****	6.5	6.5	6.6	6.3
- day 4 - pre cull [g]	10.7	**10.0***	10.1	**9.6****	10.1	10.1	10.0	9.6
- day 4 - post cull [g]	10.7	**10.0***	10.1	**9.5****	10.1	10.1	10.0	9.6
- day 7 [g]	17.1	16.4	16.6	**15.9****	16.3	16.4	16.1	15.5
- day 14 [g]	33.7	32.5	33.3	**31.8***	33.1	33.7	32.9	**31.5***
- day 21 [g]	53.9	51.9	52.6	**50.1****	52.3	53.1	51.7	**49.2****
Male body weight gain								
- day 4 to 21 [g]	43.2	41.9	42.5	**40.6***	42.2	43.0	41.7	**39.6****
[□%]		-3.0	-1.6	**-6.0**		1.9	-1.2	**-6.2**
Female pup weight [g]								
- day 1 [g]	6.5	6.1	6.2	**6.0****	6.2	6.3	6.2	6.0
- day 4 - pre cull [g]	10.4	9.8	9.8	**9.3****	9.7	9.8	9.6	9.3
- day 4 - post cull [g]	10.3	9.8	9.8	**9.3****	9.7	9.8	9.6	9.3
- day 7 [g]	16.6	16.0	16.2	**15.5***	15.8	16.0	15.4	15.1
- day 14 [g]	32.9	32.0	32.5	**31.2***	32.5	32.9	31.7	31.1
- day 21 [g]	52.0	50.8	50.6	**48.6****	51.0	51.3	49.8	**48.1****
Female body weight gain								
- day 4 to 21 [g]	41.6	41.0	40.8	**39.3***	41.3	41.6	40.1	**38.8***
[□%]		1.4	-1.9	**-5.5**		0.7	-2.9	**-6.1**

3. Body weight

Mean body weights of the high-dose F1 male and female pups (1000 ppm) were statistically significantly below control on PND 1 - 21 (ranging between -6% and -11%). Body weight gain of the high-dose F1 pups of both sexes was below control on PND 1 – 4 (-15%), PND 14 – 21 (-9%) and PND 4 – 21 (-5%).

No test compound-related influence on F1 pup body weights and pup body weight gain were noted in the low- and mid-dose groups (100 and 350 ppm).

Mean body weights of the low-dose pups were slightly but statistically significantly below control during PND 1 – 4. This phenomenon was probably caused by a high number of big litters (>14 pups) in this group and was largely counterbalanced after litter standardization. Thus, no association to treatment is assumed.

Mean body weights of the high-dose F2 male pups were statistically significantly below control on PND 14 (-5%). Mean body weights of the high-dose F2 pups of both sexes were statistically significantly below control on PND 21 (-5%). Body weight gain was statistically significantly decreased in the high-dose F2 pups during PND 14 - 21 (-8%) and PND 4 – 21 (-6%).
No test compound-related influence on F2 pup body weights and pup body weight gain were noted in the low- and mid-dose groups (100 and 350 ppm).

4. Organ weights

Mean absolute and relative pup organ weights of the F1 and F2 pups did not show statistically significant differences to the control group [see Table 5.6.1-7].

F1 pups: The decreased absolute brain and thymus weights (high dose group) are considered to be secondary to the lower body weight of the respective animals at weaning. Although the difference in body weight was only small in the mid-dose group the same cause is assumed for the marginally lower absolute thymus weights in these animals. As there was no significant effect and/or dose response in the relative brain and thymus weights in the mid and high-dose F1 pups, the changes of absolute weights and the decrease of relative male thymus weights were considered neither adverse nor toxicologically relevant. Furthermore, all values were well within the historical control range.
F2 pups: The decreased absolute thymus weights and increased relative brain weights (high-dose group) are considered to be secondary to the lower body weight of the respective animals at weaning. Although the difference in body weight was only small in the mid-dose group the same cause is assumed for the marginally lower absolute thymus weights in these animals. As there was no dose response in the relative thymus weights in the mid-and high-dose F2 pups, those changes were considered neither adverse nor toxicologically relevant. Furthermore, all values were well within the historical control range.

Table 5.6.1-7: Organ weights of F_1 and F_2 pups

Generation		F_1 (Males & Females combined)				F_2 (Males & Females combined)			
	Dose [ppm]	Absolute weight [g]	Δ%	Relative weight [% of b.w.]	Δ%	Absolute weight [mg]	Δ%	Relative weight [% of b.w.]	Δ%
Terminal weight	0	52.9				51.6			
	100	51.4	(-2.8)			52.2	(1.2)		
	350	51.6	(-2.5)			50.5	(-2.1)		
	1000	49.4	(-6.6)			48.8**	(-5.4)		
Brain[#]	0	1.515		2.855		1.492		2.901	
	100	1.491	(-1.6)	2.917	(2.2)	1.506	(0.9)	2.875	(-0.9)
	350	1.492	(-1.5)	2.897	(1.5)	1.493	(0.1)	2.950	(1.7)
	1000	1.461**	(-3.6)	2.960	(3.7)	1.475	(-1.1)	3.020*	(4.1)
Thymus	0	0.241		0.452		0.242		0.469	
	100	0.229	(-5.0)	0.447	(-1.1)	0.249	(2.9)	0.474	(1.1)
	350	0.217*	(-10.0)	0.421	(-6.9)	0.219*	(-9.5)	0.432**	(-7.9)
	1000	0.214*	(-11.2)	0.432	(-4.4)	0.212**	(-12.4)	0.432**	(-7.9)
Spleen	0	0.268		0.502		0.264		0.511	
	100	0.272	(1.5)	0.531	(5.8)	0.276	(4.5)	0.523	(2.3)
	350	0.257	(-4.1)	0.498	(-0.8)	0.254	(-3.8)	0.499	(-2.3)
	1000	0.260	(-3.0)	0.524	(4.4)	0.262	(-0.8)	0.536	(4.9)

* $p \leq 0.05$, ** $p \leq 0.01$ (Kruskal-Wallis and Wilcoxon-test (two-sided))
[#]all brain and thymus weight values were well within historical control ranges

5. Pup necropsy findings

A few gross necropsy findings were observed in F_1 [see Table 5.6.1-8]. The individual findings were observed in a low incidence, displayed no dose response and were within the historical control range. Likewise, F_2 pups displayed a low incidence of gross necropsy findings. These findings occurred without any relation to dosing and/or can be found in the historical control data at comparable or even higher incidences. Thus, all these findings were not considered to be associated to the test substance.

Table 5.6.1-8: Incidence of gross necropsy observations in F_1 and F_2 pups

Dose [ppm]	0	100	350	1000
	F_1 pups			
Litters evaluated	25	22	23	24
Pups evaluated	232	220	217	231
Live	230	216	216	230
Stillborn	2	4	1	1
Post mortem autolysis	0	0	1	0
Partly cannibalized	1	0	0	0
Incisors sloped	1	0	0	0
Microphthalmia	0	1	0	0
Hemorrhagic thymus	0	0	0	2
Supernumerary liver lobe	1	0	0	0
Dilated renal pelvis	0	0	0	1
Anorchia	0	0	0	1
Total pup necropsy observations	3	1	1	4
	F_2 pups			
Litters evaluated	23	25	25	24
Pups evaluated	261	310	287	283
Live	259	303	287	282
Stillborn	2	7	0	1
Post mortem autolysis	0	1	1	0
Partly cannibalized	0	2	0	0
Hydronephrosis	0	0	1	0
Dilated renal pelvis	6	0	0	0
Hydroureter	0	0	1	0
Hemorrhagic testis	0	0	1	0
Small testis	2	0	0	0
Mandibular agnathia	1	0	0	0
Total pup necropsy observations	9	3	4	0

6. Sexual maturation

The male and female F_1 pups selected to become F_1 parental animals were examined for sexual maturation.

The first day when vaginal opening was observed was PND 30, the last was PND 36. The mean number of days to reach the criterion and the mean body weight at the time point of the criterion is described in Table 5.6.1-9. As there was no dose response and all values were well within the historical control range, the apparent slight delay in the mid-dose females is not considered to be treatment-related. Thus, female sexual maturation is not assumed influenced by the test substance.

The first day when preputial separation was observed was PND 38, the last was PND 47. The mean number of days to reach the criterion and the mean body weight at the time point of the criterion is described in Table 5.6.1-9. The results indicate that male sexual maturation was not influenced by the test substance. All values, including the decreased body weights in the mid- and high-dose groups, were within the historical control range. Obviously the degree of the body weight difference was not high enough to influence the timing of male sexual maturation in the respective groups.

Table 5.6.1-9: Sexual maturation of F_1 and F_2 pups

Generation	F_1			
Dose [ppm]	0	100	350	1000
Animals per dose	25	25	25	25
Preputial separation				
- Days to criterion	41.6	41.5	40.8	41.6
- Body weight at criterion [g]	178.1	175.3	**166.5****	**165.3****
Vaginal opening				
- Days to criterion	31.9	32.1	**32.8***	32.4
- Body weight at criterion [g]	99.6	100.5	100.4	94.2

* $p \leq 0.05$, ** $p \leq 0.01$ (Dunnett-test, two-sided)

H. PARENTAL BLOOD ANALYSIS

1. Haematological findings

F_0 parental animals

No treatment-related, adverse changes among haematological parameters were measured.

In females of test group 3 (1000 ppm) haematocrit values were decreased and relative reticulocyte counts were higher compared to controls. The haematocrit changes were marginal (< 4 % regarding means), and not accompanied by a decrease of haemoglobin levels and red blood cell (RBC) counts. Higher reticulocyte counts reflect an adaptive reaction of the bone marrow to release new red blood cells. Therefore, the mentioned changes of red blood cell parameters may be treatment-related but were not regarded adverse.

In females of test group 1 (100 ppm) white blood cell (WBC) counts were higher compared to controls, but this increase was not dose-dependent. Some changes were measured in dosed rats regarding the differential blood cell counts, but all changed values were within the historical control ranges (see Table 5.6.1-10). Therefore all described alterations regarding total and differential white blood cell counts were regarded as incidental and not treatment-related.

F_1 parental animals

No treatment-related changes of haematological parameters were measured.

In all dosed males relative reticulocyte counts were higher compared to controls, but the means were not changed dose-dependently and they were within the historical control ranges (see Table 5.6.1-10).

In females of mid dose group (350 ppm) absolute lymphocyte counts and in females of low and mid dose groups (100 and 350 ppm) absolute and relative large unstained cell (LUC) counts were higher compared to controls, but the increases were not changed dose-dependently. Therefore, all described haematological alterations in the F1 generation rats were regarded as incidental and not treatment-related.

Table 5.6.1-10: Selected haematological findings in F_0 and F_1 parental animals

Sex	Males				Females			
Dose [ppm]	0	100	350	1000	0	100	350	1000
WBC [10^{12}/L]								
F_0	6.10±1.19	6.46±0.88	6.50±1.21	6.18±1.66	2.91±0.50	3.63±0.62**	2.93±0.72	3.26±0.72
F_1	4.88±1.07	5.13±0.90	4.98±1.01	5.41±1.01	2.80±0.51	3.16±0.68	3.42±0.61	2.82±0.62
HCT [L/L]								
F_0	0.432±0.012	0.433±0.011	0.429±0.016	0.431±0.014	0.465±0.009	0.458±0.018	0.469±0.014	0.449±0.021*
F_1	0.438±0.014	0.445±0.015	0.443±0.013	0.446±0.019	0.486±0.021	0.492±0.011	0.484±0.023	0.482±0.021
RETI [%]								
F_0	1.7±0.2	1.9±0.4	2.2±1.0	1.8±0.3	0.2±0.1	0.3±0.3	0.2±0.1	0.5±0.3*
F_1	1.4±0.2	1.7±0.4*	1.6±0.3*	1.7±0.4**	0.6±0.4	0.6±0.1	0.4±0.2	0.6±0.4
NEUT [%]								
F_0	20.6±13.4	18.8±5.1	18.5±3.6	15.9±8.8*	19.0±5.8	18.4±5.1	19.5±7.3	19.0±7.7
F_1	17.7±4.0	19.5±7.1	19.2±2.8	16.2±3.7	20.0±4.9	18.8±6.2	18.1±6.3	22.3±4.8
LUC [%]								
F_0	0.6±0.3	0.7±0.2	0.6±0.2	0.6±0.2	0.3±0.1	0.5±0.1**	0.5±0.2*	0.6±0.2*
F_1	0.4±0.1	0.4±0.2	0.5±0.1	0.4±0.1	0.3±0.1	0.6±0.2**	0.6±0.2**	0.4±0.2
MONOA [10^{12}/L]								
F_0	0.13±0.06	0.15±0.05	0.15±0.05	0.13±0.05	0.05±0.01	0.06±0.02	0.04±0.01	0.07±0.02**
F_1	0.84±0.18	0.96±0.23	0.94±0.13	0.87±0.23	0.57±0.22	0.61±0.26	0.62±0.25	0.64±0.24
LUCA [10^{12}/L]								
F_0	0.04±0.02	0.05±0.02	0.04±0.01	0.04±0.01	0.01±0.00	0.02±0.01**	0.01±0.01*	0.02±0.01**
F_1	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.01±0.01	0.02±0.01*	0.02±0.01**	0.01±0.01

* $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis and Wilcoxon-test. two sided)

2. Clinical chemistry findings

F_0 parental animals

In males of test group 3 (1000 ppm) cholesterol levels were increased. In all dosed males urea concentrations were higher compared to controls, but these values were within the historical control range (see Table 5.6.1-11). Therefore the higher urea levels were regarded as incidental and not treatment-related.

F_1 parental animals

In males of mid dose group (350 ppm) alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and additionally in males of low dose group (100 ppm) ALT activities were higher compared to controls. In rats of both sexes of mid dose group (350 ppm) and additionally in males of high dose group (1000 ppm) creatinine values were increased. In males of low dose group (100 ppm) total bilirubin levels were higher compared to controls. All mentioned values were not changed dose-dependently and therefore the alterations were regarded as incidental and not treatment-related. In males of high dose group (1000 ppm) total protein, globulin as well as cholesterol concentrations were increased.

Table 5.6.1-11: Selected clinical chemistry findings in F_0 and F_1 parental animals

Sex	Males				Females			
Dose [ppm]	0	100	350	1000	0	100	350	1000
AST [µkat/L]								
F_o	1.32±0.25	1.35±0.24	1.30±0.25	1.27±0.22	1.58±0.35	1.44±0.15	1.49±0.15	1.50±0.15
F_1	1.86±0.32	2.44±0.99	2.96±1.47*	1.98±0.80	1.49±0.29	1.49±0.35	1.57±0.25	1.43±0.28
ALT [µkat/L]								
F_o	0.80±0.16	0.87±0.14	0.78±0.17	0.74±0.16	0.65±0.08	0.64±0.10	0.59±0.06	0.66±0.12
F_1	0.76±0.10	0.98±0.23**	0.98±0.25*	0.84±0.17	0.59±0.08	0.60±0.12	0.59±0.10	0.56±0.09
UREA [mmol/L]								
F_o	5.89±0.48	6.59±0.66*	6.68±1.06*	6.50±0.98*	8.84±0.81	8.51±0.88	8.33±0.95	8.75±1.19
F_1	6.43±0.73	6.63±0.72	6.94±0.69	7.12±0.85	8.03±0.65	8.18±1.14	8.15±0.53	8.38±0.73
CREA [µmol/L]								
F_o	52.9±3.4	53.8±2.5	57.2±4.4	54.4±3.0	53.5±4.4	52.8±2.3	55.9±2.6	51.5±3.6
F_1	51.5±3.6	53.9±3.7	56.8±2.3**	55.5±3.8*	48.6±2.7	49.4±3.7	52.4±1.6*	48.6±3.4
CHOL [mmol/L]								
F_o	1.71±0.23	1.85±0.28	1.71±0.25	**2.03±0.31***	1.90±0.24	2.00±0.38	1.84±0.37	2.20±0.45
F_1	2.04±0.33	2.18±0.30	1.86±0.27	**2.42±0.36***	1.87±0.44	1.87±0.33	1.77±0.42	2.25±0.56
TBIL [µmol/L]								
F_o	1.78±0.35	1.97±0.40	2.12±0.30	2.00±0.30	2.54±0.35	2.39±0.40	2.70±0.44	2.48±0.56
F_1	1.92±0.28	2.27±0.20**	2.11±0.30	1.99±0.27	2.45±0.50	2.35±0.43	2.57±0.63	2.38±0.53
TPROT [g/L]								
F_o	66.02±2.46	66.97±2.11	65.80±2.31	67.52±2.14	66.35±1.47	65.81±2.15	66.08±1.54	67.82±2.25
F_1	63.56±2.55	63.66±2.06	63.97±2.00	**66.16±1.76****	63.06±2.35	63.79±2.73	63.22±2.37	63.95±2.08
GLOB [g/L]								
F_o	27.42±1.73	28.16±1.35	27.44±1.50	28.20±1.70	25.76±0.81	25.74±1.10	26.09±1.28	26.32±1.37
F_1	24.95±1.69	24.92±1.34	25.18±1.11	**26.63±1.16***	20.89±1.25	21.70±1.57	21.48±0.91	21.81±1.52

* $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis and Wilcoxon-test. two sided)

3. Thyroid hormones

Clinical pathological examinations revealed that changes in thyroid hormone levels were evident primarily in the adult F0 and F1 generation parental animals. In addition, higher cholesterol levels were measured in adult male F0 and F1 rats of the high dose group (1000 ppm, see chapter H.2 above), which is a well described phenomenon in hypothyroid rats (e.g., Hoogerbrugge-v.d.Linden et al., 1990; Ness and Gertz, 2004). Hypercholesterolemic males of the F1 generation also had higher levels of cholesterol transport globulins in the blood.

A summary of the thyroid hormone levels is given in the table below:

Table 5.6.1-12: Thyroid hormone levels at different time points in adults and pups (F1 & F2)

Group	F0 adults		F1 pups				F1 adults		F2 pups			
Sex	M	F	M	F	M	F	M	F	M	F	M	F
Study day	106	123	4	4	21	21	110	123	4	4	21	21
Doses [ppm]	T3 [nmol/l]											
Contr. (SD)	0.98 (0.18)	0.98 (0.16)	0.65 (0.11)	0.75 (0.14)	1.68 (0.18)	1.58 (0.25)	0.85 (0.19)	0.88 (0.15)	0.43 (0.13)	0.62 (0.20)	1.80 (0.23)	1.74 (0.22)
100 (SD)	1.07 (0.20)	1.09 (0.12)	0.66 (0.12)	0.67 (0.09)	1.64 (0.20)	1.46 (0.19)	0.97 (0.26)	0.90 (0.18)	0.59* (0.19)	0.56 (0.16)	1.72 (0.18)	1.62 (0.19)
350 (SD)	1.10 (0.23)	1.16 (0.17)	0.68 (0.09)	0.66 (0.15)	1.71 (0.25)	1.58 (0.24)	0.96 (0.18)	0.83 (0.13)	0.65* (0.16)	0.63 (0.11)	1.76 (0.11)	1.58 (0.15)
1000 (SD)	1.19 (0.27)	1.13 (0.15)	0.61 (0.14)	0.62 (0.10)	1.67 (0.24)	1.58 (0.33)	0.89 (0.12)	0.94 (0.17)	0.74* (0.18)	0.69 (0.17)	1.71 (0.12)	1.55 (0.17)
Doses [ppm]	T4 [nmol/l]											
Contr. (SD)	76.01 (13.97)	29.24 (5.16)	23.40 (2.40)	24.31 (2.30)	63.68 (7.13)	60.99 (8.29)	57.72 (13.30)	32.25 (9.94)	17.45 (3.56)	18.75 (3.93)	74.80 (16.66)	77.17 (14.61)
100 (SD)	69.72 (14.16)	33.41 (7.26)	22.47 (4.28)	22.92 (3.92)	73.42 (15.48)	66.16 (11.83)	53.12 (9.59)	26.18 (5.12)	18.28 (4.24)	16.73 (3.59)	69.86 (12.70)	76.80 (15.82)
350 (SD)	51.67** (10.87)	30.50 (6.62)	22.08 (1.95)	22.63 (4.09)	71.04 (16.70)	64.48 (12.69)	54.03 (6.32)	22.43** (5.43)	18.99 (5.66)	19.63 (4.97)	81.42 (14 39)	76.63 (9.52)
1000 (SD)	58.04** (10.00)	22.47** (4.07)	20.23 (3.69)	19.46** (2.49)	60.40 (12.30)	58.05 (8.15)	46.00** (5.48)	25.96 (5.85)	16.64 (3.43)	17.33 (3.60)	67.85 (5.69)	67.79 (6.16)
Doses [ppm]	TSH [µg/l]											
Contr. (SD)	7.29 (1.57)	5.01 (0.55)	5.35 (0.98)	5.32 (0.84)	5.78 (0.80)	4.81 (0.72)	4.52 (1.19)	3.89 (0.95)	2.68 (0.67)	2.64 (0.86)	1.71 (0.78)	1.43 (0.49)
100 (SD)	8.22 (2.57)	4.91 (1.29)	6.01 (1.02)	5.97 (0.76)	4.91 (1.52)	5.60 (1.06)	4.96 (1.43)	3.98 (1.13)	2.73 (0.72)	2.49 (0.61)	1.66 (0.62)	1.28 (0.35)
350 (SD)	8.43 (2.83)	5.35 (1.19)	5.88 (0.95)	5.12 (0.70)	5.13 (1.13)	5.08 (1.42)	6.82** (1.74)	3.99 (1.10)	3.09 (1.05)	2.76 (0.63)	1.75 (0.63)	1.49 (0.68)
1000 (SD)	12.30** (4.39)	5.29 (0.86)	6.32 (0.84)	6.48** (0.98)	5.47 (0.99)	6.16 (1.75)	9.68** (5.12)	4.64 (0.98)	2.99 (0.90)	2.74 (0.70)	2.17 (0.80)	1.99 (0.73)

*p≤0.05, **p≤0.01

F_0 parental animals

In males at ≥ 350 ppm T_4 levels were statistically significantly decreased (by -32 and -24%) and additionally, in males of the high dose group TSH concentrations were increased (170%). In females of test group 3 (1000 ppm) only T_4 levels were statistically significantly decreased by -23%.

F_1 generation (pups PND 4)

On PND 4, in female pups of high dose group (1000 ppm) T_4 levels were statistically significantly decreased (by -20%) and TSH levels were slightly statistically significantly increased (120%).

F_1 generation (pups PND 21)

No treatment-related changes of thyroid hormone levels were measured.

F_1 parental animals
At ≥ 350 ppm dose groups males showed statistically significant increases in TSH levels (1.5 and 2-fold) and, while the T4 levels were decreased only at the 1000 ppm dose group (by -20%)
The isolated finding of decreased T4 levels in females of mid dose group (350 ppm) is considered to be incidental, as no change was observed at the next higher dose level, and also because no increased TSH levels were seen at none of the administered doses

F_2 generation (pups PND 4)
No treatment-related changes of thyroid hormone levels were measured.
In males of all dose groups, T_3 levels were higher compared to controls. The reason for this increase were low levels in the control group compared to preliminary individual historical control values of two former studies (T_3 range of individual values: 0.38-1.37 nmol/L, in control group (males group 10) four individual values were below this range, whereas all T_3 values of dosed rats were within the historical control range). Further from a plausibility point of view, decreased T3 levels (in conjunction to the T4 levels) would have been expected. Therefore, the higher T_3 values in dosed male F2 generation pups on PND 4 were regarded as incidental and not treatment-related.

F_2 generation (pups PND 21)
No treatment-related changes of thyroid hormone levels were measured.

Conclusion on thyroid hormone changes:
No treatment related changes were seen in T3 levels.
The parental males were generally more affected than females: At 1000 ppm decreased T4 levels was seen in adult F0 and F1 male parental animals. They also showed increased TSH levels at the top doses of both generations. At 350 ppm the findings were less consistent over the two generations. F0 males but not F1 males showed decreased T4 levels, and F1 males showed increased TSH levels, but not the F0 males.
The only changed parameter among three hormone level measurements in parental females of the 1000 ppm dose group was a slightly (23%) decrease of T4 in the F0 generation only. No changes in the F1 generation was seen as well as TSH was not affected in parental females at none of the doses or generations. Thus, this isolated finding is of unclear biological relevance.
The F1 offspring animals were similarly weakly affected with slightly increased TSH levels (only 120%) and a 20% decrease in T4 levels at the 1000 ppm dose group in one sex at PND4 timepoint only. No changes were seen in the other generation or dose groups. Further at none of the timepoints, the thyroid of the offspring animals (PND 4 and 21) showed pathological changes.

I. PARENTAL TERMINAL INVESTIGATIONS

1. Organ weights
Organ weight determination in parental animals revealed a number of significant changes of absolute and/or relative organ weights.

F_0 parental animals

Absolute: The statistically significant terminal body weight decrease in males of the high dose group (1000 ppm) and females of mid and high dose group (350 and 1000 ppm) and increased thyroid weights in males of high dose group (1000 ppm) were considered to be treatment-related. All other statistically significant weight changes showed neither a clear dose-response relationship nor a histopathological correlate and were therefore regarded as not treatment related. All other mean absolute weight parameters did not show relevant differences when compared to the control group and were considered to be within the normal range.

Relative: The increased thyroid weights in animals of high dose group (1000 ppm) were considered to be treatment-related. They correlated histologically with thyroid follicular cell hypertrophy/hyperplasia in male animals but a histological correlate in females could not be detected. The decrease in thyroid weights of female low dose group (100 ppm) was regarded as incidental as no dose response relationship was present. A treatment related effect on liver weights in animals of both sexes of high dose group (1000 ppm) cannot be ruled out even if a histological correlate was not detected. All other statistically significant organ weight changes including the liver weight increase in male mid dose group (350 ppm) animals showed either no clear dose-response relationship or were likely due to body weight changes (e.g. brain, reproductive organs); a histopathological correlate was not detected in any of these organs. Therefore, they were regarded as not treatment-related.

All other mean relative weight parameters of male and female rats showed no statistically significant differences when compared to control group and were considered to be within the normal range.

F_1 parental animals

Absolute: The statistically significant terminal body weight decrease in males and females of the mid and high dose group (350 and 1000 ppm) and increased thyroid weights in males and females of high dose group (1000 ppm) were considered to be treatment-related. Weight changes in the pituitary gland of male animals and in the brain of female animals did not show a clear dose-response relationship or a histopathological correlate and were therefore regarded as not treatment-related. Weight changes in the adrenal gland and brain in males and kidney in females were attributed to body weight reduction and did not show a histological correlate. Therefore, they were also regarded as not treatment-related. All other mean absolute weight parameters did not show relevant differences when compared to the control group and were considered to be within the normal range.

Relative: The increased thyroid weights in animals of high dose group (1000 ppm) were considered to be treatment-related and correlated histologically with thyroid follicular cell hypertrophy/hyperplasia in male and female animals while a histological correlate in the liver of both sexes was not detected. A treatment related effect on liver weights of high dose group (1000 ppm) animals cannot be ruled out. All other statistically significant organ weight changes including the increased liver weights in male mid dose group (350 ppm) animals showed either no clear dose-response relationship or were likely due to body weight changes (brain, reproductive organs); a histopathological correlate was not detected in any of these organs. Therefore, they were regarded as not treatment-related. All other mean relative weight parameters of male and female rats showed no statistically significant differences when compared to control group and were considered to be within the normal range.

Table 5.6.1-13: Organ weights of F_0 and F_1 male parental animals

Generation	Dose [ppm]	F_0 Males Absolute weight	Δ%	F_0 Males Relative weight [% of b.w.]	Δ%	F_1 Males Absolute weight [mg]	Δ%	F_1 Males Relative weight [% of b.w.]	Δ%
Terminal weight [g]	0	413.54				422.528			
	100	416.06	(1)			415.744	(-2)		
	350	395.132	(-4)			**396.696***	**(-6)**		
	1000	**380.248****	**(-8)**			**386.776****	**(-8)**		
Adrenal gland [mg]	0	66.36		0.016		70.96		0.017	
	100	65.32	(-2)	0.016	(-2)	66.04	(-7)	0.016	(-5)
	350	64.56	(-3)	0.016	(2)	63.8*	(-10)	0.016+	(-4)
	1000	62.8	(-5)	0.017	(3)	63.44**	(-11)	0.016	(-2)
Brain [g]	0	2.082		0.506		2.11		0.502	
	100	2.098	(1)	0.507	(0)	2.107	(0)	0.51	(2)
	350	2.092	(0)	0.533	(5)	2.1	(0)	0.535*	(7)
	1000	2.066	(1)	0.547**	(8)	2.048*	(-3)	0.531*	(6)
Cauda epididymis [g]	0	0.48		0.117		0.454		0.108	
	100	0.47	(-2)	0.114	(-3)	0.46	(1)	0.111	(3)
	350	0.47	(-2)	0.119	(2)	0.468	(3)	0.12*	(11)
	1000	0.468	(-3)	0.123	(6)	0.454	(0)	0.118*	(9)
Epididymides [g]	0	1.184		0.288		1.21		0.288	
	100	1.174	(-1)	0.284	(-1)	1.204	(-1)	0.291	(1)
	350	1.163	(-2)	0.296	(3)	1.19	(-2)	0.303	(6)
	1000	1.139	(-4)	0.301	(5)	1.15	(-5)	0.298	(4)
Kidneys [g]	0	2.498		0.605		2.571		0.61	
	100	2.534	(1)	0.611	(1)	2.526	(-2)	0.608	(0)
	350	2.466	(-1)	0.624	(3)	2.468	(-4)	0.625	(2)
	1000	2.442	(-2)	0.643**	(6)	2.451	(-5)	0.634	(4)
Liver [g]	0	9.581		2.316		10.205		2.409	
	100	9.979	(4)	2.394	(3)	10.177	(0)	2.445	(2)
	350	9.639	(1)	2.437*	(5)	10.194	(0)	2.579*	(7)
	1000	9.957	(4)	**2.614***	**(13)**	10.762	(5)	**2.777***	**(15)**
HCD [liver weight]***		**7.792 – 10.013 g**		**2.125 – 2.507%**		**8.602 – 10.205 g**		**2.199 – 2.494%**	
Pituitary gland [mg]	0	10.72		0.003		12.0		0.003	
	100	10.68	(0)	0.003	(-2)	11.08*	(-8)	0.003	(-6)
	350	10.6	(-1)	0.003	(3)	10.72**	(-11)	0.003	(-4)
	1000	11.12	(4)	0.003*	(12)	11.64	(-3)	0.003	(6)
Prostate [g]	0	1.112		0.271		0.976		0.231	
	100	1.122	(1)	0.269	(-1)	1.054	(8)	0.254*	(10)
	350	1.133	(2)	0.288	(6)	1.08	(11)	0.276**	(19)
	1000	1.084	(-2)	0.285	(5)	1.074	(10)	0.279**	(20)
Seminal vesicle [g]	0	1.304		0.317		1.148		0.272	
	100	1.247	(-4)	0.301	(-5)	1.112	(-3)	0.269	(-1)
	350	1.212	(-7)	0.309	(-3)	1.116	(-3)	0.285	(5)
	1000	1.202	(-8)	0.317	(0)	1.089	(-5)	0.282	(4)
Spleen [g]	0	0.652		0.158		0.65		0.154	
	100	0.686	(5)	0.165	(4)	0.662	(2)	0.159	(3)
	350	0.657	(1)	0.167	(6)	0.647	(0)	0.163	(6)
	1000	0.614	(-6)	0.162	(2)	0.637	(-2)	0.165	(7)
Testes [g]	0	3.703		0.9		3.872		0.92	
	100	3.694	(0)	0.892	(-1)	3.886	(0)	0.939	(2)
	350	3.721	(0)	0.946	(5)	3.838	(-1)	0.976	(6)
	1000	3.655	(-1)	0.966*	(7)	3.808	(-2)	0.988	(7)
Thyroid glands [mg]	0	22.92		0.006		27.4		0.007	
	100	23.36	(2)	0.006	(2)	26.08	(-5)	0.006	(-3)
	350	21.68	(-5)	0.005	(-1)	26.52	(-3)	0.007	(3)
	1000	**27.52****	**(20)**	**0.007****	**(31)**	**31.2****	**(14)**	**0.008****	**(24)**
HCD [thyroid gland weight]****		**17.7 - 29.7 mg**		**0.005 – 0.008%**		**21.36 – 28.92 mg**		**0.007 – 0.009%**	

Table 5.6.1-13: Organ weights of F_0 and F_1 male parental animals

Generation		F0 Males				F1 Males			
	Dose [ppm]	Absolute weight	Δ%	Relative weight [% of b.w.]	Δ%	Absolute weight [mg]	Δ%	Relative weight [% of b.w.]	Δ%

* p ≤ 0.05. ** p ≤ 0.01 (Kruskal-Wallis H and Wilcoxon-test (two-sided))
*** historical control were collected in a period of 10 years before and after the study (2005 – 2015); No. of studies: F0 males: 40, F: 40, F1 M& F: 17 (see DocID 2016/1345367)
****historical control were collected in a period of 10 years before and after the study (2005 – 2015); No. of studies: F0 males: 35, F: 35, F1 M& F: 16 (see DocID 2016/1295144)

Table 5.6.1-14: Organ weights of F_0 and F_1 parental females

Generation			F0 Females				F1 Females			
		Dose [mg/kg]	Absolute weight	Δ%	Relative weight [% of b.w.]	Δ%	Absolute weight [mg]	Δ%	Relative weight [% of b.w.]	Δ%
Terminal weight	[g]	0	233.996				237.48			
		100	228.208	(-2)			235.284	(-1)		
		350	**224.288****	**(-4)**			**226.2***	**(-5)**		
		1000	**208.052****	**(-11)**			**205.2****	**(-14)**		
Adrenal glands	[mg]	0	76.08		0.033		78.68		0.033	
		100	75.32	(-1)	0.033	(2)	76.32	(-3)	0.032	(-2)
		350	72.8	(-4)	0.033	(0)	74.52	(-5)	0.033	(-1)
		1000	73.64	(-3)	0.035	(9)	70.84	(-10)	0.035	(4)
Brain	[g]	0	1.909		0.817		1.918		0.811	
		100	1.959	(3)	0.861**	(5)	1.965*	(2)	0.838	(3)
		350	1.928	(1)	0.862**	(6)	1.928	(1)	0.854**	(5)
		1000	1.913	(0)	0.922**	(13)	1.898	(-1)	0.926**	(14)
Kidneys	[g]	0	1.866		0.797		1.847		0.781	
		100	1.814	(3)	0.797	(0)	1.822	(-1)	0.776	(-1)
		350	1.867	(0)	0.832**	(4)	1.752**	(-5)	0.776	(-1)
		1000	1.945	(4)	0.941**	(18)	1.692**	(-8)	0.825*	(6)
Liver	[g]	0	7.295		3.113		7.277		3.073	
		100	6.802	(-7)	2.987	(-4)	7.008	(-4)	2.985	(-3)
		350	6.828	(-6)	3.049	(-2)	6.803	(-7)	3.016	(-2)
		1000	7.326	(0)	**3.53****	**(13)**	6.854	(-6)	**3.346***	**(9)**
HCD [liver weight]***			**5.552 – 7.711 g**		**2.436 – 3.28%**		**5.776 – 7.384 g**		**2.468 – 3.148%**	
Ovaries	[mg]	0	106.68		0.046		113.24		0.048	
		100	105.08	(-1)	0.046	(1)	113.28	(0)	0.048	(1)
		350	104.56	(-2)	0.047	(3)	112.4	(-1)	0.05	(4)
		1000	111.64	(5)	0.054**	(18)	108.32	(-4)	0.053*	(10)
Pituitary gland	[mg]	0	13.16		0.006		12.76		0.005	
		100	13.12	(0)	0.006	(2)	13.92	(9)	0.006**	(10)
		350	12.56	(-5)	0.006	(0)	13.04	(2)	0.006*	(7)
		1000	13.24	(1)	0.006**	(13)	12.56	(-2)	0.006**	(13)
Spleen	[g]	0	0.489		0.209		0.507		0.213	
		100	0.504	(3)	0.221	(6)	0.512	(1)	0.217	(6.8)
		350	0.472	(-3)	0.211	(1)	0.513	(1)	0.227	(1.5)
		1000	0.504	(3)	0.242**	(16)	0.477	(-6)	0.232**	(7.3)
Thyroid glands	[mg]	0	15.56		0.007		18.458		0.008	
		100	13.2**	(-15)	0.006*	(-13)	17.8	(2.9)	0.008	(-3)
		350	14.417	(-7)	0.006	(-3)	18.12	(5.8)	0.008	(3)
		1000	17.8	(14)	**0.009****	**(30)**	**22.4****	**(21)**	**0.011****	**(40)**
HCD [thyroid gland weight]****			14.7 – 21.957 mg		0.006 – 0.009%		15.2 – 22.72 mg		0.007 – 0.009%	
Uterus	[g]	0	0.583		0.248		0.649		0.272	
		100	0.632	(9)	0.278*	(12)	0.702	(8)	0.298	(10)
		350	0.735**	(26)	0.327**	(32)	0.634	(-2)	0.28	(3)
		1000	0.615	(5)	0.295**	(19)	0.679	(5)	0.33	(21)

* p ≤ 0.05. ** p ≤ 0.01 (Kruskal-Wallis H and Wilcoxon-test (two-sided))
*** historical control were collected in a period of 10 years before and after the study (2005 – 2015); No. of studies: F0 males: 40, F: 40, F1 M& F: 17 (see DocID 2016/1345367)
****historical control were collected in a period of 10 years before and after the study (2005 – 2015); No. of studies: F0 M: 35, F: 35, F1 M& F: 16 (see DocID 2016/1295144)

2. Macroscopic lesions

All gross lesions observed in F0 and F1 parental animals occurred singularly. They are considered to be spontaneous lesions in origin and are not related to treatment.

The mating pairs with reduced fertility, did not show relevant gross lesions.

3. Histopathology

Treatment related findings (follicular hypertrophy/hyperplasia) were noted in F0 parental males only and in male and female F1 parental animals with incidence and grading according to the Table 5.6.1-15 below.
In addition, one unilateral thyroid follicular cell adenoma was observed in a male animal of high dose group (1000 ppm). As the adenoma was thought to have arisen in the hypertrophic/hyperplastic epithelium, it was considered to be probably treatment-related.

Table 5.6.1-15: Incidence of selected histopathological lesions in F0 and F1 parental rats

	Male animals				Female animals			
Dose [ppm]	0	100	350	1000	0	100	350	1000
F0 Generation								
Follicular hypertrophy/hyperplasia	0	6	15	24	0	0	0	0
Grade 1		1	9	5				
Grade 2		3	6	9				
Grade 3		2		10				
Follicular cell adenoma	0	0	0	1	0	0	0	0
HCD [Incidence of follicular hypertrophy/ hyperplasia]*	0-32% (mean: 7.3%)				0-4% (mean: 1.3%)			
F1 Generation								
Follicular hypertrophy/hyperplasia	2	4	10	22	1	0	8	8
Grade 1	1	3	9	6	1	0	6	7
Grade 2	1	1	1	9			2	1
Grade 3				7				
Follicular cell adenoma	0	0	0	1	0	0	0	0
HCD [Incidence of follicular hypertrophy/ hyperplasia]*	0-16% (mean: 5.8%)				0-8% (mean: 1.7%)			

*historical control were collected in a period of 10 years before and after the study (2005 – 2015); No. of studies: F0 6, F: 35, F1: 5 (see DocID 2016/1295144)

It is worth to mention, that the actual substance intakes of the F1 parental generation is higher compared to the F0 parental generation. While the F0 females of the top dose have measured substance intakes of 73.3 – 112.0 (mean 92.2) mg/kg bw during premating the F1 females were exposed to 73.4 – 164.5 (mean: 102.3) mg/kg bw during the same period.

Follicular hypertrophy/hyperplasia was observed in males of the F0 and F1 generation even in the lowest dose group, however within the historical control range at the 100 ppm group in males. It is however known, that the sensitivity of the used rat strain towards developing thyroid follicular hypertrophy/hyperplasia is high, which is also indicated by the evaluation of the historical control data. The subsequently collected historical control data containing 5 (6) studies conducted during the time period between 2005 and 2015 showed, that up to 32% of the F0 parental males and up to 16% of the F1 parental males had histopathological findings in the thyroid. Based on the fact, that the findings in males at 350 and 1000 ppm were above historical controls and the observed dose response, the NOAEL of the study has been set at <100 ppm for the males. As the thyroid histopathological findings were increased in females at ≥ 350 ppm, the NOAEL for females is set to 100 ppm. A benchmark dose with 5% and 10% increase of follicular hypertrophy/hyperplasia in thyroid gland in comparison to the control group and its 95% lower confidence limit in males was calculated (see Table 5.6.1-16).

Table 5.6.1-16: Benchmark Dose Computation of follicular hypertrophy/hyperplasia in F0 and F1 males

	F0 males		F1 male	
		Grade ≥ 2		Grade ≥ 2
Specified effect	0.05			
Confidence level	0.95			
Risk type	Extra risk			
BMD	20.4536	43.6538	59.799	365.117
BMDL	13.7762	29.7171	25.5632	138.904
BMDU	38.9527	135.274	178.908	521.236
Specified effect	0.1			
Confidence level	0.95			
Risk type	Extra risk			
BMD	41.7756	89.082	113.839	523.288
BMDL	28.2974	61.0421	52.5088	280.837
BMDU	74.7031	222.357	256.412	747.039

Taken together, the values for BMDL and BMDU are a 90% two-sided confidence interval for the BMD. It is suggested to use the $BMDL_{10}$ for males for thyroid follicular hypertrophy/hypertrophy (for grades ≥ 2, because grade 1 is also occurring in untreated animals) as the relevant endpoint. This value is 61 ppm for the F0 males and 281 ppm for the F1 males. The value of 61 ppm is corresponding to a daily dose of 5.5 mg/kg bw.

The mating pairs with reduced fertility did not show relevant histopathological lesions.

4. Differential ovarian follicle count

The results of the differential ovarian follicle count (DOFC) – comprising the numbers of primordial and growing follicles, as well as the combined incidence of primordial plus growing follicles – did not reveal significant deviations between controls and the high dose group.

Table 5.6.1-17: Ovarian follicle count in F_1 maternal females

	Absolute number			Mean number		
Group	Primordial	Growing	Primordial + growing	Primordial	Growing	Primordial + growing
Control	6041	536	6577	241.64	21.44	263.08
1000 ppm	5663	583	6246	226.52	23.32	249.84

III CONCLUSION:

Thus, under the conditions of the present 2-generation reproduction toxicity study the **NOAEL** (no observed adverse effect level) for **general, systemic toxicity** is **100 ppm (approx. 9 mg/kg bw**) for the F0 and F1 parental **female rats**, based on decreased food consumption and body weight/body weight gain as well as thyroid morphology and changes in thyroid hormone levels (at 1000 ppm dose group only), observed at the LOAEL (Lowest Observed Adverse Effect Level) of 350 ppm in the F0 and F1 parental animals. The only changed parameter among three hormone level measurements in parental females of the 1000 ppm dose group was a slightly (-23%) decrease of T4 in the F0 generation (not in the F1) only. For the F0 and F1 parental **male rats** the **NOAEL** is **< 100 ppm** because, in addition to the described mid- and high-dose effects, changed thyroid morphology is still present at this dose. Therefore, a benchmark dose was calculated for F0 and F1 parental male rats which results in **$BMDL_{10}$ = 61 ppm (corresponding to a dose of 5.5 mg/kg bw).**
The **NOAEL for fertility and reproductive performance** for the F0 and F1 parental rats is **1000 ppm (92 mg/kg bw)**, the highest dose tested.
The **NOAEL** for **developmental toxicity** in the F1 and F2 progeny is **1000 ppm (approx. 92 mg/kg bw)**, the highest dose tested. The NOEL is 350 ppm for the F1 and F2 progeny based on slightly decreased pre-weaning pup body weights/pup weight gain, as well as slightly changed thyroid hormone levels in female F1 pups on PND 4, observed at the LOEL of 1000 ppm. These effects did not affect any other developmental parameters such as postnatal survival as well as post weaning development of the offspring until sexual maturity. Thus, they are not regarded as independent adverse or biologically relevant developmental toxicity.
Developmental effects do not occur in the absence of parental effects.

CA5.6.2 Developmental toxicity studies

Report: *CA 5.6.2/1*
[redacted] 1988a
Report on the study of the prenatal toxicity of Metiram-premix 95% in rabbits after oral administration (gavage)
1988/0154
Guidelines: *EPA 83-3, OECD 414*
GLP: *yes*
(certified by Ministerium fuer Umwelt und Gesundheit, Postfach 3160, 6500 Mainz)

Report: *CA 5.6.2/2*
[redacted] 1988a
Report on the study of the prenatal toxicity of Metiram-premix 95% in rabbits after oral administration (gavage) - Supplement
1988/0262
Guidelines: *EPA 83-3, OECD 414*
GLP: *yes*
(certified by Ministerium fuer Umwelt und Gesundheit, Postfach 3160, 6500 Mainz)

Deviations to the current valid OECD 414 guideline (2001):

- Due to current guideline (2001) the exposure period should cover the gestation phase from implantation to one day prior to the day of scheduled kill. In the current study metiram was only administered during the period of organogenesis (for rabbits: days 6 - 18). This dosing regime was acceptable according to the old OECD TG 414 (1981).
- According to current OECD TG 414 (2001) each dose group should contain at least 16 animals with implantation sites, whereas a number of 12 animals per group was sufficient due to previous guideline (1981).
- Due to current guideline (2001) heads of one-half of the foetuses should be examined for soft tissue alterations: in the present study only the head of those foetuses that showed severe abnormal findings in the gross-pathological examinations was evaluated for that issue
- Due to current guideline (2001) the maternal mortality should not exceed ca. 10 %: in the present study the maternal mortality in the 40 mg/kg bw/day dose group is 13 %, in the 120 mg/kg bw/day dose group is 47 %

The study is, in effect at the time of performance, conducted in accordance with OECD 414 (1981), under GLP conditions. Although the number of treated dams/dose group was lower, as now required in the current OECD TG, the study is considered to be still acceptable, as there was no indication for increased incidences of malformation or variations in the foetuses seen, even at a substantially maternally toxic dose. The deviating administration period is also considered acceptable, based on the fact, that the metiram metabolite of concern (ETU) showed no teratogenicity or embryotoxicity in rabbit when dosed during organogenesis or over the whole gestation phase. External malformations in the heads of the rabbit pups (e.g. hydrocephaly) would have been recorded during external examination of the litters, thus the above described deviation is also considered acceptable. Further a newly conducted rabbit teratogenicity study conducted with ETU in rabbits (according to the new OECD TG 414 (2001) did not give evidence for a teratogenic response in rabbits. The rabbit is not a target species for teratogenicity of metabolites of metiram (e.g. ETU). Also in the interest of animal welfare no new study was conducted.

Executive Summary

Groups of 15 female Himalayan rabbits received metiram (Batch: 765, Purity: 97.9%, <0.2% ETU) in 0.5% w/w aqueous solution of sodium carboxymethylcellulose (CMC), administered by gavage at 0, 10, 40, and 120 mg/kg bw/day on days 7 to 19 post insemination (p.i.). Controls received the vehicle alone. Animals were observed for mortality and clinical signs. Individual body weights and food consumption data were recorded. Termination and necropsy on day 29 p.i. was followed by processing and detailed visceral and skeletal examination of foetuses.

Significantly reduced food consumption was observed in the 40 and 120 mg/kg bw/day dose groups during treatment and first days of post-treatment period. Severe body weight loss was observed in dams of the high dose group during treatment and post-treatment period. Slight body weight loss was observed in dams of the 40 mg/kg bw/day dose group during treatment.
One animal of the high dose group was found dead. Two and eight animals of the 40 and 120 mg/kg bw/day dose group aborted, and showed a higher incidence of reduced or no defecation. No substance-related findings were observed in the 10 mg/kg bw/day dose group.

Thus, under the conditions of this study, the test substance caused severe signs of maternal toxicity at 120 mg/kg bw/day and was still clearly toxic to the does at 40 mg/kg bw/day. There were only discrete signs of embryo-/fetotoxicity at the highest dose level, but no indications for any teratogenic effects up to the highest dose level. The no observable effect level on the maternal organism is 10 and on the fetal organism 40 mg/kg bw/day.

(DocID 1988/0154)

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** Metiram-Premix 95%
   Description: solid, light yellow
   Lot/Batch #: 765
   Purity: 97.9% (ETU content ≤0.2%)
   Stability of test compound: stability over study period was guaranteed by the sponsor

2. **Vehicle:** 0.5% w/w aqueous solution of sodium carboxymethyl cellulose (CMC)

3. **Test animals:**
   Species: Himalayan rabbits
   Strain: Chbb:HM (outbred strain)
   Sex: Female
   Age (Day 0): 34-44 weeks
   Weight (Day 0): 2199 - 3135 g
   Source: [redacted]
   Acclimation period: at least 7 days
   Time of dosing: Days 7 to 19 p.i.
   Diet: Kliba maintenance diet, type 23-341-4 for rabbits, Klingentalmuehle AG, Kaiseraugst, Switzerland, ad libitum
   Water: tap water, ad libitum
   Housing: single housing in type K300/8 stainless steel wire mesh cages (Becker&Co, Castrop-Rauxel, Germany)
   Environmental conditions:
   Temperature: 20 - 24°C
   Humidity: 30 - 70%
   Air changes: fully air-conditioned
   Photo period: 12 h light / 12 h dark

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 09-Jun-1987 - 22-July-1987

2.´ Analyses:
Analytical determination of the stability of the test substance itself and its further characteristics (purity, homogeneity) were carried out before the beginning and after the end of the study.
Analytical verifications of the stability of the test substance in water at room temperature and at 4°C were carried out with other batches before the beginning of the study. Furthermore, samples of each preparation of the test substance in doubly distilled water with 0.5% carboxymethyl cellulose analysed twice during the study period for verification of the concentrations. Samples of the low and high concentration were additionally checked for its homogeneous distribution.

3. Preliminary study:
In the preliminary study, the test substance was administered to pregnant Himalayan rabbits (3/group) by gavage daily for a period of 13 days (days 7 - 19 p.i.) in doses of 50, 100 and 200 mg/kg bw/day. For comparison, a group of sham treated animals was used as a control.

4. Animal assignment and treatment:
After the acclimatization period, the does were fertilized by means of artificial insemination. The day of insemination was designated Day 0 (beginning of the study) and the following day as day 1 post insemination (p.i.). The animals were assigned to the different test groups (15 animals per group) according to a randomization plan and on the basis of their body weights.

The animals were dosed once daily from day 7 p.i. until day 15 p.i. by gavage with 10, 40, and 120 mg/kg bw/day metiram suspended in 0.5% aqueous CMC at a dose volume of 10 mL/kg bw.

5. Test substance preparation and analysis:
The test substance suspensions were freshly prepared each day just before treatment of the animals began. For the preparation of the suspensions, an appropriate amount of the test substance was weighed and subsequently suspended in doubly distilled water with 0.5% carboxymethyl cellulose. The homogeneity of the suspensions was maintained, because a magnetic stirrer was used to keep the suspensions homogeneous during treatment of the animals.

6. Statistics:
Examinations of dams and fetuses
The Dunnett Test was used for statistical evaluation of food consumption, body weight, body weight change, corrected body weight gain (net maternal body weight change), weight of the uterus before it was opened, weight of fetuses, weight of placentae, corpora lutea, implantations, pre-and postimplantation loss, resorptions and live fetuses.
The Fisher Exact Test was used for statistical evaluation of conception rate, mortality (of the dams) and all fetal findings.
Significances resulting from these tests have been indicated in the tables (* for $p < 0.05$, ** for $p < 0.01$).

C. METHODS

1. Observations:

The animals were examined for clinical symptoms at least once a day, or more often when clinical signs of toxicity were elicited. A check was made twice a day on working days or once a day (Saturday, Sunday or on holidays).

2. Body weight:

All animals were weighed on days 0, 2, 4, 7, 9, 11, 14, 16, 19, 21, 23, 25, 28 and 29 p.i.. The body weight change of the animals was calculated from these results.
Furthermore, after terminal sacrifice, the corrected body weight gain was calculated (body weight on day 29 p.i. minus body weight on day 7 p.i. minus weight of the uterus before it was opened).

3. Food and water consumption:

The consumption of food was determined daily during the entire study period.

4. Sacrifice and pathology:

On Day 29 p.i. all animals were sacrificed and the fetuses were delivered by cesarean section.

Dams

On day 29 p.i. the dams were sacrificed by intravenous injection of a pentobarbital and the fetuses removed by cesarean section. Moribund dams and dams showing signs of abortion or premature delivery were sacrificed by intravenous injection of a pentobarbital, too. These animals and dams which died intercurrently as well as the contents of uterus from these animals were investigated, if possible in the same way as at terminal sacrifice (exception: uterus weight).
After the dams had been sacrificed, they were necropsied and assessed by gross pathology. The uterus and the ovaries were removed and the following data were recorded:

- Weight of uterus before it was opened
- Number of corpora lutea
- Number and distribution of implantation sites classified as:
    - live fetuses
    - dead implantations:
        a) early resorptions (only decidual or placental tissues visible or according to SALWESKI from uteri from apparently non-pregnant animals and the empty uterus horn in the case of single-horn pregnancy)
        b) late resorptions (embryonic or fetal tissue in addition to placental tissue visible)
        c) dead fetuses (hypoxemic fetuses which did not breathe spontaneously after the uterus had been opened)

Furthermore, calculations of conception rate and pre- and post-implantation losses were carried out:
Conception rate: (No. of pregnant animals/ No. of fertilized animals) * 100
Pre-implantation loss: (No. of corpora lutea – No. of implantations)/ No. of corpora lutea * 100
Post-implantation loss: (No. of implantations – No. of live young)/ No. of implantations * 100

Fetuses
At necropsy each fetus was weighed, sexed and examined macroscopically for any external findings. Furthermore, the viability of the fetuses and the condition of the fetal membranes and fluids were examined. Individual placental weights were recorded.

Soft tissue examination of the fetuses
After the fetuses had been sacrificed by CO_2, the abdomen and thorax were opened in order to be able to examine the organs in situ before they were removed. Sections were made of the heart and the kidneys in order to be able to assess the opened organs. If heads of fetuses revealed severe findings (e.g. anophthalmia, microphthalmia, hydrocephalus, or cleft palate), the heads of these fetuses were severed from the trunk, fixed in BOUIN's solution and later processed and assessed according to the method of WILSON. About 10 transversal sections were prepared per head.

Skeletal examination of the fetuses
After the soft tissue examination all fetuses were placed in ethyl alcohol for staining of the skeletons (with the possible exception of the skulls according to a modified method of DAWSON. The stained skeletons were placed on an illuminated plate and examined, evaluated and assessed.

Evaluation criteria for assessing skeletons and organs of the fetuses
There are differing opinions on the classification and assessment of changes in fetuses. MUNTEFERING differentiates between malformations, which he defines as "severe formal defects outside the range of variation of the species", and macroscopic/microscopic anomalies, which he defines as "slighter formal deviations from normal". If these changes occur, they are regarded as a teratogenic effect. However, transition from variation to malformation is fluid, and the term "slighter formal deviation" is not defined. NEUBERT, on the other hand, tends to describe a change as a morphological abnormality, anomaly or functional anomaly instead of malformation since malformation refers primarily to gross-pathological changes. In English-speaking countries the terms defect, abnormality, malformation, major or minor defect or variant are used, without a clear-cut definition being given.
In the present investigations the following terms (definitions) were used for describing a change:

- Retardations: Delays in development compared with the norm at the time of the examination (cesarean section) were considered to be retardations.
- Variations: Changes which occur regularly were regarded as variations.
- Malformations: Changes which could be recorded and had progressed beyond the degree of retardations and variations were classified as malformations.
- Unclassified findings: Changes, which could not be classified as malformations, variations or retardations (e.g. focal liver necrosis in fetuses).

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

The stability of the test substance was proven. The content of active ingredient was 97.9% before the beginning and 94.9% after the end of the study. The ETU content was <0.2%.

The stability of the test substance suspensions over a period of 24 hours (at room temperature) or 3 days (at 4°C) could be demonstrated. The results of the analyses of the suspensions of test substance confirmed in general the correctness of the prepared concentrations and its homogeneous distribution.

B. PRELIMINARY STUDY

The following findings were obtained and assessed as to be possibly substance-related:

200 mg/kg group (test group 3)

- reduced food consumption of the dams during the treatment and post-treatment period
- negative body weight change (treatment and post-treatment periods) and decreased corrected body weight gain
- one doe aborted on day 27 p.i., in another doe all implants were resorbed and the third animal of this group was not pregnant

100 mg/kg group (test group 2)

- reduced food consumption of the does during the treatment period
- reduced body weight change during the treatment period one doe aborted on day 22 p.i.

50 mg/kg group (test group 1)

- one doe aborted on day 26 p.i.

No further clearly adverse effects were recorded for the dams (however, one control animal was not pregnant either) or the fetuses (e.g. fetal weights, fetal external observations).

C. OBSERVATIONS

Only pregnant dams were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant dams with scheduled sacrifice (day 29 p.i.) were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.

In this study the following females were partly or totally excluded from the above mentioned calculations:

	Not pregnant	Aborted	Died intercurrently
Test group 0	**1**	**-**	**-**
Test group 1	**-**	**-**	**-**
Test group 2	**1**	**2**	**-**
Test group 3	**-**	**8**	**1**

1. Clinical examinations

Eight does of test group 3 (120 mg/kg body weight/day) and 2 does of test group 2 (40 mg/kg body weight/day) aborted during the post-treatment period (days 21 - 28 p.i.), which is assessed as a severe substance-related effect and has to be connected with the adverse effects on food consumption and body weight in these groups. Furthermore, especially the high number of does in these two groups showing reduced or no defecation is remarkable, which is related with the reduced food consumption of these animals. The sporadic reduced defecation in 5 animals of test group 1 (10 mg/kg body weight/day), however, is regarded as an incidental finding, because this was also noted for a control group animal. In addition, another doe of the highest dose group, which was found dead on day 28 p.i., showed apathy and abdominal distension before. Some other clinical findings like conjunctivitis, slight skin lesions and alopecia, which were found in single does of all groups, including the controls are of spontaneous origin.

2. Mortality

Spontaneous mortalities were only noted in the 120 mg/kg bw/day group, where one doe died intercurrently.
Moreover, 6 of the 8 does of test group 3 (120 mg/kg body weight/day) and the two does of test group 2 (40 mg/kg body weight/day), which aborted during the post-treatment period, were sacrificed on the day or shortly after the abortion.
One doe, which showed only discrete signs of abortion on day 21 p.i., and another doe, which aborted on day 28 p.i. were sacrificed according to the planned schedule on day 29 p.i..

D. BODY WEIGHT AND BODY WEIGHT GAIN

At 120 mg/kg body weight/day (group 3), the body weight of the pregnant animals was significantly reduced during the last days of the treatment (days 16 - 19 p.i.) and the first days of the post-treatment (days 21 - 25 p.i.) periods. Body weight changes of these does were also significantly diminished (severe body weight loss) during days 7 - 19 p.i. (treatment period) and during post-treatment days 19 - 21 p.i..
In test group 2 (40 mg/kg body weight/day) no significant differences with regard to body weight or body weight change could be detected. However, a trend to reduced body weight change can also be noted, when the total body weight change during the treatment period is taken into consideration.
The influence on body weight and/or body weight changes in test groups 2 and 3 (40 and 120 mg/kg body weight/day) is clearly attributable to the administration of the test substance and corresponds with the distinctly diminished food consumption.

The results of the corrected body weight gain (body weight on day 29 p.i. minus body weight on day 7 p.i. minus weight of the uterus before it was opened) do not show any clearly dose-related differences between the groups.

Table 5.6.2-1: Mean maternal body weights during gestation

Group	Bodyweight (g) at day						
	0	**11**	**16**	**19**	**23**	**25**	**29**
Control	2721±157	2704±141	2759±160	2761±159	2759±151	2792±156	2855±187
10 mg/kg bw/day	2692±176	2878±174	2718±193	2737±176	2731±162	2772±173	2846±175
40 mg/kg bw/day	2714±231	2685±183	2716±185	2716±182	2725±167	2742±174	2836±221
120 mg/kg bw/day	2675±186	2592±146	**2577±149***	**2532±151****	**2512±205****	**2548±177****	2712±149

*:p≤0.05; **:p≤0.01

Table 5.6.2-2: Mean maternal body weight gain during gestation

Group	Bodyweight change (g) at days			
	0-7	**7-19**	**19-29**	**0-29**
Control	7.9±37.4	32.4±57.2	94.2±53.3	134.4±88.4
10 mg/kg bw/day	27.4±60.8	17.1±72.5	109.5±75.2	153.9±163.1
40 mg/kg bw/day	22.1±71.1	-20.5±114	136.1±89.6	145.7±191.9
120 mg/kg bw/day	18.6±49.3	**-161.3±163****	157.5±57.7	148.2±212.0

*:p≤0.05; **:p≤0.01

E. FOOD CONSUMPTION

Group mean food consumption of the dams of test group 3 (120 mg/kg body weight/day) was drastically and that of test group 2 (40 mg/kg body weight/day) severely reduced during the treatment period (days 7 - 19 p.i.).
During the first days post-treatment (about days 20 - 26 p.i.) the food intake of the animals of these two groups was also clearly reduced, but reached or even exceeded control levels on the last post-treatment days.
The reduced food consumption observed in the animals of groups 2 and 3 (40 and 120 mg/kg body weight/day) during the treatment period is considered to be treatment-related. The marginal, but sometimes significant reduction in food consumption of the does of the 10 mg/kg body weight group during some days of the treatment period, however, is assessed as an incidental finding, because it was mainly caused by two individuals. Moreover, mean body weights/ body weight changes of the does of this test group were not substantially affected.

Table 5.6.2-3: Mean maternal food consumption during gestation

Group	Food consumption (g/animal/day) at days						
	6-7	**7-8**	**18-19**	**21-22**	**24-25**	**27-28**	**28-29**
Control	109±15	103±22	89±31	89±27	104±17	115±16	112±17
10 mg/kg bw/day	117±21	99±22	64±36	102±32	110±20	126±31	115±25
40 mg/kg bw/day	107±26	82±38	**42±39****	73±45	92±46	126+±28	116±36
120 mg/kg bw/day	123±34	**53±27****	**18±31****	**42±63***	**57±61***	132±65	**144±11***

*:p≤0.05; **:p≤0.01

F. NECROPSY

1. Macroscopic findings

Most of the necropsy findings were recorded in all groups including the controls or without any dose-response relationship. They are spontaneous ones and/or are related to pregnancy (e.g. some amber-colored liquid in abdominal cavity; blind ending uterine horn(s), cyst(s) in the uterine mucosa).
The other necropsy findings recorded for single does of test group 2 or 3 (40 or 120 mg/kg body weight/day) are in line with the diminished food consumption and the severely reduced body weight/body weight gain of these animals (e.g. tympanic distension in large intestine; no feces in rectum; stomach filled with very hard dry food). Furthermore, fatty degeneration of the liver was recorded for some does, which aborted or died intercurrently.
The uterus weights of the animals of the substance-treated groups do not show significant differences in comparison to the controls.

2. Reproduction data of dams

The conception rate varied between 93 and 100%. As mentioned before 8 does of test group 3 and 2 out of test group 2 (120 or 40 mg/kg body weight/day) aborted in the post-treatment period, another 120 mg/kg animal was found dead on day 28 p.i. However, no further substance-related and/or statistically significant differences in the conception rate, in the mean number of corpora lutea, total implantations, resorptions and live fetuses as well as in the values calculated for the pre- and postimplantation loss could be noted. The differences evident were considered to be incidental and within the normal range of deviations for animals of this strain and age.

Table 5.6.2-4: Summary of reproduction data in the teratology study with metiram

Parameter	Dose (mg/kg bw/day)			
	0	**10**	**40**	**120**
Number of females mated	15	15	15	15
Pregnant	14	15	14	15
Aborted	0	0	2	8
Premature births	0	0	0	0
Dams with viable fetuses	14	15	12	8
Dams with all resorptions	0	0	0	0
Female mortality	0	0	2	**7****
Pregnant at C-section	14	15	12	**6****
Litter values:				
Corpora lutea/Dam	7.6±1.4	7.5±0.99	8.0±1.1	6.5±0.84
Corpora lutea/total	107	112	96	39
Implantations/Dam	5.5±2.0	5.8±2.1	6.4±1.1	5.3±1.9
Implantations/total	77	87	77	32
Pre-implantation losses (%)	28.5±21.1	23.4±24.1	19.3±12.3	18.8±24.7
Post-implantation losses (%)	21.1±24.9	11.3±19.9	11.1±12.1	17.9±16.6
Live fetuses total (mean±SD)	4.5±2.4	5.0±2.0	5.8±1.4	4.3±1.9
Live fetuses male (%)	36.8	51.2	45.9	29.2
Live fetuses female (%)	42.1	37.5	43.0	52.9
Resorptions, total (%)	21.1	11.3	11.1	17.9
Resorptions, early (%)	16.7	9.5	8.3	15.6
Resorptions, late (%)	4.5	1.8	2.7	2.4
Fetal weight (g)	44.0±3.6	43.7±5.0	41.0±7.25	38.5±8.6

3. Litter data

The sex distribution in test groups 1 - 3 (10 - 120 mg/kg bw/day) was comparable with the control group. The observable differences are without any biological relevance.

4. Placental, litter and foetal weights

The mean placental weights in groups 1, 2 and 3 (10, 40 and 120 mg/kg body weight/day) were not influenced by the administration of the test substance to the does. The differences observed in comparison to the control are without any dose-response relationship and without any biological relevance.
The mean fetal weights are slightly, but not statistically significantly lowered in test group 3 (120 mg/kg body weight/day), which might be attributed to the test substance administration to the does.

5. Fetal examinations

The external examination of the fetuses revealed no malformations or retardations in any group and only one kind of variation (pseudoankylosis) in one fetus of test groups 1 (10 mg/kg bw/day) and 3 (120 mg/kg bw/day) each.

The examination of the organs of the fetuses revealed two kinds of malformations (hypoplasia of spleen, agenesia of gallbladder) in 2 fetuses of test group 1 and one fetus in test group 3 (10 or 120 mg/kg bw/day). Variations were detected in each group including the control. The very common finding (separated origin of carotids) in the rabbit strain used in this study occurred without any dose-response relationship. No retardations were seen in any group. Moreover, only one or two fetuses out of test groups 0 - 2 (0, 10 or 40 mg/kg bw/day) showed focal liver necrosis or blood coagula around the bladder (so called unclassified findings).

Various malformations of the sternebrae were seen in one fetus of the 10 mg/kg group. No other skeletal malformations were recorded for any group. The variations exhibited were related to the ribs (accessory ribs) and the sternum (sternebra(e) bipartite, of irregular shape or fused) and were found in all groups. However, the incidence of fused sternebrae was significantly increased in test group 1 (10 mg/kg bw/day), whereas sternebra(e) of irregular shape were more often found in test group 3 (120 mg/kg bw/day). In all groups signs of retardations (incomplete or missing ossification of sternebra(e)) were found. A statistically significant increase could be seen especially in test group 2 (40 mg/kg body weight/day).

Table 5.6.2-5: Summary of fetal evaluation data in the teratology study with metiram

Fetal observations	Dose (mg/kg bw/day)			
	0	**10**	**40**	**120**
External examination (malformations, variations, retardations):				
Number examined:	63	75	69	26
Number affected:	0	0	0	0
Visceral examination malformations, variations, retardations:				
Number examined:	63	75	69	26
Number affected, malformations (foetus):	0	2	0	1
Mean %	0	2.7	0	3.8
Number affected, variations (foetus): Separated origin of carotids	37	39	**25***	12
Mean %	59	52	36	46
Number affected, retardations (foetus):	0	0	0	0
Skeletal examination:				
Number examined:	63	75	69	26
Number affected, malformations (foetus):	0	1	0	0
Mean %	0	1.3	0	0
Number affected, variations (foetus):	3	**14***	7	**6***
Mean %	4.8	19	10	23
Number affected, retardations (foetus):	24	35	**45****	11
Mean %	38	47	65	42

There were no statistically significant differences of possible biological relevance between the treated groups and the controls with respect to malformations, variations and retardations with the exception of skeletal variations and retardations.
The number of skeletal variations is slightly higher in the fetuses of the 10 and 120 mg/kg groups (significance $p < 0.05$) and skeletal retardations are increased in test group 2 (40 mg/kg bw/day). However, because a clear dose-response relationship is missing and the higher incidence of skeletal variations/retardations is still in the range of the historical control, the statistically significant increases in the relevant groups are assessed as incidental events and not related to the test substance administration.

III. CONCLUSIONS

Distinct maternal toxicity (reduced food consumption during the treatment period and shortly afterwards and pronounced loss of body weight) occurred at the highest dose level. Eight dams had abortions and a further one died in the observation period. By contrast, merely the weight was slightly reduced in the foetuses. At the mid dose of 40 mg/kg bw, there was still clear maternal toxicity. Two animals had abortions. There were however no signs of embryo- or fetotoxicity at that dose. No substance-induced changes were observed at a dose level of 10 mg/kg bw. Test substance related teratogenic changes were not detected at any dose. Under the conditions of this study, the NOAEL for maternal toxicity is 10 mg/kg bw, and the NOAEL for development is 40 mg/kg bw.

Report: *CA 5.6.2/3*
[redacted] 1979b
Effect of Metiram technical on pregnancy of the rat
1979/065
Guidelines: *none*
GLP: *no*

Deviations to the current valid OECD 414 guideline (2001):

- The ages of the animals are not documented, but a body weight range (156 – 201 g) is given and can be related to an age of 6 – 9 weeks according to Charles River
- Due to current guideline (2001) the exposure period should cover the gestation phase from implantation to one day prior to the day of scheduled kill. In the current study metiram was only administered during the period of organogenesis (for rats: days 6 - 15). This dosing regime was acceptable according to the old OECD TG 414 (1981).
- Verification of the non-pregnancy status by examination of non-gravid uteri is not documented, since non-pregnant animals were not applied for the substance application and all animals used had at least 4 living young. Nevertheless, the number of animals per dose group was at least 16 in accordance to the valid OECD guideline
- Weights of gravid uteri including the cervix are not documented, thus the net maternal body weight change cannot be calculated. In the absence of significant effects on the body weight of the foetuses, this is considered to be a minor deviation, not impacting the overall validity of the study.

Most of the deviations of the current study to the valid OECD 414 guideline (2001) can be compensated by the data given in the study report or are acceptable variations of the study procedure, according to OECD 414 guideline from 1981. Since all other important information is given in the study report and the performance of the study was according to quality specifications in effect at that time (British Standards Method, BS 6000, 6001 (1972), the quality of this study can be judged as equivalent to the current valid OECD guideline. The shorter exposure period compared to the current OECD TG 414 is considered to be a deviation of minor relevance, as the ETU-typical teratogenicity is also seen following a dosing scheme from GD 6-15. Further the technical batch used in the teratogenicity study was spiked with 2.2% ETU and can therefore be regarded as a real worst-case test substance administration, considering the teratogenic potential of ETU (see Chapter 5.8). Further, the newly conducted 2-generation toxicity study in rats, where metiram was dosed up to 1000 ppm (92 mg/kg bw) did not give any evidence for offspring effects. The $NOAEL_{development}$ in this study was ≥ 92 mg/kg bw (see above). Also in the interest of animal welfare no new study has been conducted.

Executive Summary

Groups of 20 time-mated female SPF rats (Crl:COBS CD(SD)BR) received metiram (Batch: 946H519, Purity: 96.8%, 2% ETU) in 0.5% w/w aqueous solution of sodium carboxy-methylcellulose CMC, administered by gavage at 0, 40, 80, and 160 mg/kg bw/day on Days 6 to 15 of gestation. Controls received the vehicle alone. Animals were observed for mortality or clinical signs once daily. Individual body weights and food consumption data were recorded. Termination and necropsy on Day 20 of presumed gestation was followed by processing and detailed visceral and skeletal examination of foetuses.

For the purpose of dose setting of the main study a preliminary study has been conducted in non-pregnant animals, where gavage doses of 0, 150, 300, 600 and 1200 mg/kg bw have been administered. There was severe toxicity seen at 600 and 1200 mg/kg bw (including catalepsy paralysis, lethargy. For human reasons dosing was terminated on day 7. In this preliminary study already in the 150 mg/kg bw dose group lower body weight gains in the females were observed, which was the basis for the top dose setting of 160 mg/kg bw for the main study. With the exception of a slight depression of food consumption (at 80 and 160 mg/kg bw) and reduced body weight at 160 mg/kg bw, no effects were observed in the dams. There was a marginal dosage related reduction in food consumption at 80 and 160 mg/kg, during the mid-dosing period and bodyweight gain of all test groups was slightly lower than that of controls with the greatest divergence occurring at the highest dosage of 160 mg/kg. In respect of litter values, litter size and litter weight at 160 mg/kg were significantly lower than control values due to a combination of slightly, but non-significantly higher values for pre- and post-implantation loss. Corresponding values at 40 and 80 mg/kg were comparable with those of controls. Mean foetal weight and incidences of major malformation, minor visceral and skeletal anomalies and skeletal variants were not adversely affected by treatment at any dosage.

Under the conditions of this study, the NOAEL for maternal toxicity is 80 mg/kg bw (as the slightly decreased food consumption at 80 mg/kg bw is not considered adverse). The NOAEL for embryo/fetotoxicity is 160 mg/kg bw, as the effects on litter size and total litter weight were mainly attributable to not treatment-related pre-implantation losses occurring at 160 mg/kg bw. The test substance was not teratogenic.

(DocID 1979/065)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: Metiram, technical (containing 2% ETU)

Description:	NA
Lot/Batch #:	946H159
Purity:	96.8% (as given in DocID 1986/1000164)
Stability of test compound:	NA

2. Vehicle: 0.5% w/w aqueous solution of sodium carboxymethyl cellulose (CMC)

3. Test animals:

Species:	Rat
Strain:	Crl:COBS CD(SD)BR (SPF)
Sex:	Female
Age (at mating):	NA
Weight (day 0 of gestation):	156 - 201 g
Source:	
Acclimation period:	5 days
Time of dosing:	Days 6 to 15 of gestation
Diet:	Spratts Laboratory Diet No. 1, ad libitum
Water:	tap water, ad libitum
Housing:	group housing (5 per cage) in metal cages (Bowman R) with wire mesh top, front and floor.
Environmental conditions:	
Temperature:	18 - 22°C
Humidity:	45 - 55%
Air changes:	13 per hour
Photo period:	12 h light / 12 h dark

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 07-Jun-1978 - 17-July-1978

2. Preliminary study:

Animals were assigned by weight to dose groups of 6 female animals. The animals were dosed for 10 days by gavage with 150, 300, 600, and 1200 mg/kg bw/day metiram suspended in 0.5% aqueous CMC at a dose volume of 10 mL/kg bw. Animals were observed daily for clinical signs and were weighed on days -5, -3, 1, 4, 7, and then daily until day 15. Food consumption was measured. On day 15 animals were killed by CO_2 asphyxation, dissected and examined for macroscopic pathologic changes.

3. Animal assignment and treatment:

Time-mated animals were assigned by weight to dose groups of 20 female animals. The date of mating (sperm in vaginal smear or vaginal plug) was considered day 0 of pregnancy. The animals were dosed from day 6 of gestation (GD 6) until GD 15 by gavage with 40, 80, and 160 mg/kg bw/day metiram suspended in 0.5% aqueous CMC at a dose volume of 10 mL/kg bw. The dosage volumes for individual animals were calculated according to bodyweights on days 6, 10 and 14 of pregnancy.

4. Test substance preparation and analysis:

The highest concentration (1.6% w/v) of metiram was prepared by suspending an appropriate amount of material in 0.5% sodium carboxymethyl cellulose; lower concentrations were prepared by serial dilution with 0.5% sodium carboxymethyl cellulose. Dosing formulations were prepared at intervals not greater than 3 days.

5. Statistics:

For all values expressed as a percentage or ratio values were first calculated within the litter and the group value derived as a mean of individual litter percentage. Statistical analyses were then performed using the litter as the basic sample unit and non-parametric methods since litter values rarely follow a normal distribution (Kruskal-Wallis, Jonckheere, Wilcoxon).

C. METHODS

1. Observations:
Animals were observed daily for mortality or clinical signs.

2. Body weight:
Individual body weights were measured on days 1, 3, 6, 10, 14, 17, and 20.

3. Food and water consumption:
Food consumption was recorded "weight day" to "weight day". Water consumption was measured daily commencing day 2 of pregnancy.

4. Sacrifice and pathology:
<u>Parents</u>
All animals that died, or were killed for humane reasons, were weighed and subjected to post mortem examination. On GD 20 the animals were killed by CO_2 asphyxation, dissected and examined for congenital abnormalities and macroscopic pathological changes in maternal organs; the ovaries and uteri were examined immediately to determine:

- Number of corpora lutea
- Number and distribution of live young
- Number and distribution of embryonic/foetal deaths
- Litter weight from which the mean pup weight was calculated
- Foetal abnormalities

Embryonic/foetal deaths were classified as:
- Early: only placenta visible at terminations
- Late: both placental and embryonic remnant visible at termination

Uteri or individual uterine horns without visible implantations were immersed in a 10% solution of ammonium sulphide to reveal evidence of embryonic death at very early stages of implantation.

<u>Pups</u>
Live young were examined externally and weighed. Half the pups in each litter were preserved in Bouins solution for subsequent free-hand sectioning to discover visceral abnormalities (Wilson technique); the remainder were fixed in 74OP industrial methylated spirit for subsequent macroscopic examination, evisceration and determination of sex prior to clearing and alizarin staining (modified Dawson technique) for skeletal examination. Young showing suspected abnormalities were processed by the more appropriate technique for clarification of initial observations.

Where necessary, pups were uniquely identified (toe marked) to allow correlation of initial with subsequent findings.

Structural deviations were classified as:
Major malformations: rare and/or probably lethal, eg. anencephaly, anury
Minor malformations: minor differences from "normal" that are detected relatively frequently either by free-hand sectioning, e.g. increased renal pelvic cavitation, or at skeletal examination, e.g. bipartite centrum
Variants: alternative structures occurring regularly in the control population are classified as variants. These may be permanent structures, e.g. an extra pair of ribs, or they may be transient stages of development, e.g. unossified sternebra(e).

Assessment of results
The following indices were calculated from caesarean section records of animals in the study:
Pre-implantation loss: (No. of corpora lutea – No. of implantations)/ No. of corpora lutea * 100
Post-implantation loss: (No. of implantations – No. of live young)/ No. of implantations * 100

Group mean values for litter size, embryonic death, pre and post-implantation loss were calculated in two ways:

- Mean A: includes all surviving animals that provided evidence of pregnancy including those showing total resorption.
- Mean B: includes all animals with live young at termination.

Mean B has more meaning when group size is low, in which case the mean values would be unduly influenced by the presence of a single animal with total resorption. Mean A is a more accurate index when several animals show total resorption.
For litter and mean foetal weights and abnormal values only Mean B values were calculated.

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

No information on test substance concentration control or homogeneity control was given.

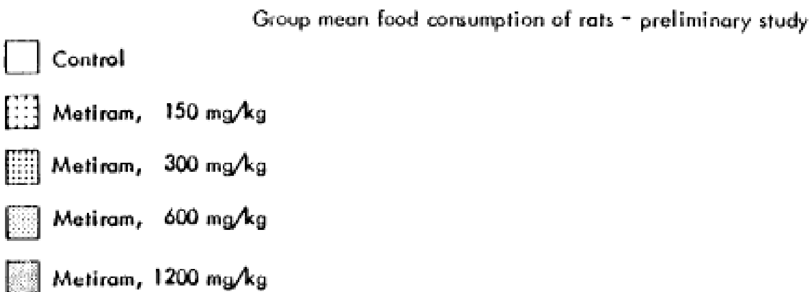
B. PRELIMINARY STUDY

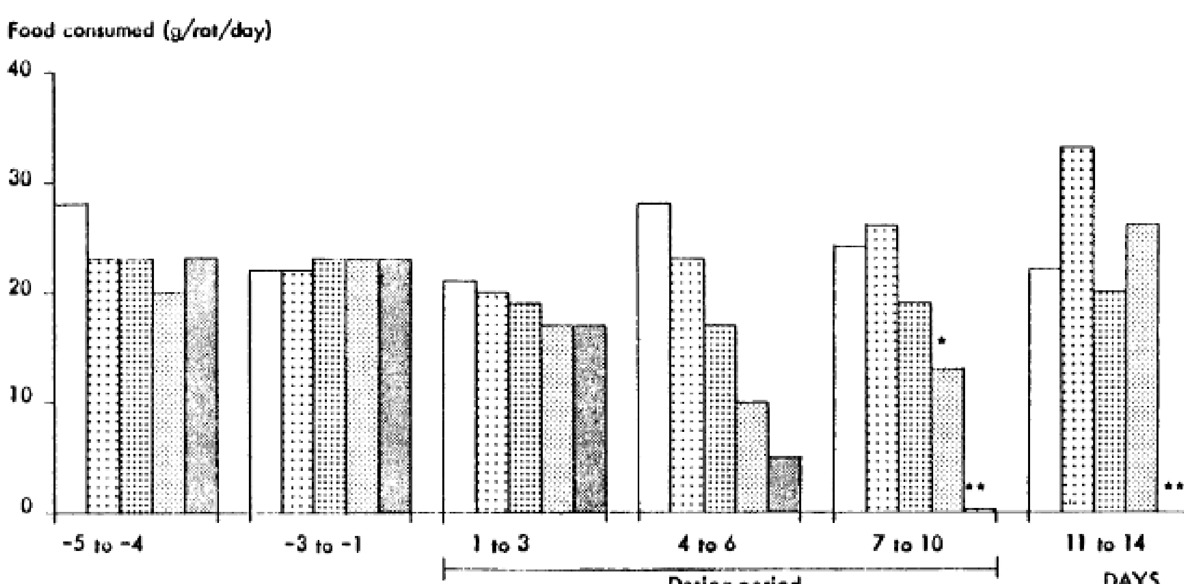
Clinical signs:
600 and 1200 mg/kg bw:
Catalepsy, paralysis, lethargy, chromodacryorrhoea, pale faeces, diuresis and death were observed. The grasping reflex and hind limb support was absent in almost all animal.
For humane reasons dosing was terminated on day 7 and because signs of reaction continued at both dosages, surviving animals at 1200 mg/kg bw (showing the most severe changes) were killed for humane reasons on day 8.

300 mg/kg bw/day:
Attenuated signs of reaction including lethargy, hypersensitivity and increased respiration were the general rule although one animal was more severely affected and was killed for humane reasons following the development of paralysis. The grasping reflex and hind limbs support was severely impaired or absent in most animals.

150 mg/kg bw/day:
Increased alertness (all animals) and reduced hind limb support (4/6) was observed. Also food consumption and body weight changes were affected in that dose group.

Food consumption
A dose-related reduction of food consumption with peak divergence between groups was observed between days 4 and 6. Despite subsequent convergence the dose-related effect persisted through the end of the dosing period. During the post-dosing period the food consumption was comparable within groups. The changes in food consumption are shown in the below figure:





* Treatment withdrawn from day 7. ** Treatment withdrawn from day 7, all animals dead or killed by day 8.

Figure 5.6.2-1: Group mean food consumption of rats – preliminary study

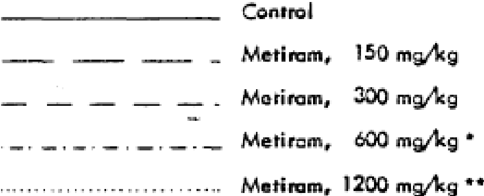
Body weight gain

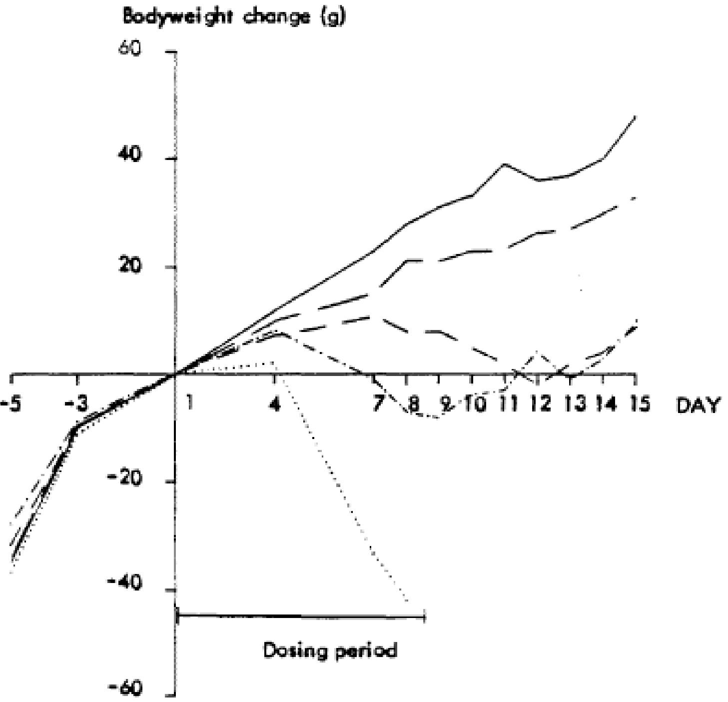
An initial retardation of weight gain was observed in all test groups (most prominent at 1200 mg/kg bw/day). From day 4 marked weight loss occurred at 600 and 1200 mg/kg bw/day and continued until all animals dies or were killed (1200 mg/kg bw/day) or until a few days after early withdrawal of treatment (600 mg/kg bw/day).

At 300 mg/kg bw/day increasing divergence from control values after day 4 was followed by a period of weight loss from day 7 until two days after cessation of dosing.

At 150 mg/kg bw/day there were no periods of weight loss but there was an overall retardation of weight gain. The changes in body weight gain are shown in the below figure:

Group mean bodyweight change of females ≠ - preliminary study





* Treatment withdrawn from day 7

** Treatment withdrawn from day 7, all animals dead or killed by day 8.

≠ For groups 1 to 4, survivors only were included in mean calculations

Figure 5.6.2-2: Group mean bodyweight change of females – preliminary study

Pathology
Among animals that died or were killed during the dosing period post-mortem examination revealed the presence of yellow (dose like) material in the caecum which was taut.
Among animals that killed at the scheduled termination date (5 days after the last dose) there were no macroscopic changes considered to be related to treatment.

C. OBSERVATIONS – MAIN STUDY

1. Clinical signs of toxicity
No signs of reaction attributable to treatment were observed.

2. Mortality
No mortality occurred.

D. BODY WEIGHT AND BODY WEIGHT GAIN

Overall weight gains of test groups were slightly lower than those of controls and although differences were not strictly related to dosage the greater divergence from control values occurred at 160 mg/kg.

Table 5.6.2-6: Group mean bodyweight change of dams with viable young

Group	Bodyweight change (g) at day					
	1	3	6	10	14	17	20
Control	-42	-23	0	26	58	92	138
40 mg/kg bw/day	-39	-20	0	23	53	81	128
80 mg/kg bw/day	-38	-20	0	24	53	83	130
160 mg/kg bw/day	-41	-21	0	22	49	75	120

E. FOOD CONSUMPTION

Table 5.6.2-7: Group mean food consumption of dams during the study

Group	Food consumed (g/rat/day) during days					
	1-2	3-5	6-9	10-13	14-16	17-19
Control	22	25	26	22	29	31
40 mg/kg bw/day	21	22	22	22	26	29
80 mg/kg bw/day	20	23	22	20	25	29
160 mg/kg bw/day	21	23	23	19	27	30

Assessment of effects on food consumption was difficult due to the generally lower food consumption of all test groups before, during and after the dosing period. However, a slight, but dosage related reduction in food consumption at 80 and 160 mg/kg during the mid-dosing period may be relevant; considering the similar chronological pattern of effect in the preliminary study.

F. NECROPSY

1. Macroscopic findings

No macroscopic changes attributable to treatment were observed at terminal post mortem examination.

2. Litter data

Pregnancy rates of parental females were 95, 85, 90, and 95% for group 1, 2, 3, and 4 animals, respectively. Due to a combination of slightly, but not significantly, higher values for pre- and post-implantation loss (the preimplantation losses are not treatment-related), litter size at 160 mg/kg was lower than that of controls the difference attaining statistical significance ($P<0.05$).
At 40 and 80 mg/kg values for litter size and post-implantation loss were comparable with those of controls.

Table 5.6.2-8: Summary of litter data in the teratology study with metiram

Parameter	Dose (mg/kg bw/day)			
	0	**40**	**80**	**160**
Number of females	**19**	**17**	**18**	**19**
Litter values:				
Corpora lutea/Dam	11.4	11.6	11.6	11.2
Implantations/Dam	10.7	10.6	10.9	9.6
Pre-implantation losses (%)	5.9	7.3	4.2	13.3
Post-implantation losses (%)	4.0	5.5	2.4	6.9
Live fetuses male	5.5	5.6	5.2	4.7
Live fetuses female	4.8	4.5	5.4	4.2
Live fetuses total	10.3	10.1	10.6	**8.9*1)**
Embryonic deaths, total	0.4	0.6	0.3	0.7
Embryonic deaths, early	0.4	0.5	0.2	0.5
Embryonic deaths, late	0.1	0.1	0.1	0.2
Litter weight (g)	38.63	37.83	40.51	**34.38***
Fetal weight (g)	3.75	3.76	3.82	3.85

*:p≤0.05

1) The decreased absolute number of total live fetuses is mainly due to increased pre-implantation losses, which is neither treatment-related nor attributable to a embryotoxic response.

3. Placental, litter and foetal weights

Mean foetal weights of test groups were comparable with, or slightly greater than that of controls; none of the differences were statistically significant (P>0.05). As a consequence of the lower litter size, litter weight at 160 mg/kg was significantly (P>0.05) lower than that of controls. Litter weights at 40 and 80 mg/kg were essentially comparable with those of controls.

4. Fetal examinations

Intergroup differences in the incidences of major malformation, minor visceral and minor skeletal anomalies were neither statistically significant nor dosage related. The only major malformations observed (4 pups) were all derived from a single litter dosed at 80 mg/kg, and the abnormality has been observed in previous studies. The abnormality is difficult to describe since an accumulation of slight changes combine to provide a characteristic "hunched" appearance.

Intergroup differences in the incidence of skeletal variants were neither statistically significant (P>0.05) nor dosage related. Separate analysis of combined values for pre- and post-implantation loss showed a significant (P<0.01) difference between controls and the high dosage group.

Table 5.6.2-9: Summary of fetal evaluation data in the teratology study with metiram

Fetal observations	Dose (mg/kg bw/day)			
	0	**40**	**80**	**160**
External examination:				
Number examined:	196	171	191	170
Number affected:	0	0	4 (2.1%)	0
Visceral examination:				
Number examined:	98	84	96	83
Number affected:	7	5	7	6
Mean %	7.1	6.0	7.3	7.2
Skeletal examination:				
Number examined:	98	87	91	87
Number affected:	8	9	19	5
Mean %	8.2	10.3	20.9	5.8
Variants:				
Sternebra variant (%)	38.8	33.5	18.0	20.9
Ribs 13 (%)	91.8	83.5	92.1	89.6
Ribs 14 (%)	8.2	16.5	7.9	10.4

III. CONCLUSIONS

With the exception of a slight depression of food consumption (at 80 and 160 mg/kg bw) and reduced body weight at 160 mg/kg bw, no changes were observed in the dams. The slight and statistical non-significant increase in pre-and post-implantation losses, led to a statistically significant decline in the litter size and total litter weight in the highest dose group. The higher percentages of pre-implantation losses (which is mainly contributing to the observed effects on litter size and total litter weight) are neither treatment-related nor an indication for teratogenicity. No other findings were observed which could be related to treatment regarding parental and developmental toxicity.
Under the conditions of this study, the NOAEL for maternal toxicity is 80 mg/kg bw (as the slightly decreased food consumption is not considered adverse), and the NOAEL for embryo/fetotoxicity is 160 mg/kg bw, as the effects on litter size and total litter weight were mainly attributable to not treatment-related pre-implantation losses occurring at 160 mg/kg bw.. The test substance was not teratogenic.

CA 5.7 Neurotoxicity Studies

Studies evaluated in the draft monograph of rapporteur member state Italy of July 2000:

No acute neurotoxicity studies are available for metiram. The potential neurotoxic effects of metiram have been investigated within the scope of a 3-month rat study and in a 12-month dog study which have been evaluated by European authorities and Italy as Rapporteur member state (European Commission Peer Review Program) and were considered to be acceptable. For the convenience of the reviewer, these are summarized in Table 5.7-1 below as extracted from the monograph and brief summaries are provided under the respective chapters.

Table 5.7-1: Summary of already peer-reviewed neurotoxicity studies as available in the monograph (2000)

Study Dose levels	Dosages (mg/kg bw/ d)	NOAEL mg/kg bw/d	Adverse effects at LOAEL	Reference
3-month feeding and neurofunctional observations Wistar rats 0, 5, 80,320, 960 ppm metiram	M&F: 0.4, 6.3, 25.4 and 81.4	81.4	No adverse effects. At highest dose signs of muscle weakness were observed which are not considered being a specific effect on the nervous system. Because: at this dose also body weight was impaired; no morphological changes in the peripheral or central nervous system were observed.	KCA 5.7.1/3 1992/11224
1-year feeding Beagle dog 0, 30, 80, 1000, 3000 ppm metiram	M&F: 0.98, 2.59, 29.9 and 84.8	84.8	No effects concerning neurotoxicity	KCA 5.7.1/4 1991/10786, KCA 5.7.1/5 1992/12594

The potential neurotoxic effects of metiram have been investigated within the scope of a 3-month rat study and in a 12-month dog study. The rat study (see chapter CA 5.3) included a functional observational battery as well as special neurohistopathology. In the dog study (see chapter CA 5.3) too, clinical neurological examinations as well as neurohistopathology were carried out. The results of these studies demonstrated that metiram did not have a neurotoxic effect and did not cause damage to the central nervous system.

Based on the available studies, the following endpoints were determined in the Annex I listing of metiram.

Delayed neurotoxicity	90-day, rat: no evidence of neurotoxicity from microscopic examination of nervous system.

Submission of not yet per-reviewed studies in this dossier:

In this dossier an acute oral neurotoxicity study in Wistar rats and a 90-day neurotoxicity study are submitted. The new 90-day neurotoxicity has been conducted, because

- The existing study has been conducted prior to the adoption of the OECD TG 424
- There were some questionable effects on fore limb grip strengths (without any evidence for neuropathological effects, as confirmed by an additional external neuropathological review of additional tissue investigations) seen the existing 90-day study including neurofunctional parameter in females down to the lower mid dose. The objective was to identify a clear NOAEL in a study conducted according to the most recent OECD TG.
- A NOAEL (for thyroid hormone changes) should be identified. Therefore additional thyroid hormone measurements were included in this 90-day study to be able to identify a NOAEL for thyroid hormone changes, as in the existing studies varying effects on TSH levels of questionable treatment relationship were seen at all doses.
- After consulting with an expert in neuropathology (Prof. Peter Spencer) some additional tissues were fixed and examined in the new study (rami distales musculares of nervus tibialis) in order to fully elucidate metiram´s potential for neurotoxicity. An external pathological review has been conducted on the full set of neuropathological tissues.
- The dose setting was based on the doses used in the existing 90-day repeated dose toxicity study (incl. neurofunctional parameter) in rats conducted in 1992 (doses were 5, 80. 320, 960 ppm) and the 2-generation toxicity study conducted in the year 2010/2011 (doses were 100, 350, 1000 ppm). The objective was to stay below the 1000 ppm top dose of the 2-generation toxicity study, as the findings in the thyroid in rats were significant in that study.

Study **Dose levels**	**NOAEL** **mg/kg bw/d**	**Adverse effects at LOAEL**	**Reference**
Acute oral neurotoxicity study Wistar rats M&F: 0, 125, 500, 2000 mg/kg bw metiram	2000 mg/kg bw/d	No effects	2010/7008373
90-day neurotoxicity study Crl:WI(Han) rats 0, 40, 200, 900 ppm M: 2.6, 13, and 59 mg/kg bw/d metiram F: 3.6, 17 and 71 mg/kg bw/d metiram	Neurotoxicity: 59 (M) and 71 (F) mg/kg bw/d Systemic toxicity: 59 (M) and 17 (F) mg/kg bw/d	Neurobehavior/Neurohistopathology: No effects Systemic toxicity in females: Impaired body weight development, increased thyroid weight, hyperplasia/hypertrophy of thyroid and thyroid hormone changes Decreased T4 levels in males considered treatment-related, but not adverse	2014/1315300

CA 5.7.1 Neurotoxicity studies in rodents

Acute neurotoxicity studies

Report:	CA 5.7.1/1 [redacted] 2010a BAS 222 29 F (Metiram TC) - Acute oral neurotoxicity study in Wistar rats - Administration via gavage (Including amendment no. 1) 2010/7008373
Guidelines:	EPA 870.6200, OECD 424, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.43
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

The acute neurotoxicity of metiram (batch 300015, purity: 91.5%) was investigated in groups of 10 male and female Wistar rats (Crl:WI(Han) after a single administration by gavage at dose levels of 0, 125, 500, and 2000 mg/kg bw/d.
No test substance-related adverse findings were observed during the whole observation period of 14 days. There also was no indication of neurotoxicity up to the limit dose of 2000 mg/kg body weight in animals of both sexes seen.
Therefore, the no observed adverse effect level (NOAEL) under the conditions of the present study was 2000 mg/kg bw for male as well as female Wistar rats.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Metiram TC (BAS 222 29 F)
Description:	solid, beige/yellow
Lot/Batch #:	300015
Purity:	91.5%
Stability of test compound:	The test substance was stable which was proven by reanalysis. Homogeneity was given.
2. Vehicle and/or positive control:	1% aqueous carboxymethylcellulose (CMC) Positive controls: Diazepam Carbaryl Carbaryl (1-Naphthylmethylcarbamate) Acrylamide Trimethyltinchloride Nomifensin 3,3'-Iminodipropionitrile

3. Test animals:

Species:	Rat
Strain:	Crl:WI(Han)
Sex:	Male and female
Age:	Males: 35-36 (Section A), 33-34 (Section B) Females: 36-37 (Section A), 32-33 (Section B) 49 days at administration
Source:	[redacted]
Acclimation period:	13 – 17 days
Diet:	ad libitum (ground Kliba maintenance diet, rat-mouse-hamster pellets, supplied by Provimi Kliba SA, Kaiseraugst, Switzerland)
Water:	ad libitum
Housing:	single housing in Polycarbonate cage type MIII (floor area about 800 cm²). For enrichment wooden gnawing blocks (Typ NGM E-022) supplied by Abedd® Lab. And Vet. Services GmbH, Vienna, Austria were added.
Environmental conditions:	
Temperature:	20-24°C
Humidity:	30-70%
Air changes:	fully air conditioned
Photo period:	12 hours light from 6:00 - 18:00 h, 12 hours dark from 18:00 - 6:00 h

B. STUDY DESIGN AND METHODS

1. Dates of work: 10-Mar-2009 - 25-Nov-2009
(In life dates: 17-Mar-2009 (first day of FOB and MA determinations on day -7) to 10-Apr-2009 (necropsy of last subset)

2. Animal assignment and treatment:

Metiram was applied as a suspension. It was administered once to groups of 10 male and 10 female Wistar rats by oral gavage at dose levels of 0, 125 (low dose), 500 (mid dose) and 2000 mg/kg (high dose). The application volume was 10 mL/kg bw.
Each group per sex was subdivided into 2 subsets (Section A males and Section A females = first 5 animals of each dose group and Section B males and Section B females = second 5 animals of each dose group) in order to balance the groups for FOB and motor activity measurements. The animals were assigned to the treatment groups by means of computer generated randomization lists based on body weights.

3. Test substance preparation and analysis:

The dosing suspensions were prepared by mixing weighed amounts of test substance with appropriate amounts of the vehicle (1% aqueous CMC) with a magnetic stirrer. During administration of the test substance, preparations were kept homogeneous using a magnetic stirrer. The test-substance preparations were made once before the first administration and were divided into daily aliquots and stored closed in a freezer at -18°C.

The stability of the test substance in 1% carboxymethylcellulose in drinking water at +4°C over a period of a maximum 4 hours was proven during the study.

Homogeneity analyses of suspension preparation were performed for all concentrations administered. For this - as laid down in the SOP - samples were taken from the top, middle and bottom of the storage containers. The homogeneity samples were also used for concentration control analysis. Furthermore three samples of each dose was taken to confirm the correctness of the concentration.

Table 5.7.1-1: Analysis of diet preparations for homogeneity and test-item content

Concentration expected [g/100 ml]	Sampling	Concentration found [g/100 mL]
1.25	23.03.2009	1.29
1.25	23.03.2009	1.29
1.25	23.03.2009	1.29
5	23.03.2009	5.0
5	23.03.2009	5.0
5	23.03.2009	5.0
20	23.03.2009	18.7
20	23.03.2009	18.7
20	23.03.2009	18.7

For all samples the relative deviations between observed and expected concentration are far below 20% (max. 6.5%). Homogeneity for all samples can be stated.

4. Statistics:

Means and standard deviations of each test group were calculated for several parameters. Further statistical analyses were performed according to following tables:

Table 5.7.1-2: Statistics of clinical examinations

Parameter	Statistical test
body weight, body weight change	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means
feces, rearing, grip strength forelimbs, grip strength hindlimbs, footsplay test, motor activity	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided).If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using Wilcoxon-test (two-sided) for the equal medians

Table 5.7.1-3: Statistics of pathology

Parameter	Statistical test
Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair wise comparison of each dose group with the control group was performed using the WILCOXON test for the hypothesis of equal medians

C. METHODS

1. Clinical observations:

The animals were checked daily for evident signs of toxicity. Abnormalities and changes were recorded for each animal individually.
A check for moribund and dead animals was made twice on each working day and once on Saturdays, Sundays, and public holidays. Animals that were in a moribund state were sacrificed and necropsied.

2. Body weight:

Body weight was determined before the first neurofunctional tests in order to randomize the animals. During the study body weights were determined on the days FOBs were conducted, i.e. Days -7, 0, 7 and 14. The difference between the body weight on the respective day of weighing and the body weight on day 0 was calculated as body weight change. On study day 15 the body weights of the fasted animals were used for determination of the relative organ weights.

3. Food consumption, food efficiency, and compound intake:

Individual food consumption was checked daily by visual inspection. No food consumption data were recorded.

4. Water consumption:

Water consumption was observed daily by visual inspection of the water bottles for any overt changes in volume. No water consumption data were recorded.

5. Ophthalmoscopy:

Not performed in this study

6. Functional observation battery (FOB):

FOBs were performed in all animals prior to administration (day -7) and on study days 0, 7 and 14. The FOBs were performed prior to the test substance administration. The FOB started with passive observations without disturbing the animals (home cage observations), followed by removal from the home cage and open field observations in a standard arena. Thereafter, sensorimotor tests and reflex tests were conducted. The examinations were carried out by trained technicians being not aware of the group allocation of the animals. The findings were ranked according to the degree of severity, if applicable.

During the home cage observation attention was paid to posture, tremors, convulsions, abnormal movements, impairment of gait and (if applicable) other findings.

For open field observation the animals were transferred to a standard arena (50 x 50 cm with sides 25 cm high) and observed for at least 2 minutes. The following parameters were assessed:

1. behavior when removed from cage	10. respiration
2. fur	11. tremors
3. skin	12. convulsions
4. salivation	13. abnormal movements
5. nose discharge	14. impairment of gait
6. lacrimation	15. activity/arousal level
7. eyes/pupil size	16. feces (no. fecal pellets/appearance/consistency) within 2 minutes
8. posture	17. urine (appearance/quantity) within two minutes (Q)
9. palpebral closure	18. number of rearings within two minutes (Q)

(Q) quantitative parameter	

For sensorimotor tests and reflexes the animals were removed from the open field. The following tests were performed:

1. approach response	8. behavior during "handling"
2. touch response	9. vocalization
3. vision ("visual placing response")	10. pain perception ("tail pinch")
4. pupillary reflex	11. grip strength of forelimbs (Q)
5. pinna reflex	12. grip strength of hind limbs (Q)
6. audition ("startle response")	13. landing foot-splay test (Q)
7. coordination of movements ("righting response")	14. other findings
-----------	
(Q) quantitative parameter	

7. Motor activity measurement:

Motor activity examinations (MA) were performed in all animals prior to administration (day -7) and on study days 0, 7 and 14. MA was performed using the TSE Labmaster System (TSE Systems GmbH, Bad Homburg, Germany) with 18 infrared beams per cage. For the measurements animals were placed in new clean polycarbonate cages with absorbent material. Motor activity measurements started at 14:00 h. The numbers of beam interrupts were counted over 12 intervals of 5 minutes each. Measurement started individually for each animal when the 1st beam was interrupted and lasted exactly 1 hour. No food or water was offered during the measurements.

8. Neuropathology:

The first five surviving animals per sex and test group were selected for neuropathology evaluation. These animals were sacrificed by perfusion fixation under deep Isoflurane anesthesia. SOERENSEN's phosphate buffer was used as the rinsing solution and fixation was performed with a solution according to KARNOVSKY.

The remaining animals were sacrificed using CO_2 without further examination.

The sacrificed animals were necropsied and the visible organs or organ sections were assessed by gross pathology as accurately as it is possible for perfused animals. The weight of the brain (without olfactory bulb) was determined in all perfused animals after removal of the brain but before any other preparation. For determination of the relative brain weights the terminal body weights were uses

Additionally to organ/tissues listed in paragraphs below, the following organs/tissues were preserved in neutral buffered 4% formaldehyde:

Brain (remaining material after trimming)
Spinal cord (parts of cervical and lumbar cord)
Gross lesions

Various peripheral nerves, parts of the brain and brain-associated organs, parts of the spinal cord and muscles were embedded and histologically examined. The remaining organ material and the animal bodies were stored in neutrally buffered 4% formaldehyde solution. Details are given below:

The following organ samples were embedded in paraffin, sectioned and stained with hematoxylin-eosin (H&E) and assessed by light microscopy. (✓: all dose groups; # only control and high dose - low and mid dose organs were stored in 4% formaldehyde)	
#	Brain (cross sections): - Frontal lobe - Parietal lobe with diencephalon - Midbrain with occipital and temporal lobe - Pons - Cerebellum - Medulla oblongata
#	Brain-associated organs/tissues - Eyes with retina and optical nerve
#	Spinal cord (cross and longitudinal sections): - Cervical cord (C3-C6) - Lumbar cord (L1-L4)
#	Peripheral nervous system: - Gasserian ganglia with nerve - Gastrocnemius muscle

The following nerves were embedded in an epoxy resin, semi thin sectioned and stained with Azure II - Methylene blue basic Fuchsine (AMbf) and assessed by light microscopy. (✓: all dose groups; # only control and high dose, - low and mid dose organs/tissues were stored in buffer solution)	
#	Dorsal root ganglion, (3 of C3-C6)
#	Dorsal root fiber (C3-C6)
#	Ventral root fiber (C3-C6)
#	Dorsal root ganglion, (3 of L1-L4)
#	Dorsal root fiber (L1-L4)
#	Ventral root fiber (L1-L4)
#	Proximal sciatic nerve
#	Proximal tibial nerve (at knee)
#	Distal tibial nerve (at lower leg)

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity

No treatment-related clinical observations were noted during the daily standard clinical observation.

2. Mortality

No mortality was observed in this study.

3. Ophthalmoscopy

No ophthalmoscopy examinations were performed in this study.

B. BODY WEIGHT AND BODY WEIGHT GAIN

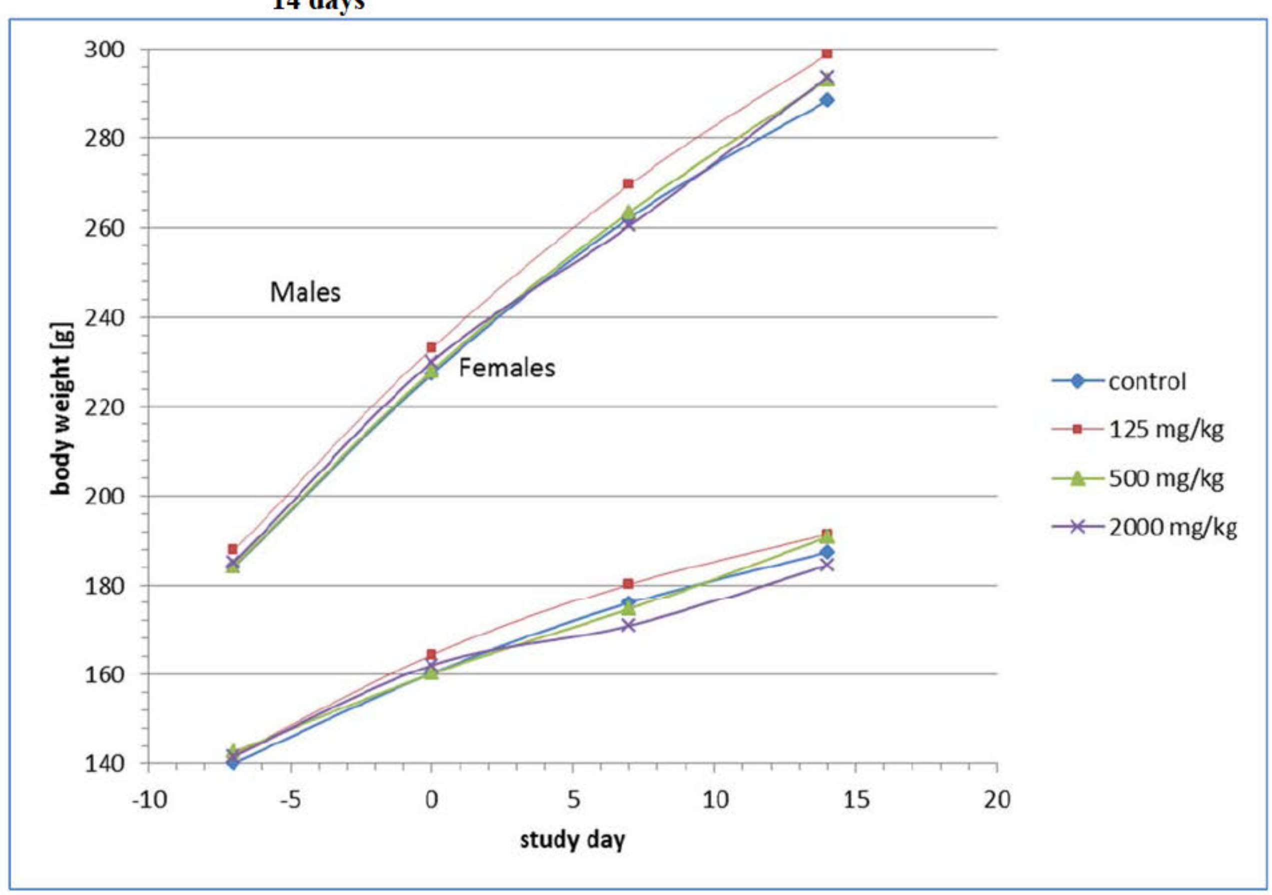
Only body weight change was significantly decreased on day 7 in female animals of dose group 3 (2000 mg/kg). This single occurrence without any influence on other parameter noted in the present study was assessed as incidental and toxicologically irrelevant. [Figure 5.7.1-1, Table 5.7.1-4].

Table 5.7.1-4: Mean body weights and body weight gain of rats administered metiram once and observed for 14 days

	Males				Females			
Dose level [mg/kg bw]	0	125	500	2000	0	125	500	2000
Body weight [g]								
- Day 0	184.2	187.9	184.4	185.2	140.01	141.9	142.6	141.6
- Day 14	288.4	298.7	293.0	293.6	187.5	191.5	190.9	184.6
Δ% (compared to control)		3.6	1.6	1.8		2.1	1.8	-1.6
body weight gain day 7 [g]	35.0	36.7	35.6	30.6	15.8	15.9	14.5	8.8**
Overall body weight gain (day 14) [g]	61.1	65.7	64.9	63.6	27.3	27.2	30.9	22.6
Δ% day 14 (compared to control)		7.5	6.3	4.1		-0.5	13.1	-17.0

** $p \leq 0.01$ (Dunnett's test two-sided)

Figure 5.7.1-1: Body weight development of rats administered metiram once and observed for 14 days



C. FOOD CONSUMPTION

Not determined quantitatively for this study. No test-substance related effects on food consumption were observed

D. FUNCTIONAL OBSERVATION BATTERY

Deviations from (rank) "zero values" were obtained in several animals. However, most findings were either equally distributed between treated groups and controls or displayed no dose-response relationship or occurred in single animals only, thus these observations were considered incidental.

1. Home cage observations

No significant deviations from controls were observed.

2. Open-field observations

No significant deviations from controls were observed.

3. Sensorimotor tests / reflexes

No significant deviations from controls were observed.

E. MOTOR ACTIVITY

Regarding the overall motor activity no test substance-related findings or significant deviations were observed.
Comparing the single intervals with the control groups, the following significantly changed values were measured:

- in low dose males (125 mg/kg bw/day) the value on day -7 of interval 7 was significantly increased
- in mid dose males (500 mg/kg bw/day) the value on day -7 of interval 7 was significantly increased
- in mid dose males (500 mg/kg bw/day) the value on day 0 of interval 9 was significantly increased
- in high dose males (2000 mg/kg bw/day) the value on day 0 of interval 2 was significantly decreased
- in low dose females (125 mg/kg bw/day) the value on day 7 of intervals 11 was significantly increased

These single and inconsistently changed values were without any dose-response relationship and did not significantly affect the overall motor activity. Therefore, these findings were assessed as incidental and not related to treatment with the test substance.

F. NECROPSY

1. Terminal body and brain weight

No treatment-related changes of terminal body weights or absolute and relative brain weights were noted.

2. Gross and neuro-histopathology (Perfusion fixed animals)

No treatment related gross pathology or neuro-histopathological findings were observed. The single (grade 1) findings of an “axonal degeneration” were recorded in peripheral nerve branches of control and treated animals. This finding is regarded as incidental or spontaneous in nature.

G. POSITIVE CONTROLS

No concurrent positive control was employed in this study.

However, in several positive control studies, behavioral and neuro-pathological sequelae of substances with nervous system effects were evaluated using Functional Observational Batteries (FOB), Motor Activity Measurements and Neuropathology. Clinical signs of peripheral neuropathy (e.g. ataxia, limb weakness), central neuropathy (e.g. tremors) and autonomic signs (e.g. salivation) could be shown. Histopathologically, changes in the peripheral nervous system (e.g. Wallerian-like degeneration) and central nervous system (e.g. neuronal necrosis) were seen. The motor activity device was able to show both increased and decreased activity. The inter-observer reliability of the technicians performing FOBs was proven. Thus, the ability of the methods used to detect signs of neurotoxicity was demonstrated.

Positive control studies employed single or repeated administration of the following neurotoxicants: 3,3-Iminodipropionitrile, Carbaryl, Nomifensin, Diazepam, Acrylamide and Trimethyltinchloride, Carbaryl (1-Naphtylmethylcarbamat). Study summaries are attached to the report.

III. CONCLUSIONS

Single oral gavage of metiram to rats at dose levels of 0, 125, 500 and 2000 mg/kg bw did not result in any signs of general systemic. Moreover, regarding functional observational battery as well as measurement of motor activity, no test substance-related findings were noted. Additionally, no treatment-related neuropathological findings were noted, i.e. no brain weight changes or neurohistopathological findings were observed.

Based on the afore mentioned findings the NOAEL for neurotoxicity and systemic toxicity was 2000 mg/kg bw in males and female rats.

Subchronic neurotoxicity studies:

The potential neurotoxic effects of metiram have been investigated within the scope of a 3-month rat study (BASF DocID 1992/11224) and in a 12-month dog study (BASF DocID 1991/10786). The rat study included a functional observational battery as well as special neurohistopathology (for details please refer to chapter CA 05.03 of this dossier). In the dog study (for details please refer to chapter CA 05.03 of this dossier) too, clinical neurological examinations as well as neurohistopathology were carried out. The results of these studies demonstrated, that metiram did not have a neurotoxic effect and did not cause damage to the central nervous system.
A new 90-day neurotoxicity study in rats was carried out with metiram and is described below.

Report:	CA 5.7.1/2 2014 BAS 222 29 F (Metiram TK) - Repeated dose 90-day oral neurotoxicity study in Wistar rats - Administration via the diet 2014/1315300
Guidelines:	OECD 424, EPA 870.6200, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.43
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

The aim of this study was to determine the effect of metiram on neurotoxicity to rats after administration via the diet for 90 days. The test substance was administered to groups of 10 male and female Wistar rats at dietary concentrations of 40, 200, and 900 ppm, corresponding to 2.6, 13, and 59 mg/kg bw/day for males and 3.6, 17, and 71 mg/kg bw/day for females. Clinical signs were recorded daily and functional observational battery (FOB) and measurements of motor activity, forelimb and hindlimb grip strength and hindlimb landing footsplay were made before the start of dosing and on study days 1, 22, 50 and 85. Five animals per sex and test group were fixed by *in situ* perfusion and subjected to neuropathological examinations. The remaining five animals were sacrificed and assessed by gross pathology, followed by organ weight determinations and histopathological examinations. Thyroid hormone levels were also determined in the serum of these animals.
The oral administration of metiram over a period of 3 months revealed signs of systemic toxicity at a concentration in the diet of 900 ppm taking into account impaired body weight development and pathological findings (thyroid follicular hypertrophies) in female animals as well as decreased T4 levels in animals of both sexes. No adverse neurobehavioral effects were observed in male and female Wistar rats at any concentration. In addition, no test substance-related effects were observed in the neurohistopathological investigation at any concentration.

Under the conditions of this study the no observed adverse effect level (NOAEL) for neurotoxicity was 900 ppm for male (59 mg/kg bw/d) and female animals (71 mg/kg bw/d).
The NOAEL for systemic toxicity was 900 ppm (59 mg/kg bw) for males (13 mg/kg bw/d) with a NOEL of 200 ppm (13 mg/kg bw) based on decreased T4 levels. The NOAEL for female animals was (17 mg/kg bw/d) based on decreased body weights and thyroid effects.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 222 29 F (Metiram TK)
Description: solid / beige
Lot/Batch #: 300015
Purity: 91.5%
Stability of test compound: The test substance was stable over the test period as guaranteed by the sponsor (expiry date 31 Jan 2016).

2. Vehicle: diet was used as carrier

3. Test animals:
Species: Rats
Strain: Crl:WI(Han)
Sex: Male and female
Age: 48-50 days (start of administration)
Source: [redacted]
Acclimation period: about 14 days
Diet: Ground Kliba mouse/rat maintenance diet "GLP", meal, supplied by Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water: drinking water, ad libitum
Housing: in groups (5 animals per cage) in polysulfonate cages (H-Temp.), TECNIPLAST, Hohenpeissenberg, Germany single housing during FOB and MA in Makrolon cages (type MIII)

Environmental conditions:
Temperature: 20 - 24°C
Humidity: 30 - 70%
Air changes: 15/h
Photo period: 12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 29-July-2014 - 14-Nov-2014

2. Animal assignment and treatment:
Metiram was administered to groups of 10 male and 10 female animals at dietary concentrations of 0, 40, 200 and 900 ppm for 13 weeks. The dietary concentrations of BAS 222 29 F resulted in doses of 2.6, 13, and 59 mg/kg bw/day for males and 3.6, 17, and 71 mg/kg bw/day for females.

On the day of arrival the animals were subjected to an acclimatization period during which they received ground food and drinking water ad libitum. Prior to the first functional observational battery the animals were distributed according to weight among the individual test groups, separated by sex. The weight variation of the animals used did not exceed 20 percent of the mean weight of each sex. The list of randomization instructions was compiled with a computer.

In order to balance the groups for functional observational batteries and motor activity, the study was conducted with several subsets (Section A males and Section A females = first 5 animals of each test group; Section B males and Section B females = second 5 animals of each test group). For functional observational batteries and measurements of motor activity, the animals were tested in randomized order. Thus, it was ensured, that

- all animals were examined within the same day after start of test substance administration. Time of testing was therefore identical for all animals;
- for each examination day animals from all test groups (including controls) could be used;
- the examinations for all subsets could be performed on the same time of the day, thus potential diurnal effects could be negated.

3. Test substance preparation and analysis:
The stability of metiram in the diet over a period of 42 days at a concentration of 50 ppm was verified before the study (project No. 09L00361).
The stability of metiram in the diet at room temperature at a concentration of 40 ppm for a period of 35 days was verified during the administration period.

Homogeneity analyses of the test substance preparations were performed in samples of the highest and lowest concentrations at the start of the administration period. These samples also served for concentration control analyses. In samples of the mid concentration only concentration control analyses were performed.

4. Statistics:
Means and standard deviations (S.D.) of each test group were calculated for several parameters.
In addition the following statistical analyses were carried out:

Parameters	Statistical test	Markers in the tables	References
Body weight, body weight change	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means	* for p ≤ 0.05 ** for p ≤ 0.01	DUNNETT, C.W. (1955): A multiple comparison procedure for comparing several treatments with a control. JASA, Vol. 50, 1096-1121 DUNNETT, C.W. (1964). New tables for multiple comparisons with a control. Biometrics, Vol. 20, 482-491
Rearing, grip strength forelimbs, grip strength hindlimbs, foot-splay test, motor activity	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians	* for p ≤ 0.05 ** for p ≤ 0.01	SIEGEL, S. (1956): Non-parametric statistics for the behavioural sciences. McGraw-Hill New York

C. METHODS

1. Observations:

The animals were examined for clinical changes and for abnormal pattern of behavior daily. A check for moribund and dead animals was performed twice daily (from Monday to Friday) or once daily at weekends or public holidays.

2. Body weight and body weight change:

Body weight was determined before the start of the administration period and thereafter in weekly intervals. The difference between the body weight on the respective day of weighing and the body weight on day 0 was calculated as body weight change.

3. Food consumption and compound intake:

Individual food consumption was determined in weekly intervals (as representative value over 3 days) and calculated as mean food consump

4. Detailed clinical observations:

All animals were subjected to detailed clinical observations (DOC) on day 0 and weekly thereafter.

DOC includes the following examinations:

1. Abnormal behavior in handling
2. Fur
3. Skin
4. Posture
5. Salivation
6. Respiration
7. Activity/arousal level
8. Tremors
9. Convulsions
10. Abnormal movements
11. Gait abnormalities
12. Lacrimation
13. Palpebral closure
14. Exophthalmus
15. Assessment of the feces discharged during the examination (appearance, consistency)
16. Assessment of the urine discharged during the examination
17. Pupil size

5. Functional observational battery:
The functional observational battery (FOB) was carried out in all animals on study days -7, 1, 22, 50, and 85. At least one hour before the start of FOB the animals were transferred singly to polycarbonate cages. Drinking water but no food was provided ad libitum.

Home cage observations
The animals were observed in the rack for 10-30 seconds in their cages with the lids closed. The following parameters were determined:

- Posture
- Tremors
- Convulsions
- Abnormal movements
- Gait

Open field observations
The animals were observed in a standard arena for the following parameters:

Behaviour on removal from cage	Lacrimation	Tremors	Feces excreted within 2 min (number, appearance, consistency)
Fur	Eyes, pupil size	Convulsions	Urine excreted within 2 minutes (amount, color)
Skin	Posture	Abnormal movements/stereotypies	Rearing within 2 min
Salivation	Palpebral closure	Gait	
Nasal discharge	Respiration	Activity/arousal level	

<u>Sensory motor tests/reflex tests</u>
The animals were removed from the open field and were subjected to the following examinations:

<table>
<tr><td>Approach response</td><td>Pinna reflex</td><td>Vocalisation</td><td>Grip strength of hindlimbs</td></tr>
<tr><td>Touch response</td><td>Auditory startle response)</td><td>Pain perception (Tail pinch)</td><td>Behaviour during handling</td></tr>
<tr><td>Visual placing response</td><td rowspan="2">Righting response</td><td rowspan="2">Grip strength of forelimbs</td><td rowspan="2">Landing foot-splay test</td></tr>
<tr><td>Pupillary reflex</td></tr>
</table>

6. Measurement of motor activity:
Measurement of motor activity was carried out in all animals on study days -7, 1, 22, 50, and 85. The examinations were performed using the TSE Labmaster System and 18 beams located per cage. The number of beam interruptions was counted over 12 intervals for 5 minutes in each case. No food or water was provided during the measurements.

7. Clinical pathology:
Blood samples were taken from fasted animals by puncturing the retrobulbar venous plexus under isoflurane anesthesia. Hormone levels of T3, T4, and TSH were determined.

8. Urinalysis:
Urine was sampled for the analysis of certain metabolites, i.e. ethylenethiourea (ETU) and ethyleneurea (EU). The sampling procedure was carried out in all animals of all test groups. In the afternoon preceding the day fixed for urine sampling, the animals were transferred individually into metabolism cages (no food or drinking water provided). Urine was sampled at 2-8°C overnight. On the following morning, the individual urine specimens were frozen and sent to the analytical laboratory.

9. Sacrifice and pathology:
Animals scheduled for perfusion fixation
Five males and five females of each group were subjected to deep anesthesia with isoflurane and sacrificed by perfusion fixation. The animals fixed by perfusion were necropsied with regard to the question of neuropathology.

Absolute and relative brain (without olfactory bulb) weights were determined.

The following organs/tissues were preserved in neutrally buffered 4% formaldehyde solution:
- Brain
- Spinal cord (sections from cervical and lumbar cords)
- All gross lesions

<u>Paraplast embedding and preservation</u>
The following organs/tissues were sampled:

Organ samples :	**Test group**			
	0	**1**	**2**	**3**
Brain (cross sections):				
• Frontal lobe	A1	B1	B1	A1
• Parietal lobe with diencephalon	A1	B1	B1	A1
• Midbrain with occipital and temporal lobe	A1	B1	B1	A1
• Pons	A1	B1	B1	A1
• Cerebellum	A1	B1	B1	A1
• Medulla oblongata	A1	B1	B1	A1
• Eyes with retina and optical nerve	A1	F1	F1	A1
Spinal cord (cross and longitudinal sections):				
• Cervical cord (C3–C6)	A1	F1	F1	A1
• Lumbar cord (L1–L4)	A1	F1	F1	A1
Peripheral nervous system:				
• Gasserian ganglia with nerve	A1	F1	F1	A1
• Gastrocnemius muscle	A1	A1	A1	A1
All gross lesions	A2	A2	A2	A2

A = hematoxylin and eosin (HE) stain
B = Paraplast embedding
F = preservation in 4% formaldehyde
1 = all perfused animals/test group
2 = all animals affected/test group

Plastic embedding and storage

The following organ specimens were removed after perfusion. The histotechnical processing, examination by light microscopy and assessment of findings was performed according to the table below:

Organ samples:	Test group			
	0	1	2	3
Peripheral nervous system:				
• Dorsal root ganglion, (3 of C3–C6)	T1	P1	P1	T1
• Dorsal root fiber (C3-C6)	T1	P1	P1	T1
• Ventral root fiber (C3-C6)	T1	P1	P1	T1
• Dorsal root ganglion, (3 of L1-L4)	T1	P1	P1	T1
• Dorsal root fiber (L1-L4)	T1	P1	P1	T1
• Ventral root fiber (L1-L4)	T1	P1	P1	T1
• Proximal sciatic nerve *(longitudinal and cross sections)*	T1	P1	P1	T1
• Proximal tibial nerve (at knee) *(longitudinal and cross sections)*	T1	P1	P1	T1
• Distal tibial nerve (at lower leg) *(longitudinal and cross sections)*	T1	P1	P1	T1
• Tibial nerve (rami musculares distales to M. gastrocnemius) *(cross sections)*	T1	P1	P1	T1

P = Storage of fixed specimen in buffer solution
T = Plastic embedding (epoxy resin), semithin sectioning and staining with Azure-II-Methylene blue-basic fuchsin (AMBF)
1 = all perfused animals/test group

After completion of the histopathological assessment by the study pathologist and the internal peer review an external peer review was performed by Prof. Dr. Peter Spencer (Oregon Health Sciences University, USA) including cervical and lumbar spinal cord, musculus gastrocnemius, cervical and lumbar spinal ganglia, cervical and lumbar dorsal and ventral root fibers, proximal and distal tibial nerve cross sections and rami musculares of the tibial nerve of control and test group 3 female animals. No treatment-related findings were noted.

Animals not scheduled for perfusion fixation
The animals were sacrificed by decapitation under isoflurane anesthesia and were assessed by gross pathology.
Weights of the anesthetized animals and of the thyroid glands were determined. Furthermore, thyroid glands were fixed in 4% formaldehyde solution and H&E stain of the thyroid glands of animals were examined histopathologically.

Additional examinations

- Measurement of thyroid hormones (T3, T4 and TSH) was carried out in the serum samples taken at final sacrifice in each 5 male and female animals of all dose groups.
- Urine was sampled for the analysis of potential metabolites, i.e. ethylenethiourea (ETU) and ethyleneurea (EU). The sampling procedure will be carried out individually, by transferring the animals to metabolism cages (no food or drinking water provided). Urine will be samples at 2-8°C overnight. On the following morning, the individual urine specimens will be frozen and sent to the analytical laboratory.

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

The various analyses confirmed

- the stability of the test-substance preparations for a period of 35 days at room temperature,
- the homogeneous distribution of the test substance in the vehicle,
- the correctness of the prepared concentrations.

In addition, ethylenethiourea (ETU) and ethyleneurea (EU) could be detected and quantified in the urine samples of the treated animals of test groups 1-3 (40, 200 and 900 ppm).

B. OBSERVATIONS

1. Clinical signs of toxicity

No test substance-related, adverse findings were observed at none of study days. Especially also **no** hind limb weaknesses or ataxia were seen.

2. Mortality

No animal died prematurely during the study.

C. BODY WEIGHT AND BODY WEIGHT GAIN

No test substance-related effects on body weight development, i.e. mean body weight and body weight change values, were observed for male animas of test groups 1-3 (40, 200 and 900 ppm) as well as for female animals of test groups 1 and 2 (40 and 200 ppm).
Significantly lower mean body weight was observed for female animals of test group 3 (900 ppm) from study day 14 onwards until the end of the administration period, reaching a maximum deviation of -15% on study day 77. The same was true for mean body weight change values of female animals of test group 3 (900 ppm) showing a maximum deviation of -63% on study day 21. These deviations to the control were assessed to be related to treatment and adverse.

On the study days scheduled for FOB and MA measurements, female animals of test group 3 (900 ppm) had significantly lower body weights on study days 22 (-13%), 50 (-10%) and 85 (-13%) whereas for male animas of test groups 1-3 (40, 200 and 900 ppm) as well as for female animals of test groups 1 and 2 (40 and 200 ppm) no changes were observed. A tabulated summary of the body weight/body weight gain values are given in Table 5.7.1-5 below. As already stated, the deviations to the control were assessed to be related to treatment and adverse.

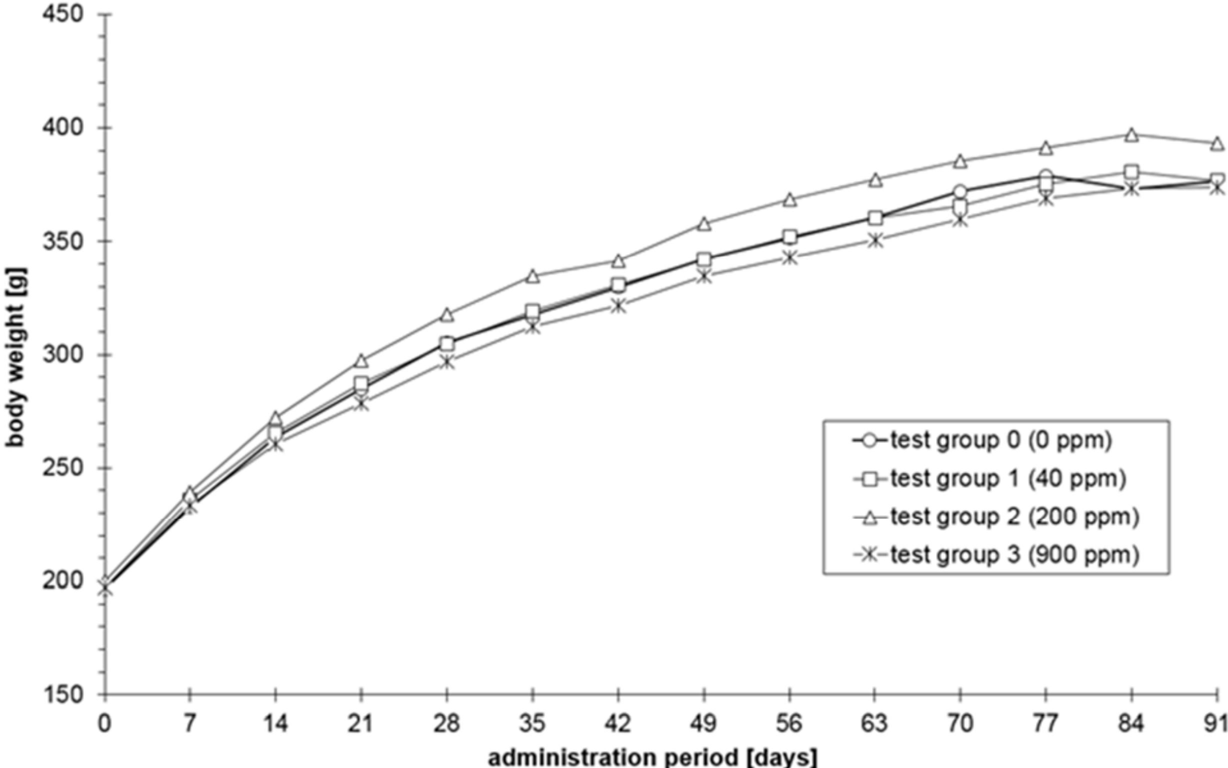


Figure 5.7.1-2: Mean body weights of male animals administered metiram for 90 days

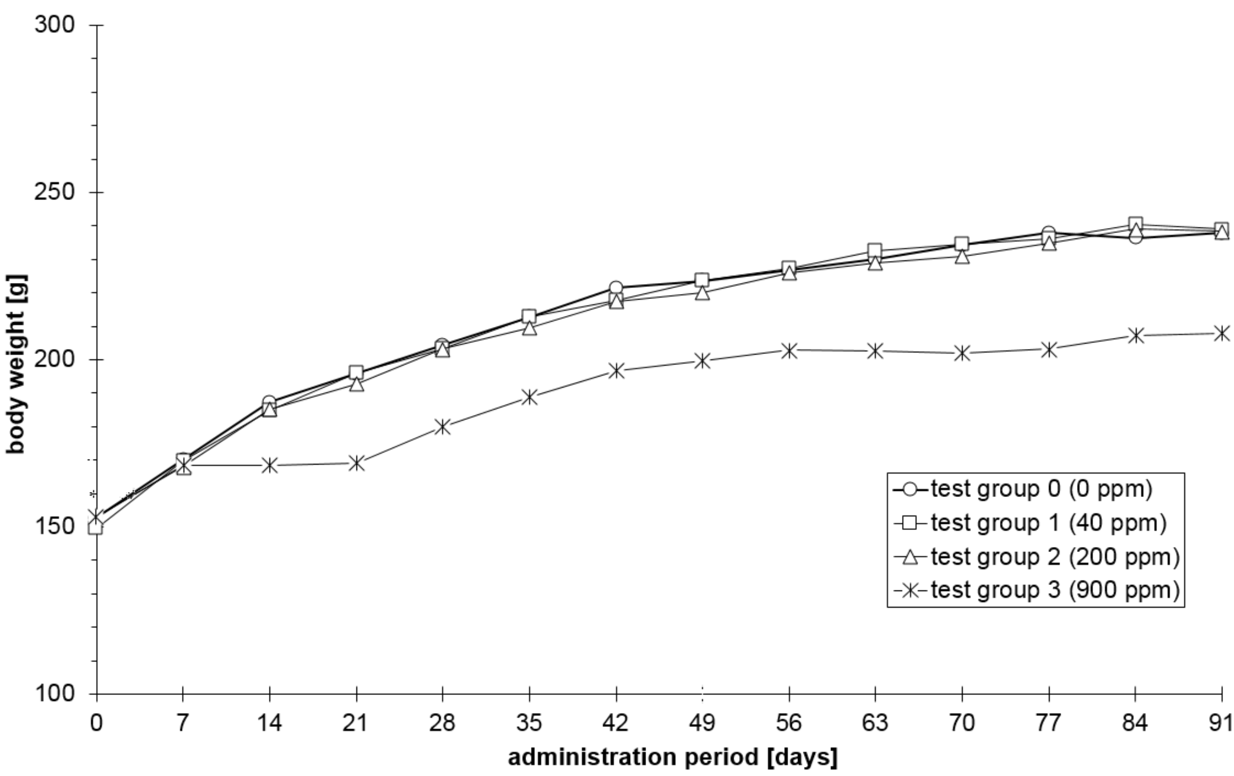


Figure 5.7.1-3: Mean body weights of female animals administered metiram for 90 days

Table 5.7.1-5: Body weight/body weight gain data of males and females administered metiram at the different study days and dose groups

Study day	Males [g] (SD)				Females [g] (SD)			
	Controls	40 ppm	200 ppm	900 ppm	Controls	40 ppm	200 ppm	900 ppm
0	196.6 (7.3)	197.4 (9.7)	199.9 (7.1)	197.2 (6.6)	152.7 (10.3)	149.7 (5.6)	153.4 (6.7)	153.1 (8.2)
7	232.9 (12.1)	235.8 (14.3)	239.4 (10.1)	233.2 (8.9)	170.1 (10.5)	169.8 (6.4)	168.0 (8.9)	168.4 (9.0)
14	263.3 (17.4)	265.3 (18.1)	272.4 (14.8)	260.6 (10.6)	187.2 (12.3)	184.9 (9.0)	185.2 (9.5)	168.6** (13.2)
21	284.8 (23.6)	287.0 (20.7)	297.5 (18.4)	278.6 (15.6)	195.9 (13.8)	196.1 (8.5)	192.8 (9.5)	169.2** (13.3)
28	305.2 (27.0)	304.4 (23.3)	317.8 (21.0)	296.8 (15.8)	204.3 (15.6)	203.4 (10.0)	203.2 (11.3)	180.1** (15.9)
35	317.4 (30.7)	319.2 (22.2)	334.4 (22.9)	312.5 (17.1)	212.7 (14.0)	212.8 (9.3)	209.4 (12.4)	188.8** (12.5)
42	329.7 (32.5)	330.9 (24.9)	341.5 (24.3)	321.6 (18.7)	221.4 (15.0)	217.8 (11.5)	217.4 (13.8)	196.8** (12.4)
49	342.1 (35.1)	342.1 (25.6)	358.0 (26.1)	334.7 (17.4)	223.6 (15.4)	223.7 (10.9)	220.1 (14.4)	199.7 (12.4)
56	351.5 (36.4)	352.0 (26.8)	368.3 (28.6)	343.0 (17.4)	226.9 (17.4)	227.4 (10.4)	226.1 (14.1)	202.9** (15.9)
63	360.3 (37.2)	360.1 (28.1)	377.4 (29.6)	350.7 (18.9)	230.0 (16.3)	232.5 (11.2)	229.0 (15.0)	202.8** (14.6)
70	371.9 (39.2)	365.6 (28.2)	385.6 (31.3)	359.7 (19.5)	234.4 (17.4)	234.5 (14.2)	231.0 (16.1)	202.1** (15.3
77	378.8 (38.6)	375.2 (27.8)	391.4 (31.7)	368.8 (18.6)	238.0 (17.9)	236.1 (12.8)	234.8 (15.3)	203.2** (14.0)
84	373.2 (37.7)	380.6 (29.3)	397.3 (31.9)	373.2 (19.0)	236.4 (17.1)	240.4 (13.4)	239.0 (15.0)	207.4** (14.0)
91	376.7 (39.4)	376.6 (28.5)	393.4 (34.1)	373.8 (20.6)	237.9 (16.3)	239.0 (12.6)	238.4 (15.2)	207.9** (11.0)

**p<0.01

D. FOOD/WATER CONSUMPTION AND SUBSTANCE INTAKE

No test substance-related, adverse findings were observed for male animas of test groups 1-3 (40, 200 and 900 ppm) as well as for female animals of test groups 1 and 2 (40 and 200 ppm).
Reduced food consumption was observed for female animals of test group 3 (900 ppm) from study days 14 to 42 and from study days 56 to 91. These deviations to the control were assessed to be related to treatment.
No overt changes with respect to water consumption were observed visually for animals treated with the test substance.

The approximate, mean daily test-substance intake in mg/kg bw/d over the entire study period is shown in the following table:

Test group	Concentration in the diet [ppm]	Mean daily test substance intake [mg/kg bw/d]	
		Males	Females
1	40	2.6	3.6
2	200	13	17
3	900	59	71

E. CLINICAL PATHOLOGY

Concerning clinical pathology the following treatment-related thyroid hormone value changes were observed in the blood samples from day 92:

Test group 1 (40 ppm):
No treatment-related changes of T3, T4 and TSH values were observed (see table below).

Test group 2 (200 ppm):
No treatment-related changes of T3, T4 and TSH values were observed (see table below).

Test group 3 (900 ppm):
Significantly decreased T4 values were observed in both sexes (see table below). The percent changes were -30% in males and -31% in females.

Table 5.7.1-6: Thyroid hormone changes in males and females measured at day 92

	Males				Females			
	Controls	**40 ppm**	**200 ppm**	**900 ppm**	**Controls**	**40 ppm**	**200 ppm**	**900 ppm**
T3 (SD) [nmol/l]	1.01 (0.10)	0.92 (0.12)	1.06 (0.13)	1.01 (0.06)	1.06 (0.12)	1.13 (0.14)	1.01 (0.08)	1.03 (0.08)
T4 (SD) [nmol/l]	70.46 (9.89)	60.67 (10.86)	68.20 (10.19)	49.12* (9.56)	43.73 (7.60)	55.33 (19.00)	40.03 (4.41)	30.36* (4.97)
TSH (SD) [µg/l]	5.08 (1.59)	4.73 (1.65)	5.41 (1.57)	5.71 (2.33)	3.47 (0.44)	5.04 (1.41)	4.72 (1.03)	4.65 (0.66)

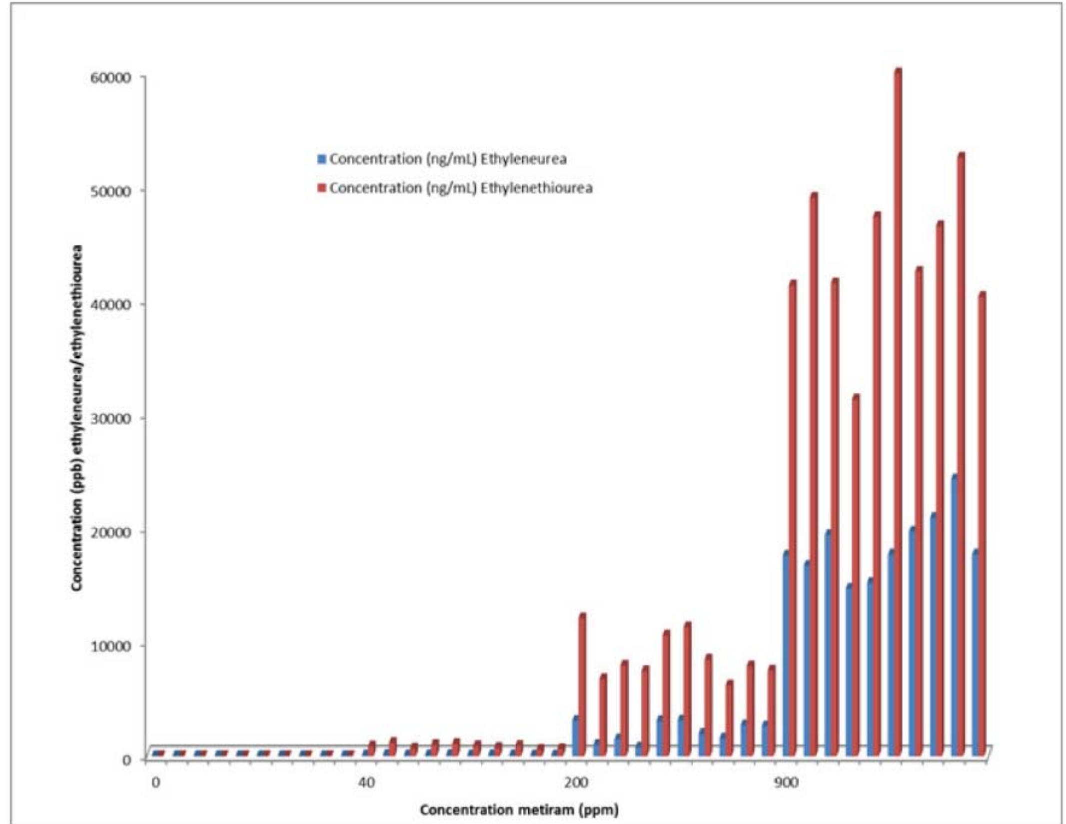
*$p \leq 0.05$

In conclusion, concerning clinical pathology parameters, the no observed effect level (NOEL) for this test compound in rats of both sexes is 200 ppm. However in males of the 900 ppm dose group (=59 mg/kg bw) the T4 value was the only changed parameter among three hormone level measurements and without anatomical pathological finding in the thyroids of these individuals. Therefore, in males of the top dose the T4 value decrease was regarded treatment-related but not adverse.

F. URINALYSIS

Based on the analytical results it can be concluded that ethylenethiourea (ETU) and ethyleneurea (EU) could be detected and quantified in the urine samples of the treated animals of test groups 1-3 (40, 200 and 900 ppm).

Clear dose-responses are seen for the urinary levels of ethylene urea and ethylene thiourea in male and female rats (see Figure below).



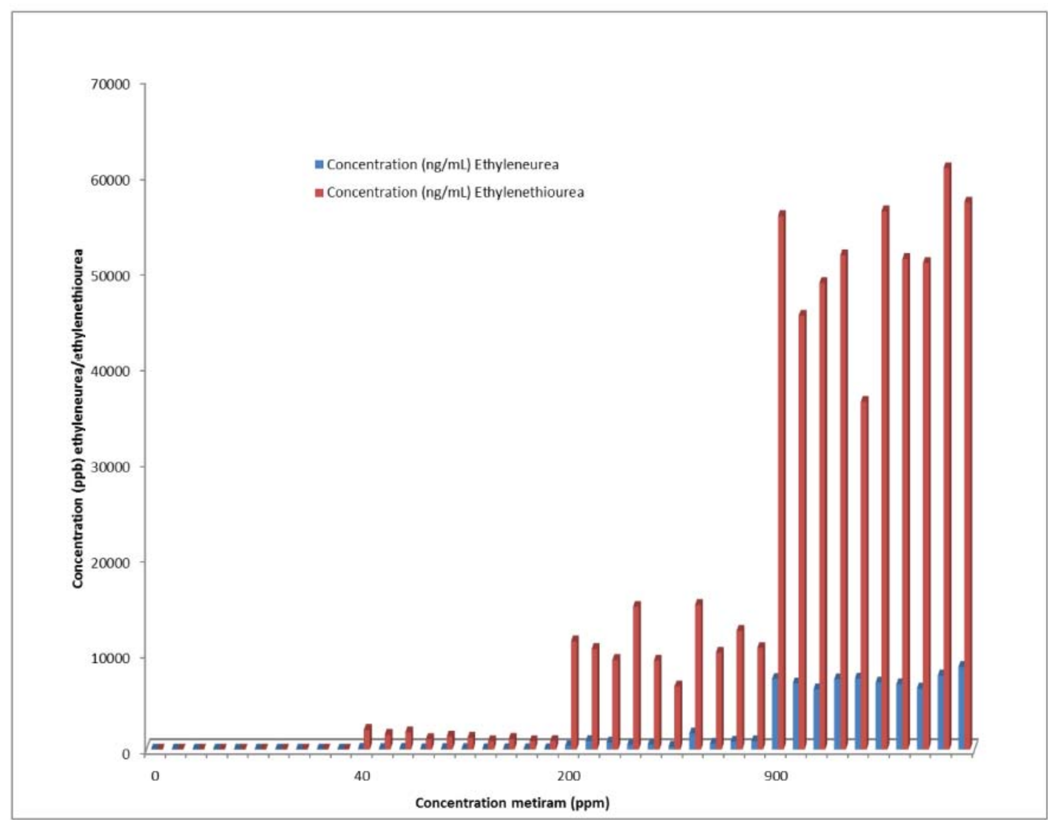


Figure 5.7.1-4: Concentrations of EU and ETU in urine of male (top) and female (bottom) rats

G. FOB

Home cage observations:
No test substance-related, adverse findings were observed for treated animals of test groups 1-3 (40, 200 and 900 ppm) on study days 1, 22, 50 and 85.

Open field observations:
No test substance-related, adverse findings were observed for treated animals of test groups 1-3 (40, 200 and 900 ppm) on study days 1, 22, 50 and 85.

Sensorimotor tests/reflexes:
No test substance-related, adverse findings were observed for treated animals of test groups 1-3 (40, 200 and 900 ppm) on study days 1, 22, 50 and 85.

Quantitative parameters:
No test substance-related effects were observed for male animals of test groups 1-3 (40, 200 and 900 ppm) as well as for female animals of test groups 1 and 2 (40 and 200 ppm). The table below summarizes the measurements of fore limb grip strengths (GSF), hind limb grip strengths (GSH) and foot splay test (FST).

Table 5.7.1-7: Fore limbs (GSF) and hind limbs (GSH) grip strengths and foot splay test (FST) values at different time points measured in males and females (each 10 animals/sex)

	GSF [Nm]					GSH [Nm]					FST [cm]				
Doses [ppm]	Day -7	Day 1	Day 22	Day 50	Day 85	Day -7	Day 1	Day 22	Day 50	Day 85	Day -7	Day 1	Day 22	Day 50	Day 85
	Males														
0	6.0 (1.2)	6.6 (1.3)	8.0 (1.5)	10.3 (2.2)	8.1 (1.7)	4.1 (0.7)	5.5 (0.6)	7.1 (1.0)	9.3 (1.2)	9.7 (1.7)	7.4 (0.7)	9.1 (0.9)	9.4 (1.1)	10.2 (0.9)	10.9 (1.8)
40	5.3 (1.3)	6.7 (1.2)	8.5 (1.1)	8.9 (2.5)	9.1 (2.7)	4.1 (0.8)	5.3 (0.8)	7.7 (1.6)	8.7 (1.7)	9.0 (1.7)	7.9 (1.4)	9.3 (1.5)	10.3 (1.3)	10.7 (1.5)	10.7 (1.1)
200	5.7 (1.2)	6.3 (1.2)	8.4 (1.0)	9.9 (1.4)	8.5 (2.1)	4.2 (0.4)	5.3 (1.0)	7.7 (1.4)	9.2 (1.6)	9.5 (2.1)	8.1 (1.8)	9.2 (1.5)	9.4 (0.9)	9.9 (1.1)	10.2 (1.2)
900	5.8 (1.0)	6.3 (1.1)	8.5 (1.5)	9.0 (2.4)	8.3 (2.8)	4,3 (0.5)	5.2 (0.4)	7.2 (1.0)	8.7 (1.3)	9.0 (1.6)	8.5 (0.8)	9.6 (1.3)	10.5 (1.5)	10.9 (1.0)	10.7 (1.5)
	Females														
0	6.1 (0.3)	7.1 (0.6)	8.1 (1.1)	6.9 (1.8)	7.8 (1.3)	3.4 (0.5)	4.3 (0.7)	5.4 (1.1)	5.6 (0.6)	6.1 (1.0)	8.6 (0.9)	9.3 (1.2)	9.6 (1.0)	9.7 (1.3)	9.9 (0.9)
40	5.9 (0.5)	6.7 (0.9)	8.1 (0.8)	7.0 (1.7)	7.5 (1.2)	3.4 (0.5)	3.9 (0.8)	4.9 (0.7)	5.1 (1.0)	5.4 (0.8)	8.5 (0.9)	8.9 (1.0)	9.1 (0.7)	9.6 (1.3)	10.6 (1.3)
200	5.5 (1.1)	7.0 (0.7)	7.6 (2.0)	7.3 (1.0)	7.6 (1.3)	3.5 (0.6)	4.2 (0.6)	5.3 (0.7)	5.2 (1.0)	6.0 (1.0)	8.0 (0.9)	8.7 (0.7)	8.9 (0.9)	9.5 (1.1)	9.8 (1.7)
900	6.0 (0.5)	7.1 (1.0)	4.7** (1.3)	6.8 (0.8)	6.3** (1.1)	3.1 (0.2)	4.2 (0.7)	3.4** (0.8)	5.1 (0.6)	5.1 (0.7)	8.9 (1.4)	9.4 (1.0)	8.6 (1.1)	10.1 (1.4)	9.7 (1.3)

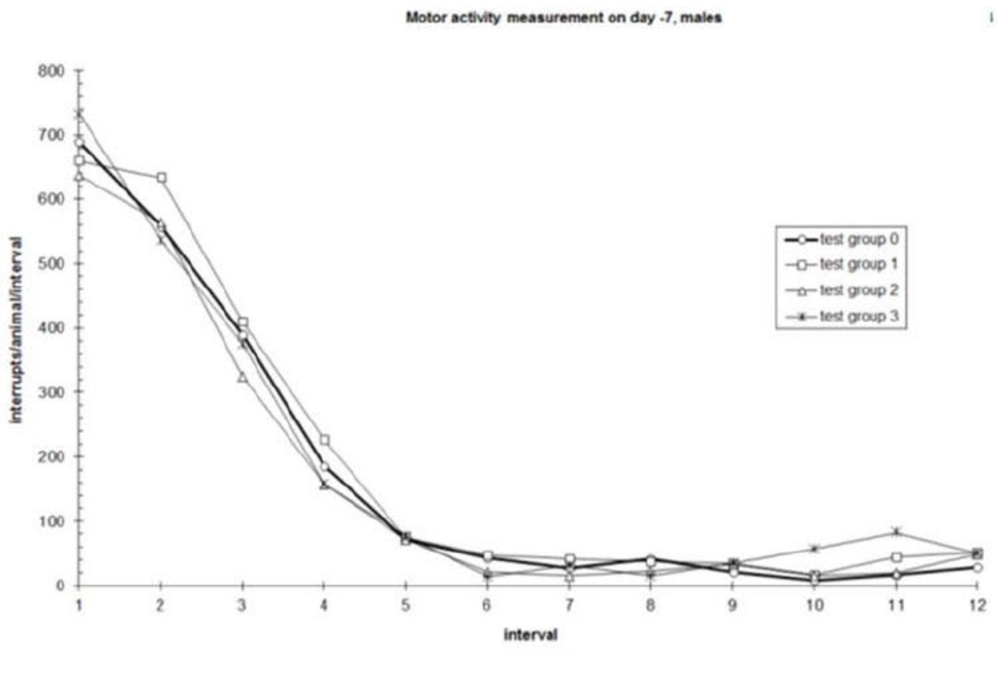
*p<0.01

In female animals of test group 3 (900 ppm), grip strength of fore limbs was significantly reduced on study days 22 and 85 (max. reduction of -42.4% on study day 22). Grip strength of hind limbs was reduced on study days 22 (-37%, significantly) and 85 (-17%, not significantly). These changes were assumed to be related to the reduced body weight rather than to neurofunctional changes. Thus, the findings were assessed as being treatment-related but only caused by the impaired overall condition in female animals of test group 3 (900 ppm). This assessment is further supported by a detailed look into the body weight data presented in Table 5.7.1-5, where the females of the top dose group gain almost no body weight between the time points 7 and 21, which is closest to study day 22, where the grip strengths of fore and hind limbs was affected most. Also the fact, that fore and hind limb grip strengths were equally affected supports the assessment of a general body-weight-related effect. Further the overall number of rearings was not affected at none of the timepoints assessed.

H. MOTOR ACTIVITY

Regarding the overall motor activity as well as single intervals, no test substance-related deviations were noted for male and female rats of test groups 1-3 (40, 200 and 900 ppm) when compared to the control animals.
On study day 22, interval 5 was significantly increased in male animals of test group 2 (200 ppm) and on study day 85, the values of interval 6 were significantly increased in female animals of test groups 3 (900 ppm). These findings were assessed to be incidental because no dose-response relationship and no changes in overall activity were observed at any day of examination.

Figure 5.7.1-5: Motor activity measurement (day -7) of male and female animals before start of treatment



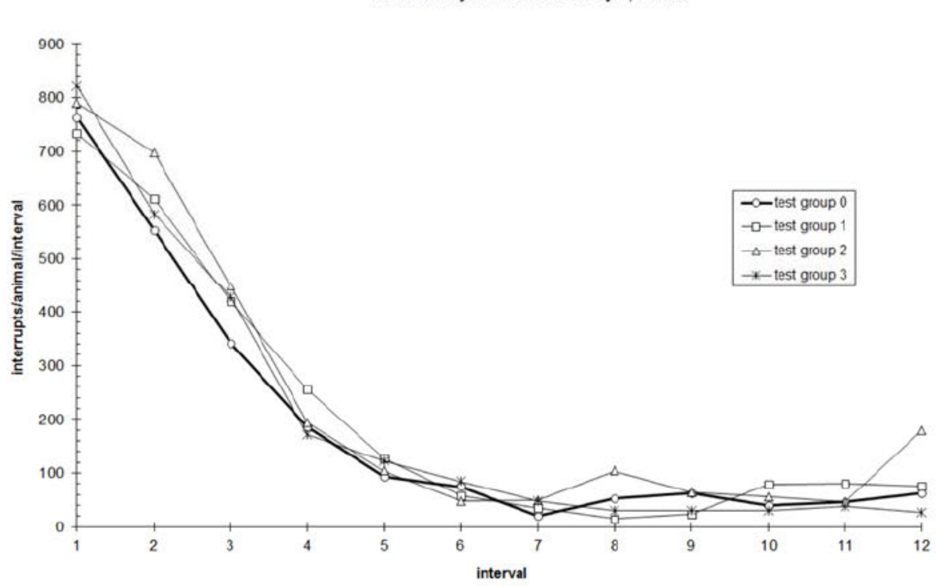


Figure 5.7.1-6: Motor activity measurement of male animals during treatment (days 1, 22, 50, 85)

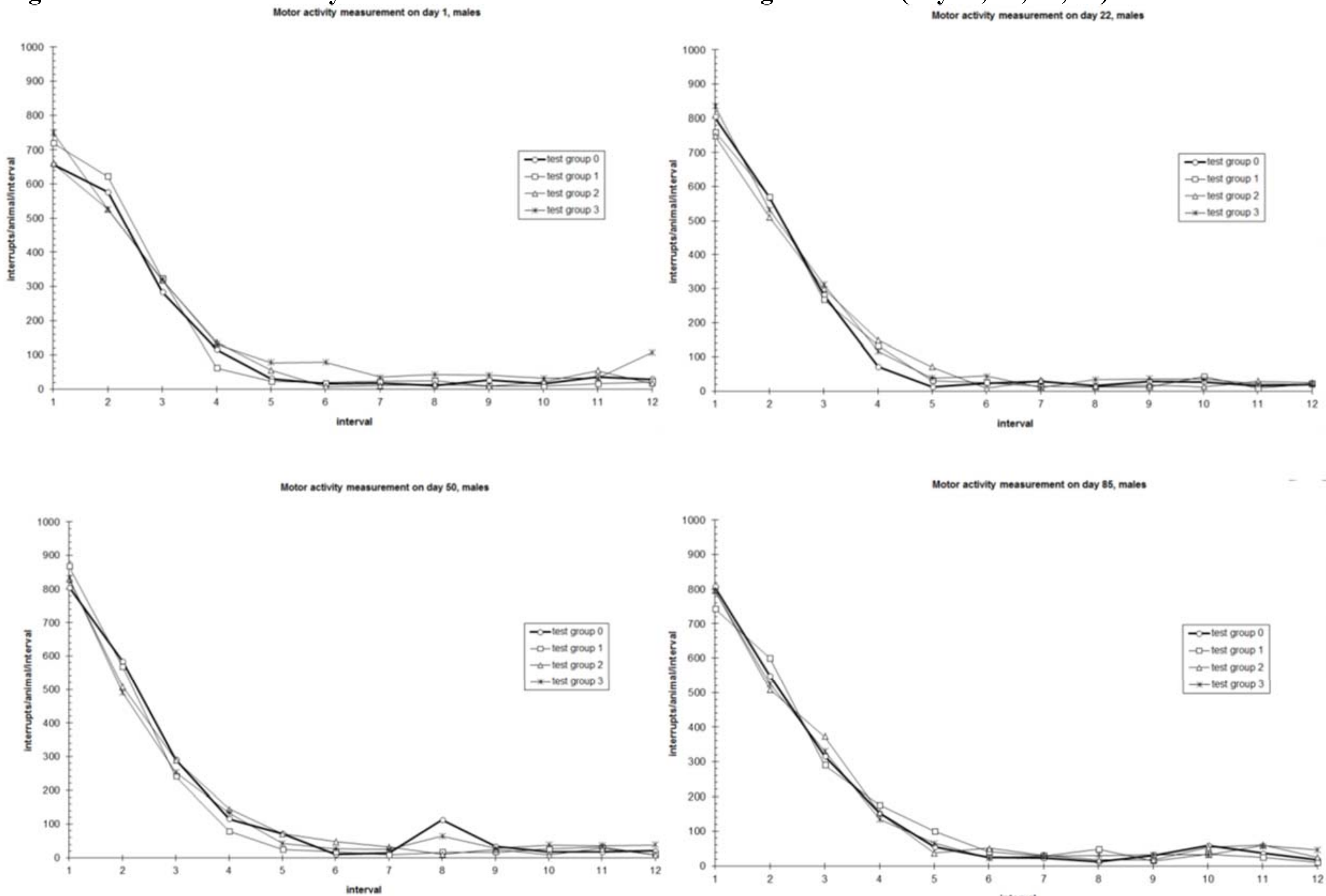
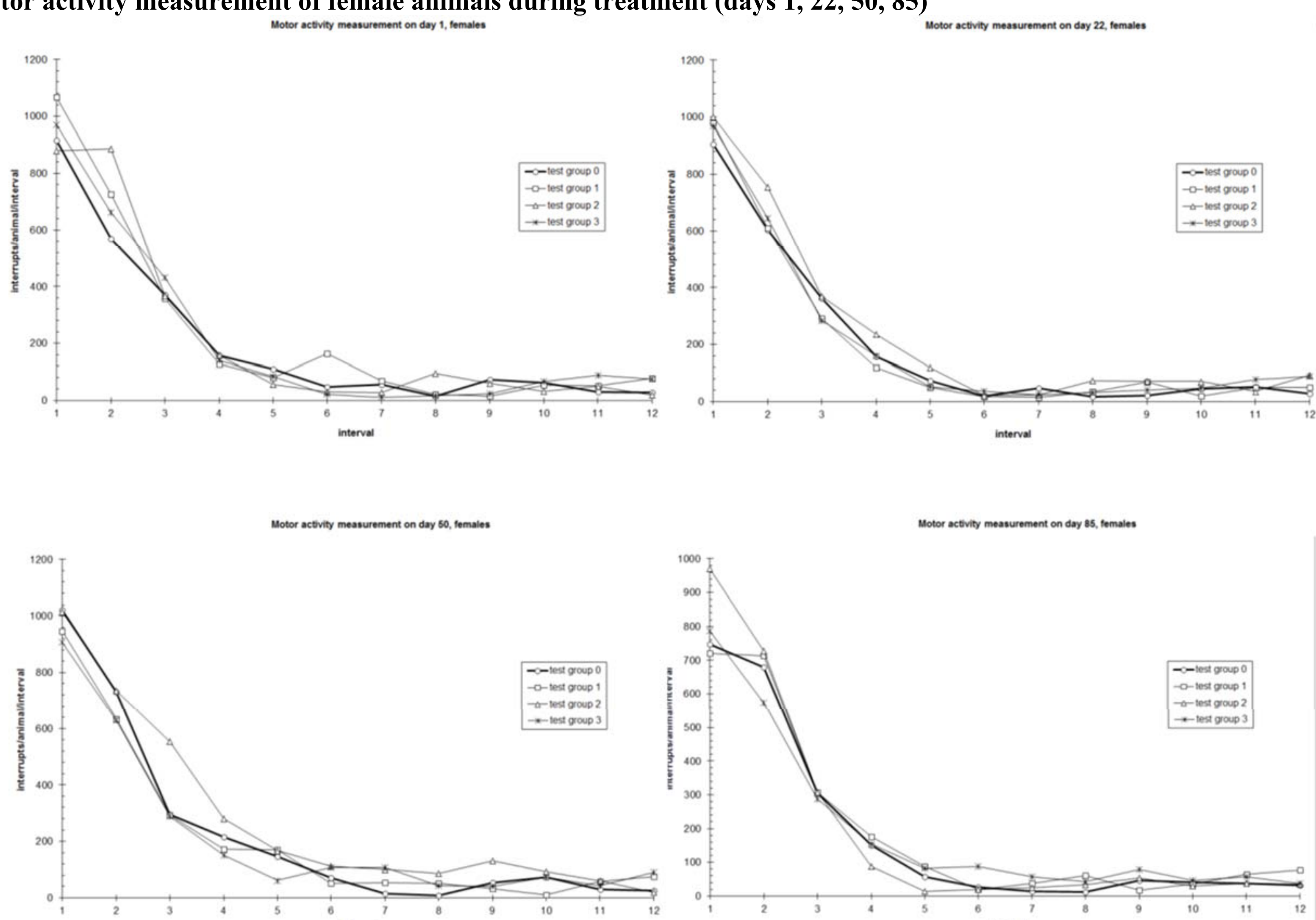


Figure 5.7.1-7: Motor activity measurement of female animals during treatment (days 1, 22, 50, 85)



I. NECROPSY FINAL SACRIFICE GROUP

1. Weight parameters

Absolute weights

When compared with control group 0 (=100%), the terminal body weight of test group 3 (900 ppm) females was significantly decreased (-14%, $p \leq 0.05$), which was considered to be treatment-related.

None of the other mean absolute weight parameters showed significant differences when compared to the control group 0.

Relative organ weights

When compared with control group 0 (=100%), the mean relative organ weight of the thyroid glands was significantly increased in female animals of test group 3 (900 ppm) (+41%, $p \leq 0.05$). This was considered to be treatment-related.

None of the other mean relative weight parameters showed significant differences when compared to the control group 0.

Table 5.7.1-8: Absolute and relative thyroid weights of males and females (Mean ± SD#)

Dose group [ppm]		0	40	200	900
	Males				
Terminal bw [g]	absolute	361 ± 43	351 ± 27	379 ± 35	356 ± 13
Thyroid [g]	absolute	21.4 ± 4.39	22.0 ± 4.06	22.2 ± 3.70	22.8 ± 4.55
	relative	0.006 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.006 ± 0.001
	Females				
Terminal bw [g]	absolute	221 ± 21	225 ± 10	221 ± 19	**189 ± 11***
Thyroid [g]	absolute	17.0 ± 1.0	19.0 ± 5.87	16.4 ± 3.78	20.8 ± 3.56
	relative	0.008 ± 0.001	0.008 ± 0.001	0.007 ± 0.002	**0.011 ± 0.001***

#: numbers were rounded and thus may not exactly reflect the numbers given in the study report
*: $p \leq 0.05$

2. Gross lesions

No macroscopic lesions were recorded.

3. Histopathology

No test substance-related, adverse findings were observed in male and female animals of test groups 1-3 (40, 200 and 900 ppm), except one female animal of test group 3 (900 ppm) which showed moderate hypertrophy/hyperplasia of follicular cells of the thyroid gland.

J. NECROPSY NEUROTOXICITY PART

1. Weight parameters

When compared with control group 0 (=100%), the terminal body weight of test group 3 (900 ppm) females was significantly decreased (-13%, $p \leq 0.01$), which was considered to be treatment- related.

None of the other mean absolute weight parameters showed significant differences when compared to the control group 0.

There were no changes in relative organ weights.

2. Gross lesions

No macroscopic lesions were recorded.

3. Histopathology

No neuropathological findings were noted. This was confirmed by an external reviewer.

III. CONCLUSIONS

In conclusion, the oral administration of metiram over a period of 3 months revealed signs of systemic toxicity at a concentration in the diet of 900 ppm taking into account impaired body weight development and pathological findings in the thyroid of female animals as well as decreased T4 levels in animals of both sexes. No adverse neurobehavioral effects were observed in male and female Wistar rats at any concentration. The observed lower grip strengths in females at the top dose at study days 22 and 85 are considered to be related to lower body weights and not an indicator for a specific neurotoxic effect. In addition, no test substance-related effects were observed in the neurohistopathological investigation at any concentration.
Under the conditions of this study the no observed adverse effect level (NOAEL) for neurotoxicity was 900 ppm for male (59 mg/kg bw/d) and female animals (71 mg/kg bw/d).
The NOAEL for systemic toxicity was 900 ppm for male (59 mg/kg bw/d) and 200 ppm for female animals (17 mg/kg bw/d).

CA 5.7.2 Delayed polyneuropathy studies

As metiram does not belong to a chemical class suspected to induce delayed neuropathies, no study is considered to be necessary and thus no further study was conducted.

CA 5.8 Other Toxicological Studies

Toxicological studies have been conducted with a number of metabolites of metiram. An overview over the available studies and the nature of the metabolites is given in the following table:

CA 5.8.1 Toxicity studies of metabolites

Studies evaluated in the draft monograph of rapporteur member state Italy of July 2000:

During Annex I inclusion the following metabolites of metiram were evaluated: ethylene thiourea (ETU), ethylene urea (EU) and ethylenebisisothiocyanate sulphide (EBIS). Studies submitted for Annex I listing consisted of a complete set of toxicity studies for ETU, a carcinogenicity study for EU and a 90-day oral feeding study for EBIS. These already peer reviewed studies are summarized below and the following new studies for the metabolites are conducted:

Table 5.8.1-1: Overview of new studies conducted with metiram metabolites,

Metabolite Reg.No./Cas.No.	Studies available	DocID
Ethylene thiourea (ETU)	In vitro comparative metabolism study	2005/1043820, 2015/1161955
146099/96-45-7	Acute toxicity	2001/1014630, 20031029080, 2002/1005548, 2003/1029079
	Skin/eye irritation	2001/1014631, 20031029082, 2001/1014629, 2003/1029081
	Extended-One-Generation toxicity	2013/7002198
	Teratogenicity in rabbits	2010/1050667, 2010/1050668, 2010/1050669
	Immunotoxicity	2010/1050669
Ethylene urea (EU) 27270/120-93-4	Acute toxicity	2011/1293133, 2012/1360603
	Skin and eye irritation	2012/1360608, 2011/1293135
	Skin sensitization	2009/1129202
	Genotoxicity	2012/1360606
	28-day study	2002/1026377
	Analogue approach justification	~~2013/1065851~~ 2016/1321628
	OECD 422 study (Studies conducted under REACH (Tier 2)	2013/1089127
EBIS 243959/ 33813-20-6	Genotoxicity (Ames, In vitro micronucleus)	2014/1134381, 2014/1172991
	Reproduction and subchronic rat study available and summarized	
Jaffe´s base 6002546/484-92-4	Genotoxicity (Ames, In vitro micronucleus)	2014/1315335, 2014/1315332

Metabolite Reg.No./Cas.No.	Studies available	DocID
TDIT 4670450/-	Genotoxicity (Ames, In vitro micronucleus)	2015/1042058, 2014/1315331
Ethylene diamine (EDA) 4183259/107-15-3	Full set of toxicological studies available (HPV chemical) and summarized	

Table 5.8.1-2: Overview of literature data derived with metiram metabolites

Metabolite Reg.No./Cas.No.	Peer reviewed literature	DocID
Ethylene thiourea (ETU)	Reproductive toxicity	2013/1419083
146099/96-45-7	Developmental toxicity	2012/1368184
	Mechanistic studies	2006/1051537, 2011/1297251, 2013/1419082, 2005/1043780, 2006/1051535, 2006/1051534, 2002/1027618, 2006/1051537, 2014/1323273, 2011/1293971, 2012/1362403, 2004/1040994, 2004/1040995
Hydantoin	Genotoxicity (Ames)	1992/1005457

Based on the available data the following assessment was drawn in the Annex I listing of metiram:

Other toxicological studies	Metabolite EBIS: oral LD50 in rats: 240 mg/kg bw; effects on thyroid, evidence of neurotoxicity in rats and mice.

Presence of Structural alerts – QSAR evaluation of metabolites

For all metabolites where no relevant toxicological information on the compound was available, presence for potential structural alerts was evaluated with different QSAR models. Models used were the OASIS TIMES and VEGA (Caesar, SarPy and Toxtree).

OASIS TIMES

OASIS TIMES is a hybrid statistical and knowledge-based model for toxicity prediction. The Tissue Metabolism Simulator (TIMES), developed by LMC (Bourgas University, Bulgaria; http://oasis-lmc.org/) integrates on the same platform a metabolic simulator and QSAR models for predicting toxicity of selected metabolites. The metabolic simulator generates plausible metabolic maps from a comprehensive library of biotransformations and abiotic reactions. It allows prioritization of chemicals according to toxicity of their metabolites. Of OASIS TIMES the prediction model for Ames test was considered and therefore predictivity is limited to this test system only.

The report for the evaluation made is available under DocID 2015/1094106 for prediction of Ames mutagenicity.

The reactivity model describing interactions of chemicals with DNA is based on an alerting group approach. Only those toxicophores extracted from the training set having clear interpretation for the molecular mechanism causing the ultimate effect included in the model. The mechanistic interrelation between alerts and related parametric ranges generalizing the effect of the rest of the molecules on the alert is also considered. The structural component of the model is based on the structural similarity between chemicals in the training set which were correctly predicted by the model. The structural neighborhood of atom-centered fragments is used to determine this similarity. The training set consists of 1514 chemicals for Ames and 808 chemicals for chromosomal aberration.
The derived model is combined with metabolic simulator TIMES used for predicting metabolic activation of chemicals with the S9 mix. The metabolic simulator is trained to reproduce documented maps for mammalian liver metabolism for 261 chemicals. Parent chemicals and each of the generated metabolites are submitted to a battery of models to screen for a general effect and mutagenicity mechanisms. Thus, chemicals are predicted to be mutagenic as parents only, parents and metabolites, and metabolites only. Mutagenicity could be due to the parent chemical only or as a result of its metabolic activation (i.e., the parent is inactive but it is transformed to a mutagenic metabolite), or both parent structure and metabolites could be mutagenic.

This OASIS QSAR system is also included in the OECD Toolbox (but not in combination with TIMES), in order to make use of (Q)SAR approaches also in the assessment of chemicals under REACH (OECD, 2008). The BASF-internal version has the advantage that it is capable to consider metabolic transformation.

VEGA
Using the VEGA platform, access to a series of QSAR (quantitative structure-activity relationship) models for regulatory purposes was obtained. Of the models offered by VEGA [http://www.vega-qsar.eu/] the three independent statistical/rules based prediction models for mutagenicity (Ames) were selected.

The data obtained for metiram and its metabolites can be found under DocID 2015/1094107.

The first one is an implementation of **CAESAR**, which makes predictions based on the comparison of the structure of interest to the CAESAR database of mutagenicity data of substances in the structure database. A score is provided for the match of the structures, and the mutagenicity data of the closest related substances compared to the structure of interest. Consequently, if a structure is not adequately presented in the database, the prediction is only of very limited validity.

The second algorithm **SarPy** searches for isolated structural alerts of substructures in the molecule. Again this is based on the mutagenicity data provided in the structure database. Only predictivity for mutagenicity based on the Ames test was generated.

The third algorithm **Toxtree** is based on Benigni-Bossa Mutagenicity rules of structural alerts for mutagenicity. It works as a decision tree for estimating carcinogenicity, based on a list of structural alerts (SAs). The SAs for mutagenicity are molecular functional groups or substructures known to be linked to the mutagenic activity of chemicals. As one or more SAs embedded in a molecular structure are recognised, the system flags the potential mutagen of the chemical. The model goes through a first step in which a set of 12 SAs related to mutagenicty is checked The SAs are the following (SA numbers refer to the original Benigni/Bossa study):
- SA 1: Acyl halides
- SA 6: Propiolactones or propiosultones
- SA 11: Simple aldehyde
- SA 12: Quinones
- SA 13: Hydrazine
- SA 14: Aliphatic azo and azoxy
- SA 16: alkyl carbamate and thiocarbamate
- SA 18: Polycyclic Aromatic Hydrocarbons
- SA 21: alkyl and aryl N-nitroso groups
- SA 22: Azide and triazene groups
- SA 25: Aromatic nitroso group
- SA 28bis: Aromatic mono- and dialkylamine
- SA 29: Aromatic diazo

CAVEAT on reliability of QSAR modules implied

With regard to the QSAR evaluations as implied in OECD TIMES and in VEGA it should be noted that for nearly all analysis the algorithm reported an out of structural domain error. Each of this QSAR models is built on a set of chemicals that forms its chemical domain, space or applicability domain. That means that the prediction is best if a structure of interest is represented in the original baseline dataset. Substances outside of the dataset are evaluated in comparison to the chemical space, and only in case that the chemical space adequately covers all structural elements or the queried structure, the prediction is considered to be adequately covered by experimental data. Predictions outside of the applicability domain have far lower predictability. In addition all mentioned QSAR models check for structural alerts, like those identified by the Benigni-Bossa rules that have been implicated in mutagenic actions.

As a consequence, the predictivity is solely based on the proposed DNA-interaction via the structural alert, not (VEGA) or not appropriately (OASIS TIMES) taking into account possible functional group interaction and stereochemical hindrance. It is well established that structure elements have to be evaluated within the context of a structure.

In general, the predictivity of various QSAR models for genotoxicity equivalent to the Ames test has been considered to be reasonable accurate. Predictivity rates expressed as accuracy and specificity are usually >80%. This is in particular true, if information from more than one QSAR model is combined.

Threshold of toxicological concern concept

A further approach to assess whether chemical structures are of concern for which no or only limited information on the toxicological profile is available, is to consider whether the predicted exposure is above or below a threshold of toxicological concern. The proposed threshold levels are 0.0025 µg/kg bw/day for potentially genotoxic compounds and 1.5 µg/kg bw/day for non-genotoxic Cramer Class III compounds (see MCA 6.7).

The available toxicological database for metabolites will be discussed in the following paragraphs. This starts with an assessment of the QSAR predictions of the parent metiram and is followed by the metabolites as indicated below.

Table 5.8.1-3: Overview on QSAR evaluation of relevant metiram metabolites

	QSAR prediction of mutagenicity (Ames)				
	OASIS Times (v2.27.16.8), Ames, with S9		VEGA		
			VEGA, Caesar 2.1.12	VEGA, SarPy 1.0.6-DEV	VEGA, TOXTREE 1.0.0-DEV
	Prediction (compound evaluated)	Overall prediction*	Prediction (compound evaluated)	Prediction (compound evaluated)	Prediction (compound evaluated)
Metiram	Negative Out of domain	-	Positive Out of domain	Negative Out of domain	Negative Out of domain
Ethylenethiourea (ETU) Reg.No. 146099	Negative Out of domain	Negative Out of domain	Negative Out of domain (experimental value: positive)	Negative Out of domain (experimental value: positive)	Negative In domain (experimental value: negative)
Ethyleneurea (EU) Reg.No. 27270	Negative In domain	-	Negative Could be out of domain	Negative Could be out of domain	Negative Could be out of domain
Ethylene bis-isothiocyanante sulfide (EBIS) Reg.No. 243959	Negative Out of domain	Negative Out of domain	Positive Out of domain (experimental value: negative)	Negative Could be out of domain (experimental value: negative)	Negative Out of domain
Jaffes Base Reg.No. 6002546	Negative Out of domain	Negative Out of domain	Positive Out of domain	Negative Out of domain	Negative In domain
TDIT Reg.No. 4670450	Negative Out of domain	Negative Out of domain	Positive Out of domain	Negative Out of domain	Negative Out of domain
Ethylene diamine (EDA) Reg.No. 4183259	Negative In domain	Negative In domain	Negative In domain	Negative In domain	Negative Out of domain

*: Overall prediction of compound evaluated including presumed in silico metabolites.

Metiram:

$$\left(\left(\text{[C}_2\text{H}_4(\text{NHCS}_2)_2]\text{Zn(NH}_3)\right)_3 \; \text{[C}_2\text{H}_4(\text{NHCS}_2)_2\text{]}\right)$$

QSAR predictions on metiram:

OASIS TIMES (V.2.27.16.8; Mutagenicity S-9 activated v09.09) [see molecule 1 of reports DocID 2015/1094106]
There were **no** Ames mutagenicity alerts for metiram and no structural alerts were received. The parent structure was out of the applicability domain. No data were received for or in-silico generated metabolites.

VEGA: Mutagenicity model (CAESAR, version 2.1.12) [see molecules 1/2 of report DocID 2015/1094107]
Metiram was out of the model applicability domain. The prediction is **'mutagen'** with no specific structural alerts and a **low** overall reliability. Deficiencies result from:

- only moderately similar compounds with known experimental value in the training set have been found
- some similar molecules found in the training set have experimental values that disagree with the predicted value
- accuracy of prediction for similar molecules found in the training set is not adequate

VEGA: Mutagenicity model (SarPy; version 1.0.6-DEV) [see molecule 1/2 of report DocID 2015/1094107]
Metiram was out of the model applicability domain. The prediction is **'non-mutagen'** with no specific structural alerts and a **low** overall reliability. Deficiencies result from:

- only moderately similar compounds with known experimental value in the training set have been found
- some similar molecules found in the training set have experimental values that disagree with the predicted value
- accuracy of prediction for similar molecules found in the training set is not adequate

VEGA: Mutagenicity model (Toxtree; version 1.0.0-DEV) [see molecule 1/2 of report DocID 2015/1094107]
Metiram was in the model applicability domain. The prediction is **'non-mutagen'** with no specific structural alert. The overall reliability is low.
Deficiencies result from:

- only moderately similar compounds with known experimental value in the training set have been found
- some similar molecules found in the training set have experimental values that disagree with the predicted value
- accuracy of prediction for similar molecules found in the training set is not optimal
- some atom centered fragments of the compound have not been found in the compounds of the training set or are rare fragments

Toxicity studies with Metiram:

Study type	Batch/Purity	Result	Reference
In vitro Mutagenicity in bacterial cells (Ames test)	- / 98% Metiram	Negative Negative	1977/027
In vitro Mutagenicity in bacterial cells (Ames test) OECD 471	- / 99% Metiram 2.2% ETU	Negative Negative Negative	1985/020
ex vivo Host-mediated assay	- / 99% Metiram 2.2% ETU	negative	1985/210
In vitro Mutagenicity in mammalian cells CHO/HGPRT Based on OECD 476	- / 99% Metiram 2.2% ETU	Weakly positive (borderline significance Negative	1985/238
In vitro Mutagenicity in mammalian cells CHO/HGPRT OECD 476	64-9668 / 93% Metiram	Negative Negative	1990/0285
In vitro Mutagenicity in mammalian cells SCE	- / 99% Metiram 2.2% ETU	Weakly positive Negative Weakly positive	1986/082
In vitro DNA damage & repair UDS test	- / 99% Metiram 2.2% ETU	negative	1984/209
In vivo Cytogenicity assay Chromosome analysis	- / 99% Metiram 2.2% ETU	Negative	1986/265
In vivo Mutagenicity test SCE OECD	64-9668 / 93% Metiram	Negative	1990/0569
In vitro Mutagenicity in bacterial cells (Ames test) OECD 471	FRE-001136 / 15.52 g/L Metiram 2.43 g/L Reg.No. 247214 0.88 g/L Reg.No. 251072 (Technical Metiram in DMSO) 1.35 g/L ETU (Reg.No. 146098)	Negative	2014/1313080
In vitro MNT	300015 / 91.5% Metiram <0.05% ETU (Reg.No. 146098)	Positive (-S9) Negative (+S9)	2014/1315333
In vivo MNT	300015 / 91.5% Metiram <0.05% ETU (Reg.No. 146098) Bioavailability confirmed	Negative	2014/1315334

Conclusion on Metiram:

No conclusive structural alert was identified for metiram regarding mutagenicity. The prediction was “non-mutagen” in three out of four models used and is thus in concordance with available in vitro data (see MCA 5.4).
Furthermore, in a weight of evidence approach the available in vitro and in vivo studies suggest no genotoxic properties of metiram.

It is concluded that metiram has no genotoxic properties based on the results from several in vitro and in vivo genotoxicity studies.

Ethylenethiourea (ETU)

QSAR predictions on ETU:

OASIS TIMES (V.2.27.16.8; Mutagenicity S-9 activated v09.09) [see molecule 12 of report DocID 2015/1094106]
There were **no** Ames mutagenicity alerts for ETU or in-silico generated metabolites and no structural alerts were received. The parent substance was out of the applicability domain.

VEGA: Mutagenicity model (CAESAR, version 2.1.12) [see molecule 10 of report DocID 2015/1094107]
ETU was out of the model applicability domain. The prediction is **‘non-mutagen’** with no specific structural alerts and a **low** overall reliability (experimental value: **‘mutagen’**).

VEGA: Mutagenicity model (SarPy; version 1.0.6-DEV) [see molecule 10 of report DocID 2015/1094107]
ETU was out of the model applicability domain. The prediction is **‘non-mutagen’** with no specific structural alerts and a **low** overall reliability (experimental value: **‘mutagen’**).

VEGA: Mutagenicity model (Toxtree; version 1.0.0-DEV) [see molecule 10 of report DocID 2015/1094107]
ETU was in the model applicability domain. The prediction is **‘non-mutagen’** with no specific structural alert. The overall reliability is good (experimental value: **‘non-mutagen’**)

Toxicological information on ETU:

Ethylenethiourea (ETU) is a metabolite, environmental degradation product and a minor technical impurity of metiram and other ethylene bisdithiocarbamate (EBDC) fungicides, a class of nonsystemic fungicides used worldwide for the control of a variety of diseases on a wide range of fruit, vegetable, cereal, and ornamental crops [Hurt SS, Nave VA, 1992 Ethylenethiourea: Toxicology monograph addendum prepared for the WHO Experts Group on pesticide residues; BASF DocID 1992/11595]. The assessment of all relevant toxicological endpoints of ethylene thiourea has been performed using peer-reviewed literature data. *Since the last review of ETU in the context of a pesticide registration, new in vivo and in vitro toxicological studies have been conducted (US EPA data requirement, mechanistic studies).*

Metabolism (ETU)

Oral doses of ETU are rapidly and almost completely (about 80%) absorbed and rapidly excreted, primarily in the urine and more quickly in mice than in rats. *After a 0.5 mg/kg bw oral dosage of radioactive ethylene thiourea to rats, 96% of radioactivity was excreted in urine within 72 hours (█████ 1985; BASF DocID1985/0470)* In most species the greater majority (70% or more) of an oral dose is eliminated via the urine within 48 hours. Concentrations in blood and tissues are generally similar with the exception of somewhat higher levels in the thyroid; levels in maternal and fetal tissues were similar 3 hours after dosing. Half-lives for elimination from maternal blood were 5.5 and 9.4 hr in mice and rats, respectively *(Ruddick, 1977; BASF DocID 1977/10228)*. Unchanged ETU was the primary metabolite in rats and guinea pigs, with small amounts of ethylene urea. In mice the principal identified metabolites were ETU and imidazolinyl sulfenate, and in cats, S-methyl-ETU was the principal metabolite. A maximum of 26% of a 10 hour dermal dose to rats was absorbed.

Report: CA 5.8.1/1
Saghir S.A. et al., 2005a
Ethylenethiourea: Interspecies comparison of in vitro metabolism by female rat, female mouse and female human liver S9 fractions
2005/1043820
Guidelines: none
GLP: yes
(certified by United States Environmental Protection Agency)

Objectives of the study:
The study was designed to determine relative in vitro metabolism of ETU by the liver S-9 fraction of female rats, female mice and female humans for comparison. The metabolism was determining by measuring loss of ETU added to the reaction mixtures.

Materials and Methods:
Relative metabolism capacity of ETU was determined in liver S-9 fractions of CD rats (uninduced females and Aroclor 1254 induced males), CD-1 mice (uninduced females and Aroclor 1254 induced males) and compared with female human donors by quantifying the disappearance of ETU. Rat and mouse liver S-9 fractions were prepared from young adult animals (8-12 weeks of age). Human liver S-9 fractions were obtained from female donors of childbearing age (17-41 years). Triplicate liver S-9 fractions were incubated with ETU (5 µg ETU/mg protein) and cofactors at 37°C for 1 hour in a shaking bath. The reaction was stopped by adding 100 µl of 10% (v/v) formic acid, wthen internal standard (2.5 µg D_4-ETU) was added and the incubates were passed through a Nylon membrane (0.45 µm pore size) filter and analyzed for ETU by LC/MS. Three types of controls were also incubated to determine non-enymatic loss of ETU and to compare with the actual reactions in order to determine the percentage loss of the added ETU.

Results:
Concentrations of ETU in the control (incubated with ETU and all other cofactors with the exception of liver S-9 fraction) incubations remained unchanged (10.1 ± 0.1 µg/ml) from what was added (10 µg/ml) to each vial, indicating that no non-enzymatic conversion/hydrolysis or binding of ETU to the glass vials or filtering apparatus occurred during the incubation and processing of samples.

The concentration of ETU in the uninduced rat and mouse liver S-9 incubations was 9.89 ± 0.11 and 9.60 ± 0.50 µg/ml, respectively, indicating a loss of 2 and 5% #ETU after 1 hour of incubation which remained statistically unchanged from controls. The liver S-9 fraction obtained from the Aroclor 1254 induced rats and mice were more active in metabolizing ETU than the liver S-9 fraction from the uninduced rats and mice amounting to the loss of 10 and 9% ETU, respectively. The loss was significantly different from the control however, not different from each other.

Human liver S-9 fractions were most active in metabolizing ETU than either uninduced or Aroclor 1254 induced rats and mice. The amount of ETU in the human liver S-9 pools was between 8.81 ± 0.03 and 9.01 ± 0.17 µg/ml accounting to a loss of 11-13% of ETU when compared to the control. The difference was statistically significant from control and uninduced rats and mice, but not from Aroclor 1254 induced rats and mice. The loss of ETU among different human liver S-9 pools (#1, #2, and #3) were not statistically different from each other. The below table summarizes the data. The results indicate that the metabolism of ETU will be in the order of humans > induced rat ≈ induced mouse > uninduced mouse > uninduced rat.

Table 5.8.1-4: Concentration (mean ± SD of 3 replicates) of ETU in the control (no liver S-9 protein), uninduced rat and mouse, three human pools and Aroclor 1254 induced rat and mouse liver S-9 reaction mixtures after 1 hour of incubation, % loss in ETU in different liver S-9 incubation compared to no-liver S-9 protein control

	Concentration of ETU (µg/ml) after 1 h of incubation						
Sample ID	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Mean**	**SD**	**% of control[2]**	**% loss[3]**
Control[1]	10.10	10.00	10.20	10.10	0.10	-	-
Rat (uninduced)	9.87	9.79	10.00	9.89	0.11	97.89	2.11
Mouse (uninduced)	9.86	9.91	9.02	9.60	0.50	95.02	4.98
Human (pool #1)	8.87	8.97	9.20	9.01	0.17	89.24	10.76
Human (pool #2)	8.80	8.78	8.84	8.81	0.03	87.19	12.81
Human (pool #3)	8.85	8.65	9.25	8.92	0.31	88.28	11.72
Rat (induced)[4]	8.84	9.32	9.22	9.13	0.25	90.36	9.64
Mouse (induced)[4]	9.25	9.25	9.18	9.23	0.04	91.35	8.65

[1] Control incubations contained buffer, cofactors, and ETU without the addition of liver S-9 fraction
[2] Calculated by dividing the mean ETU concentration in each sample with ETU concentration in control
[3] Represent the loss of ETU due to metabolism by each liver S-9 fraciton compared to control (100% minus % of control)
[4] Animals were induced with Aroclor 1254 prior to procuring liver of S-9 preparations

Conclusion:
After a 1 h incubation, the loss of ETU was between 11 and 13% in a liver S-9 fraction collected from 3 pools of female human donors, while the loss of ETU was only 2 % in rat liver S-9 mix.

Report:	CA 5.8.1/2 Zhu W., 2015 Ethylene thiourea (ETU): Comparative in vitro metabolism using mouse, rat, rabbit, dog and human hepatocytes 2015/1161955
Guidelines:	none
GLP:	yes (certified by The Department of Health of the Government of the United Kingdom)

Executive Summary

Ethylene thiourea (ETU) is a metabolite of ethylene bisdithiocarbamate fungicides. The objective of this study was to compare the metabolism of ETU in different experimental animals (mouse, rat, rabbit and dog) with that in humans.
LC-MS analysis of ETU (2 µM or 200 µM) incubated with mouse, rat, rabbit, dog and human cryopreserved hepatocytes was carried out using an YMC-Pack ODS-AQ column with a 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in acetonitrile gradient system. A Micromass Q-tof mass spectrometer was operated in positive electrospray ionisation mode for the analysis.
Following incubation of ETU with mouse, rat, rabbit, dog and human cryopreserved hepatocytes, unchanged ETU and ethylene urea (EU) were detected and measured semi-quantitatively.
The extent of metabolism as determined by the peak area of ETU remaining after 3 h incubations was least in mouse and rat at both concentrations compared with rabbit, dog and human.
Overall under the experimental conditions used, there was no notable evidence of metabolites of ETU. EU was detected at trace levels (typically < 0.5%) in all species but was also detected in the no hepatocyte control samples at similar levels.
(BASF DocID 2015/1161955)

II. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Ethylene thiourea (ETU)
Description:	powder, white
Lot/Batch #:	BCBM9015V
Purity:	99.9%
Specific activity:	16.86 mCi/mmol
Source:	Sigma-Aldrich [Fluka Analytical brand]
Stability of test compound:	The stability of the test substance was guaranteed by the sponsor until October 2015.

2. Vehicle:	water

3. Cell culture:

Cell type:	Hepatocytes
Species:	mouse, rat, rabbit, dog, and human
Sex:	female donor
Source:	Bioreclamation IVT (cryopreserved hepatocytes were delivered frozen in liquid nitrogen)
Culture medium:	Williams' Medium E supplemented with 10 mM dexamethasone (5 µl) and cell maintenance 'cocktail B' (20 mL)
Culture conditions:	in an orbital shaking water bath (60 rpm) at 37°C, under an atmosphere of humidified 95% oxygen: 5% carbon dioxide

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 11-November-2014 to 05-January-2015

2. Objective:

The objective of this study was to compare the metabolism of ETU in different experimental animals (mouse, rat, rabbit and dog) and humans. Hepatocytes were used, as the main site of metabolism of ETU is the liver. Hepatocytes from females were chosen because one of the aims in conducting this study was to investigate whether metabolism may be a factor in the species differences in teratogenicity of ETU. Previous studies have indicated that species differences in metabolism between rats and mice contribute to the fact that ETU is teratogenic in rats but not mice. A previous in vitro metabolism study indicated that metabolism was most efficient in humans and mouse >rat (Saghir et al, 2005; DocID 2005/1043820).

3. Incubation of cells:

Thawing procedure for cryopreserved hepatocytes:

Vials were removed from liquid nitrogen and the caps unscrewed slightly to allow the release of nitrogen gas. The vials were then partially immersed in a water bath set at 37°C for approximately 2.5 min until the majority of the contents had thawed. The contents of the vials were then decanted directly into warmed 32.4% (v/v) Percoll®, a commercially available solution composed of colloidal silica coated with polyvinylpyrrolidone, (mouse, rat, rabbit and dog) or 28.8% (v/v) Percoll® (human) in supplemented Williams' Medium E supplemented with 10 mM dexamethasone (5 µL) and cell maintenance 'cocktail B' (20 mL). The resulting cell suspensions were mixed by gentle inversion and then centrifuged (168 × g for 20 min for rat, rabbit, dog and human and 60 × g for 20 min for mouse) in order to pellet the cells. The majority of the resulting medium was removed and the cells were re-suspended by gentle swirling.

The cell suspensions were then made up to appropriate volumes (ca. 1 mL) with supplemented Williams' Medium E.

Incubation procedures:
For each species, components were mixed together in the following order, to give total incubation volumes of 2.0 mL for 0.5, 1 and 3 h incubation, or 0.5 mL for 0 h incubation with final concentrations of ETU of 2 µM and 200 µM:

- Hepatocyte suspension (0.3 × 10^6 viable cells/mL for mouse and 1 × 10^6 viable cells/mL for rat, rabbit, dog and human) in supplemented Williams' Medium E containing 10% of Foetal calf serum
- Test substance (Solutions (40 µL for 0.5, 1 and 3 h or 10 µL for 0 h incubation) either 0.2 mM or 20 mM ETU in water)

The hepatocytes were incubated for 0.5, 1 and 3 h in polyethylene vials in an orbital shaking water bath (set at 60 rpm and 37°C), under an atmosphere of humidified 95% oxygen : 5% carbon dioxide. Reactions were terminated after the appropriate incubation time by removal of 0.5 mL of incubates from the water bath to appropriately labelled tubes containing chilled water/acetonitrile/formic acid (92/5/3, v/v/v) (50 µL), and transferring the samples to ice for at least 10 min.
For 0 h incubation samples, ice cold water/acetonitrile/formic acid (92/5/3, v/v/v) (50 µL) was added concurrently with the test substance, with gentle swirling and transferring the samples to ice for at least 10 min.
Samples were then homogenised by ultrasonic disruption (approximately 30 s) using an ultrasonic water bath.
Control incubations in the absence of hepatocytes and control incubations with hepatocytes in the absence of test substance were conducted for 3 h only to check for the stability of ETU under the incubation conditions and for hepatocyte-related material that could interfere with the analysis of ETU and its metabolites. All the incubations were conducted in duplicate.
The resulting samples were stored at approximately -20°C (± 10°C) pending processing for analysis.
Samples were removed from the storage and allowed to thaw at room temperature. Once thawed, samples were centrifuged at 18659 × g for 15 minutes at 4°C. Following centrifugation, aliquots (ca 200 µL) of the subsequent supernatants were transferred to HPLC vials for LC-MS analysis.

4. Determination of hepatocyte viability and metabolic activity:
Viability
Hepatocyte viability was determined by the trypan blue exclusion test.

Metabolic activity
In parallel incubations, positive control incubations were conducted with 7-ethoxy[^{14}C]coumarin (7-EC) as a substrate, together with control incubations in the absence of hepatocytes. 7-EC in dimethylformamide (DMF) solution (5 mM, 10 µL) was added to give a final concentration of 50 µM (the final concentration of DMF was 1%, v/v).
The 7-EC incubates were incubated for 3 hours, in polyethylene vials in an orbital shaking water bath (set at 60 rpm and 37°C), under an atmosphere of humidified 95% oxygen : 5% carbon dioxide. Reactions were terminated after 3 h by addition of ice cold acetonitrile (2 mL), transferring the samples to ice for at least 10 min. Samples were then homogenised by ultrasonic disruption (approximately 30 s) using an ultrasonic water bath.

Following storage at approximately -20°C, the 7-EC samples were centrifuged (18620 × g, 15 min, 4°C) and the resulting supernatants were transferred to clean tubes. The supernatants were concentrated to dryness under a centrifugal evaporator at room temperature.
The dried residues were reconstituted in 40 mM ammonium formate, pH 5 (1 mL). An aliquot (0.45 mL) of each was taken into a clean tube for deconjugation and the remainder was transferred to a HPLC vial prior to analysis. Deconjugation was conducted by incubating (1 h, at 37°C) the 0.45 mL portion of the reconstituted supernatant with β-glucuronidase enzyme (50 µL, 2000 units, type H1 from Helix pomatia, also containing sulfatase activity) in a water shaking bath. Samples were then transferred to HPLC vials prior to analysis.
Positive controls for β-glucuronidase and sulfatase enzyme activities were determined by the production of free phenolphthalein from phenolphthalein glucuronic acid and p-nitrocatechol from p-nitrocatechol sulfate, respectively, upon the addition of 1 M sodium hydroxide, after incubation (1 h, 37°C). Both the phenolphthalein glucuronic acid and p-nitrocatechol sulfate were prepared in 40 mM ammonium formate, pH 5.
The percentage of sample radioactivity associated with 7-EC and its known metabolite 7-Hydroxycoumarin (7-HC) was determined for untreated and deconjugated samples.

5. Analytics:
Analysis of ETU and metabolites:
These were analysed by LC-MS. Each chromatogram was evaluated in order to determine peak areas for each metabolite and parent compound. This enabled the peak area of parent compound to be expressed as a percentage of peak area of the parent compound at 0.5 h for each sample, the peak areas for each metabolite and parent compound were summed and the percentage of the total peak area associated with each metabolite or parent compound was determined. It was assumed that each metabolite and parent compound was detected with equal response, although this may not be the case. The analytical method in this study was considered to be semi-quantitative.
Analysis of 7 EC:
The 7-EC metabolism data presented were derived from evaluations of the HPLC radiochromatograms, which enabled the amount of radioactivity attributed to each regions of interest (ROI) in the chromatogram to be expressed as a percentage of total sample radioactivity. The percentage ROI values were used to determine the percentage metabolism of 7-HC and 7-EC. Control values (in the absence of hepatocytes) were taken into account.

II. RESULTS AND DISCUSSION

The disappearance of the parent was measured by comparing ETU remaining following incubation of ETU (2 µM and 200 µM for 0.5, 1 and 3 h with 0.5 h incubation rather than the 0 h incubation.
This is because:
• The non-incubated samples (ie t = 0 h) and ETU samples incubated in the absence of hepatocytes did not give data consistent with the incubated samples possibly due to the reduced opportunity for non-enzymic processes to occur at 37°C. Protein binding may not occur or non-specific binding (e.g. to the sample container) may be increased.
• The 0.5, 1, 3 h time points were sampled from the same container at the specific times, whereas the samples for zero time point were taken from different containers to ensure termination at time zero and therefore may not be directly comparable.

A summary of the peak areas for ETU and proportions remaining following incubations of 2 µM and 200 µM ETU with mouse, rat, rabbit, dog and human hepatocytes for 0.5, 1 and 3 h are shown in Table 5.8.1-5. The proportion of ETU remaining during the incubation was measured by comparing the peak areas after 0.5, 1, 3 h incubations with the peak area at 0.5 h for each species at the concentrations of 2 µM and 200 µM of ETU.

A. VIABILITY OF HEPATOCYTES

The metabolic viability of the hepatocytes used was assessed by measurement of the metabolism of radiolabelled 7-EC in parallel with the ETU incubations. The percentage of sample radioactivity associated with 7-HC and 7-EC was determined for both untreated and enzyme deconjugated samples.
Overall, the changes in the levels of the Phase I metabolite 7-HC following deconjugation indicated that all hepatocytes were metabolically viable and were capable of integrated Phase I/II metabolism under the incubation conditions used on this study. Therefore, the results generated for the incubation of these hepatocytes with ETU are considered to be valid.

B. METABOLISM

Mouse hepatocytes:
The proportions of ETU remaining after 0.5 h, 1 h and 3 h incubation with mouse hepatocytes compared to time 0.5 h were 100%, 117% and 113%, respectively at 2 µM ETU. At 200 µM ETU, 89.5% of the parent was left unchanged after 3 h incubation with hepatocytes. The results suggest that after 3 h incubation, the levels of ETU remained broadly unchanged at both concentrations.
EU was detected in the 200 µM incubations only at all time points, both with and without hepatocytes. This result suggests that EU may be a trace metabolite and/or an impurity in ETU. No other metabolites of ETU were detected.

Rat hepatocytes:
The proportions of ETU remaining after 0.5 h, 1 h and 3 h incubation with rat hepatocytes compared to time 0.5 h were 100%, 110% and 88.8%, respectively at 2 µM ETU. At 200 µM ETU, 87.8% of the parent was left unchanged after 3 h incubation with hepatocytes. The results suggest that after 3 h incubation, the levels of ETU remained broadly unchanged at both concentrations.
EU was detected in the 200 µM incubations only at all time points, both with and without hepatocytes. This result suggests that EU may be a trace metabolite and/or an impurity in ETU. No other metabolites of ETU were detected.

Rabbit hepatocytes:
The proportions of ETU remaining after 0.5 h, 1 h and 3 h incubation with rabbit hepatocytes compared to time 0.5 h were 100%, 101% and 76.6%, respectively at 2 µM ETU. At 200 µM ETU, 86.7% of the parent was left unchanged after 3 h incubation with hepatocytes.
EU was detected in the 200 µM incubations only at all time points, both with and without hepatocytes. This result suggests that EU may be a trace metabolite and/or an impurity in ETU. No other metabolites of ETU were detected.

Dog hepatocytes:
The proportions of ETU remaining after 0.5 h, 1 h and 3 h incubation with dog hepatocytes compared to time 0.5 h were 100%, 91.7% and 74.2%, respectively at 2 µM ETU. At 200 µM ETU, 77.5% of the parent was left unchanged after 3 h incubation with hepatocytes.
EU was detected in the 200 µM incubations only at all time points, both with and without hepatocytes. This result suggests that EU may be a trace metabolite and/or an impurity in ETU. No other metabolites of ETU were detected

Human hepatocytes:
The percentages of ETU remaining after 0.5 h, 1 h and 3 h incubation with human hepatocytes compared to time 0.5 h were 100%, 86.5% and 77.9%, respectively for the concentration of 2 µM. At 200 µM, 83.0% of the parent was left unchanged after 3 h incubation with hepatocytes.
EU was detected in the 200 µM incubations only at all time points, both with and without hepatocytes. This result suggests that EU may be a trace metabolite and/or an impurity in ETU. No other metabolites of ETU were detected.

Table 5.8.1-5: Proportions of ETU remaining following in vitro incubation of ETU (2 µM or 200 µM) with mouse, rat, rabbit, dog and human cryopreserved hepatocytes for up to 3 h

	Mouse		Rat		Rabbit		Dog		Human	
Conc / incubation time	**Mean peak area**	**% to T=0.5h**	**Mean peak area**	**% to T=0.5h**	**Mean peak area**	**% to T=0.5h**	**Mean peak area**	**% to T=0.5h**	**Mean peak area**	**% to T=0.5h**
2 (µM)										
0.5 h	55.7	100	55.9	100	58.7	100	63.0	100	63.0	100
1.0 h	65.3	117	61.4	110	59.6	101	57.8	91.7	54.5	86.5
3.0 h	62.7	113	49.6	88.8	45.0	76.6	46.7	74.2	49.1	77.9
200 (µM)										
0.5 h	1582	100	1563	100	1497	100	1576	100	1637	100
1.0 h	1584	100	1565	100	1482	99.0	1384	87.8	1464	89.4
3.0 h	1417	89.5	1373	87.8	1298	86.7	1222	77.5	1359	83.0

III. CONCLUSION

Following incubation of ETU with mouse, rat, rabbit, dog and human cryopreserved hepatocytes, EU and unchanged ETU were detected and measured semi-quantitatively.
The extent of metabolism as determined by the peak area of ETU remaining after 3 h incubations was least in mouse and rat at both substrate concentrations (2 and 200 µM ETU) compared with rabbit, dog and human.
Overall under the experimental conditions used, there was no notable evidence for metabolism of ETU observed. EU was detected at trace levels (typically < 0.5%) in all species and was also detected in the no hepatocyte control samples at similar levels.

Acute Toxicity (ETU)

ETU is only slightly toxic after acute oral administration to mammalian species with measured LD_{50} values ranging from 545 mg/kg bw in pregnant rat to 4000 mg/kg bw in adult mice [Hurt SS, Nave VA, 1992 Ethylenethiourea: Toxicology monograph addendum prepared for the WHO Expert Group on pesticide residues; BASF DocID 1992/11595]. For the convenience of the reviewer the studies are summarized in Table 5.8.1-6 as extracted from the monograph.

Table 5.8.1-6: Summary of already per reviewed acute toxicity studies

Type of test Test species	Test substance purity	Results [mg/kg bw]	Reference
Oral LD_{50} mouse	Unknown	4000	1984/10114
Oral LD_{50} mouse	Unknown	3000	1978/10175
Oral LD_{50} mouse (pregnant)	Unknown	>3000	1987/10325
Oral LD_{50} mouse	Unknown	ca. 2400	1980/10213
Oral LD_{50} rat	Unknown	ca. 2400	1980/10212
Oral LD_{50} rat	Unknown	1832	1972/10122
Oral LD_{50} rat	Unknown	940	1984/10114
Oral LD_{50} rat (pregnant)	unknown	600	1987/10325
Oral LD_{50} rat	unknown	545	1978/10175
Oral LD_{50} hamster	unknown	>3000	1978/10175
Oral LD_{50} hamster (pregnant)	unknown	>2400	1987/10325
Skin sensitization guinea pig	Wet powder	Weak sensitizer	1976/10131

Report:	*CA 5.8.1/3*
	[redacted] 2001a
	Acute toxicity study of ETU (Ethylene thiourea) in Sprague-Dawley rats by dermal administration
	2001/1014630
Guidelines:	*OECD 402, EEC 92/69 B 3*
GLP:	*yes*
	(certified by Behoerde fuer Arbeit, Gesundheit und Soziales, Freie und Hansestadt Hamburg, Hamburg)

Report:	*CA 5.8.1/4*
	[redacted] 2003a
	Acute toxicity study of ETU (Ethylene Thiourea) in Sprague-Dawley rats by dermal administration according to EC guideline B.3 and OECD 402 - First revision
	2003/1029080
Guidelines:	*OECD 402, EEC 92/69 B 3*
GLP:	*yes*
	(certified by Freie und Hansestadt Hamburg, Behoerde fuer Arbeit, Gesundheit und Soziales, Hamburg, Germany)

Note: First Revision was done in order to prepare the report according to FIFRA guidelines. No changes concerning the results were performed.

Executive Summary

In an acute dermal toxicity study 10 Sprague-Dawley rats (5/sex) were dermally exposed to a single dose of ‘Ethylene Thiourea’ (ETU, batch L33-99, purity: 99.6%) as suspension in sesame oil at a dose level of 2000 mg/kg bw. The treated skin was clipped and exposed under occlusive dressing for 24 hours.

No mortality occurred. Accordingly, the dermal LD50 was found to be greater than 2000 mg/kg bw for rats.

Rat, dermal: LD50 > 2,000 mg/kg bw

No signs of systemic toxicity or skin effects were observed in the animals during the 14 day observation period. The expected body weight gain was observed in the course of the study. No abnormalities were noted at necropsy of animals sacrificed at the end of the study.

(BASF DocID 2001/1014630)

II. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	ethylene thiourea (ETU)
Description:	solid powder, light beige
Lot/Batch #:	L33-99
Purity:	99.6%
Stability of test compound:	The stability of the test substance was guaranteed by the sponsor until September 2005.
2. Vehicle:	sesame oil
3. Test animals:	
Species:	Rat
Strain:	Sprague-Dawley / Crl: CD®BR
Sex:	males and females
Age:	female: 54 days, male: 42 days
Weight at dosing (mean):	female: 208 - 227 g; male: 203 - 235 g
Source:	
Acclimation period:	five days
Diet:	Altromin 1324 (Altromin GmbH, 32791 Lage/Lippe, Germany)
Water:	tap water ad libitum
Housing:	Single housing in Makrolon cage, type III with granulated textured wood (Granulate A2, J. Brandenburg, 49424 Goldenstedt, Germany)
Environmental conditions:	
Temperature:	19 – 25°C
Humidity:	40 – 70%
Air changes:	no data
Photo period:	12-hour light-dark cycle (06:00 – 18:00, 18:00 – 06:00)

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 03-April-2001 to 17-April-2001 (day of first administration and day of last observation)

2. Animal assignment and treatment:

Twenty four hours before treatment the fur was clipped. Ten rats (5 per sex per group) received a single dose of 2000 mg/kg bw of test substance in sesame oil to the clipped epidermis (dorsal and dorsolateral parts of the trunk). Test substance was covered with a occlusive dressing (8 layers of gauze which was covered with a plastic sheet and secured with adhesive plaster) for 24 hours. Afterwards the occlusive dressing was removed and possible residual substance was removed. The application area was about 30 cm² (5x6 cm) which corresponds to at least 10% of the body surface. The observation period lasted 14 days. Individual body weights were recorded shortly before application (day 0) and weekly thereafter and at the end of the study. Clinical signs and symptoms were recorded several times on the day of administration, at least once each workday for the individual animals. The Scoring of skin findings were evaluated daily after start of application. The evaluation of skin reactions was performed according to Draize (1959) (Appraisal of the safety of chemicals in food, drugs and cosmetics. The association of food and drug officials of the United States Austin, Texas.) After the observation period a necropsy of all animals with gross pathology followed.

II. RESULTS AND DISCUSSION

A. MORTALITY

No deaths occurred after dermal administration of 2000 mg/kg bw of the test substance.

B. CLINICAL OBSERVATIONS

No systemic clinical signs or no local effects were observed during clinical examination. No skin reaction was observed.

C. BODY WEIGHT

Normal body weight gain was observed during the 14 days of the observation period.

D. NECROPSY

No macroscopic anomaly was noted in the organs examined after dermal treatment.

III. CONCLUSION

Under the experimental conditions of this study the dermal LD_{50} of 'Ethylene thiourea' in rats was determined to be greater than 2000 mg/kg bw for males and females. Based on the results of this study, ethylene thiourea does not warrant classification as to acute dermal toxicity according to EU criteria (28th ATP of Council Directive 67/548/EEC by Commission Directive 2001/59/EC / Council Regulation 1272/2008/EC) and according to the OECD Globally Harmonized System (GHS, revision 2, 2007).

Report:	*CA 5.8.1/5*
	2002a
	Acute inhalation toxicity study of milled Ethylene thiourea (ETU) in Sprague-Dawley rats
	2002/1005548
Guidelines:	*OECD 403, EEC 67/548, Official Journal of the European Communities L3833 (1992) Part A: Acute toxicity (inhalation) B.2, EPA, JMAFF*
GLP:	*yes (certified by Behoerde fuer Arbeit, Gesundheit und Soziales, Freie und Hansestadt Hamburg, Hamburg)*

Report:	*CA 5.8.1/6*
	2003d
	Acute inhalation toxicity study of milled Ethylene Thiourea (ETU) in Sprague-Dawley rats - Limit test - First revision
	2003/1029079
Guidelines:	*OECD 403, EEC 67/548, EPA, JMAFF, Official Journal of the European Communities L3833 (1992) Part A: Acute toxicity (inhalation) B.2*
GLP:	*yes (certified by Behoerde fuer Arbeit, Gesundheit und Soziales, Freie und Hansestadt Hamburg, Hamburg)*

Note: First Revision was done in order to prepare the report according to FIFRA guidelines. No changes concerning the results were performed.

Executive Summary

In an acute inhalation toxicity study, groups of 5 male and 5 female Sprague dawley rats were exposed to ethylene thiourea as a dust (batch: 01815-177; Purity: 99.9%) at a concentration of 10.40 mg/L air for 4 hours. The animals were observed for 14 days after exposure.

No mortality occurred at the tested concentration, which was the highest technical possible concentration. Accordingly, the acute inhalation LC_{50} for after dust inhalation exposure of ethylene thiourea was determined to be

LC_{50} (male and female rats) > 10.40 mg/L

Clinical signs of toxicity consisted of slight to moderated reduced motility and ataxia, slightly reduced muscle tone and dyspnea. Findings were observed immediately after exposure and lasted 30 minutes. No clinical signs and findings were observed from one hour after end of treatment onwards. The mean body weights of the animals increased throughout the study period. No gross pathological abnormalities were noted during the necropsy at termination of the post exposure observation period.
Cascade impactor measurements resulted in particle size distributions with mass median aerodynamic diameters (MMADs) of 2.065 µm.

According to the EU and GHS classification criteria, no classification is warranted as to acute inhalation toxicity for ethylene thiourea.

(DocID 2002/1005548)

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:**	milled ethylene thiourea (ETU)
Description:	white powder
Lot/Batch #:	01815-177
Purity/content:	99.9%
Stability of test compound:	The stability was guaranteed for the duration of the study. (Expiry date: 09/2010)
2. **Vehicle:**	none, the test substance was doses unchanged
3. **Test animals:**	
Species:	Rat
Strain:	Sprague Dawley / Crl:CD^{R}BR
Sex:	male and female
Age:	males: 45 days, female animals: 68 days
Weight at dosing (mean):	males: 237 - 252 g; females: 202 - 211 g
Source:	
Acclimation period:	at least 5 days
Diet:	ssniff® R/M-H V1530 (ssniff Spezialdiaeten GmbH, 59494 Soest, ad libitum
Water:	Tap water, ad libitum
Housing:	2-3 animals per cage Makrolon cage (type III), granulated textured wood was used as bedding material supplied by J. Brandenburg, Goldenstedt, Germany
Environmental conditions:	
Temperature:	19 - 25 °C
Humidity:	40 - 70%
Air changes:	not indicated in the report
Photo period:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 27-Feb-2002 - 9-May-2002

2. Animal assignment and treatment:

For determination of the acute inhalation toxicity (single head-nose inhalation, 4-hour-exposure) groups of five male and five female rats were exposed to 10.40 ± 0.33 mg/L of the test substance ethylene thiourea, that was dosed unchanged as a dust aerosol. The animals were randomly selected from a pool of animals. Food was withdrawn approximately 16 hours before the start of the exposure. After exposure, animals were observed for at least 14 days.
Individual body weights were recorded shortly before exposure (day 0), weekly thereafter, and at the end of the study.

Detailed clinical observations were recorded for each animal separately at least once on each workday of the observation period. No comprehensive clinical examination was performed on public holidays or weekends unless symptoms were still observed.
A check for any dead or moribund animal was made at least one daily. At the end of the observation period the surviving animals were sacrificed and were subjected to gross-pathological examination.

3. Statistics/calculations:
The calculation of the particle size distribution was carried out in the inhalation laboratory on the basis of mathematical methods for evaluating particle measurements. [DIN 66141: Darstellung von Korngrößenverteilungen, DIN 66151: Partikelgrößenanalyse (Beuth-Vertrieb GmbH, Berlin 30, FRG und Köln 1, Germany)]

4. Generation of the test atmosphere and exposure:
The study was carried out using a dynamic inhalation apparatus (air changes/h (≥ 12)) with a nose-only exposure of the animals according to KIMMERLE & TEPPER (Rhema-Labortechnik, 65719 Hofheim, Germany). The apparatus consists of a cylindrical exposure chamber (volume 40 L) which holds a maximum of 20 animals in pyrex tubes at the edge of the chamber in a radial position. The dust was generated with a dust generator (9101-51, Fa. Heinrich Burghart, Technische Laborgeraete, 22559 Wedel, Germany).
The generator was fed with compressed air from a compressor (air was taken from the surrounding atmosphere of the laboratory room and filtered using an in-line disposable gas-filter). At the bottom of the exposure chamber, the air was sucked off at a lower rate than it was created by the dust generator in order to produce a homogenous distribution and a positive pressure in the exposure chamber.
Supply airflow (compressed air) of 900 L/h was used for the exposure. The exhaust airflow was set at 800 L / h. An air change of about 22.5 times per hour can be calculated by dividing the supply air flow by the volume of the inhalation system. The animals were exposed to the inhalation atmosphere for 4 hours plus an equilibration time for the inhalation system of at least 15 minutes.

5. Analytical investigation:
The nominal concentration was calculated from the amount of substance dosed and the supply air flow.

The concentration of the test substance in the inhalation atmosphere was determined via gravimetrical measurement. Dust samples were taken at least once every hour during the exposure Pre-weighed filters were placed into the filtration equipment. By means of a vacuum pump metered volumes of the dust were drawn through the filter. For each sample the dust concentration in mg/L was calculated from the difference between the pre-weight of the filter and the weight of the filter after sampling with reference to the sample volume of the inhalation atmospheres. Mean and standard deviation for the concentration were calculated based on the results from individual measurements.

6. Particle Size Analysis:

An analysis of the particle size distribution was carried out twice during the exposure period using a cascade impactor according to MAY (May, K.R. Aerosol impaction jets, J. Aerosol Sci. 6, 403, 1975, Research engineers Ltd., London N1 5RD, UK).
The dust from the exposure chamber was sucked through the cascade impactor for 5 minutes at a constant flow rate of 5 L/min. The slides were removed from the impactor and were weighed on an analytical balance (SARTORIUS, type 1601 004, precision 10 µg).
The mass median aerodynamic diameter (MMAD) was estimated by means of nonlinear regression analysis (LITCHFIELD & WILCOXON). The 32 µm particle size range and the filter (particle size range < 0.5 µm) were not included in the determination of the MMAD in order not to give undue weight to these values. The geometric standard deviation (GSD) of the MMAD was calculated from the quotient of the 84%- and the 50%- mass fractions.

II. RESULTS AND DISCUSSION

A. MORTALITY

No lethality occurred at the tested concentration of 10.40 mg/L during the study period of 14 days. Therefore, the study satisfies the criteria of a limit test.
Based on the absence of mortality the following LC_{50} value was determined:

LC_{50} (both sexes combined):	> 10.40 mg/L
LC_{50} (male rats)	> 10.40 mg/L
LC_{50} (female rats)	> 10.40 mg/L

B. CLINICAL OBSERVATIONS

Clinical signs of toxicity comprised slight to moderate reduced motility and ataxia and slightly reduced muscle tone and dyspnea. Findings were observed immediately after end of exposure and lasted up to 30 minutes. One hour after end of treatment the animals were free of symptoms. The nature and duration of the observations are indicated in Table 5.8.1-7.

Table 5.8.1-7: Nature and duration of clinical signs observed in rats exposed for 4 hours to ETU as a dust aerosol

Test group 1 (10.40 mg/L)	Males	Females
Reduced motility	+ - ++ 0 – 30 min (5/5)	+ - ++ 0 – 30 min (5/5)
ataxia	+ - ++ 0 – 30 min (5/5)	+ - ++ 0 – 30 min (5/5)
Reduced muscle tone	+ 0 – 30 min (5/5)	+ 0 – 30 min (5/5)
dyspnea	+ 0 – 30 min (5/5)	+ 0 – 30 min (5/5)

+: slight, ++: moderate; (number of animals affected/number of animals treated)

C. BODY WEIGHT

The animals gained the expected weights throughout the study period.

D. NECROPSY

No gross pathological abnormalities were detected in the animals that underwent necropsy at termination of the study.

E. ANALYTICAL MEASUREMENTS

The result of the analytical concentration measurements are presented in Table 5.8.1-8.

Table 5.8.1-8: Measurement of the analytical concentration

Mean concentration (mg/L)	Standard deviation	Nominal concentration (mg/L)
10.40	0.33	123.6

The measurements of particle-size distribution revealed mass median aerodynamic diameters (MMAD) in the respirable range of 2.065 µm with geometric standard deviations of 3.351 µm. The geomentric mean diameter of the test substance was 2.16 µm as determined with a Malvern Mastersizer. The respirable amount particle size smaller or equal to 4 µm was 5.67 mg/L.

III. CONCLUSION

Under the conditions of this study the 4 hour inhalation LC_{50} of ethylene thiourea for male and female rats was estimated to be > 10.40 mg/L. Based on the results of this study, ethylene thiourea does not warrant classification as to acute inhalation toxicity according to EU criteria (28th ATP of Council Directive 67/548/EEC by Commission Directive 2001/59/EC / Council Regulation 1272/2008/EC) and according to the OECD Globally Harmonized System (GHS, revision 2, 2007).

Irritation/corrosion

Report:	*CA 5.8.1/7*
	[redacted] 2001b
	Acute skin irritation test (Patch test) of ETU (Ethylene thiourea) in rabbits
	2001/1014631
Guidelines:	*EEC 92/69 B 4, OECD 404*
GLP:	*yes*
	(certified by Behoerde fuer Arbeit, Gesundheit und Soziales, Freie und Hansestadt Hamburg, Hamburg)

Report:	*CA 5.8.1/8*
	[redacted] 2003b
	Acute skin irritation test (patch test) of ETU (Ethylene Thiourea) in rabbits according to EC guideline B.4 and OECD guideline 404 - First revision
	2003/1029082
Guidelines:	*EEC 92/69 B 4, OECD 404*
GLP:	*yes*
	(certified by Freie und Hansestadt Hamburg, Behoerde fuer Arbeit, Gesundheit und Soziales, Hamburg, Germany)

Note: First Revision was done in order to prepare the report according to FIFRA guidelines. No changes concerning the results were performed.

Executive Summary

In a primary dermal irritation study the skin irritation/corrosion potential of ethylene thiourea (ETU, batch: L33-99, purity: 99.6%) was tested. The clipped intact skin of three Himalayan rabbits was exposed to 0.5 g of the test-substance for four hours. Observation of local reactions was performed 1, 24, 48 and 72 hours after removal of the patch. No erythema or edema of intact skin was noted after 24 to 72 hours in the 3 rabbits treated with ETU. Based on the findings of this study ETU does not show a skin irritation potential.

(BASF DocID 2001/1014631)

J. MATERIAL AND METHODS

B. MATERIALS

4. **Test Material:** ethylene thiourea (ETU)
   Description: solid powder, light beige
   Lot/Batch #: L33-99
   Purity: 99.6%
   Stability of test compound: The stability of the test substance was guaranteed by the sponsor until September 2005.

5. **Vehicle:** test substance was moistened with water

6. Test animals:

Species:	Rabbit
Strain:	Himalayan
Sex:	male
Age:	5 - 6 months
Weight at dosing (mean):	2.2 - 2.5 kg
Source:	[redacted]
Acclimation period:	at least 20 days
Diet:	Altromin 2023 (Altromin GmbH, 32791 Lage/Lippe, Germany), ad libitum
Water:	tap water ad libitum
Housing:	Single housing in special restrainers with dimensions of 425 mm x 600 mm x 380 mm (manufacturer: Dipl. Ing. W. EHRET GmbH, 16352 Schönewald, Germany)
Environmental conditions:	
Temperature:	17 – 23°C
Humidity:	40 – 70%
Air changes:	no data available
Photo period:	12-hour light-dark cycle (06:00 – 18:00, 18:00 – 06:00)

B. STUDY DESIGN AND METHODS

4. Dates of experimental work: 15-March-2001 (day of application)

5. In-vitro pre-test: No *in vitro* pre-test was conducted.

6. Animal assignment and treatment:

The potential of ETU to cause acute dermal irritation or corrosion was assessed by a single topical application of the unchanged test substance (moistened with water) to the clipped intact dorsal skin of three Himalayan rabbits. A dose of 500 mg ETU, moistened with aqua ad iniectabilia, was applied to the test site (area: approx. 6 cm²). The test substance was applied to the test site and then covered with a gauze patch. The patch was held in place with non-irritating tape for the duration of 4 hours. Since this study was performed according to OECD 404 a semi-occlusive cover is assumed. Observation of local reactions was performed 1, 24, 48 and 72 hours after removal of the patch.

II. RESULTS AND DISCUSSION

No erythema or edema of intact skin was noted after 1, 24 to 72 hours in the 3 rabbits treated with ETU (Table 5.8.1-9).

Table 5.8.1-9: Skin irritation study of ETU in Himalayan rabbits

Readings	Animal	Erythema	Edema	Additional findings
1 h	01 (♂)	0	0	
	02 (♂)	0	0	
	03 (♂)	0	0	
24 h	01	0	0	
	02	0	0	
	03	0	0	
48 h	01	0	0	
	02	0	0	
	03	0	0	
72 h	01	0	0	
	02	0	0	
	03	0	0	
Individual means 24 – 72 h	01	0.0	0.0	
	02	0.0	0.0	
	03	0.0	0.0	
24-72 h mean	all	0.0	0.0	

IV. CONCLUSION

Based on the findings of this study ethylene thiourea does not show a skin irritation potential under the test conditions chosen. Based on the results of this study, ethylene thiourea does not warrant classification as to skin irritation according to EU criteria (28th ATP of Council Directive 67/548/EEC by Commission Directive 2001/59/EC / Council Regulation 1272/2008/EC) and according to the OECD Globally Harmonized System (GHS, revision 2, 2007).

Report:	*CA 5.8.1/9*
	██████ 2001c
	Acute eye irritation study of ETU (Ethylene thiourea) by installation into the conjunctival sac of rabbits
	2001/1014629
Guidelines:	*OECD 405, EEC 92/69 B 5*
GLP:	*yes*
	(certified by Behoerde fuer Arbeit, Gesundheit und Soziales, Freie und Hansestadt Hamburg, Hamburg)

Report:	*CA 5.8.1/10*
	██████ 2003c
	Acute eye irritation study of ETU (Ethylene Thiourea) by instillation into the conjunctival sac of rabbits according to EC guideline B.5 and OECD guideline 405 - First revision
	2003/1029081
Guidelines:	*OECD 405, EEC 92/69 B 5*
GLP:	*yes*
	(certified by Freie und Hansestadt Hamburg, Behoerde fuer Arbeit, Gesundheit und Soziales, Hamburg, Germany)

Note: First Revision was done in ordert o prepare the report according to FIFRA guidelines. No changes concerning the results were performed.

Executive Summary

In an eye irritation study, the eye irritation/corrosion potential of ethylene thiourea (ETU; batch: L33-99, purity: 99.6%) was determined by instillation of 100 mg of the test substance into the conjunctival sac of the right eye of three Himalayan rabbits.

The ocular reactions were assessed approximately 1, 24, 48 and 72 hours after the administration of the test substance.

No reaction concerning the corneal opacity, iritis and conjunctival chemosis was observed during the observation period. No additional findings were noted in the animals during the observation period.

Eye irritation scores (24 to 72 hours) for each animal were 0.0 for corneal opacity, iris, conjuctival discharge and conjunctival redness. Consequently, mean average scores for irritation were calculated to be 0.0 for corneal opacity, iris, chemosis and conjunctival redness.

(BASF DocID 2001/1014629)

II. MATERIAL AND METHODS

B. MATERIALS

1. **Test Material:** ethylene thiourea (ETU)
   Description: solid powder, light beige
   Lot/Batch #: L33-99
   Purity: 99.6%
   Stability of test compound: The stability of the test substance was guaranteed by the sponsor until September 2005.

2. **Vehicle:** test substance was used unchanged

3. **Test animals:**
   Species: Rabbit
   Strain: Himalayan
   Sex: male
   Age: 7-8 months
   Weight at dosing (mean): 2.2 – 2.3 kg
   Source: [redacted]
   Acclimation period: at least 20 days
   Diet: Altromin 2023 (Altromin GmbH, 32791 Lage/Lippe, Germany), ad libitum
   Water: tap water ad libitum
   Housing: Single housing in special restrainers with dimensions of 425 mm x 600 mm x 380 mm (manufacturer: Dipl. Ing. W. EHRET GmbH, 16352 Schönewald, Germany)

   Environmental conditions :
   - Temperature : 17 – 23°C
   - Humidity : 40 – 70%
   - Air changes: no data available
   - Photo period: 12-hour light-dark cycle (06:00 – 18:00, 18:00 – 06:00)

B. STUDY DESIGN AND METHODS

4. **Dates of experimental work:** 20-March-2001 (day of application)

5. **In-vitro pre-test:** No *in vitro* pre-test was conducted

6. **Animal assignment and treatment:**

The potential of ETU to cause acute eye irritation/corrosion was assessed by instillation of 100 mg) of the undiluted test substance into the conjunctival sac of the right eye. The left eye, which remained untreated, served as the negative control. The eyes of the animals were not rinsed.

The ocular reactions were assessed approximately 1, 24, 48 and 72 hours after the administration of the test substance with a slit lamp. 24 hours after administration the eyes were treated additionally with fluorescein and examined.

II. RESULTS AND DISCUSSION

No ocular reaction on the cornea, conjunctiva and iris were observed (see Table 5.8.1-10). The fluorescein test did not show any pathological findings.

Table 5.8.1-10: Individual and mean eye irritation scores after ocular application of ETU

Readings	Animal	Cornea	Iris	Conjunctiva		Additional finding
		Opacity		Redness	Chemosis	
1 h	01 (♂)	0	0	0	0	
	02 (♂)	0	0	0	0	
	03 (♂)	0	0	0	0	
24 h	01	0	0	0	0	
	02	0	0	0	0	
	03	0	0	0	0	
48 h	01	0	0	0	0	
	02	0	0	0	0	
	03	0	0	0	0	
72 h	01	0	0	0	0	
	02	0	0	0	0	
	03	0	0	0	0	
Mean 24 – 72 h	01	0.0	0.0	0.0	0.0	
	02	0.0	0.0	0.0	0.0	
	03	0.0	0.0	0.0	0.0	
Mean		0.0	0.0	0.0	0.0	

III. CONCLUSION

Based on the findings of this study ethylene thiourea does not have an eye irritation potential under the test conditions chosen. Based on the results of this study, ethylene thiourea does not warrant classification as to eye irritation according to EU criteria (28th ATP of Council Directive 67/548/EEC by Commission Directive 2001/59/EC / Council Regulation 1272/2008/EC) and according to the OECD Globally Harmonized System (GHS, revision 2, 2007).

One case of allergic contact dermatitis to the rubber additive material ethylene thiourea (ETU) has been published. The patch testing was positive with 1%, 0.1% and 0.01% ETU (Bruze M, Fregert S, 1983; BASF DocID 1983/10301).

Short term toxicity (ETU)

Short-term toxicity is summarized in Table 5.8.1-11 below as extracted from the monograph.

Table 5.8.1-11: Summary of already peer reviewed short term toxicity studies on ETU

Type of test Test species	Test substance purity	Dose tested	NOAEL	Reference
Rat 90-day feeding study	96.8%	0, 1.0, 5.0, 25, 125, 625 ppm	25 ppm, equivalent to **1.44 mg/kg bw**	1977/10212
Mouse 90-day feeding study	98%	0, 125, 250, 500, 1000, 2000 ppm	250 ppm, equivalent to **38 mg/kg bw**	1992/12022 1992/1004097
Dog 13-week feeding study	98%	0, 10, 150, 2000 ppm	150 ppm, equivalent to **6.02 mg/kg bw**	1991/5117
Additional information on ETU				
Rat 28-day drinking water	-	0, 10.6, 17.6, 23.4 mg/kg bw	No NOAEL	1986/10347 1991/11527
Rat 13-week feeding study	-	0, 60, 125, 250, 500, 750 ppm	No NOAEL	1992/12022 1992/1004097
Rat 30, 30,90,120-day feeding study	-	0, 50, 100, 500, 750 ppm	No NOAEL	1972/10122

No new studies are available concerning short-term toxicity of ethylene thiourea.

Summaries of Freudenthal 1977 (BASF DocID 1977/10212) and of the 13-week dog study (BASF DocID 1991/5117) (as provided in the Monograph) are included here, as some results of the Freudenthal, 1977 are used later and the dog study is the proposed study to be used for AOEL setting.

Report:	*CA 5.8.1/11* *Freudenthal R.I. et al., 1977a* *Dietary subacute toxicity of Ethylene thiourea in the laboratory rat* *1977/10212*
Guidelines:	*none*
GLP:	*no*

Material and methods:
ETU (96.8% pure) was administered in corn oil (1%) to 5 groups (60/sex/group) of rats at dietary concentrations of 0 (control 72/sex/group), 1.0, 5.0, 25, 125 or 625 ppm for 90 consecutive days. At 30-day intervals, 10 rats/sex/group were sacrificed and serum T_3, T_4, TBG (thyroid binding globulin) and TSH (thyroid stimulating hormone) levels were measured. The remaining 10 rats of each sex per group were used for thyroid ^{125}I uptake studies. The free thyroxin index (TFI) was calculated. Necropsy was also performed. The remaining 10 animals/sex/group were used to evaluate the thyroid uptake of ^{125}I. Microscopic examination of tissues was restricted to thyroid and liver. PTU (propylthiouracil) and amitrole were used as positive controls.

Findings:
Rats exposed at the highdose level showed an increased mortality rate and a marked body weight decrease. Clinical signs were observed as early as the 8th day of treatment and included excessive salivation, loss of hair, rough and bristly hair coat and scaly skin texture. Microfollicular hyperplasia was seen in the thyroid at ≥ 125, ppm but also at the 25 ppm level at observation day 30 only. At 625 ppm adenomatous hyperplasia of follicles to solid adenomas were present. Liver changes (mild centrilobular changes) were not dose-related.
Thyroid – to – brain ratio was significantly increased at ≥ 125 ppm at all observation intervals.
At 125 ppm, serum T_4 was decreased and TSH (measured only at 30 d timepoint) was 3-fold increased (compared to controls) At 625 ppm radiolabeled iodine uptake, T_3, bound TBG, and T_4 values were significantly decrased at all time points only. TSH was 2-fold increased at that dose (30 day timepoint).
With the exception of a statistically significant decrease in serum T_4 at 25 ppm at day 60, but not at day 30 and 90 and not accompanied by a change in free thyroxin index (FTI), there was no measurable difference in thyroid function parameters at 25, 5 and 1 ppm.

Conclusion:
A NOAEL has been determined at 25 ppm (1.44 mg/kg bw in male and 2.12 mg/kg bw in female rats) at week 12 based on the observation of altered thyroid function and follicular hyperplasia.

Report:	*CA 5.8.1/12*
	█████ 1991a
	ETU. 13 week oral (dietary) toxicity study in the beagle dog
	1991/5117
Guidelines:	*EPA 82-1*
GLP:	*yes*

Material and methods:
ETU (purity 98.0%) was administered daily in the diet to four groups of each four male and female Beagle dogs for 13 weeks at dietary concentrations of 0 (control), 10, 150 and 2000 ppm. Overall mean test article intake (in mg/kg body weight/day) was as follows: 0, 0.39 for males and 0.42 for females; 6.02 for males and 6.51 for females; 66.23 for males and 71.62 for females. Clinical examinations were performed daily. Body weight was recorded weekly for each animal. Food consumption was evaluated daily for each animal. Ophtalmological examinations were performed pretest and at week 13. Clinical pathology investigations (haematology, blood, biochemistry and urine analysis) were performed pretest and at week 4, 8 and 13. Measurement of the blood levels of thyroid hormones T3 and T4 was performed for all animals pretest and at weeks 4, 8 and 13. All surviving animals were killed and after organ weighed necroscopy examination performed at week 13. Tissues of all animals were examined histopathologically.

Findings:
<u>Mortality</u>
Two group 4 males were killed moribund on day 51.

In one of the two sacrificed male animals decreased activity, tremor, thin appearance, wounds and/or swelling of the eyelids were observed. Recurring hyperthermia was noted as from day 36 (up to 40.5°C) in one male. An infection was suspected in one sacrifices animal and thus antibiotic treatment was administered. No clinical improvement was observed. Anorexia or marked dysorexia was noted on several days between day 33 and 50. A progressive reduction in body weight was noted as from week 5 (-30% between day 29 and 51). Moderate changes in haematology and clinical chemistry parameters and a dark coloration of urine collected on day 50 was observed in both sacrificed animals. Necropsy revealed effects on bone (thickening, fibrous), submaxillary lymph node (enlarged), kidneys (pelvic dilatation), prostate (small), and thyroid (enlarged). Histopathology revealed hypotrophy of the epididymides, hypertrophy of the basophilic cells with microvacuolisation in the pituitary, glandular hypotrophy of the prostate, moderate lymphoid hyperplasia in the submaxillary lymph node, slight focal atrophy/degeneration of the testes, moderately severe involution of the thymus, and increase in colloid size in the thyroid.

In the second sacrificed male animal very marked pallor, decreased activity, rough coat, and dyspnea were observed on day 51. Dysorexia was noted as from day 48. A slight reduction in body weight was noted during weeks 7 and 8 (-8% between day 43 and 51). Necropsy revealed effects on kidneys (pelvic dilatation), spleen (enlarged), and thyroid (enlarged). Histopathology revealed minimal, multifocal necrosis in the liver, increased extramedullary haematopoiesis in the spleen, hypertrophy of the basophilic cells with microvacuolisation in the pituitary, glandular hypotrophy of the prostate, minimal focal atrophy/degeneration of the testes (partial atrophy of some seminiferous tubules with the presence of giant multinucleated cells), and severe diffuse follicular hyperplasia in the thyroid.

Clinical signs
No treatment-related clinical signs were observed in group 2 and 3 animals. In the surviving group 4 animals pale mucous membrane in one male and female animal. Decreased activity was noted in all group 4 females. Blood was observed in the faeces of two females throughout the treatment period. A bilobed swelling in the pharyngeal area in 2 females of group 4 was observed. Further clinical signs were not attributed to treatment.

Body weight
No statistically significant changes were observed in any of the treated groups. A biologically significant decrease in body weight was noted in the two group 4 males killed moribund during the treatment period. During the treatment period a body weight gain, biologically similar to that of the controls, was noted in all surviving, treated animals.

Food consumption
No treatment-related effects were observed in group 2 and 3 animals. A slight to moderate reduction in the mean food consumption was noted in group 4 animals from week 5 for males and week 3 for females. The reduction was statistically significant at weeks 12 and 13 for group 4 males and at weeks 11 and 12 for group 4 females. The reduction in food consumption was associated with a biologically significant decrease in food efficiency in group 4 males during weeks 5, 6, and 7. Episodes of anorexia/marked dysorexia were noted only in the two group 4 males, which were killed moribund.

Table 5.8.1-12: Mean maternal food consumption in males

Group mg/kg bw/day	**Food consumption (g/animal/day) at week**				
	3	**5**	**11**	**12**	**13**
Control	352±37	302±26	317±47	315±35	335±45
10	333±33	292±23	297±42	280±12	317±41
150	337±28	304±36	337±23	333±14	319±28
2000	322±98	253±31	247	**217****	**223***

*:p≤0.05; **:p≤0.01

Table 5.8.1-13: Mean maternal food consumption in females

Group mg/kg bw/day	**Food consumption (g/animal/day) at week**				
	3	**5**	**11**	**12**	**13**
Control	333±29	272±31	301±16	278±14	289±28
10	310±68	274±63	308±49	286±35	310±65
150	306±19	288±14	326±25	306±13	300±10
2000	247±55	245±62	**239±26***	**223±29***	235±24

*:p≤0.05; **:p≤0.01

Ophthalmology
Ophthalmological examination revealed no treatment related changes.

Haematology
A slight to moderate decrease in haemoglobin (Hb), packed cell volume (PCV) and red blood cell count (RBC) was noted at weeks 8 and 13 in group 4 animals. The reduction in haemoglobin was statistically significant at week 8 for group 4 females and at week 13 for group 4 males and females.
At week 13, the reduction in Hb and RBC noted in group 4 was associated with a slight to moderate increase in the reticulocyte count. A very high number of reticulocytes was noted in one male before killing.

Table 5.8.1-14: Summary of clinical haematology (week 13)

Dose (ppm)	Males				Females			
	Hb (g/dL)	**PCV (%)**	**RBC (M/mm^3)**	**RETI (/1000)**	**Hb (g/dL)**	**PCV (%)**	**RBC (M/mm^3)**	**RETI (/1000)**
0	14.1±1.5	41.6±2.7	6.5±0.3	3±1	14.9±0.6	42.9±1.8	6.5±0.4	4±3
10	13.6±0.4	40.1±1.6	6.1±0.1	5±3	13.6±1.2	40.2±2.8	6.1±0.5	7±3
150	14.6±1.3	43.8±5.1	6.7±0.8	8±4	13.5±0.9	39.1±2.8	5.9±0.5	7±2
2000	**11.4±0.1***	**33.5±1.5***	**4.9±0.2****	9±7	**11.3±0.3*****	**33.1±1.3*****	**4.9±0.1*****	**14±6****

*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$

Clinical chemistry
A slight decrease in sodium and chloride levels and moderate decreases in phosphorus, alkaline phosphatase, ASAT and ALAT activity, and blood urea nitrogen were observed. Except for one animal (phosphorus) all individual values of these parameters were within the normal limits. Slight to moderate increases of potassium, serum protein, creatinine, and total cholesterol levels were observed. Increases of protein (globuline) and cholesterol in individual animals were above the normal limits.

Urine analysis
Urine volume and specific gravity were not influenced by treatment. Orange or dark colored urine was noted in four group 4 animals at week 8 and/or 13.

Thyroid hormones
No treatment-related effects were observed in group 2 and 3 animals. A marked reduction in blood levels of T_3 and T_4 was noted in all group 4 animals at weeks 4, 8, and 13 (apart from the reduction in T_3 levels which was noted only at weeks 8 and 13 in group 4 females). The lowest blood levels were noted at weeks 8 and 13, blood levels below the limits of detection were noted for most of the group 4 animals.

Table 5.8.1-15: Serum T_3 and T_4 Levels in male and female animals

Dose (ppm)	Males				Females			
	0	10	150	2000	0	10	150	2000
T_3								
-Pretest	74.5±2.5	81.9±12.9	81.0±8.8	78.6±7.8	91.1±6.8	**70.5±5.9***	**63.1±13.7****	82.0±12.2
-Week 4	66.9±8.7	71.5±14.0	71.6±19.1	41.2±16.2	68.7±11.2	72.4±15.6	64.1±11.6	66.5±21.3
-Week 8	69.1±14.1	61.4±13.0	79.9±16.2	**20.0****	59.7±12.9	64.6±26.4	66.9±17.4	**20.0****
-Week 13	69.4±23.1	63.5±7.7	103.4±23.9	31.7	71.5±11.8	60.2±18.3	71.4±3.7	**25.4±5.3*****
T_4								
-Pretest	2.2±0.1	2.5±0.6	2.1±0.3	2.2±0.5	2.2±0.3	2.1±0.3	2.0±0.4	2.4±0.4
-Week 4	1.9±0.3	1.7±0.5	1.5±0.5	**0.6±0.4****	1.9±0.2	2.2±0.6	1.7±0.6	1.1±0.6
-Week 8	2.2±0.7	2.0±0.6	1.9±0.4	**0.2****	2.0±0.3	2.4±1.1	2.1±0.4	**0.2****
-Week 13	2.1±0.8	1.5±0.4	2.1±0.3	**0.2****	1.9±0.3	2.1±0.5	2.0±0.4	**0.2*****

*: p≤0.05; **: p≤0.01; ***: p≤0.001

Pathology

Organ weights

A marked increase in thyroid weight (absolute, body weight and brain weight ratios) was noted at week 13 in group 4 males and females, being statistically significant for the females.

A slight increase in liver weight was noted at week 13 in group 4 males (absolute and brain weight ratios) and females (absolute, body weight and brain weight ratios), being statistically significant for the brain weight ratio in females.

A slight increase in adrenal weight (absolute, body weight and brain weight ratios) was noted at week 13 in group 4 males and females, being statistically significant for the body and brain weight ratio in females.

Other changes observed were not considered to be biologically significant.

Macroscopic findings

Exophthalmia was noted in 2 males and 3 females from group 4 and in 1 group 3 male. A very marked enlargement of the thyroid gland was noted in all surviving group 4 animals.

Microscopic findings

No treatment-related microscopic changes were noted in the animals of groups 2 and 3. Hypertrophy of the basophilic cells of the pituitary with microvacuolisation was observed in all group 4 animals. Severe follicular hyperplasia of the thyroid gland was observed in all surviving group 4 animals. A moderate involution of the thymus was observed in 1 male and 2 females from group 4.

Discussion
Treatment-related effects were limited to the high dose level of 2000 ppm. At this level, two male dogs lost weight and had to be sacrificed on day 51 of treatment (one had developed an infection and the other a severe regenerative anaemia). Females were subdued and less active but gained weight normally. Food consumption was moderately depressed in both sexes. Food efficiency factor was moderately depressed in males.
A moderate to marked increase in the cholesterol level was observed throughout the duration of the study and was attributed to the alteration of the thyroid functions. A regenerative, non-haemolytic, anaemia was seen at the week 8 and 13 examination. Changes in the thyroid included, in both sexes, a significant increase in the size and weight of the gland and severe follicular hyperplasia. This damage manifested as a marked reduction in blood levels of T3 and T4 at weeks 4, 8 and 13. A slight increase in liver and adrenal weight without histopathological alteration was observed. Hypertrophy and microvacuolisation of the basophilic cells of the pituitary of some animals of the high dose group was noted.

Conclusion:
The **NOAEL** was determined at 150 ppm, equivalent to **6.02 mg/kg bw/day** in males and to **6.51 mg/kg bw/day** in females, on the basis of mortality and thyroid effects, registered at 2000 ppm.

Genotoxicity (ETU)

A total of more than 90 individual studies are presently available, either in the peer-reviewed published literature or as technical reports of studies conducted in compliance with Good Laboratory Practices and OECD and/or other applicable national regulatory testing guidelines. Inevitably among such a large database, there are a few positive results. These conflicting reports highlight a generic problem in genotoxicity safety assessment: although individual test results typically yield either a positive or a negative response, the overall evaluation of an extensive battery of tests for a particular chemical rarely yields an unambiguous conclusion. In 1992, Mendelsohn, *et al*. (BASF DocID 1992/1004078) showed that the response of a chemical to a battery of genotoxic tests is not a dichotomous (i.e., either positive or negative) property, but rather, appears to be a continuous property that ranges from strongly negative to strongly positive. This result together with a four-step weight of evidence procedure Elia et al. (DocID 1994/11032) evaluate ETU. The proposed methodology, a four-step weight of the evidence procedure which can be used to guide a rational evaluation of a chemical's potential to cause genotoxicity in humans, was described in detail in the monograph of the Annex I inclusion of metiram. Based upon this evaluation, in higher organism test systems ETU is not mutagenic in the two major endpoints used to assess genotoxicity, gene mutation and chromosomal damage, and, further, it does not cause adverse effects in ancillary test of genotoxic damage. In summary ETU is not mutagenic in mammalian systems.

The conclusion derived using this four-step, weight of the evidence procedure concurs with the evaluation of most other responsible authorities who have generally agreed that ETU is not a mammalian genotoxin. These authorities include:

· the UK MAFF who concluded that:
"the mutagenicity studies... show no evidence that ETU. . . is genotoxic *in vivo*." (UK MAFF, January, 1990, BASF DocID DK-901-005),

· the US National Toxicology Program (NTP) who concluded that:
"Ethylene thiourea has been tested extensively for genotoxicity in a variety of *in vitro* and *in vivo* systems, and the results, with few exceptions, are negative." (Chhabra et al,1992, BASF DocID 1992/12022, NTP, 1992 BASF DocID1992/1004097).

· the International Agency for Research on Cancer, who concluded that for ETU:
"No data were available on the genetic and related effects of ethylene thiourea in humans. Ethylene thiourea did not induce dominant lethal mutations, micronuclei or sister chromatid exchanges in mice or chromosomal aberrations in rats treated *in vivo*. It did not induce unscheduled DNA synthesis in human fibroblasts *in vitro* or chromosomal aberrations, sister chromatid exchanges, mutation or unscheduled DNA synthesis in rodent cells *in vitro*. Ethylenethiourea did not induce sex-linked recessive lethal mutations in *Drosophila*, but it induced aneuploidy and mutation in yeast. Studies on gene conversion and DNA damage in yeast and on mutations in bacteria have given conflicting results." (IARC, 1987, BASF DocID 1987/1002154), and

· the FAO/WHO, who concluded that:
"ETU has been the subject of many *in vitro* and *in vivo* studies for genotoxicity. It induced mutations in bacteria at very high doses but variable responses have been obtained in other types of mutation assays. Acceptable assays for other genotoxicity endpoints *in vitro* were generally negative, while all *in vivo* assays were negative. The Meeting concluded that ETU was not genotoxic (FAO/WHO, 1993, BASF DocID 1993/11609)."

Thus, the weight of the evidence of this comprehensive data base indicates that ETU is not genotoxic in mammalian systems and is unlikely to pose a genotoxic risk to humans.

Long-Term Toxicity and Carcinogenicity (ETU)

Long-term toxicity is summarized in Table 5.8.1-19 below as extracted from the monograph.

The rat has generally been the most sensitive species to ETU-induced thyroid effects, followed by the dog and the monkey (monkey study is described under FAO/WHO, 1993, BASF DocID 1993/11609), with the mouse being relatively insensitive. A NOAEL for the induction of thyroid tumors was demonstrated in a combined perinatal and adult exposure 2-year feeding studies in rats at 25 ppm, equivalent to 1.1 to 1.2 mg/kg bw, and an overall NOAEL for the effects of ETU on the thyroid-pituitary axis has been demonstrated in 2-year chronic feeding studies in the rat at 5 ppm equivalent to 0.37 to 0.49 mg/kg bw in males and females, respectively.

Induction of liver tumors by ETU has been observed only in the mouse, and only at dietary levels of 330 ppm, equivalent to 55 to 61 mg/kg bw, or higher. These dose levels were also associated with centrilobular hepatocellular cytomegaly and other typical indications of generalized work-related stress to the liver, in addition to thyroid inhibition and TSH-induced follicular hyperplasia and neoplasia. Hepatocellular tumors have not been seen in rats or hamsters. In the mice, no increase in the incidence of the liver tumors was noted after 2 years of dietary feeding at 100 ppm, equivalent to 17 to 18 mg/kg bw. With respect to human evidence IARC has concluded in 2001 (IARC, 2001, Volume 79, BASF DocID 2001/1028635), that there is sufficient evidence for carcinogenicity seen in experimental animal studies, but that there is inadequate evidence in humans for the carcinogenicity of ethylene thiourea. A classification into IARC category 3 (not classifiable as to its carcinogenicity to human) has been concluded. In making its evaluation, the Working Group concluded that ethylene thiourea produces thyroid tumors in mice and rats by a non-genotoxic mechanism, which involves interference with the functioning of thyroid peroxidase resulting in a reduction in circulating thyroid hormone concentrations and increased secretion of thyroid-stimulating hormone. Consequently, ethylene thiourea would not be expected to produce thyroid cancer in humans exposed to concentrations that do not alter thyroid hormone homeostasis. An additional consideration of the Working Group, based on the lack of genotoxicity of ethylene thiourea, was that the liver tumors and benign pituitary tumors in mice were also produced by a non-genotoxic mechanism. Evidence from epidemiological studies and from toxicological studies in experimental animals provides compelling evidence that rodents are substantially more sensitive than humans to the development of thyroid tumors in response to thyroid hormone imbalance (IARC, 2001, BASF DocID 2001/1028635).

The liver is subject to metabolic regulation by thyroid hormones, and liver neoplasms have also been produced in mice by two other thionamides, 2-thiouracil and 6-methyluracil, which also produced thyroid neoplasms in both rats and mice. There have been no reports that the use of propylthiouracil or other clinically-administered thionamides associate with an excess incidence of primary liver cancer in humans.

There is a wide variability in the incidence of liver tumors among various strains of mice, which is partly dependent on hormonal and/or nutritional factors, in addition to genetic factors. Genetic factors are particularly operative in the case of the B6C3F1 and other C3H-derived strains whose high and variable background tumor incidences indicate the presence of a significant population of "initiated" or latent tumor cells whose potential is readily expressed under stressed conditions of various origins. That the induction of these types of tumors is of questionable relevance for assessment of oncogenic potential in human populations, where the background incidence of liver cancer is extremely low, has been acknowledged by numerous authorities.

Since the liver tumor response with ETU occurs at dose levels with previously established significant thyroid toxicity and is similar to that of other thionamide drugs, it is believed to be related secondary to the primary thyroid effect. Thus, if relevant at all to human risk assessment, the liver tumor response would have a threshold at a level equivalent or higher than that for thyroid effects.

Report: CA 5.8.1/13
Mattioli F. et al., 2006a
DNA fragmentation and DNA repair synthesis induced in rat and human thyroid cells by chemicals carcinogenic to the rat thyroid
2006/1051536
Guidelines: none
GLP: no

Executive Summary:

Chemicals, including ethylene thiourea, a metabolite of mancozeb, known to induce thyroid follicular-cell adenomas and carcinomas in rats were assessed for their ability to induce DNA damage and DNA repair synthesis in primary cultures of human thyroid cells. DNA fragmentation was also assessed *in vivo* using the Comet assay in kidney, liver and thyroid cells of rats given a single oral dose of ethylene thiourea, equivalent to ½ LC50.
Significant dose-dependent increases in the frequency of DNA single-strand breaks and alkali-labile sites, as measured by the same Comet assay, were obtained after a 20-h exposure to ethylene thiourea from 1.25 to 5 mM. These concentrations were not cytotoxic.
DNA repair synthesis, as evaluated by quantitative autoradiography, was present in thyroid cells exposed to ethylene thiourea from two of three donors, but did not match the criteria for a positive response in thyroid cells.

Ethylene thiourea, administered orally to rats as a single dose of 916 mg/kg (corresponding to ½ LD50), induced a statistically significant degree of DNA fragmentation in thyroid cells but not in liver or kidney cells. These findings suggest that the compound might be carcinogenic to thyroid in humans.

Objective:
To investigate the ability of ethylene thiourea to induce DNA damage and DNA repair synthesis in primary cultures of human thyroid cells.

Materials and Methods:

Test material	Ethylene thiourea (ETU)
Description	None
Source	Sigma-Aldrich, Milan, Italy
Batch	No data
Purity	98%

Test Animals	
Species	Rat
Strain	Sprague Dawley
Age/weight requested from supplier	120-150 g
Source	Harlan (Correzzana. Italy).
Acclimatization period	1 week
Diet	TRM rat chow supplied by Harlan, *ad libitum*
Water	Tap water, *ad libitum*
Environmental conditions	Temperature: 22±2.0°C Humidity: 50±10% Air changes: no data Photoperiod: 12 hours light / 12 hours dark

Preliminary assay: A cytotoxicity assay provided evidence that a 20-h exposure to serial concentrations increasing for the factor 2 produced a dose-dependent reduction in the fraction of trypan-blue-excluding primary human thyroid cells. The highest concentration producing less than 30% reduction in relative survival was 5 mM. To avoid DNA damage due to toxicity this was the maximum concentration tested.

Cells and treatments: Human thyroid samples were obtained from discarded surgical material during the course of prescribed surgery. Thyroid cells were isolated within 2 h from thyroidectomy. Viable cells, as measured by the trypan blue exclusion test, were 90 to 95%. Morphologically, the cells appeared to be more than 90% follicular cells; the other cells were mainly fibroblasts. Aliquots of cell suspensions (2 x 10^5 cells/well) were plated in 24-well plates for the Comet assay and in 35-mm dishes coated with rat tail collagen (1 x 10^6 cells/dish) for cytotoxicity and DNA repair synthesis determinations. After an attachment period of 3 h at 37°C in an atmosphere of 95% air-5% CO^2, cell cultures were washed and incubated for 20 h with serial concentrations of ethylene thiourea and the positive control methyl methanesulfonate (MMS) in serum-free medium.
The media containing ethylene thiourea was prepared from stock solutions in dimethyl sulfoxide ≤0.5% in corresponding control cultures. MMS was dissolved in the medium just prior to use. [Methyl-^{3}H]thymidine (10 µCi/mL) was added to thyroid cell cultures to be used for the DNA repair assay and left in the culture for the entire treatment period (20h). At the end of treatment, cells were immediately examined for cytotoxicity by trypan blue exclusion, for DNA fragmentation by the Comet assay, and for DNA repair synthesis by quantitative autoradiography. DNA damage and repair were assessed in three independent experiments with cells from 3 three different donors.

DNA-damaging activity and DNA repair: The Comet assay was used to assess DNA fragmentation. DNA repair synthesis was done by autoradiograph to the criteria indicated by the guidelines for the performance of UDS test *in vitro*. One hundred cells per data point were counted manually in two autoradiographs. Net nuclear grains (NNG) of each cell were determined by subtracting the grains of an equal size area in the cytoplasm (CYT) from silver grains over the nucleus (NUC). Cell in S-phase were had very dense labelling of silver grains over the nucleus. A positive response was a dose-dependent increase of NNG over at least two consecutive concentrations. The increase was considered positive if both NNG values exceeded the lab-specific threshold (i.e., NNG treated - NNG control > 5). This was confirmed by a dose-dependent increase in the percentage of repairing cells. The data are expressed as the mean ± S.D. of the 100 net nuclear counts.

***In vivo* studies**:Rats were given a single dose of 916 mg/kg ethylene thiourea corresponding to half the LD50. The dose volume was 0.01 mL/g body weight. Controls received an equal volume of the vehicle (DMSO). Rats were killed 16 h after treatment. Thyroid, liver and kidneys excised. Thyroid, kidney and liver cells were isolated.
The cells of the three organs were finally resuspended in a suitable volume of Merchant's solution and counted in a haemocytometer. The fraction of viable cells, determined with the trypan blue exclusion method, higher than 80% in each animal and organ. The degree of DNA fragmentation was evaluated with the Comet assay. The pooled mean ± S.D. was based on the mean data generated from individual animals.
Statistical analysis: Statistical analysis of Comet assay data was performed by the use of ANOVA and the two samples compared were values of tail length and tail moment in 50 cells from each dose point. The $p<0.05$ level was considered to be statistically significant.

Results:
Comet assay with human thyroid cells: In three independent experiments on cells from three different donors a statistically significant dose-dependent increase of both tail length and moment, indicating DNA single-strand breaks and/or alkali-labile sites was consistently produced by a 20-h exposure to concentrations which were not cytotoxic.
In cultures of thyroid cells exposed to ethylene thiourea a dose-dependent increase over control of both NNGs and the percentage of repairing cells was constantly observed but was not positive by the criteria i.e. increase over control of NNGs was consistently dose-dependent, and at two consecutive concentrations and exceeded threshold (NNGs treated - NNGs control > 5). Positive response criteria were matched in cultures from only two of three patients.

Table 5.8.1-16: Damage of nuclear DNA and DNA repair synthesis induced in primary cultures of human thyroid cells by 20 h exposure to ETU

Treatment condition	D	Relative survival	Comet assay		DNA repair synthesis			%Repair
			tail length µm	tail moment	NUC	CYT	NNG	
Control (DMSO)	11	(0.96)	1.6 ± 0.4	196 ± 31	5.2 ± 2.7	3.6 ± 2.1	1.6 ± 1.8	3
ETU 1.25 mM	11	1.01	2.9 ± 1.0	262 ± 87a	7.5 ± 2.7	2.9 ± 1.6	4.6 ± 2.2	18
ETU 2.5 mM	11	0.99	2.8 ± 1.1 a	257 ± 100a	6.1 ± 2.3	1.8 ± 1.3	4.3 ± 1.8	32
ETU 5 mM	11	0.98	3.7 ± 1.4a	334 ± 117a	7.8 ± 2.5	1.9 ± 1.4	5.9 ± 1.9	54
MMS 75 µM	11	0.98	22.2 ± 8.1a	1998 ± 724a	30.8 ± 8.4	4.6 ± 2.9	26.2 ± 8.3	100
Control (DMSO)	12	(0.96)	1.7 ± 0.3	160 ± 29	10.1 ± 3.3	9.7 ± 2.9	0.4 ± 1.7	34
ETU 1.25 mM	12	1.01	2.4 ± 0.6a	215 ± 58a	13.6 ± 4.4	9.9 ± 4.5	3.7 ± 2.4	42
ETU 2.5 mM	12	0.99	2.7 ± 1.0a	249 ± 94a	9.5 ± 2.7	3.7 ± 2.1	5.8 ± 2.0b	76
ETU 5 mM	12	0.98	3.3 ± 1.4a	247 ± 100a	13.0 ± 4.2	4.0 ± 3.6	9.0 ± 2.5b	96
MMS 75 µM	12	0.98	19.5 ± 6.8a	1766 ± 530a	43.1 ± 9.4	13.0 ± 3.7	30.1 ± 9.3	100
Control (DMSO)	13	(0.95)	1.7 ± 0.5	57 ± 47	9.7 ± 3.2	8.0 ± 3.1	1.7 ± 2.3	12
ETU 1.25 mM	13	1.01	2.5 ± 0.7a	224 ± 66a	2.9 ± 3.1	7.1 ± 3.1	5.8 ± 2.3	76
ETU 2.5 mM	13	1.02	3.1 ± 0.8a	255 ± 64a	12.6 ± 3.4	4.8 ± 2.7	7.8 ± 2.0b	98
ETU 5 mM	13	1.01	3.8 ± 1.2a	318 ± 100a	14.7 ± 3.8	6.5 ± 3.1	8.2 ± 2.0b	100
MMS 75 µM	13	0.99	9.9 +2.9a	1711+230a	34.2 +7.6	11.0 ± 5.1	23.2 +5.3	100

mean ± standard deviation
D = donor ref.
Relative survival: values in parentheses indicate the fraction of viable cells in controls: relative survival was calculated from the ratio (fraction of viable cells in treated cultures/fraction of viable cells in control cultures).
DNA repair synthesis: NUC, nuclear grain count; CYT, cytoplasmic grain count: NNG. net nuclear grains. The % repair is the percentage of cells with net nuclear labelling >5 grains.
a Comet assay: significance level was determined by the use of ANOVA (p <0.05).
b Positive response. i.e. NNG treated - NNG control ≥ 5 over at least two consecutive concentrations.

***In vivo* study:** Ethylene thiourea was tested in three rats, and three rats were vehicle controls. None of the rats died or showed marked signs of toxicity. Ethylene thiourea induced a statistically significant marked increase of DNA lesions in thyroid cells. The ratio treated/control of tail length was 8.1. The presence or absence of DNA fragmentation in liver and kidney was positively correlated with the capability of the test compounds of inducing the development of tumors in these two organs DNA fragmentation was absent or of minimum degree in the liver and kidney of rats treated ethylene thiourea. This is consistent with the finding that, in rats, ethylene thiourea causes only thyroid tumors.

Table 5.8.1-17: Comet assay results in cells from rats given a single dose of ETU

	Thyroid		Liver		Kidney	
Rat number	tail length µm	tail moment	tail length µm	tail moment	tail length µm	tail moment
1	13.7 ± 9.4	1181 ± 846	1.3± 0.5	131 ± 52	7.4 ± 4.2	575 ± 313
2	13.3 ± 6.8	1085± 578	2.8 ± 1.5	258 ± 136	4.5 ± 2.2	379± 170
3	18.8 ± 4.2	1863± 502	3.1 ± 1.0	316± 101	5.6 ± 2.6	411 ± 185
pooled	15.3 ± 3.1a	1376± 424a	2.4 ± 1.0	235± 95	5.8 ± 1.5a	455 ± 105a

a: significant ANOVA ($p < 0.05$).

Conclusions:

Ethylene thiourea, administered orally to rats in a single dose corresponding to 1/2 LD50, induced a statistically significant degree of DNA fragmentation in the thyroid. These findings suggest that the compound might be carcinogenic to thyroid in humans.

Report:	*CA 5.8.1/14*
	[redacted] 1992a
	ETU. 52 week oral (dietary) toxicity study in the beagle dog
	1992/5082
Guidelines:	*EPA 83-1, OECD 416*
GLP:	*yes*
	(certified by Ministere de la Solidarite, de la Sante et de la Protection Sociale, Paris, France)

Material and methods:
Groups of Beagle dogs (4 per group and sex) were orally exposed to dietary concentrations of ETU (98% pure) of 0 (control), 5, 50 or 500 ppm for 52 consecutive weeks equal to a test article intake of 0, 0.18 and 0.19; 1.99 and 1.79; 20.13 and 20.15 mg/kg bw/day for males and females, respectively. Clinical examinations were performed daily, and physical examinations were performed pretest and monthly. Food consumption was measured daily for each animal. Body weight was recorded weekly for each animal. Ophtalmological examinations were performed pretest and at weeks 26 and 52. Clinical pathology investigations were performed twice pretest and at week 13, 26 and 52. Measurement at the blood levels of thyroid hormones T3 and T4 was performed for all animals pretest and at weeks 13, 26 and 52. All surviving animals were killed and necropsied after 52 weeks, selected organs were weight at necropsy. Selected tissues from all animals killed after 52 weeks and animals sacrificed moribund or found dead were examined histopathologically.

Findings:
<u>Mortality</u>
A high incidence of mortality was recorded in the high dose group: one female died during week 6 of treatment, one male during week 7 and another male during week 17. The cause of death was attributed to severe non-haemolytic regenerative anaemia. The anaemia was associated with leucopenia, occasional, transient thrombocytopenia and centrolobular hepatocytic necrosis. Bone marrow smears confirmed the diagnostic of regenerative anaemia. Bilirubinemia, occasional bilirubinuria were also recorded in those animals with anaemia.

<u>Clinical signs</u>
No treatment-related clinical signs were observed in group 2 and 3 animals. Pale mucous membranes were observed in 5 animals of group 4, and was associated with a subdued behavior and a change in the colour of the feces (yellow orange). Signs of vomiting and diarrhea were noted with an incidence, which was similar in all groups.

<u>Body weight</u>
A treatment related reduction in mean body weight gain was noted in group 50 ppm males (slight) and in group 500 ppm males and females (moderate). Reductions were not statistically significant but were considered to be treatment-related. Food consumption was not statistically different between groups.

Haematology
A statistically significant decrease in red blood cell count (RBC) was noted at weeks 13 and 26 in group 4 animals and at week 52 for combined group 2 males and females. This change was associated with a statistically significant decrease in haemoglobin (Hb) and packed cell volume (PCV) in group 2 animals.

Clinical chemistry
A slight decrease in sodium and potassium levels was observed at week 13 in group 2 and/or group 4 animals. All individual values were within the normal values. Although not statistically significant, slightly elevated blood urea nitrogen or total cholesterol levels were noted mainly in group 3 and 4 animals. Triglycerides and globulin were statistically significantly elevated in animals of group 4. Total bilirubin was statistically significantly elevated in animals of all dose groups. Creatinine level was moderately but significantly increased at week 26 for group 4 females. No effect on ASAT or ALAT activity was observed. Gamma glutamyltranspeptidase activity was significantly lower at week 52 in group 4 animals.

Urine analysis
Urine volume and specific gravity were not influenced by treatment. No other effects have been observed.

Thyroid changes
Thyroid changes were noted in group 50 and 500 ppm males and females. They were characterised by:

- increased weight, both absolute and relative to body and brain weights (all animals)
- follicular dilatation (hypertrophy) with colloid retention for 1 male and 1 female from group 50 ppm and for 3 males and 2 females from 500 ppm. This change was occasionally associated with increased number of follicles
- reduced T3/T4 hormone levels were detected in the 500 ppm group 4 animals showing anemia.

Table 5.8.1-18: Serum T_3 and T_4 levels in male and female animals

Dose (ppm)	Males				Females			
	0	5	50	500	0	5	50	500
T_3								
-Pretest	79±17	72±19	74±11	83±7	78±10	70±8	75±7	88±17
-Week 13	77±16	86±25	91±25	60±7	90±18	72±9	86±21	96±15
-Week 26	57±13	57±15	65±25	65±6	71±13	65±14	68±15	63±15
-Week 52	67±29	54±13	74±26	61±31	124±106	67±29	73±17	95±5
T_4								
-Pretest	2.7±0.9	2.4±0.6	2.5±0.3	3.5±0.5	2.5±0.8	2.6±0.6	2.3±0.5	2.4±0.9
-Week 13	2.8±0.3	2.3±0.8	2.6±0.6	1.5±0.9	3.1±0.5	2.7±0.6	2.4±0.7	2.8±0.3
-Week 26	2.1±0.4	1.8±0.5	2.3±0.7	1.7±0.4	2.5±0.5	2.3±0.6	2.4±0.6	2.1±0.4
-Week 52	2.2±0.3	1.9±0.6	2.5±0.6	1.6±0.2	2.5±0.6	2.6±1.2	2.2±0.6	2.7±0.4

Pathology

Organ weights

A dose-related increase in thyroid weight (absolute, body weight and brain weight ratios) was noted at week 52 in group 3 and 4 animals.

Macroscopic findings

At week 52 an enlargement of the thyroid gland was noted in one group 4 male. The other macroscopic findings did not indicate an effect of treatment.

Microscopic findings

Animals found dead or were killed in a moribund state showed centrilobular hepatocellular necrosis (2 males and 1 female of group 4). Furthermore, pigment accumulation in Kupffer's cells was observed. Hypertrophy of follicular cells with dilatation of follicles was seen in the thyroid of one male.

Principal lesions of the animals killed at the end of treatment included hepatic lesions (pigment accumulation in Kupffer's cells and hepatocytes) in group 3 and 4 animals. Thyroid changes (follicular dilatation with colloid retention) was also observed in group 3 and 4 animals.

Summary:

Based on the following effects observed in the 50 and 500 ppm dose groups, 5 ppm was determined as NOAEL of the study.

The concentration of 500 ppm was associated with:
- A moderate to severe anemia in 3 males and 1 female. The anemia was regenerative and non-haemolytic. The anemia resulted in the death of 3 animals. This anemia was associated with liver necrosis.
- Leucopenia and/or thrombopenia in some occasions (males and females)
- A moderate increase in serumglobulin (males and females)
- An increase in ASAT/ALAT activity in some males
- A moderate reduction in mean body weight (males and females)
- Enlargement and follicular dilatation of the thyroid gland (males and females)
- Reduction in T3/T4 hormones was only observed in animals which presented anemia
- Pigment accumulation in Kuppfer's cells and occasionally in hepatocytes.

The concentration of 50 ppm was associated with:
- a slight reduction in body weight gain (males)
- a moderate, transient increase in serumglobulin (females)
- enlargement and follicular dilatation of the thyroid gland (males and females)
- pigment accumulation in the liver (Kuppfer's cells and occasionally hepatocytes)

Conclusion:

The highest dietary concentration of 500 ppm (approximately equivalent to 20 mg/kg bw/day) was definitely above the Maximum Tolerated Dose. The NOAEL was determined at 5 ppm, equivalent to 0.18 mg/kg bw/day in males and 0.19 mg/kg bw/day in females, based on increased thyroid weight, hypertrophy with colloid retention at 50 ppm.

ETU is not classified for carcinogenicity.

The US-EPA has classified ethylene thiourea as a Group B2, probably human carcinogen (http://www.epa.gov/ttnatw01/hlthef/ethyl-th.html), based on the NTP study, which showed increased incidence of thyroid tumors in rats, and thyroid, liver, and pituitary gland tumors in mice exposed to ethylene thiourea (NTP, 1992, DocID 1992/11603), however it has been stated, that in a study of female workers occupationally exposed to ethylene thiourea did not report an increasedincidence of thyroid cancer (http://www.epa.gov/ttnatw01/hlthef/ethyl-th.html).

Table 5.8.1-19: Summary of already peer reviewed long-term toxicity studies on ETU

Type of test Test species	Test substance purity	Dose tested	NOAEL	Reference
Dog 1-year feeding study	98%	0, 5, 50, 500 ppm	5 ppm, equivalent to 0.18 mg/kg bw based on increased thyroid weight, hypertrophy with colloid retention at 50 ppm	1992/5082
Rat 2-year feeding study	96.2%	0, 0.5, 2.5, 5.0, 125 ppm	5 ppm, equivalent to **0.37 mg/kg bw**	1992/11621
Mouse 2-year feeding study (perinatal exposure)	97.0%	0, 33, 330 ppm (perinatal phase) 0, 100, 330, 1000 ppm (adult phase)	No NOAEL due to decreased TSH and diffuse thyroid cytoplasmic vacuolisation. Clear evidence of carcinogenicity (thyroid follicular cell neoplasms, hepatocellular neoplasms, adenomas of the pituitary.	1992/12022 1992/1004097
Rat 2-year feeding study (perinatal exposure)	97.0%	0, 9, 30, 90 ppm (perinatal phase) 0, 25, 83, 250 ppm (adult phase)	No NOAEL due to decreased T4, increased TSH and follicular hyperplasia. Clear evidence of carcinogenicity (follicular cell tumors of thyroid).	1992/12022 1992/1004097
Additional information on ETU				
Rat 2-year feeding study	97%	0, 175, 350 ppm	No NOAEL	1972/10092
Rat 2-year feeding study	-	0, 5, 25, 125, 250, 500 ppm	5 ppm equivalent to 0.25 mg/kg bw	1975/10144
Rat 2-year feeding study, 24 month males, 20 month females	-	0, 50, 17, 60, 200 ppm	No NOAEL	1976/10115
Hamster, feeding study 24 month males, 20 month females	-	0, 5, 17, 60, 200 ppm	No NOAEL	1976/10115

No new studies are available concerning long-term toxicity of ethylene thiourea.

The overall lowest NOAEL to be considered for reference value setting is ***5 ppm (corresponding to 0.18 mg/kg bw****) coming from the 1-year dog study (BASF DocID 1992/5082), supported by the NOAELs derived in the 2-year carcinogenicity studies in rats, which corresponds to 0.37 and (BASF DocID 1975/10144 and 1992/11621).*

Reproductive toxicity (ETU)

In the existing Monograph a two-generation toxicity study of ETU in Sprague-Dawley rats was summarized. This study was conducted in 1992 according to the OECD TG 416 (1981). No compound related effects were observed on the reproductive parameters. Effects attributable to the properties of ETU were seen on the target organ, the thyroid. The overall NOAEL was 2.5 ppm, equivalent to 0.11 – 0.43 mg/kg bw. Two peri-postnatal studies in female rats and mice confirm that ETU has no effects on fertility, pregnancy and parturition (BASF DocIDs 1992/12022 and 1992/1004097).
An extended one-generation toxicity study has been conducted, as required by US-EPA Data-call-in. This study, which was not yet peer-reviewed, included a developmental thyroid cohort and 2 neurotoxicity cohorts (PND 22 and PND 70) and is summarized in the following.

Report: CA 5.8.1/15
[redacted] 2013b
Ethylenethiourea (ETU): An F1 extended one generation reproductive toxicity study in Crl:CD(SD) rats
2013/7002198
Guidelines: EPA 870.3800, EPA 870.6300, OECD 443 (2011)
GLP: yes
(certified by United States Environmental Protection Agency)

Report: CA 5.8.1/16
Zablotny C.L. et al., 2011a
Ethylene Thiourea (ETU): Dietary reproduction probe study in Crl:CD(SD) rats
2011/7009688
Guidelines: OECD 421 (1995), EPA 870.3550 (2000)
GLP: yes
(certified by United States Environmental Protection Agency)

Executive Summary

In a dose-finding study rats were treated with ETU prior to breeding, continuing during breeding and post-breeding for assessment of general toxicity, reproduction and developmental toxicity data. Furthermore, the bioavailability of ETU e.g. plasma kinetics were determined.
In an extended one generation toxicity study, ethylenethiourea (ETU; batch: BCBC9199V; purity 100%) was administered in the diet to groups of 27 male and 27 female Crl:CD(SD) Sprague Dawley rats at nominal dose levels of 0, ~0.2, 2.0 and 10 mg ETU/kg bw/day (0, 2.8, 28, 140 ppm). Nominal dose levels were achieved across both P1 and F1 generations; however, during some study intervals (e.g. PND 35-36), F1 offspring had greater ETU intake per kg body weight than P1 males and pre-breeding females. F1 offspring diets, which were adjusted to one-half normal concentration from PND 21-35, returned to full concentration diets on PND 35.
F1 offspring were divided into Cohorts 1A, 1B, 2A, and 2B at weaning (postnatal day (PND) 21) as follows:

- Cohort 1A (26/sex/dose) were used to evaluate reproductive toxicity.
- Cohort 1B animals (26/sex/dose) were known as the reproductive/endocrine group
- The Cohort 2A and 2B animals (22/sex/dose) were used to assess potential developmental neurotoxicity (DNT).

Systemic toxicity was assessed across life stages. In the parental generation, high-dose ETU females had decreased body weight (7-8%) on lactation days (LD) 4 and 7, which coincided with decreased feed consumption (5-10%) and decreased high-dose F1 pup body weights on PND 14 and 21 (decreased by 11-12% and 8-9%, respectively). The F1 body weights in the high-dose males were significantly different from the controls from PND 21 through cohort termination or until PND 112 (7.4% decrease in body weight in Cohort 1B). The F1 high-dose female body weights recovered to control values by ~PND 56.

This ETU extended one-generation study confirmed that the thyroid is the most sensitive target organ for ETU-induced toxicity. Treatment-related thyroid effects included significant changes in thyroid hormone profile, thyroid weights, and thyroid histopathology (follicular cell hyperplasia) at doses ≥ 28 ppm ETU in both males and females at multiple life stages. At 2.8 ppm ETU, there was an adaptive change in thyroid histopathology (follicular cell hypertrophy) in P1 males (20 of 27 animals affected) and F1 Cohort 1A males (15 of 26 animals affected), which was deemed not to be adverse, but rather, related to the re-establishment of thyroid homeostasis. There was corresponding dose-related hypertrophy of individual cells in the pars distalis region of the pituitary at all dose levels (P1 and F1 males; ≥4 animals affected/dose/generation) or mid- and high-dose levels (P1 females; ≥7 animals affected/dose/generation). This slight pituitary hypertrophy denoted an adaptive response to stimulation of the hypothalamic pituitary- thyroid axis in order to reestablish thyroid hormone homeostasis; therefore, this finding was not considered adverse.
ETU exposures at 28/38 and 140/190 ppm resulted in decreased thymus weights in both P1 and F1 males and females (≥ 20% in males and ≥11% in females) with associated atrophy of lymphoid tissue in the thymus in the high-dose animals (12 of 26 animals affected in Cohort 1A males). This effect was judged to be treatment related.
There was no evidence of treatment-related reproductive toxicity or effects on the estrogen- or androgen-related endocrine pathways at any dose of ETU. The a priori triggers for producing a second generation were not met; therefore, the F1 animals were not mated in this study.
For developmental neurotoxicity, there were no effects on brain weights in the Cohort 2B animals (PND 22); however, there was an exposure-related decrease in overall brain size (6-7% decrease in brain weight) at the highest dose of ETU (140 ppm) in the Cohort 2A animals (PND 78). Commensurate with this change, overall brain macroscopic and microscopic measurements were decreased in Cohort 2A high-dose animals (1-4% decrease across all gross brain measurements; ≤ 5% change in microscopic measurements in high-dose animals), but there were no treatment-related changes in these parameters at ≤ 28 ppm. Given the small changes in overall brain size in Cohort 2A high-dose animals and the absence of effects on brain weight in the Cohort 2B and unselected (PND 22) weanlings, it is possible that these effects occurred during the post-weaning period when exposures to ETU per kg body weight were greater than nominal. There were no treatment-related effects on neuropathology at any dose levels in either Cohort 2A or 2B animals. There were no effects on neurobehavioral endpoints at any dose of ETU, despite exposures during critical windows of development.

This ETU F1-extended one generation study established a no-observed-adverse-effect level (NOAEL), based on thyroid toxicity, of 2.8 ppm in male and female rats (0.2 mg/kg/day) across all life stages.

(BASF DocID 2013/7002198)

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** Ethylenethiourea (ETU)

Description:	not reported
Lot/Batch #:	BCBC9199V
Purity:	100%
Stability of test compound:	The test substance was stable over 14 days at dietary concentration ranging from 0.34 to 100000 ppm. Test diets were prepared and used within these stability limits.

2. **Vehicle and/or positive control:** rodent diet

3. **Test animals:**

Species:	Rat, Sprague Dawley
Strain:	Crl:CD(SD)
Sex:	Male and female
Age:	8 weeks at initiation of treatment
Weight at day -2:	♂: 251.8 ± 7.6 g, ♀: 190.4 ± 9.2 g
Source:	
Diet:	LabDiet Certified Rodent Diet #5002 (PMI Nutrition International, St. Louis, Missouri) in meal form, ad libitum
Water:	tap water, ad libitum
Housing:	individual housing in stainless steel cages with following exceptions: • During mating male and female mating pairs were housed together • pregnant animals and their litters housed together until PND 21 (end of lactation) in plastic cages provided with ground corn cob nesting material; during the early post-weaning period (PND 21-28) pups were housed with littermates of the same sex in plastic cages

Enrichment for rodents included the use of a non-woven gauze pad per animal in caging, a stainless steel object in the cage for manipulation, and periodic rotation of each rack of cages in the animal room to allow animals a variety of visual experiences. Non-woven gauze pads were not added during the breeding, gestation, and lactation phases of the study. In addition the stainless steel object was not added during the lactation phase.

Environmental conditions:

Temperature:	21-23°C (and a maximum permissible excursion of ± 3°C)
Humidity:	40 - 70%
Air changes:	12-15 times per hour
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: In-life dates: 11-Nov-2011 (start of administration of F_0 parental animals) to 20-May-2012 (sacrifice of last F_1 offspring animals

2. Plasma kinetics of ETU

Groups of 12 male and 12 female Crl:CD(SD) rats were fed diets supplying 0 (control), 0.2, 2.0, and 10 mg/kg/day ethylene thiourea (ETU). Males were exposed for at least two weeks prior to breeding and continuing throughout breeding and post-breeding for approximately 8 weeks of exposure. The females were exposed for two weeks prior to breeding, continuing through breeding (up to two weeks), gestation (three weeks), and lactation (three weeks). Plasma ETU levels were assessed at steady state in P1 males at termination, P1 females on gestation day (GD) 20, lactation day (LD) 4 and LD 21, and F1 offspring on postnatal day (PND) 4 and PND 21. ETU levels were single point measurements except for P1 GD 20 dams, where 3 samples were collected for 24h AUC determination. To evaluate ETU systemic exposure and plasma concentration linearity across treatment groups, blood samples were collected from six rats/sex/group (0, 0.2, 2, and 10 mg/kg/day dose groups). Three blood samples from adult females were collected from the jugular vein without anesthesia at 6 AM, 9 AM, and 5 PM on GD 20 and placed into heparinized tubes. Blood samples from adult females and culled pups were also collected at 8-9 AM on LD 4 and LD 21. Blood samples from adult females on LD 21 and all culled pups were collected under anesthesia, from the left ventrical of the heart in heparinized capillary tubes for culled pups, and from the vena cava and placed into heparinized tubes for adult females. Blood samples from adult males were collected from the vena cava at necropsy (8-9 AM) after 8 weeks of exposure and placed into heparinized tubes.

3. Animal assignment and treatment:
In the main study, groups of 27 male and 27 female Crl:CD(SD) rats/dose were fed diets supplying approximately 0, 2.8, 28, or 140 ppm (0, 0.2, 2, 10 mg/kg bw/day) ETU for approximately four weeks prior to breeding and continued through breeding (up to two weeks). P1 males received increased dietary concentrations (3.8, 38, and 190 ppm ETU) starting on test day 44 for approximately 7 additional weeks until termination to ensure that nominal dose levels were achieved. P1 females received the original dietary concentrations through gestation (three weeks) and lactation (three weeks with one-half the dietary concentrations given from LD 14-21). P1 females were exposed until LD 22 (the end of the lactation period). Selected F1 offspring were maintained on the test diet until PND 22 (Cohort 2B), PND 78 (Cohort 2A), PND 90 (Cohort 1A), or PND-120 (Cohort 1B). Test diets for the F1 generation were one-half the dietary concentrations (0, 1.4, 14, and 70 ppm) from PND 21-35 and offspring received full dietary concentrations (0, 2.8, 28, and 140 ppm) from PND 35 until termination.

F1 offspring were divided into Cohorts 1A, 1B, 2A, and 2B at weaning (postnatal day (PND) 21) as follows:

- Cohort 1A (26/sex/dose) were used to evaluate reproductive/endocrine toxicity, which included estrous cycle evaluation and post-mortem evaluations focused on reproductive organs, sperm assessment, and ovarian follicle counts on PND 90. This group also was used to assess general systemic and thyroid toxicity, which included clinical chemistry/hematology parameters, thyroid hormone assessment, and urinalysis. Post-mortem evaluations in Cohort 1A (PND 90) also included gross pathology, organ weights, and histopathology on a wide range of tissues, including thyroids.
- Cohort 1B animals (26/sex/dose) were known as the endocrine group and designated to clarify any equivocal responses seen in the Cohort 1A animals. This group also was used to assess general systemic and thyroid toxicity, which included thyroid hormone assessments. Post-mortem evaluations in Cohort 1B (PND 120) included gross pathology and organ weights with a primary focus on tissues affected in Cohort 1A, including thyroids.
- The Cohort 2A and 2B animals (22/sex/dose) were used to assess potential developmental neurotoxicity (DNT) as follows:
    - Cohort 2A (12/sex/dose) were used for developmental neurotoxicity (DNT) assessments, which included functional observational battery (FOB), motor activity, and acoustic startle response (ASR). On PND 78, Cohort 2A F1 animals were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry.
    - Cohort 2B (10/sex/dose) underwent necropsy on PND 22, which included brain weight collection in these weanlings and immersion fixation of tissues for examination of neuropathology.

Mating procedure: Breeding of the P1 adults commenced after approximately 4 weeks of treatment. Each female was placed with a single male from the same dose level (1:1 mating). Animals were paired until mating occurs or two weeks had elapsed. During each breeding period, daily vaginal lavage samples were evaluated for the presence of sperm as an indication of mating. The day on which sperm were detected or a vaginal copulatory plug was observed in situ was considered GD 0. The sperm- or plug-positive (presumed pregnant) females were then separated from the male and returned to their home cage. If mating had not occurred after two weeks, the animals were separated without further opportunity for mating.

Standardization of litters: To minimize variation in pup growth due to differences in litter size, all litters were standardized to ten pups per litter on PND 4. This was accomplished by randomly ordering the pups in each litter by sex. Pups were culled then randomly selected using a computer generated randomization procedure, so that five males and five females remained in each litter. If it was not possible to have five pups/sex in each litter, unequal numbers of males and females were retained (e.g., six males, four females). Litters with fewer than ten pups were not culled. Preferential culling of runts was not performed.

Set Assignment: All litters were weaned on PND 21. Three male and three female F1 pups/litter were randomly selected and one male and one female each was assigned to Cohort 1A, 1B, or Cohort 2. The following prioritization plan was used (highest to lowest priority): Cohort 1A, 2A, 1B, and 2B. One male and one female per litter were assigned to Cohort 1A. For Cohorts 2A and 2B, one male or one female per litter were assigned: the developmental neurotoxicity groups (n = 10 males + 10 females/dose level with 20 litters represented in each Cohort). If available, one male and one female per litter were assigned to Cohort 1B: the reproductive endpoints group (n > 20 males and 20 females/dose level). If there were insufficient litters from which to select both male and female offspring for groups 1 or 2, additional animals were randomly selected from available litters as needed in order to obtain the required number of animals/dose level. Use of same sex littermates in the Cohort 2A or 2B was avoided whenever possible. If there were insufficient pups to fill all of the designated pup assignments, pups were assigned in accordance with the prioritization plan outlined above.

4. Test substance preparation and analysis:
Test material was used for preparation of a concentrated test material-feed mixture (premix). Each pre-mix was prepared by mixing test material with ground feed to achieve the targeted pre-mix concentration. Diets were prepared by diluting pre-mixes with ground feed. Control diets were prepared by mixing ground diet using a similar procedure. Premixes and test diets were not adjusted for purity and were prepared every 14 days based on stability data. In addition, sieving of the test material as well as a Quadro Co-mil was used during preparation of the premix to facilitate homogeneous distribution of the test material.

The following nominal dose levels were selected for the study:

2.8	ppm	as low dose
28	ppm	as intermediate dose
140	ppm	as high dose

ETU concentrations in the diet of the F_0 females were reduced to 50% during the lactation period. This dietary adjustment, derived from historical body weight and food consumption data, maintained the dams at constant dose-levels of ETU during this period of increased food intake. To account for the large amount of feed consumed per kg body weight in weanling pups, from PND 21-35, male and female weanlings received a diet containing the same concentration of ETU that was given to the P1 females during the third week of lactation (one-half the adult concentration). On PND 35, F1 male and female offspring received adult dietary concentrations of ETU until termination.

Dose confirmation analyses of all dose levels, plus control and premix, were determined pre-exposure and during the lactation phase. The homogeneity of all test diets was determined concurrently with dose confirmation. ETU concentrations in feed were analyzed by high performance liquid chromatography (HPLC), positive ion electrospray ionization (+ESI), with mass spectrometry detection operating in multiple reaction ion monitoring (MRM) mode.
Stability of ETU in the diet was determined to be 14 days at dietary concentrations ranging from 0.34 to 100,000 ppm. Test diets were prepared and used within these stability limits.

Table 5.8.1-20: Dose confirmation and homogeneity of test-item in vehicle

Nominal Dose level [ppm]	Sampling	Target Concentration [w/w%]	Mean measured concentration [w/w%]	% of Target Concentration	Homogeneity % R.S.D.#
Males					
2.8	09.11.2011	0.000280	0.000270	96.5	9.9#
2.8	20.12.2011	0.000380	0.000333	87.6	11.6#
2.8	10.01.2012	0.000399	0.000337	84.5	4.0#
28	09.11.2011	0.00280	0.00276	98.6*	-
28	20.12.2011	0.00380	0.00342	89.9*	-
28	10.01.2012	0.00380	0.00335	88.1*	-
140	09.11.2011	0.0140	0.0128	91.8	2.1#
140	20.12.2011	0.0190	0.0169	89.1	2.4#
140	10.01.2012	0.0190	0.0167	87.7	1.6#
0.1% premix	09.11.2011	0.100	0.0928	92.8*	-
0.1% premix	20.12.2011	0.100	0.0880	88.0*	-
0.1% premix	10.01.2012	0.100	0.0924	92.4*	-
Lactating Dams					
2.8	09.11.2011	0.000280	0.000270	96.5	9.9#
2.8	10.01.2012	0.000294	0.000264	89.9	8.4#
28	09.11.2011	0.00280	0.00276	98.6*	-
28	10.01.2012	0.00280	0.00247	88.2*	-
140	09.11.2011	0.0140	0.0128	91.8	2.1#
140	10.01.2012	0.0140	0.0124	88.9	1.2#
0.1% premix	09.11.2011	0.100	0.0928	92.8*	-
0.1% premix	10.01.2012	0.100	0.0924	92.4*	-
One-half normal dam (LD14-21) and Pups (PND 21-35)					
2.8	10.01.2012	0.000147	0.000138	93.9	8.8#
28	10.01.2012	0.00140	0.00123	87.7*	-
140	10.01.2012	0.00700	0.00605	86.4	2.5#
0.1% premix	10.01.2012	0.100	0.0924	92.4*	-

based on mean values of the six individual samples
* based on mean values of the two individual samples

5. Statistics:

Means and standard deviations of each test group were calculated for several parameters. Further statistical analyses were performed according to following tables:

Statistics

Parameter	Statistical test
Parental and F1 post-weaning body weights, body weight gains, litter mean body weights, litter mean age at vaginal opening (females), litter mean age at preputial separation (males), interval from vaginal opening to first estrus, feed consumption, anogenital distance (absolute and relative to the cubed root of body weight), sperm count, follicle count, percent total and progressively motile sperm, mean estrous cycle length, T4, TSH, and organ weights (absolute and relative)	Bartlett's test for equality of variances with either a parametric or nonparametric analysis of variance (ANOVA); If the ANOVA was significant at alpha = 0.05, a Dunnett's test or the Wilcoxon Rank-Sum test with Bonferroni's correction was performed.
Urine volume, urine specific gravity, clinical chemistry data (excluding globulin and albumin to globulin ratio), coagulation and hematologic data (excluding differential WBC counts and RBC indices)	Bartlett's test for equality of variances with either a parametric or nonparametric analysis of variance (ANOVA); If significant at alpha = 0.05, the ANOVA was followed respectively by Dunnett's test or the Wilcoxon Rank-Sum test with a Bonferroni correction for multiple comparisons to the control.
Gestation length, average time to mating, and litter size	nonparametric ANOVA. If the ANOVA was significant, the Wilcoxon Rank-Sum test with Bonferroni's correction was performed
Sperm morphology	Arcsine transformed and analyzed using a parametric ANOVA
Mating, conception, fertility, and gestation indices, nipple retention	Fisher exact probability
Neonatal sex ration on postnatal day 1	Binomial distribution test
Survivial indices, post-implantation loss, and other inicidence data among neonates	Censored Wilcoxon test as modified by Haseman and Hoel (1974) with Bonferroni's correction
DCO and FOB incidence data (scored observations only)	z-Test of proportions comparing each treated group to the control group.
FOB body weights, grip performance, rectal temperature, landing foot splay data	2-way analysis of variance (ANOVA) with the factors of sex and treatment. Differences between the groups were primarily detected by the treatment factor. For these parameters the first examination were whether the sex- treatment interaction was significant at alpha = 0.05; if it was, a one-way ANOVA was done separately for each sex. Comparisons of individual treatment groups to the control group were made with Dunnett's test only when a statistically significant treatment effect existed at alpha = 0.05. Dunnett's test corrects for multiple comparisons to the control and the experiment-wise error rate of alpha = 0.05 were reported.
Motor activity, Acoustic startle response habituation	Factorial repeated-measure design (multivariate approach); the pillai's trace p-values were used.
Data used to examine quantitative structural brain differences including brain weight, brain macroscopic measurements, and brain microscopic measurements inCohort 2A	One-way ANOVA and adjacent MANOVA

C. Methods

1. Observations:

A cage-side examination was conducted at least twice daily, at approximately the same time each day. This examination was typically performed with the animals in their cages and was designed to detect significant clinical abnormalities that were clearly visible upon a limited examination, and to monitor the general health of the animals. The animals were not hand-held for these observations unless deemed necessary.

Clinical Observations: Clinical examinations were conducted on all males pre-exposure and weekly throughout the study. Clinical examinations were conducted on all females pre-exposure and weekly throughout the pre-breeding and breeding periods. Mated (sperm-positive or plug-positive) females received clinical examinations on GD 0, 7, 14, and 20. Females were observed for signs of parturition beginning on or about GD 20 (see litter data). Females that delivered litters were subsequently evaluated on LD 0, 1, 4, 7, 14, and 21. Dams were examined for signs of abnormal nursing behavior. Pups that died or appeared moribund were examined for the presence of milk bands. Clinical observations were not conducted on females that failed to mate or deliver a litter during the gestation and lactation phases of the study, unless deemed appropriate based on cage side observations. F1 offspring received weekly clinical observations until necropsy. Clinical observations included a careful, hand-held examination of the animal with an evaluation of abnormalities in the eyes, urine, feces, gastrointestinal tract, extremities, movement, posture, reproductive system, respiration, skin/hair-coat, and mucous membranes, as well as an assessment of general behavior, injuries, or palpable mass/swellings.

Detailed Clinical Observations: Detailed clinical observations (DCO) were conducted on the P1 animals pre-exposure and weekly during the exposure period. DCO observations were not conducted on females that failed to mate or deliver a litter during the gestation and lactation phases of the study. F1 offspring were given weekly DCO evaluations after weaning. The DCO was conducted at approximately the same time each examination day, according to an established format. The examination included cage-side, hand-held, and open-field observations, which were recorded categorically or using explicitly defined scales (ranks).

2. Body weight:

All **parental** rats were weighed during the pre-exposure period and weekly during the 4-week pre-breeding periods. Males continued to be weighed weekly during breeding, after breeding until termination. Mated females were weighed on GD 0, 7, 14, and 20. Lactating females were weighed on LD 1, 4, 7, 14, and 21. Females that failed to mate and/or deliver a litter were not weighed during the subsequent gestation and/or lactation segments of the study. Weekly body weight gains were calculated for males throughout the study and for females before and after breeding. During gestation and lactation, female body weight gains were calculated for the following intervals: GD 0-7, 7-14, 14-20, 0-20, and LD 1-4, 4-7, 7-14, 14-21, and 1-21. The F1 **offspring** were weighed weekly beginning on PND 21, on the day of puberty onset (i.e., vaginal patency or preputial separation), and at termination. The Cohort 2A offspring also were weighed on the day of the Functional Observational Battery (FOB) and acoustic startle measurement.

3. Food consumption, food efficiency and compound intake:

Feed consumption was determined weekly during the 4-week pre-breeding period for all animals by weighing feed containers at the start and end of a measurement cycle. Feed consumption was not measured during breeding due to co-housing. Following breeding, feed consumption for males continued to be measured weekly until termination. For mated females, feed consumption was measured on GD 0, 7, 14, and 20. For females delivering litters, feed consumption was measured on LD 1, 4, 7, 11, 14, 17, 19, and 21. Feed consumption was not measured for females that failed to mate or failed to deliver a litter. Feed consumption was determined weekly in the F1 offspring beginning when the animals were individually housed on PND 28 until the day prior to necropsy.

Test material intake (TMI, expressed as mg/kg/day) was calculated using feed concentrations, body weights, and feed consumption data using the following equation:

$$\text{TMI}= \frac{(\text{feed consumption [g/day]}) * (1000\ \text{mg/g}) * ((\ \%\ \text{test material feed})/100)}{(\text{current bw [g]}+\text{previous bw [g]} / 2) / 1000\ \text{g/kg}}$$

Test material intake was not calculated during the breeding period due to co-housing.

4. Ophtalmoscopy:

Not performed in this study.

5. Hematology, clinical chemistry and hormone measurements

P1 and Cohort 1A animals were fasted overnight prior to terminal blood collection. Blood samples were obtained from the orbital sinus following anesthesia with O_2/CO_2 at the scheduled necropsy (after ~11 weeks of treatment in P1 males, on LD 22 in P1 females, and on ~ PND 90 in Cohort 1A offspring). Blood were not obtained from animals that died or were euthanized in a moribund condition prior to their scheduled necropsy. The following hematological and clinical chemistry parameters were determined:

Clinical chemistry:		
Electrolytes	*Metabolites and Proteins*	*Enzymes*
Calcium (CA)	Albumin (ALB)	Alanine aminotransferase (ALT)
Chloride (CL)	Bilirubin (total) (TBIL)	Aspartate aminotransferase (AST)
Phosphorus (inorganic) (PHOS)	Cholesterol (CHOL)	Alkaline phosphatase (ALP)
Potassium (K)	Creatinine (CREA)	□-glutamyl transpeptidase (GGT)
Sodium (NA)	Globulin (by calculation) (GLOB)	
	Glucose (GLU)	
	Protein (total) (TP)	
	Triglycerides (TRIG)	
	Urea nitrogen (UN)	
Hematology:		
Hemoglobin (HGB)	Total white blood cell (WBC) count	Prothrombin time (PT)
Hematocrit (HCT)	Differential WBC count	
Platelets (PLAT)	Mean corpuscular hemoglobin (MCH)	
Reticulocyte (RET) count	Mean corp. volume (MCV)	
Red blood cell count (RBC)	Mean corp. hemoglobin conc. (MCHC)	

Urinalysis
Urine samples were obtained from P1 males (approximately TD 70) and Cohort 1A offspring (PND 80-85 males and females) during the week prior to the scheduled necropsy. Samples were collected from the first 10 animals/sex/dose that were available. Animals were housed in metabolism cages and the urine collected overnight (approximately 16 hours). Feed and water were available during this procedure. No urine collection was performed on P1 (LD 22) females. Color, appearance, specific gravity (refractometer), and urine volume were assayed. Semiquantitative analyses of pH, bilirubin, glucose, protein, ketones, blood/blood cells and urobilinogen were conducted. Urine samples were collected from each animal by manual compression of the urinary bladder. The urine samples were pooled from each group, and the microsediment was characterized microscopically.

Hormone measurements:
Blood from P1 males (15/dose), P1 females (10/dose), culled pups (first 15 litters/dose if possible; male and female samples pooled by litter), PND 22 assigned for termination (non-selected weanlings; 15/sex/dose), and Cohort 1A offspring (15/sex/dose) were collected under O_2/CO_2 or isoflurane anesthesia and placed on ice immediately after collection. Blood from pups culled from the same litter were pooled in plastic serum separator tubes and placed on ice. A terminal blood sample from PND 22 weanlings assigned for termination (non-selected weanlings) and culled pups was used, whereas P1 males, P1 females, and Cohort 1A offspring were anesthetized a few days prior to necropsy (~11 weeks after initiation of exposure, ~LD 18, and ~PND 85, respectively) for in-life sample collection to avoid the effects of fasting on thyroid hormone levels.

6. Male reproduction data
Sperm parameters as sperm motility, morphology and sperm count were evaluated in P1 and Cohort 1A males at termination. Histopathological examination of the testes included a qualitative assessment of stages of spermatogenesis. Microscopic evaluation included a qualitative assessment of the relationships between spermatogonia, spermatocytes, spermatids, and spermatozoa seen in cross sections of the seminiferous tubules. The progression of these cellular associations defined the cycle of spermatogenesis. In addition, sections of both testes were examined for the presence of degenerative changes (e.g., vacuolation of the germinal epithelium, a preponderance of Sertoli cells, sperm stasis, inflammatory changes, mineralization, and fibrosis). When possible, sections of the rete testis were examined in the Cohort 1A offspring. Examination of the epididymis was included the caput, corpus and cauda and the vas deferens was examined when possible.

Reproductive indices were calculated for all dose levels in the P1 animals. Reproductive indices were calculated as follows:

Male mating index = (No. males with evidence of mating / No. paired) x 100
Male conception index = (No. males siring a litter / No. mated) x 100
Male fertility index = (No. males siring a litter/No. paired) x 100

7. Female reproduction and delivery data

Estrous Cycle Evaluation: Vaginal lavage samples from all P1 females were collected daily beginning two weeks after the initiation of dosing. Estrous cycle was evaluated for 2 weeks prior to mating, and during cohabitation until each female was sperm- or plug-positive or until the two week mating period has elapsed. Lavage samples were collected by gently irrigating the vagina with water and transferring lavage fluid to a microscope slide. Vaginal lavage slides were examined microscopically to determine estrous cycle length and pattern. Vaginal smears were collected in Cohort 1A offspring after vaginal opening until the first cornified smear was recorded to determine the time interval between vaginal patency and first estrus. Vaginal smears were inadvertently collected and read in Cohort 1B and Cohort 2A offspring after vaginal opening until the first cornified smear was recorded. The data is contained in the study file. The estrous cycle was also evaluated in Cohort 1A females for 2 weeks from approximately PND 75 through PND 88. On the day of scheduled necropsy, the stage of the estrous cycle was also determined for all P1 and adult F1 female rats in Cohort 1A and Cohort 1B.

Uterus examination: Examination of the ovaries from P1 control and high-dose females (10/dose) included a qualitative assessment of follicle stages with an emphasis on the possible depletion of primordial follicles. For Cohort 1A females, examination of the ovaries included enumeration of primordial (small) follicles, growing follicles, and corpora lutea using a method similar to Bucci et al. (1997) with additional recommendations from Heindel (1999) and Bolon et al. (1997). All Cohort 1A females in the control and high-dose groups terminated at scheduled necropsy were selected for this examination.

Reproductive indices were calculated for all dose levels in the P1 animals. Reproductive indices were calculated as follows:

Female mating index = (No. females with evidence of mating/No. paired) x 100
Female conception index = (No. females with evidence of pregnancy/No. mated) x 100
Female fertility index = (No. females with evidence of pregnancy/No. paired) x 100
Gestation index = (No. females delivering a viable litter/No. females with evidence of pregnancy) x 100
Gestation survival index = percentage of delivered pups alive at birth

The total number of pups delivered and the number of liveborn and stillborn pups were noted, and the survival index was calculated:

Day 1 or 4 pup survival index = (No. viable pups on day 1 or 4/No. born live) x 100

Day 7, 14, or 21 pup survival index = (No. viable pups on day 7, 14 or 21/No. after culling) x 100

The uteri of all P1 females were examined for the presence and number of implantation sites.

Post-implantation loss = (No. implants – No. viable offspring)/(No. implants) x 100

8. Litter data

The day of parturition was recorded as the first day that one or more delivered fetuses were noted, and was designated as LD 0. The following information was recorded for each litter: the date of parturition, the number of live and dead pups on LD 0, 1, 4, 7, 14, and 21, and the sex and body weight of each pup on LD 1, 4 (before and after culling), 7, 14, and 21. Any pup found dead or sacrificed in moribund condition was sexed and examined grossly, to the extent possible, for external and visceral defects. These pups were preserved in neutral, phosphate-buffered 10% formalin.
Anogenital distance (absolute and relative to the cube root of body weight) was measured in all F1 pups on PND 1. The data collection was counterbalanced.
To minimize variation in pup growth due to differences in litter size, all litters were standardized to ten pups per litter on PND 4. This was accomplished by randomly ordering the pups in each litter by sex. Pups were culled then randomly selected using a computer generated randomization procedure, so that five males and five females remained in each litter. If it was not possible to have five pups/sex in each litter, unequal numbers of males and females were retained (e.g., six males, four females). Litters with fewer than ten pups were not culled. Preferential culling of runts was not performed.
All non-selected F1 weanlings were given a gross necropsy examination on PND 22, which included a gross pathologic assessment of reproductive organs. Blood was collected from a subset of weanlings (15/sex/dose) for thyroid hormone analyses. Thyroid glands were harvested from the same subset of the pups used for thyroid hormone analyses. Body weight and organ weights (brain, spleen, and thymus) were collected from 10 pups/sex/dose (up to 20 litters represented, if possible) with preservation of these tissues plus mammary tissues for possible histopathological examination.
All offspring were evaluated for the presence of nipple/areolae on PND 12. The average number of nipples/areolae in male and female offspring in each litter was determined. The mean number of nipples/areolae for males and females in each dose level was calculated from these litter means. Observers were blind to treatment group when evaluating pups for the presence of nipples/areolae. There was no statistically or biologically significant, treatment-related increases in retained nipples/areolae in males on PND 12; thus Cohort 1A males were not examined for nipple/areolae retention at necropsy.
All F1 animals (Cohorts 1-2) were observed daily for vaginal opening beginning on PND 26 or for balano-preputial separation beginning on PND 35. Age and body weight of the animals on the day these markers of puberty onset were acquired and recorded. Examination for puberty onset ceased upon acquisition, or on PND 43 (females) or 53 (males), whichever came first. Any abnormalities of genital organs (e.g., persistent vaginal or preputial threads) were noted.

9. Developmental Neurotoxicity – Cohort 2A:

Functional Observational Battery (FOB)

The FOB was conducted between PND 63 and 75 under red light conditions by the same observer on Cohort 2A rats (12 males and 12 females representing 24 litters) at approximately the same time each test day. The FOB included cage-side, hand-held, and open-field observations and measurements of body weight, rectal temperature, fore- and hindlimb grip performance, and landing foot splay. The FOB was conducted on rats randomly selected and presented to the observer who was blind to the treatment status of the animal. FOB Parameters are described in Table 5.8.1-21.

Table 5.8.1-21: FOB Parameters

Cage-side Observations:
Abnormal movements or behavior
Resistance to removal from cage
Hand-held Observations:
Palpebral closure
Lacrimation (non-colored periocular wetness)
Pupil size
Pupil reactivity
Salivation (non-colored perioral wetness)
Muscle tone
Extensor-thrust response
Reactivity to handling
Open-field Observations:
Level of activity (ambulatory and rearing)
Responsiveness to sharp noise
Responsiveness to touch
Responsiveness to tail pinch
Gait evaluation
Urination
Defecation
Categorical Observations:
Abnormal behavior
Abnormalities of the eye
Abnormal urine or feces
Abnormalities of the gastrointestinal (GI) tract
Injury
Missing extremity
Abnormal muscle movements
Palpable mass/swellings
Abnormal posture
Abnormalities of the reproductive system
Abnormal respiration
Abnormal skin or hair-coat/mucous membranes
Excessive soiling
General abnormalities
Measurements:
Rectal temperature
Hindlimb grip performance
Forelimb grip performance
Landing foot splay

Acoustic Startle Response (ASR) Habituation:

The ASR of Cohort 2A rats was tested on PND 24 ± 1. Body weights were collected on the day of ASR assessment. The ASR system was commercially available (Med Associates, Inc., St. Albans, Vermont) package consisted of 8 acoustically insulated chambers and hardware to generate the auditory stimuli and measure the resulting startle responses.

10. Sacrifice and pathology:

Non-selected PND 22 Weanlings:

On PND 22, weanlings (non-fasted) not assigned to Cohorts 1 or 2 were assigned to the weanling necropsy group. In the non-selected weanling group, pups were submitted for a complete necropsy. Pups were anesthetized with isoflurane or O_2/CO_2, weighed, blood collected from a subset of weanlings by orbital sinus or cardiac puncture, and euthanized by decapitation. Weanlings were given a gross necropsy examination, including an assessment of reproductive organs. Weights of the thymus, brain, and spleen were recorded from 10 pups/sex/dose (representing 20 litters/dose, if possible), and the organ:body weight ratios calculated. Thyroid weights (post-fixation) were recorded from 15 pups/sex/dose using the same animals as those bled for thyroid hormone analyses. Thyroid weight:body weight ratios were calculated. The above organs, as well as mammary tissue from male and female weanlings, were preserved in neutral, phosphate-buffered 10% formalin.
Histopathological examination of the thyroid gland was performed in males and females from all dose levels.

P1 Adults and Cohort 1A Offspring:

P1 males (fasted) were necropsied after ~ 11 weeks of exposure. Adult P1 females (fasted) were terminated on LD 22 after weaning of their litters, or at least 24 days after the end of the mating period for females not producing a litter. F1 male and female adults (fasted) from Cohort 1A were submitted for necropsy on approximately PND 90. Animals were weighed in the animal room and vaginal lavage smears were prepared from all surviving P1 and Cohort 1A females prior to transportation to the necropsy room. The animals were anesthetized by the inhalation of O_2/CO_2 and blood was collected from the orbital sinus for 10/sex/dose for clinical chemistry and hematology (both P1 and Cohort 1A animals). The tracheas were exposed and clamped, and the animals were euthanized by decapitation. The necropsy included an examination of the external tissues and all orifices. The head was removed, the cranial cavity opened and the brain, pituitary and adjacent cervical tissues was examined. The eyes, skin, viscera, nasal cavity, lungs were examined. The uteri of all P1 females were examined for the presence and number of implantation sites. Weights of the ovaries, uterus (with oviducts and cervix), testes, epididymides, seminal vesicles with coagulating glands (and fluids), prostate, brain, pituitary (weighed after fixation), liver, kidneys, heart, thymus, adrenal glands, spleen, and thyroid with parathyroid glands (weighed after fixation) were recorded, and the organ:body weight ratios calculated for P1 and Cohort 1A animals. In addition, weights of the left testis and left cauda epididymis were collected in P1 males and Cohort 1A males. These data were used to calculate sperm counts (cauda epididymis).

Histopathologic examination was conducted on the control and high-dose groups of P1 adults and Cohort 1A offsprings. Following tissues were preserved for histopathology: adrenals, aorta, bone (including joint), bone marrow, brain (cerebellum, brainstem, cerebrum), cecum, cervix, coagulation glands, colon, cranial nerve – optic, duodenum, epididymides, esophagus, eyes, gross lesions, heart, ileum, jejunum, kidneys, lacrimal/harderian glands, larynx, liver, lungs, mammary gland, mediastinal lymph node, mediastinal tissues, mesenteric lymph node, mesenteric tissues, nasal tissues, pharynx, oral tissues, ovaries, oviducts, pancreas, parathyroid glands, peripheral nerve-tibial, pituitary, prostate, rectum, salivary glands, seminal vesicles, skeletal muscle, skind and subcutis, spinal cord (cervical, thoracic, lumbar), spleen, stomach, testes, thyroid gland, tongue, trachea, urinary bladder, uterus, vagina, vas deferens. Examination of tissues from the remaining groups were limited to those tissues that demonstrated treatment-related histopathologic effects at the high dose group (thyroid and pituitary glands for both sexes of P1 and Cohort 1A animals, liver for male P1 animals, and thymus for both sexes of Cohort 1A animals), relevant gross lesions, and reproductive organs from animals that failed to mate or exhibit reduced fertility.

Cohort 1B Offspring:
F1 male and female adults (fasted) from Cohort 1B were submitted for necropsy on approximately PND 120. Animals were weighed in the animal room for all surviving Cohort 1B animals prior to transportation to the necropsy room. The animals were anesthetized by the inhalation of CO_2, the tracheas were exposed and clamped, and the animals were euthanized by decapitation. A complete necropsy was conducted. The necropsy evaluation and the histopathology was done as describe for P1 Adults and Cohort 1A Offspring above.

Cohort 2A Offspring:
Cohort 2A males and females (fasted) were submitted for necropsy on PND 78. Animals were weighed in the animal room prior to transportation to the necropsy room. The Cohort 2A animals underwent perfusion for fixation of nervous system tissues for neuropathology. tissues (see below) for neuropathologic evaluation were prepared from all animals in the control and high-dose groups, and the pituitary glands were prepared for neuropathologic evaluation in low- and mid-dose males. In addition, appropriate brain sections from the low and intermediate dose groups were taken to slide for morphometry evaluation.

Following tissues were evaluated:
Brain
olfactory bulb
cerebrum (frontal, parietal, temporal, occipital)
thalamus/hypothalamus
midbrain, pons
cerebellum
medulla including nucleus gracilis/cuneatus
pituitary gland
trigeminal ganglia with nerve
spinal cord (cross and oblique section)
 cervical swelling (C3-C6)
 lumbar swelling (L1-L4)
dorsal root ganglia
 cervical and lumbar
dorsal and ventral roots
 cervical and lumbar
peripheral nerves (cross and longitudinal section)*
 proximal sciatic
 proximal and distal tibial (at the knee and calf muscle branches)
 peroneal (saved)
 sural
 caudal (saved)
nasal tissue with olfactory epithelium
eyes - with optic nerve (longitudinal section only)
Skeletal muscle
 anterior tibial and gastrocnemius

Brain weight and gross measurements were recorded on all dose groups. Linear measurements consisted of the: 1) cerebral length (L2 – anterior to posterior, excluding olfactory lobes) and width (L3 – maximum), and 2) cerebellar length (L10 – anterior to posterior) and width (L5 – maximum). Microscopic brain measurements were recorded on all animals in the control and high-dose groups.

Cohort 2B, PND 22 Weanlings – Neurotoxicity Group:
Weanlings selected for neuropathology (non-fasted) were anesthetized with O_2/Isoflurane. While under deep anesthesia, animals were weighed and tissues were examined for gross pathologic alterations. The brain (excluding the olfactory lobes) was weighed and brain weight:body weight ratios calculated. The brain and head were immersed in neutral, phosphate-buffered 10% formalin. No additional tissues were saved. For neuropathological evaluation following tissues were prepared from all animals in the control and high-dose groups: brain, olfactory bulb, pituitary, trigeminal nerve and ganglion, gross lesions. Nine cross-sections of the brain were prepared from the following structures: olfactory bulb, cerebrum (frontal, parietal, temporal and occipital lobes, including the hippocampus, and basal ganglia), thalamus/hypothalamus, midbrain, pons, medulla oblongata, and cerebellum. In addition, sections were prepared from the trigeminal ganglion and nerve, and pituitary gland.

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

See Section B 3. Above

B. PLASMA KINETICS OF ETU

ETU concentrations in plasma were quantifiable (above the lower limit of quantification) by LC/MS in all samples obtained from the exposed animals. Mean plasma concentrations from a single sampling time point, daily systemic exposure (AUC24h, for GD 20 dams only) and kinetics for all groups are summarized in . Gestation AUC24h values were based on plasma concentrations on GD 20 and test material intake from GD 14-20. Mean plasma concentration for dams and pups on LD 21/PND 21 were based on nominal dose levels (mg/kg/day), because both dams and pups were consuming test diets at this stage, making measurement of individual animal feed consumption inaccurate.

Plasma concentrations in all groups were dose-proportional (linear) across all dose levels (0.2, 2 and 10 mg/kg/day, target doses) as illustrated in Figure 5.8.1-1 and confirmed by statistical analysis. On GD 20, blood samples were taken from each of six pregnant rats, at three time points (6AM, 9AM, and 5PM) for calculation of the ETU daily systemic exposure (AUC24h, as per Saghir et al., 2006). Plasma AUC24h values also were dose proportional (linear) across all ETU-treated dose groups as confirmed by statistical analysis. There were no observed sex- or lactation-related differences in ETU kinetics. In LD 4 culled pups, mean plasma ETU concentrations were approximately 22% of dam plasma levels (± 5%). Plasma concentrations of ETU in LD 21 pups were approximately 65% of dam levels (± 4%). The higher pup-to-dam ETU plasma concentration ratio at LD 21 was likely due to pup consumption of ETU-containing diets, in addition to suckling at that time.

Figure 5.8.1-1: Graphical overview of ETU plasma kinetics in GD20 dams (a), male Crl:CD(SD) rats (b), LD21 dams and pups (c) and LD4 dams and pups (d).

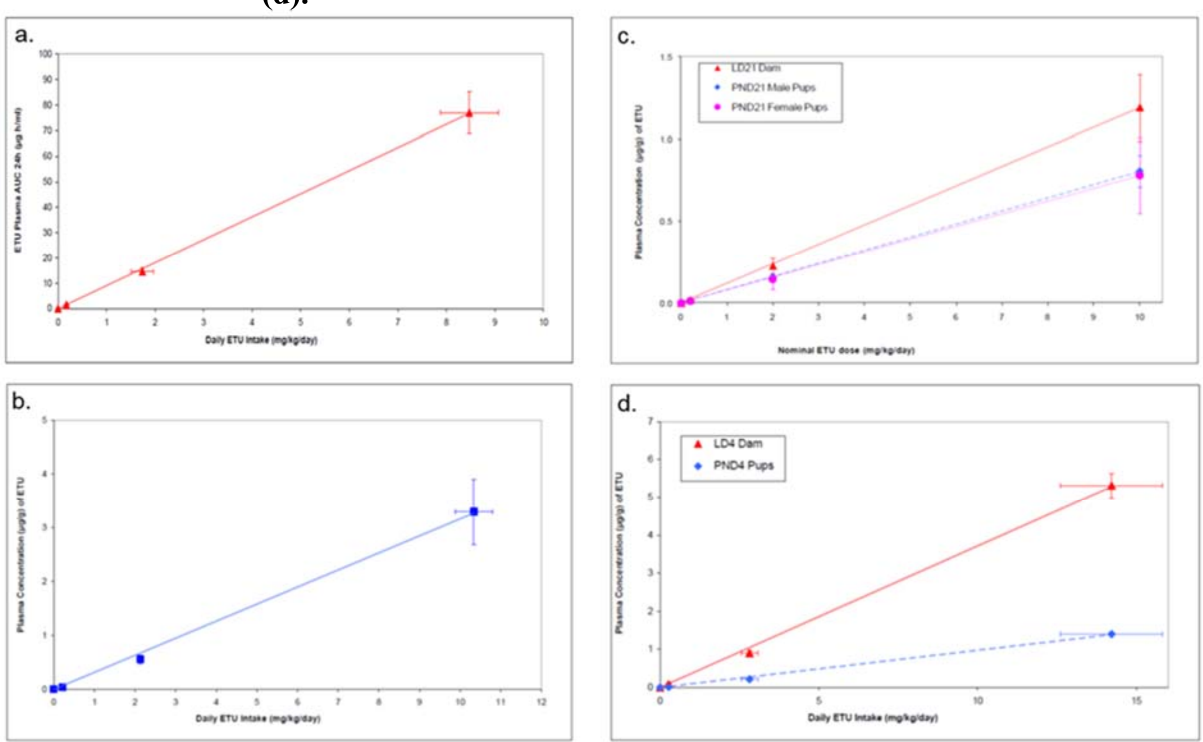


Table 5.8.1-22: Overview on plasma concentrations or AUC values

Nominal Dose (mg/kg/day)				0	0.2	2	10	Dose-Proportionality
Plasma Concentration (µg/g) or AUC_{24h} (µg h/mL)*		Males	Mean	NQ	0.040	0.552	3.295	Linear
			SD		0.006	0.088	0.608	
	GD20 (AUC_{24h})	Dams	Mean	NQ	1.403	14.692	77.028	Linear
			SD		0.121	1.388	8.202	
	LD4	Dams	Mean	NQ	0.084	0.900	5.312	Linear
			SD		0.012	0.072	0.323	
		Pups	Mean	NQ	0.014	0.216	1.399	Linear
			SD		0.003	0.021	0.197	
	LD21	Dams	Mean	NQ	0.018	0.227	1.192	Linear
			SD		0.003	0.047	0.202	
		Male Pups	Mean	NQ	0.011	0.159	0.806	Linear
			SD		0.002	0.094	0.236	
		Female Pups	Mean	NQ	0.011	0.146	0.780	Linear
			SD		0.002	0.063	0.231	

C. OBSERVATIONS

1. In-Life Observations

P1 males and females did not exhibit any treatment-related observations throughout the term of the study. Clinical observations recorded for P1 adults during the study were isolated occurrences and/or deemed to be spontaneous occurrences common to this strain/age of rat and unrelated to exposure. Observations in F1 pups, which included two low-dose pups from the same litter with small limbs (dam #6258), one low-dose pup with decreased activity (dam #6253), three dead, high-dose pups with their placenta attached (dam #6323), and one high-dose pup with cleft lip (dam #6325), were isolated occurrences and judged to be unrelated to treatment. Other litter observations did not exhibit a dose-response relationship, were of low incidence and of minimal severity; these findings were considered incidental and unrelated to ETU treatment.

2. Detailed Clinical Observations – P1 Males and Females

P1 males and females did not exhibit any treatment-related changes in detailed clinical observations during any phase of the study. One observation, excessive hair loss, was not dose related and is a common occurrence in this strain of rats. One high-dose female (#6319) had a small sunken eye, which was observed after a mechanical injury to the eye had occurred (i.e., scratched cornea) and was deemed unrelated to treatment.

3. Survival – P1 Males and Females

There were no effects of ETU exposure on p1 male or female survival. All P1 animals survived to scheduled necropsy.

D. PARENTAL BODY WEIGHT AND BODY WEIGHT GAIN

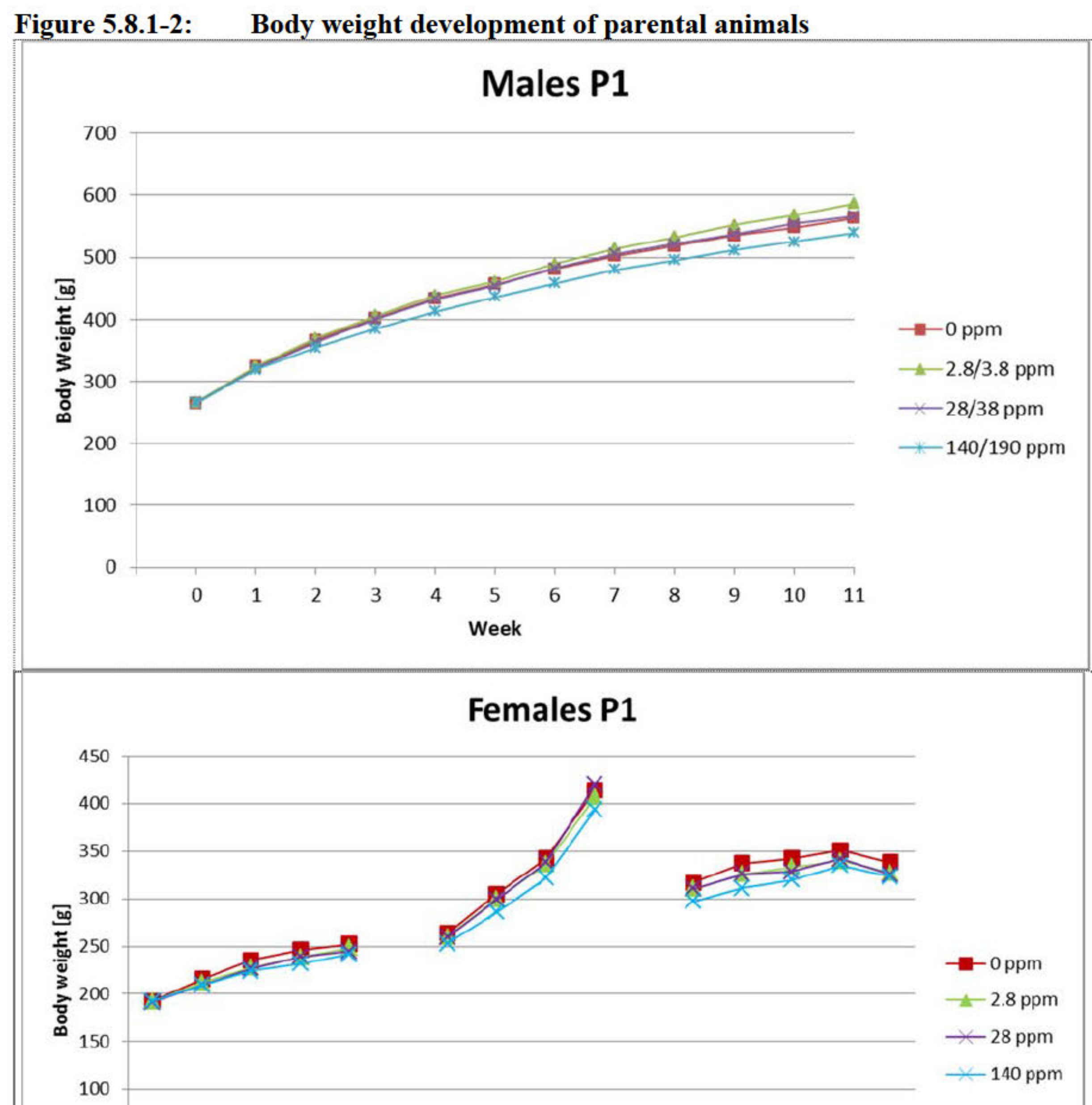
Male
P1 males had similar body weights across dose groups prior to initiation of dosing. Body weights were decreased by approximately 4-5% in the high-dose P1 males during the pre- and post-mating periods; however, these differences were not statistically different. P1 male body weight gains were decreased in the high-dose group by 8-13% throughout the exposure period with statistically significant effects on body weight gains on TD 1-22 and 1-29. There were no effects on body weight or body weight gain at any intervals at lower dose levels of ETU.

Female Pre-mating
P1 females had similar body weights across dose groups prior to initiation of dosing. Body weights were decreased by approximately 5% in the high-dose P1 females during the pre-breeding period; however, these differences were not statistically different. P1 female body weight gains were decreased in the high-dose group by 18-27% throughout the pre-mating period with statistically significant effects on body weight gain on TD 1-8, 1-15, and 1-22. Body weight gains also were significantly decreased (22%) in the mid-dose group on TD 8, but this effect was reduced at subsequent time points. There were no effects on body weight or body weight gains at 2.8 ppm ETU.

Female Gestation
ETU-treated P1 females had similar body weights to control animals during gestation; however, gestation body weight gains were significantly decreased by 19% in high-dose dams on GD 0-7. Body weight gains were not significantly altered in high-dose dams during the remainder of gestation and were not affected during any gestation interval at lower dose levels of ETU.

Female Lactation
During lactation, female body weights in the high-dose group were significantly decreased by 8% on LD 4, and 7% on LD 7. These body weight decreases were associated with a decrease in lactation body weight gain (30%) on LD 1-4 in the high-dose dams. Lactation body weights were not significantly affected in 140 ppm dams after LD 7. For the remainder of the lactation period, the high-dose dams gained more weight (or lost less weight) than the control group, resulting in a 25% increase in lactation body weight gain (LD 1-21) in the high-dose dams. There were no effects on lactation body weight/body weight gain at 2.8 or 28 ppm ETU.

Figure 5.8.1-2: Body weight development of parental animals



E. PARENTAL FOOD CONSUMPTION AND COMPOUND INTAKE

Targeted dose levels for dietary ETU exposures were 0.2, 2, and 10 mg/kg/day ETU for the low- mid- and high-dose groups, respectively. Overall, dietary exposures were close to nominal levels for the P1 generation, although P1 females received doses that were 71-87.5% higher than nominal doses during the lactation period.

Male

There were treatment-related decreases in feed consumption (4-12%) in high-dose P1 males throughout the exposure period, which corresponded with the decreased body weight gains seen at this dose level. The decreases in feed consumption in the high dose group were statistically significant during most of exposure intervals. There were no treatment-related differences in P1 male feed consumption during the premating or post-mating period at doses ≤ 28 ppm.

Test material intake in P1 males were fairly close to predicted values: 0.205 mg/kg/day at 2.8/3.8 ppm, 2.02 mg/kg/day at 28/38 ppm, and 9.69 mg/kg/day at 140/190 ppm.

Female Pre-mating

Overall, pre-mating feed consumption was similar between the ETU-treated and control females. There were slight decreases in feed consumption in the high-dose females (2-7%), but these decreases were not statistically identified, despite significant effects on body weight gains in high-dose females during the pre-mating period.
During the pre-mating period, test material intake in P1 females were close to predicted values: 0.204 mg/kg/day at 2.8 ppm, 2.07 mg/kg/day at 28 ppm, and 10.2 mg/kg/day at 140 ppm.

Female Gestation

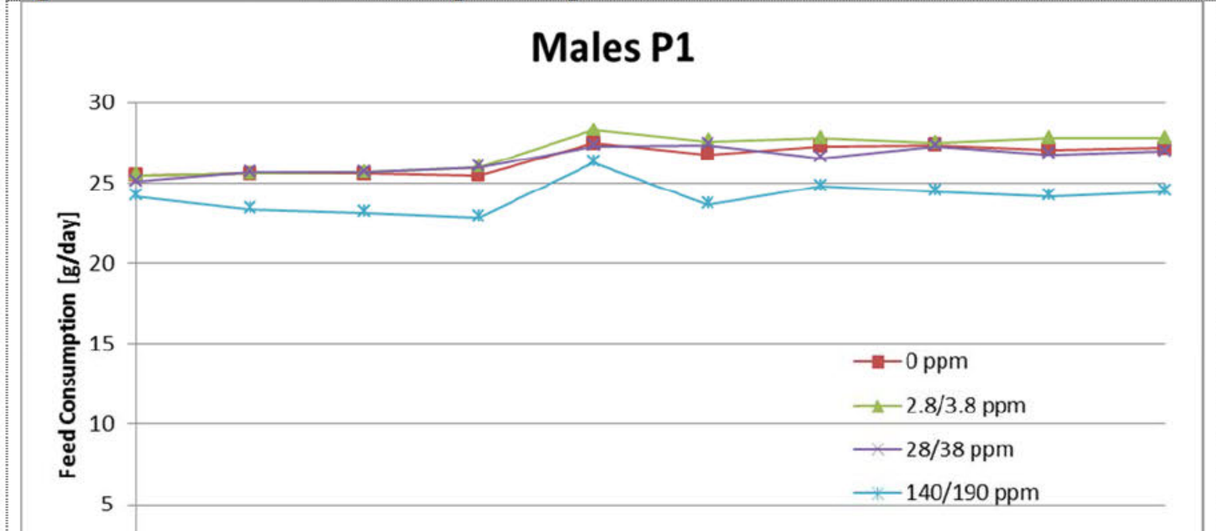
There were no significant differences in gestation feed consumption with ETU exposure. Feed consumption was decreased slightly (7%) in the high-dose dams on GD 0-7, the interval during which gestation body weight gains were significantly decreased in this dose group.
The test material intake for P1 females during gestation were fairly close to predicted values: 0.204 mg/kg/day at 2.8 ppm, 2.09 mg/kg/day at 28 ppm, and 10.3 mg/kg/day at 140 ppm.

Female Lactation

During lactation, maternal feed consumption in high-dose dams was decreased by 10% on LD 1-4, which corresponded with an interval of decreased lactation body weights/gains in this dose group. Feed consumption was decreased slightly (5%) on LD 4-7 in the 140 ppm group relative to controls; however, these decreases became more pronounced (12-14%) and were statistically different in these dams on LD 7-11, 11-14 and 14-17. The relative difference in feed consumption improved for high-dose dams during LD 17-21, which may be related to concurrent pup feed consumption and/or the lower dietary concentration of ETU (one-half concentration) that was introduced on LD 14. There were no effects on lactation feed consumption at ETU doses ≤ 28 ppm.

In the P1 females, test material intake (TMI) exceeded the targeted low dose levels of 0.2, 2, and 10 mg/kg/day, particularly during the second week of the lactation period (LD 7-14), when TMI was double the targeted dose level. The test material intake for P1 females during lactation was 0.375 mg/kg/day at 2.8 ppm, 3.58 mg/kg/day at 28 ppm, and 17.1 mg/kg/day at 140 ppm; thus, test material intake was 87.5%, 79%, and 71% greater than nominal at 0.2, 2.0, and 10 mg/kg/day dose levels, respectively. Increases in test material intake are not unusual during lactation as rats consume larger amounts of feed during this period. Dietary concentrations were not adjusted until LD 14 in this study. Without dietary adjustments, test material intake on LD 1-14 was approximately twice the targeted values in the ETU-treated groups. During LD 14-21, ETU dietary concentrations were adjusted to one-half the normal concentration, such that test material intake was 42-55% greater than targeted dose levels. Overall, dietary adjustments used during the lactation phase of this study were conservative such that dams still received a greater dose/kg body weight/day than was administered during the pre-mating period.

Figure 5.8.1-3: Food consumption of parental animals



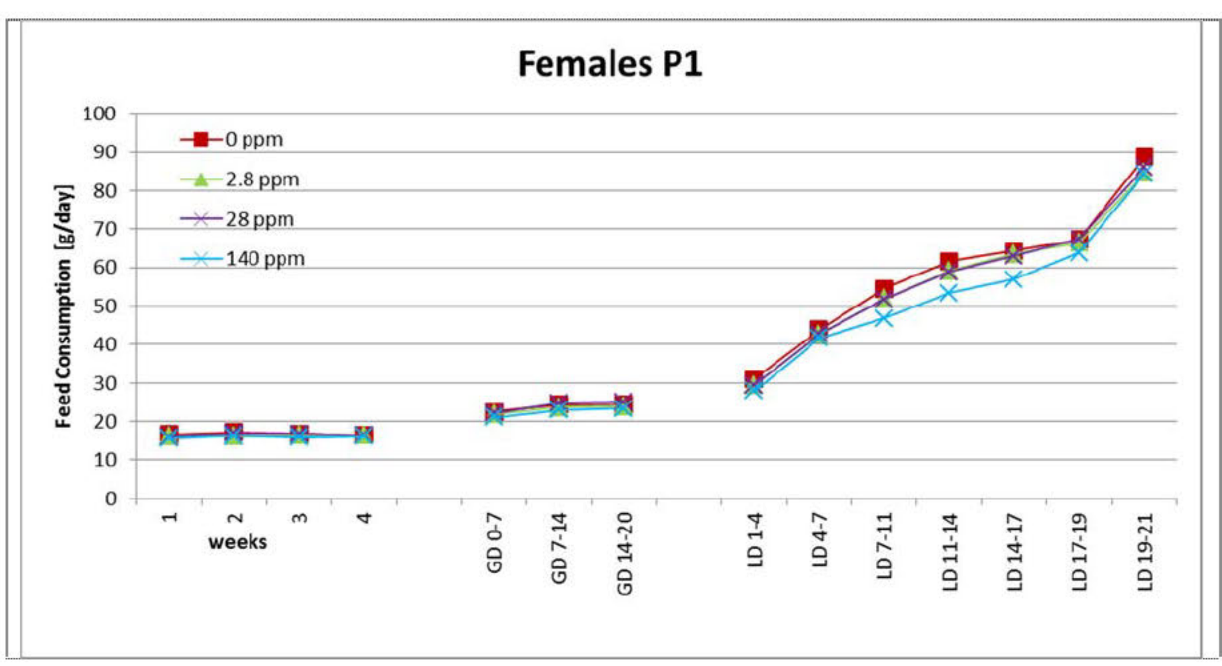


Table 5.8.1-23: Average ETU intake (mg/kg bw/d) in parental animals

Dose Group & sex	2.8 ppm* Average ppm	 Average MKD	28 ppm* Average ppm	 Average MKD	140 ppm* Average ppm	 Average MKD
P1 males	3.38±1	0.205±0.021	33.4±12	2.02±0.659	167±59	9.69±3.189
P1 females (premating)	2.80±0	0.204±0.0148	28.0±0	2.07±0.145	140±0	10.2±0.623
P1 females - gestation period - lactation period	 2.80±0 2.43±0.786	 0.204±0.0198 0.375±0.0934	 28.0±0 23.1±7.48	 2.09±0.207 3.58±0.886	 140±0 116±37.4	 10.3±0.936 17.1±4.07

* for males dose = 2.8/3.8, 28/38 and 140/190 ppm, respectively
MKD = (ppm * food consumption (mg/day) for that period) / (Average body weight (kg) for that period)

F. REPRODUCTION DATA

Dietary exposure to ETU had no significant effect on any of the reproductive indices, including male and female mating, conception, fertility, and gestation indices, or percent post-implantation loss.

1. Male reproductive performance

Reproductive Indices: For all F_0 parental males, which were placed with the females to generate F_1 pups, copulation was confirmed. Thus, the male mating index was 100% in all groups including the controls. Fertility was proven for nearly all parental males within the scheduled mating interval for F_1 litter. One male in each group did not generate F_1 pups. Thus, the male fertility index was 96.3% in all test groups including the control. This reflects the normal range of biological variation inherent in the strain of rats used for this study.

Parental generation				
Dose [ppm]	0	2.8/3.8	28/38	140/190
Males placed with females	27	27	27	27
Mated [n]	27	27	27	27
Male mating index [%][a]	100	100	100	100
Male conception index [%][b]	96.3	96.3	96.3	96.3
Male fertility index [%][c]	96.3	96.3	96.3	96.3

[a] # males with evidence of mating/total # males cohoused with females x 100%
[b] # males which sired a litter / # males mated x 100%
[c] # males which sired a litter / # males cohoused with females x 100%

Sperm analysis: There were no significant, treatment-related effects on sperm motility or progressive motility when P1 males were exposed to concentrations of < 190 ppm ETU in the diet for 11 weeks.
With respect to epididymal sperm counts, there were no significant, treatment-related differences between control and 190 ppm males. Sperm counts were not conducted for the lower dose levels due to the lack of effect at the highest dose.
The proportion of abnormal sperm was not significantly different between control and high-dose (190 ppm) males. While the proportion of abnormal sperm was slightly higher in the 190 ppm males (0.042 vs. 0.018 in controls), this mean was influenced by an outlier value (0.475 for one high dose animal #6202). When this one outlier was removed from the data (n = 26), the high dose males had 2.5% abnormal sperm compared to 1.8% in the controls. Values less than 5.0% are considered typical for control male rats (Stump et al., 2012; Linder et al., 1992 as cited in the study report [DocID2013/7002198]). Sperm morphology was not examined in samples from the lower dose levels due to the lack of effect at the highest dose.

2. Female reproductive performance

Estrous Cycle Length – P1 females: There was no significant difference in mean estrous cycle length in the ETU-treated groups compared with the control group. The mean estrous cycle length for all groups ranged from 4.0-4.3 days. There was no indication of persistent estrus (i.e., greater than two consecutive days in estrus) in either the P1 control or ETU-treated animals. There was no apparent difference in the percentage of time spent in estrus or diestrus in ETU-treated females compared with controls.

The female mating index calculated after the mating period for F_1 litter was 100% in all test groups. The mean duration until sperm was detected (GD 0) varied between 2.3 and 3.4 days without any relation to administered doses. All sperm positive rats delivered pups or had implants in utero with the exception of one female in each group (including control), that did not become pregnant.
The female fertility index was for all groups 96.3%.
The mean duration of gestation was identical in all test groups: 22 days for control, low-, mid- and high-dose groups. The gestation index was 100% in all test groups.
Furthermore, there were no indications for test substance-induced intrauterine embryo-/fetolethality since the postimplantation loss did not show any statistically significant differences between the groups, and the mean number of F_1 pups delivered per dam remained unaffected (13.4, 14.0, 14.0, and 13.7 pups/dam at 0, 2.8, 28 and 140 ppm, respectively).
The rate of liveborn pups was also not affected by the test substance, as indicated by live birth indices of 98.6%, 99.2%, 97.8 and 98.3% in group 0, 1, 2 and 3, respectively.
Dietary exposure to ETU at doses up to 140 ppm had no effect on number of live pups born / litter or subsesequent litter size measurements on LD 1, 4, 7, 14 or 21.

Table 5.8.1-24: Summary of female reproduction and delivery data

Parental generation	P1			
Dose [ppm]	**0**	**2.8**	**28**	**140**
Animals per dose	27	27	27	27
Female fertility				
- placed with males	27	27	27	27
- mated [n]	27	27	27	27
- mating index [%]	100	100	100	100
- pregnant [n]	26	26	26	26
- **Fertility index** [%]	96.3	96.3	96.3	96.3
Pre coital interval [days]	2.3±1.1	3.4±2.4	2.3±1.1	2.8±1.2
Duration of gestation [days]	21.5±0.5	21.5±0.5	21.7±0.5	21.8±0.5
Post implantation loss - dto per litter [mean %]	8.86±10.13	6.13±6.19	6.60±10.11	9.67±10.20
Females with liveborn	26	26	26	26
- **Gestation index** [%]	100	100	100	100
Pups delivered [n]	348	363	365	357
- per dam [mean n]	13.4	14.0	14.0	13.7
- liveborn [n]	343	360	357	351
- **Live birth index** [%]	98.6	99.2	97.8	98.3

G. PUP DATA

1. Survival

Dietary exposure to ETU had no significant effect on F1 offspring survival (see Table 5.8.1-25). The survival index indicating pup mortality during lactation (PND 1-21) varied between 95.8% and 100%.

The mean number of delivered F_1 pups per dam and the rates of liveborn and stillborn F_1 pups were evenly distributed about the groups. The respective values reflect the normal range of biological variation inherent in the strain used in this study.

Table 5.8.1-25: Summary of litter data

Pup generation	F_1			
Dose [ppm]	**0**	**2.8**	**28**	**140**
Number of litters	26	26	26	26
- with liveborn pups	26	26	26	26
- with stillborn pups	3	3	4	3
Pups liveborn [n]	343	360	357	351
Pups dead at birth [n]	5	3	8	6
Pups died [n]	8	15	7	9
Pups day 4 - post cull [n]	296	287	314	309
Pups survival index				
- Day 1 survival index [%][a]	99.1	99.2	98.6	98.0
- Day 4 survival index [%][a]	97.7	95.8	98.0	97.4
- Day 7 survival index [%][b]	99.6	99.6	100.0	99.6
- Day 14 survival index [%][b]	99.6	98.8	100.0	99.6
- Day 21 survival index [%][b]	99.6	98.8	100.0	99.6
Sex ratio [males female] - Day 1	51:49	49:51	52:48	48:52

[a] [# of live pups on day 1 or 4 / # of live pups on day 0] x 100
[b] [# of live pups on day 7, 14, or 21 / # of live pups after culling on day 4] x 100

2. Sex ratio

Dietary exposure to ETU had no significant effect on offspring sex ratio. All differences were within the historical control range and not indicative of a treatment-related effect [see Table 5.8.1-25]

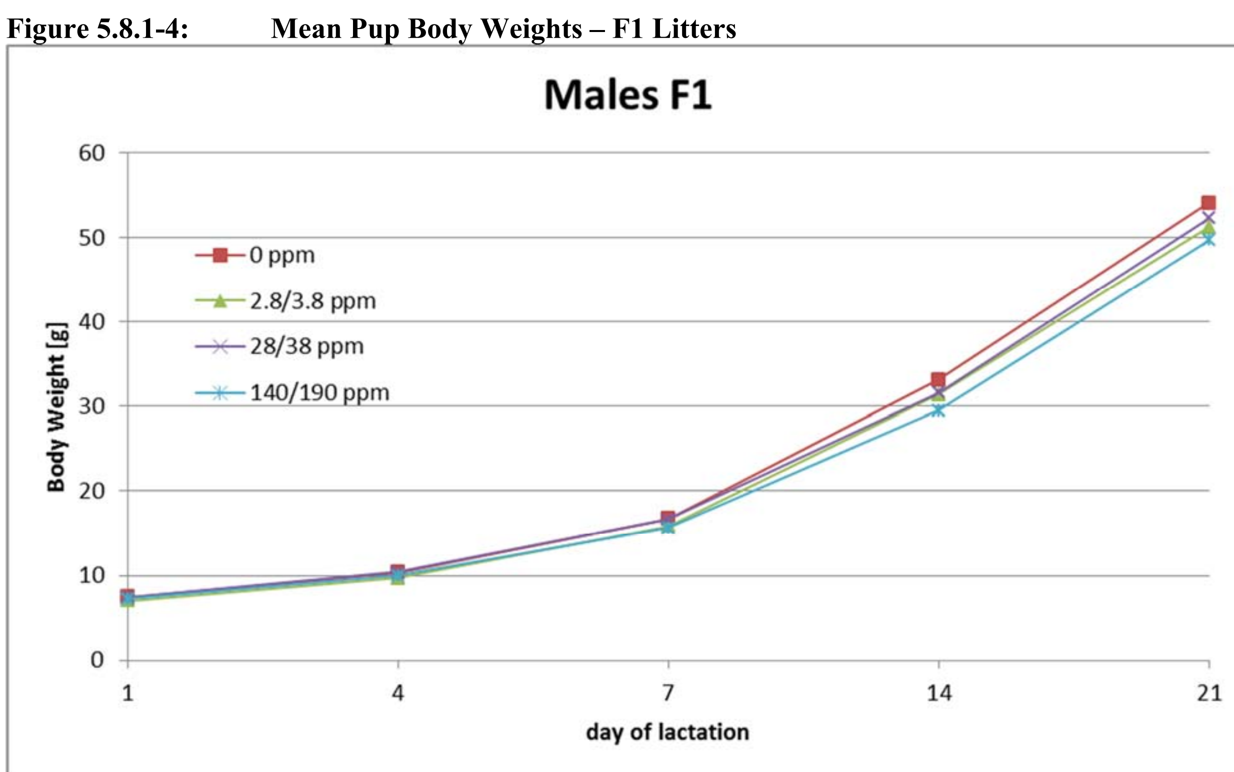
3. Pup clinical observations

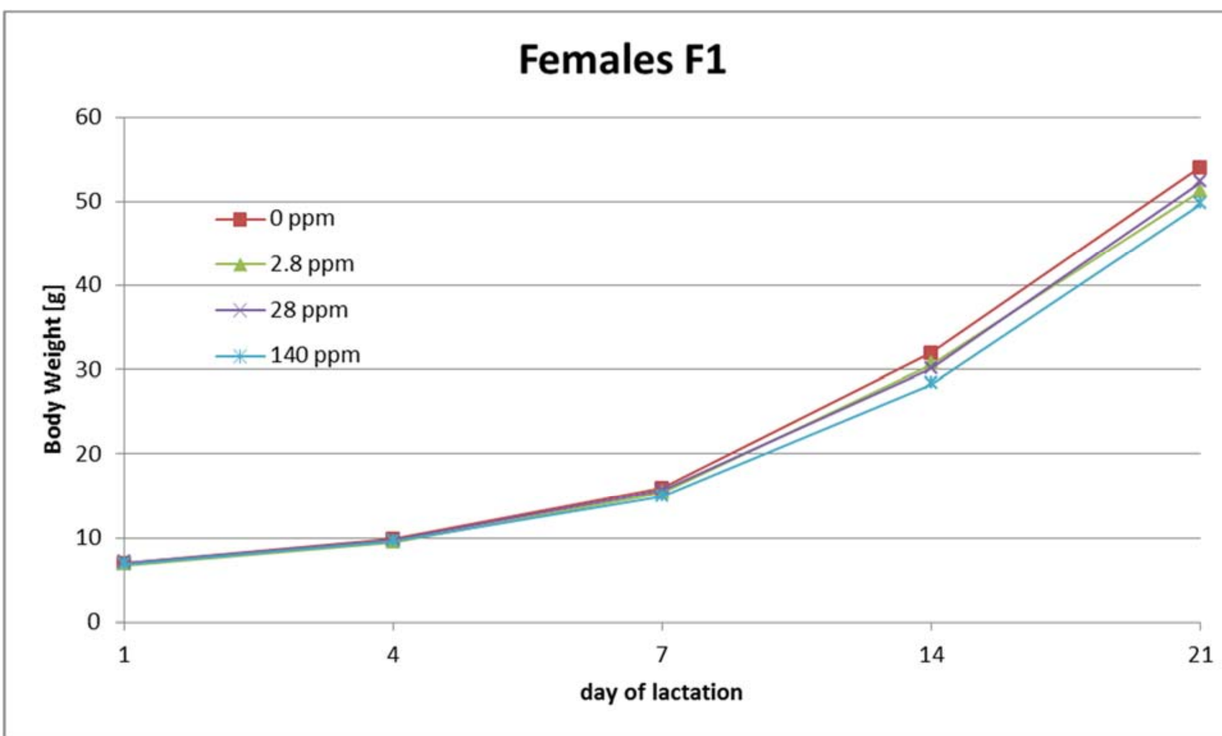
PND1 to PND 21 no clinical observations were reported. Clinical observations later than PND 21 are to be found in the individual cohorts below.

4. Body weight

On PND 1, body weights of the male and female pups in the 140 ppm group were similar to the control pup body weights (see Figure 5.8.1-4 and Table 5.8.1-26). As the lactation period progressed, the differences in pup body weights in the high-dose group began to increase relative to the control pups. By PND 14, high-dose male and female pups weighed significantly less (11-12%) than the corresponding control pups. This period of lactation (LD 0-14) corresponded with the period of highest test material intake by the P1 dams as there were no dietary concentration adjustments during this period to offset the large increases in maternal feed consumption that occur during lactation. On PND 21, high-dose male and female pups still weighed significantly less (8-9%) than control pups, although the magnitude of the body weight decrement was less than on LD 14. This body weight improvement may be due to the lower dietary concentration as diets were adjusted to one-half normal concentration during the LD 14-21 period, and/or it may be related to the pups beginning to consume diet directly during this interval. These decreases in pup body weight at 140 ppm were considered treatment related. There were no effects on pup body weights at ≤ 28 ppm ETU during lactation.

Figure 5.8.1-4: Mean Pup Body Weights – F1 Litters





Day 4 until day 21 of lactation body weights are taken after culling

Table 5.8.1-26: Pup body weights

Dose (ppm)	PND1		PND7		PND14[a]		PND21[a]	
	Females	Males	Females	Males	Females	Males	Females	Males
0	6.9	7.4	15.9	16.7	32.0	33.1	52.3	54.1
2.8	6.7	7.0	15.3	15.8	30.6	31.4	50.2	51.2
% change	-2.9	-5.4	-3.8	-5.4	-4.4	-5.1	-4.0	-5.4
28	7.0	7.4	15.6	16.6	30.1	31.6	49.9	52.3
% change	+1.4	0	-1.9	-0.6	-5.9	-4.5	-4.6	-3.3
140	6.8	7.2	14.9	15.6	**28.3***	**29.5***	**47.6***	**49.7***
% change	-1.4	-2.7	-6.3	-6.6	**-11.6**	**-10.9**	**-9.0**	**-8.1**

[a] One-half normal dietary concentration (1,4, 14, 70 ppm) on LD 14-21
* statistically different from control mean by Dunnet's test
Bolded values interpreted to be treatment related

5. Hormone measurements

F1 Offspring on PND 4: For measurements of serum levels of T4 and TSH, samples were pooled by litter to insure sufficient sample volume to assess 15 litters for each dose group. There was a significant, treatment-related increase (65%) in serum TSH in high-dose PND 4 culled pups (see Table 5.8.1-27). T4 was decreased by 28% at this dose level; this difference was not statistically identified, but was considered treatment related. It is possible that activation of the hypothalamic-pituitary-thyroid axis resulted in an increase in TSH levels, which subsequently increased T4 levels in high-dose pups; therefore, a significant decrease in serum T4 was not detected at this dose level. There was a significant decrease in T4 in the low-dose PND 4 culled pups; however, this decrease was judged to be incidental as there was no dose-response relationship and no corresponding increase in TSH in these animals.

Table 5.8.1-27: Serum T4 and TSH levels in male and female PND 4 Pups

Dose (ppm)	Males and Females			
	T4 (µg/dL)	% Change	TSH (ng/mL)	% Change
0	0.82	NA	0.89	NA
2.8	0.48*	-41	0.92	-3
28	0.72	-12	1.05	+18
140	**0.59**	**-28**	**1.47***	**+65**

NA = not applicable
* statistically different from control mean by Wilcoxon's test
Bold type indicates the effects were interpreted to be treatment related

F1 Offspring on PND 21: There were significant decreases in serum T4 in both male and female weanlings at 140 ppm (see Table 5.8.1-28). Serum T4 also was significantly decreased at 28 ppm in female weanlings, but there was no significant decrease in T4 in male weanlings in the 28 ppm dose group. There were corresponding significant increases in TSH levels in both male and female weanlings at 140 ppm. Females also had significant increases in TSH in the 28 ppm group; TSH was not significantly increased in male weanlings at this dose level. Thus, a dose-related pattern of thyroid hormone changes was present in PND 22 weanlings and considered related to ETU treatment.

Table 5.8.1-28: Serum T4 and TSH Levels in male and female PND 22 weanlings

Dose (ppm)	Males				Females			
	T4 (µg/dL)	% change	TSH (ng/mL)	% change	T4 (µg/dL)	% change	TSH (ng/mL)	% change
0	3.73	NA	0.95	NA	3.11	NA	0.87	NA
2.8	3.71	-0.5	0.89	-6	3.13	+0.6	1.03	+18
28	3.23	-13	1.14	+20	**2.35***	**-24**	**1.32$**	**+52**
140	**1.76***	**-53**	**2.68$**	**+182**	**1.57***	**-50**	**2.40$**	**+176**

NA = not applicable
* statistically different from control mean by Dunnett's test
$ statistically different from control mean by Wilcoxon's test
Bold type indicates the effects were interpreted to be treatment related

6. Organ weights

Both male and female PND 22 weanlings from the 140 ppm group had treatment-related higher absolute and relative thyroid gland weights, which corresponded to diffuse hyperplasia and hypertrophy of thyroid follicular cells in males and females at this dose level (see Table 5.8.1-29); however, only the increase in the male relative thyroid weight achieved statistical significance. There were no changes in thyroid weights at doses ≤ 28 ppm in either males or females and no treatment-related changes in final body weights, brain, spleen, or thymus weights at any dose level.

Table 5.8.1-29: Thyroid weight effects – non-selected PND 22 Weanlings

Generation		Males				Females			
	Dose [mg/kg]	Absolute weight [g]	□%	Relative weight [g/100g bw]	□%	Absolute weight [g]	□%	Relative weight [g/100 g bw]	□%
Thyroid	0	0.0048	NA	0.0084	NA	0.0052	NA	0.0094	NA
	2.8	0.0047	-2.1	0.0084	0	0.0051	-1.9	0.0092	-2.1
	28	0.0049	+2.1	0.0088	+4.8	0.0050	-3.8	0.0094	0
	140	**0.0057***	**+18.8**	**0.0105***	**+25**	**0.0055**	**+5.8**	**0.0105**	**+11.7**

NA = not applicable
* statistically different from control mean by Dunnett's test
Bold type indicates the effects were interpreted to be treatment related

7. up necropsy findings

There were no gross observations attributed to ETU exposure in the non-selected PND 22 weanlings.
Males and females from the 140 ppm group had treatment-related very slight diffuse follicular cell hyperplasia and very slight or slight diffuse follicular cell hypertrophy of the thyroid gland. Treatment-related very slight diffuse follicular cell hypertrophy of the thyroid gland was also present in 3/15 males and 3/15 females from the 28 ppm group. The hyperplasia was characterized by papillary infolding and/or stratification of the follicular epithelial cells. A generalized decrease in the amount of colloid accompanied the hypertrophy of the follicular epithelial cells.

8. Sexual maturation

Male and female F1 pups selected for Cohorts 1 and 2 were examined for sexual maturation. No treatment-related effects on sexual maturation were observed.

There were no significant, treatment-related effects on age at vaginal opening in F1 female rats; however, the body weight at the time of vaginal opening was significantly lower (7%) in the high-dose females (Table 5.8.1-30). This does not indicate an effect on the estrogen pathway, because the age at vaginal opening was not altered. Instead, these data indicate that the decreased growth rate of the F1 high dose females was insufficient to delay vaginal opening. There was no evidence of retained vaginal threads.

Puberty onset was delayed by 1.9 days in the high dose group of males (Table 5.8.1-30), but this effect was considered to be related to body weight decrements and slightly delayed growth, not directly due to ETU exposure. Body weight at the time of puberty onset was similar in 140 ppm males and controls (239.5 g compared with 245.4 g in controls). Because high-dose males weighed the same as controls 1.9 days later when puberty onset occurred, these data indicate that 140 ppm ETU had an effect on the rate of growth of peri-pubescent male rats. This result was confirmed by examining F1 juvenile male body weights on PND 28, 35, and 42. Male pups in the high-dose group weighed 7% less than controls at PND 28, and 8% less than controls on PND 35 and 42. These results are consistent with ████ (2003, as cited in the study report [DocID 2013/7002198]), who reported that a similar body weight decrement (10%) in juvenile male rats delays puberty up to 1.8-days. Furthermore, the age at preputial separation in the high-dose males was within the range of historical control values (43.0-45.7 days). There was no evidence of retained preputial threads and no effects on age/body weight at preputial separation for ETU doses ≤ 28 ppm.

Table 5.8.1-30: Sexual maturation of F1 pups

Sex	Male		Female	
	Age days	Body weight [g]	Age days	Body weight [g]
Dose				
0	43.5 ± 1.7	245.4 ± 19.4	31.7 ± 1.2	112.4 ± 9.8
2.8	44.4 ± 2.1	243.2 ± 20.1	31.9 ± 0.9	109.9 ± 9.0
28	44.5 ± 2.7	242.4 ± 17.5	31.8 ± 1.0	111.1 ± 9.5
140	45.4* ± 1.9	239.5 ± 20.4	31.9 ± 0.9	104.1* ± 7.1

* statistically different from control mean by Dunnett's test

There were no significant, treatment-related differences in absolute or relative anogenital distance and nipple/areolae retention in either male or female offspring.

H. CLINICAL CHEMISTRY AND HEMATOLOGY

P1 generation (adults)
Clinical chemistry parameters were examined in 12 P1 animals/sex/dose group. Statistically identified changes in clinical parameters are summarized in Table 5.8.1-31. The only treatment-related clinical chemistry alteration was a statistically significant increase in the cholesterol concentration of females given 140 ppm dietary ETU. Males given 190 ppm had a statistically significant lower alanine aminotransferase (ALT) activity, and males given 38 or 190 ppm had statistically significant higher creatinine concentrations. Females given 2.8 ppm had statistically significant lower total protein and albumin concentrations and females given 28 ppm had a statistically significant lower albumin concentration. The alterations in ALT, creatinine, total protein, and albumin were interpreted to be unrelated to treatment because the values for these parameters were of minimal difference from controls, and did not progress in a clear dose-responsive manner.
Urinalysis parameters were examined in 12 P1 males/dose group. There were no statistically significant or treatment-related effects on urinalysis parameters for males at any dose level.

Table 5.8.1-31: Clinical Chemistry Difference – P1 Adults

Sex	Males			
Dose (ppm)	0	3.8	38	190
Alanine Aminotransferase (u/L)	34	35	33	27[$]
Creatinine (mg/dL)	0.3	0.3	0.3*	0.3*
Sex	**Females**			
Dose (ppm)	0	2.8	28	140
Total Protein (g/dL)	6.9	6.5*	6.6	6.8
Albumine (g/dL)	4.6	4.2*	4.3*	4.5
Cholesterol (mg/dL)	64	70	72	**87***

*statistically different from control mean by Dunnett's test, as taken from the study report, however also when evaluating the individual data, the statistical significance remains unclear.
[$] statistically different from control mean by Wilcoxon's test
Bold type indicates the effects were interpreted to be treatment related

Hematological parameters were examined in 12 P1 animals/sex/dose group. Statistically identified changes in hematology parameters are summarized in Table 5.8.1-32. Males given 190 ppm dietary ETU had a statistically significant lower reticulocyte count, and females given 28 or 140 ppm had statistically significant higher reticulocyte counts. Females from all treatment groups had statistically identified lower red blood cell counts. These hematologic alterations were interpreted to be unrelated to treatment because of the lack of a dose response in either sex. In addition, there were no treatment-related histologic effects on the bone marrow of high-dose males or high-dose females, and microscopic evaluation of peripheral blood smears of high-dose females revealed no significant differences from controls. Differential white blood cell counts and prothrombin times were similar across groups for both males and females.

Table 5.8.1-32: Hematology – P1 Adults

Sex	Males			
Dose (ppm)	0	3.8	38	190
Reticulocyte Count (E9/µL)	145.4	153.0	126.6	126.3*
Sex	**Females**			
Dose (ppm)	0	2.8	28	140
Red Blood Cell Count (E6/µL)	8.32	7.91*	7.88*	7.91*
Reticulocyte Count (E9/µL)	139.9	156.6	182.7*	182.8*

* statistically different from control mean by Dunnett's test

I. PARENTAL TERMINAL INVESTIGATION

1. Thyroid Hormone levels

High-dose males had significant decreases in serum concentrations of T4 (67%) and a coincident significant increase in serum TSH levels (326%; see Table 5.8.1-33). At 38 ppm ETU, males had a significant decrease in serum T4 levels (23%), but TSH levels were not significantly altered. There were no significant effects on serum T4 or TSH levels in male rats given 3.8 ppm ETU in the diet. For high-dose parental females on LD 22, there was a significant decrease (76%) in serum T4 levels and a corresponding significant increase (323%) in serum TSH concentrations (see Table 5.8.1-33). At 28 ppm ETU, females had a significant decrease in serum T4 levels (36%), but TSH levels were not significantly altered. There were no significant changes in serum T4 or TSH in LD 21 dams given 2.8 ppm ETU in the diet.

Table 5.8.1-33: Thyroid hormone analyses – P1 adults

Dose	Males		Females	
(ppm)	T4 (µg/dL)	TSH (ng/mL)	T4 (µg/dL)	TSH (ng/mL)
0	4.09	3.24	1.60	1.59
3.8/2.8[a]	3.88	2.62	1.41	1.71
38/28[a]	**3.15***	4.27	**1.03[$]**	2.10
190/140[a]	**1.34***	**13.81[$]**	**0.39[$]**	**6.72[$]**

[a] dose in ppm for males/females
* statistically different from control mean by Dunnett's test
[$] statistically different from control mean by Wilcoxon's test
Bold type indicates the effects were interpreted to be treatment related

2. Organ weights

Alterations in final body weights and organ weights are summarized in Table 5.8.1-34. The mean final body weight of males given 190 ppm was 3.6% lower than controls, but was not statistically identified. Males given 38 or 190 ppm had treatment-related, statistically significant lower absolute and relative thymus weights.
Males given 190 ppm had treatment-related, statistically significant higher absolute and relative thyroid weights, which corresponded with hypertrophy and hyperplasia of thyroid follicular cells. Thymus and thyroid organ weight changes were considered to be treatment related.

The weights of the following organs were statistically different from controls, but these organ weight changes were deemed unrelated to treatment and/or not considered toxicologically significant.

Statistically significant organ weight changes that were interpreted to be reflective of lower body weights and/or were considered not toxicologically significant included lower absolute adrenal, heart, and kidney weights in males given 190 ppm, because:
1) Weights of the adrenal, heart, and kidneys previously have been shown to be sensitive to body weight changes (OECD, 2001, as cited in the study report [DocID 2013/7002198]). In the current study, relative weights for these organs in the high-dose P1 males were not significantly different from the control group, indicating an impact of body weight on these endpoints.
2) Each of these absolute organ weights also was affected in the Cohort 1A high-dose males (PND 90 with a 10% change in terminal body weight), but not in the Cohort 1B high-dose males (PND 120 with a 7.5% decrease in terminal body weight) (see below).
3) High-dose P1 females did not exhibit any changes in absolute or relative heart or kidney weights. As with the P1 males, relative adrenal weights in high-dose females were not statistically different from controls (see below).
4) There was no associated histopathological changes in these organs (see below).
Males given 3.8 ppm had significantly lower relative pituitary weights than control animals; however, this finding did not exhibit a dose-response relationship and there were no significant differences in absolute pituitary weights. This finding was judged to be incidental and unrelated to ETU treatment.

Alterations in final body weights and organ weights are summarized in Table 5.8.1-34. The mean final body weight of females given 140 ppm was 3.0% lower than controls, but was not statistically identified.
Females given 28 and 140 ppm had treatment-related lower absolute and relative thymus weights, which were statistically identified in the high-dose group. Females given 140 ppm had treatment-related, statistically significant higher absolute and relative thyroid weights, which corresponded with hypertrophy and hyperplasia of thyroid follicular cells. Absolute thyroid weights were significantly lower at 2.8 ppm; however, this finding did not exhibit a dose-response relationship and relative thyroid weight was not affected at this dose level.
The weights of the following organs were statistically different from controls, but these organ weight changes were deemed unrelated to treatment and/or not considered toxicologically significant.

A statistically significant decrease in adrenal weights in the 140 ppm P1 females was interpreted to be reflective of lower body weights and was considered not toxicologically significant, because:
1) Adrenal weights previously have been shown to be sensitive to body weight changes (OECD, 2001). In the current study, relative weights for the adrenal in the 140 ppm P1 females were not significantly different from the control group, indicating an impact of body weight on this endpoint.
2) Absolute adrenal weights were not significantly affected in the Cohort 1A or Cohort 1B high-dose females (PND 90 and PND 120) (see below).
3) There was no effect on relative adrenal weights in the high-dose P1 males.
4) There was no associated histopathological changes in the adrenal glands from high-dose females (see below).

Brain weight has previously been shown to be conserved in the presence of body weight changes (OECD, 2001), whereas absolute brain weights were significantly decreased in high-dose P1 females. Typically relative brain weights are increased in response to body weight decreases; in this study, relative brain weights were similar to the control group. However, brain weight findings in the P1 high-dose females were considered incidental. Brain weights were not affected in Cohort 1A or Cohort 1B high-dose females (n = 25 or 26/dose), despite exposure during critical windows of development. Absolute brain weights were decreased significantly in high-dose Cohort 2B females, although the sample size was less in this group (n = 12/dose). Furthermore, there were no effects on brain weight in the high-dose P1 males and no associated histopathological changes in either high-dose P1 males or females.
Relative pituitary weights were significantly increased in the 140 ppm P1 females, a change that was interpreted to be reflective of lower body weights and was considered of questionable toxicological significance, because:
1) Absolute pituitary weight in the 140 ppm P1 females was not significantly different from the control group.
2) Neither absolute nor relative pituitary weights were significantly affected in the Cohort 1A or Cohort 1B high-dose females (PND 90 and PND 120), despite exposure during critical windows of development (see below).
3) While both the high-dose P1 males and high dose P1 females had histopathological findings in the pituitary (hypertrophy), there was no corresponding change in pituitary weights in the high-dose P1 males.
Relative liver weights were statistically increased in high-dose P1 females. Liver weights previously have been shown to be sensitive to body weight changes with relative liver weights typically remaining unchanged or decreasing in feed restriction studies (OECD, 2001; Carney et al., 2004). In the current study, the increase in relative liver weight also was seen in the Cohort 1A high-dose females (PND 90), but was not present in the Cohort 1B high-dose females. Relative liver weights were not increased in the P1 high-dose males and there were no histopathological changes in high-dose P1 female livers; therefore, the minimal increase (5.5%) in relative liver weights in high-dose P1 females was judged to be not toxicologically significant.

Table 5.8.1-34: Selected organ weights of parental animals

Sex		Males				Females			
	Dose [m/f ppm]	Absolute weight	□%[&]	Relative weight [g/100g bw]	□%[&]	Absolute weight [mg]	□%[&]	Relative weight g/100g bw]	□%[&]
Terminal weight [g]	0	538.0	NA	NA	NA	297.1	NA	NA	NA
	3.8/2.8	563.1	+4.7	NA	NA	289.5	-2.6	NA	NA
	38/28	541.2	+0.6	NA	NA	292.5	-1.5	NA	NA
	190/140	518.6	-3.6	NA	NA	288.3	-3.0	NA	NA
Thymus [g]	0	0.271	NA	0.051	NA	0.186	NA	0.063	NA
	3.8/2.8	0.290	+7.0	0.052	+2.0	0.180	-3.2	0.063	0
	38/28	**0.215***	**-20.7**	**0.040***	**-21.6**	**0.165**	**-11.3**	**0.056**	**-11.1**
	190/140	**0.198***	**-26.9**	**0.038***	**-25.5**	**0.144***	**-22.6**	**0.050***	**-20.6**
Thyroid	0	0.0221	NA	0.041	NA	0.0155	NA	0.0052	NA
	3.8/2.8	0.0209	-5.4	0.037	-9.8	0.0135*	-12.9	0.0047	-9.6
	38/28	0.0209	-5.4	0.039	-4.9	0.0155	0	0.0053	+1.9
	190/140	**0.0360[$]**	**+62.9**	**0.0069***	**+68.3**	**0.0209***	**+34.8**	**0.0073[$]**	**+40.4**
Adrenals [g]	0	0.065	NA	0.012	NA	0.075	NA	0.025	NA
	3.8/2.8	0.064	-1.5	0.011	-8.3	0.076	+1.3	0.026	+4.0
	38/28	0.062	-4.6	0.011	-8.3	0.074	-1.3	0.026	+4.0
	190/140	_0.056*[a]_	_-13.8_	0.011[a]	-8.3	_0.066[$]_	_-12.0_	0.023	-8.0
Heart [g]	0	1.513	NA	0.282	NA				
	3.8/2.8	1.534	+1.4	0.273	-3.2				
	38/28	1.487	-1.7	0.277	-1.8				
	190/140	_1.380*_	_-8.8_	0.266	-5.7				
Kidneys [g]	0	3.559	NA	0.664	NA				
	3.8/2.8	3.578	+0.5	0.636	-4.2				
	38/28	3.501	-1.6	0.649	-2.3				
	190/140	_3.256*_	_-8.5_	0.629	-5.3				
Liver [g]						11.841	NA	3.989	NA
						11.991	+1.3	4.140	+3.8
						12.168	+2.8	4.154	+4.1
						12.135	+2.5	_4.210*_	_+5.5_
Brain [g]						1.970	NA	0.664	NA
						1.942	-1.4	0.675	+1.7
						1.940	-1.5	0.666	+0.3
						_1.895*_	_-3.8_	0.660	-0.6
Pituitary [g]	0	0.0151	NA	0.0028	NA	0.0147	NA	0.0050	NA
	3.8/2.8	0.0142	-6.0	0.0025[$]	-10.7	0.0144	-2.0	0.0050	0
	38/28	0.0145	-4.0	0.0027	-3.6	0.0150	+2.0	0.0051	+2.0
	190/140	0.0146	-3.3	0.0028	0	0.0162	+10.2	0.0056*	+12.0

* statistically different from control mean by Dunnett's test
[$] statistically different from control mean by Wilcoxon's test
Bold type indicates the effects were interpreted to be treatment related
Italics type indicates the values were interpreted to be secondary to lower final body weights.
[a] Historical control data ranges for absolute and relative adrenal gland weights in adult male CD rats were 0.055-0.067 g and 0.010-0.011 g/100 g body weight, respectively

3. Macroscopic lesions

There were no treatment-related gross pathologic observations in males and females from any dose level. All gross pathologic observations were interpreted to be spontaneous alterations, unrelated to dietary administration of ETU.

4. Histopathology

Complete histopathological examinations were conducted on 27 control and high-dose animals/sex. In addition, the thyroid glands and pituitary glands of all low-and mid-dose males and females, the livers of all low- and mid-dose males, all relevant gross pathologic observations, and the reproductive tracts of males and females that failed to deliver offspring were examined microscopically.

Males

Treatment-related histopathologic effects are summarized in Table 5.8.1-35. The thyroid gland was the primary target organ of P1 males administered ETU. Males from all dose groups had treatment-related increases in the incidence of diffuse follicular cell hypertrophy of the thyroid gland, relative to controls. The severity of the hypertrophy increased from very slight in 20/27 males given 3.8 ppm to slight in 23/27 males given 190 ppm. A generalized decrease in the amount of colloid accompanied the hypertrophy of the follicular epithelial cells. There were no adverse pathologic observations (i.e., degeneration, necrosis etc.) associated with follicular cell hypertrophy, suggesting that these alterations were adaptive rather than being pathologic and consistent with a more rapid colloid turnover. These thyroid alterations were considered treatment related but not adverse. Males given 38 or 190 ppm had treatment-related diffuse follicular cell hyperplasia of the thyroid gland. There was a dose-responsive increase in the severity of follicular cell hyperplasia, with most affected males at 38 ppm having very slight hyperplasia, and most affected males at 190 ppm having slight hyperplasia. The hyperplasia was characterized by papillary infolding and/or stratification of the follicular epithelial cells. Additional treatment-related thyroid effects consisted of focal slight nodular hyperplasia of follicular cells in one male given 190 ppm, and follicular cell adenoma in two males given 190 ppm. Follicular cell hyperplasia was considered treatment related and adverse.

Males from all dose levels had a treatment-related dose-responsive increase in the incidence of slight hypertrophy of individual cells in the pars distalis of the pituitary gland. The hypertrophic cells were scattered throughout the pars distalis, and had pale eosinophilic cytoplasm with round intracytoplasmic vacuoles of variable size. These hypertrophic cells were interpreted to represent thyrotropes that were responding to low serum thyroxine levels via the hypothalamic – pituitary – thyroid axis, with a resultant increase in the production of thyroid stimulating hormone by the pituitary gland.

Males given 190 ppm had an increase in the incidence of slight multifocal vacuolization (consistent with fatty change) of individual hepatocytes. The vacuolization was characterized by microvesiculation of hepatocellular cytoplasm that was most prominent in periportal hepatocytes, and macrovesiculation of individual hepatocytes that were randomly scattered in all portions of liver lobules. The increase in fatty change of the liver may have been reflective of an alteration in lipid metabolism associated with hypothyroidism in high-dose males.

Males that mated but failed to sire a litter included one control animal (#6126), one 3.8-ppm animal (#6152), one 38-ppm animal (#6166), and one 190-ppm animal (#6204). There were no histopathological changes in any of these males that explained the failure to sire a litter. The control male that failed to sire a litter (#6126) had the lowest absolute epididymal sperm count (279.1 x 10^6) of the 53 animals sampled (control and high-dose groups); this epididymal sperm count was 40% below the control group mean value. There were no other corresponding sperm parameter changes to explain the inability to sire litters by the remaining three males.
One high-dose male (#6202) was observed to have moderate unilateral degeneration of the seminiferous tubules of the testis and bilateral slight decreased spermatic elements in the epididymides (i.e., round and multinucleated spermatids present). This animal also had slightly lower sperm motility and progressive motility and a higher proportion of abnormal sperm (largest outlier value noted above) than other high-dose males; however, this male sired a normal size litter with 13 live born pups and 1 pup born dead.

Table 5.8.1-35: Incidence of selected histopathological lesions in parental rats

Dose [mg/kg]	0	3.8	38	190	0	2.8	28	140
Sex	male				female			
Animals in group	27	27	27	27	27	27	27	27
Thyroid gland # examined	27	27	27	27	27	27	27	27
- hyperplasia, follicular cell, diffuse								
-very slight	0	0	**14**	**4**	0	0	**7**	**1**
-slight	0	0	**1**	**23**	0	0	0	**26**
- hyperplasia, nodular, follicular cell, focal								
-slight	0	0	0	**1**	0	0	0	**1**
- hypertrophy, follicular cell, diffuse								
-very slight	4	**20**	**21**	**4**	2	2	**12**	1
-slight	0	0	**1**	**23**	0	0	0	**26**
- adenoma, follicular cell, benign, primary	0	0	0	**2**	0	0	0	0
Pituitary # examined	27	27	27	27	27	27	27	27
- hypertrophy, pars distalis, individual cells								
-very slight	0	0	0	0	1	2	4	3
-slight	0	**4**	**5**	**24**	0	0	3	**22**
Liver # examined	27	27	27	27	27	27	27	27
- vacuolization, consistent with fatty change, hepatocyte, individual cells, multifocal								
-very slight	23	24	26	18	3	0	0	2
-slight	0	2	0	**8**	0	0	0	0

Bolded values interpreted to be treatment related

Females
Treatment-related histopathologic effects are summarized in Table 5.8.1-35. The thyroid gland was the primary target organ of P1 females administered ETU. Females given 28 or 140 ppm had treatment-related diffuse follicular cell hyperplasia and hypertrophy of the thyroid follicular cells. There was a dose-responsive increase in the severity of follicular cell hyperplasia and hypertrophy, with most affected females at 28 ppm having very slight alterations, and most affected females at 140 ppm having slight alterations. The hyperplasia and hypertrophy of the thyroid follicular epithelial cells was of the same character as described above in males. An additional treatment-related thyroid effect consisted of focal slight nodular hyperplasia of follicular cells in one female given 140 ppm.

Females given 28 or 140 ppm had a treatment-related dose-responsive increase in the incidence of slight hypertrophy of individual cells in the pars distalis of the pituitary gland. The microscopic appearance of the hypertrophic cells, and their relationship to low serum thyroxine levels are the same as described above for males.
Females that mated but failed to conceive a litter included one control animal (#6234), one 2.8-ppm animal (#6260), one 28-ppm animal (#6274), and one 140-ppm animal (#6312). There were no histopathological changes in any of these females that explained the failure to conceive a litter.
One low-dose female (#6267) and one high-dose female (#6323) had malignant mammary gland adenocarcinomas without metastases. These tumors were considered spontaneous occurrences common to this strain/age of rat and judged to be unrelated to ETU treatment.

J. COHORT 1A – REPRODUCTIVE TOXICITY

This cohort was used for further examinations concerning reproductive toxicity. Animals were sacrificed on PND 90.

1. Clinical Observations

Cohort 1A males and females did not exhibit any treatment-related observations throughout the term of the study. Clinical observations recorded during the study were isolated occurrences and/or deemed to be spontaneous occurrences common to this strain/age of rat and unrelated to exposure.

There were no effects of ETU exposure on Cohort 1A male or female survival. All Cohort 1A animals survived to scheduled necropsy.

2. Body weights

There were significant decreases in body weights of high-dose Cohort 1A males at multiple time points (Table 5.8.1-36 and Figure 5.8.1-5). High-dose Cohort 1A males weighed 8% less than controls on PND 21. Despite a slight improvement in body weight decrement on PND 28, the body weight differential in these high-dose males was again 8% on PND 35. Beginning on PND 35, body weights in the high-dose pups were significantly decreased relative to the controls at all time points through the remainder of the study. During the interval from PND 35 to 56, the decrease in body weight in the high-dose males reached 11% and the body weight decrement remained at this level throughout the remainder of the monitoring period (i.e., to PND 84). Consistent with these body weight decrements, body weight gains were significantly decreased by 10-12% in high-dose males at all intervals from PND 49-84. Thus, 140 ppm ETU resulted in sustained, treatment-related effects on body weights/body weight gains in Cohort 1A males. There were no significant effects on either body weight or body weight gains at ≤ 28 ppm ETU.

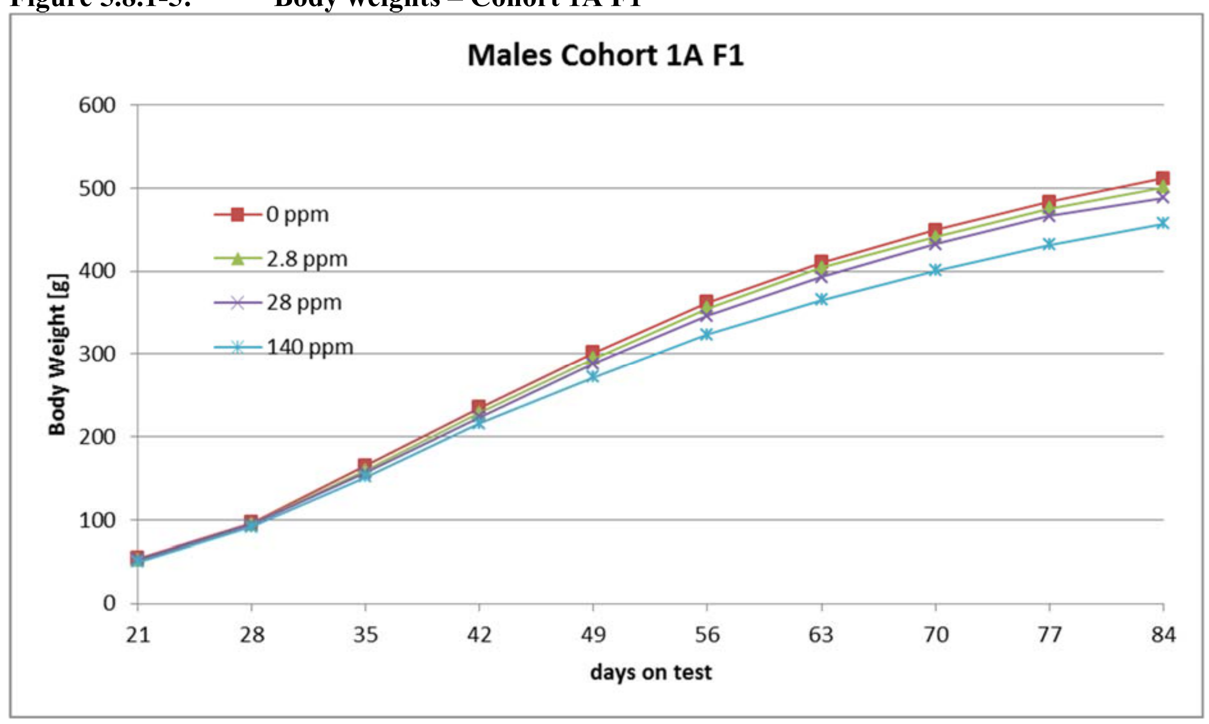
There were significant decreases in body weights of high-dose Cohort 1A females at early time points during the post-weaning period from PND 21-49; however, from PND 56-84 body weights in the high-dose females did not differ significantly from controls (Table 5.8.1-36 and Figure 5.8.1-5). High-dose Cohort 1A females weighed 9% less than controls on PND 21, but there was a continued diminution in this body weight differential as the study progressed. By PND 56, body weights were decreased by 7% at 140 ppm and by PND 84, body weights were decreased by only 3% in high-dose females. Body weights were not significantly altered at ETU doses ≤ 28 ppm. Body weight gains did not differ at any dose level of ETU at any interval throughout the monitoring period in the Cohort 1A females.

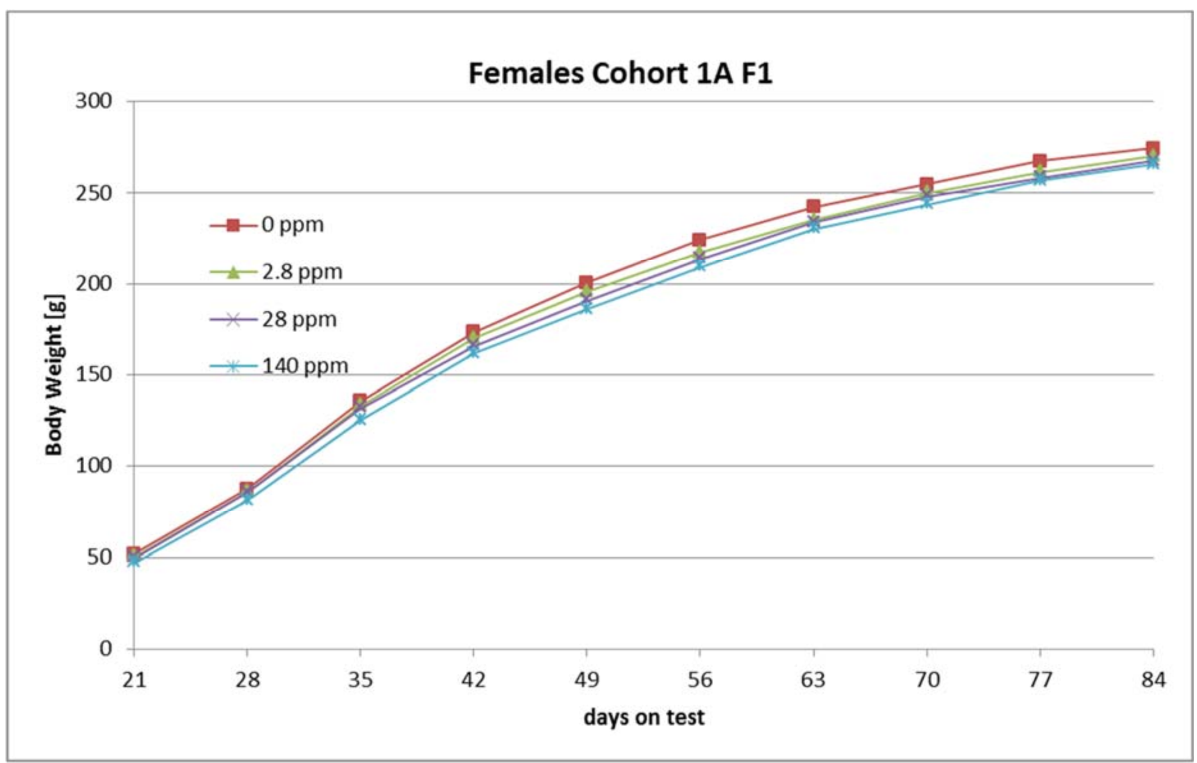
Table 5.8.1-36: Cohort 1A Body weights and body weight gains

Dose (ppm)	BW PND 21	BW PND 35	BWG PND 21-35[a]	BW PND 49	BWG PND 21-49	BW PND 56	BWG PND 21-56	BW PND 84	BWG PND 21-84
Males									
0	54.1	164.8	110.6	301.1	246.9	361.9	307.7	511.6	457.5
2.8	51.5	159.1	107.5	294.5	242.6	355.1	303.1	500.8	448.0
% change	-48.	-3.5	-2.8	-2.2	-1.7	-1.9	-1.5	-2.1	-2.1
28	52.6	156.8	104.2	288.0	235.4	345.8	293.2	488.3	435.7
% change	-2.8	-4.9	-5.8	-4.4	4.7	-4.4	-4.7	-4.6	-4.8
140	**50.0**	**151.4***	**101.4**	**271.9***	**221.9***	**323.4***	**273.4***	**457.2***	**407.2***
% change	**-7.6**	**-8.1**	**-8.3**	**-9.7**	**-10.1**	**-10.6**	**-11.1**	**-10.6**	**-11.0**
Females									
0	51.7	135.8	84.1	200.4	148.8	224.3	172.7	274.5	222.9
2.8	50.3	133.3	83.0	195.5	145.2	217.0	166.7	270.2	219.9
% change	-2.7	-1.8	-1.3	-2.4	-2.4	-3.3	-3.5	-1.6	-1.3
28	49.5	132.0	82.5	190.5	141.0	213.5	164.0	267.5	218.0
% change	-4.3	-2.8	-1.9	-4.9	-5.2	-4.8	-5.0	-2.6	-2.2
140	**47.1***	**125.3***	**78.2**	**185.8***	**138.8**	209.2	162.1	265.6	218.5
% change	**-8.9**	**-7.7**	**-7.0**	**-7.3**	**-6.7**	-6.7	-6.1	-3.2	-2.0

BW = Body weight (g); BWG = Body weight gain (g)
[a] On PND 35, animals were given full concentrations of ETU in the diet. From PND 21-35, they received one-half normal dietary concentrations (1.4, 14, 70 ppm)
* Statistically different from control mean by Dunnett's test
Bolded values interpreted to be treatment related.

Figure 5.8.1-5: Body weights – Cohort 1A F1





3. Food consumption

In high-dose males, feed consumption values were less than controls at all intervals examined with significant differences (10-15%) in feed consumption from PND 42-49 through the remainder of the monitoring period (i.e. PND 88) (Table 5.8.1-37). These decreases in feed consumption were consistent with the decreases in body weight/body weight gain seen in the high-dose Cohort 1A males from PND 35 to PND 84. Feed consumption was not significantly altered in Cohort 1A males given ≤ 28 ppm ETU except during the final study interval (PND 84-88) where feed consumption was significantly decreased at all ETU doses. This finding was considered incidental.

There were significant treatment-related decreases in Cohort 1A female feed consumption during the early post-weaning intervals on PND 28-49 (Table 5.8.1-37). These decreases in feed consumption were consistent with the decreases in body weight/body weight gain seen in the high-dose Cohort 1A females from PND 21 to PND 49. Feed consumption was not significantly altered in Cohort 1A females given ≤ 28 ppm ETU at any time interval.

Table 5.8.1-37: Cohort 1A Food consumption (g/day)

Dose (ppm)	PND 28-35[a]	PND 35-42	PND 42-49	PND 49-56	PND 77-84	PND 84-88
Males						
0	17.8	23.9	27.2	29.0	29.6	30.0
2.8	17.5	23.8	26.6	28.5	28.9	27.7*
% change	-1.7	-0.4	-2.2	-1.7	-2.4	-7.7
28	17.4	23.1	26.3	27.6	28.4	26.8*
% change	-2.2	-3.3	-3.3	-4.8	-4.1	-10.7
140	**16.6**	**22.1**	**23.8***	**24.7***	**26.5***	**26.0***
% change	**-6.7**	**-7.5**	**-12.5**	**-14.8**	**-10.5**	**-13.3**
Females						
0	15.1	18.4	18.5	18.8	18.5	18.3
2.8	14.9	17.7	17.6	17.9	18.8	17.3
% change	-1.3	-3.8	-4.9	-4.8	+1.6	-5.5
28	15.0	17.5	17.7	18.3	18.4	17.4
% change	-0.7	-4.9	-4.3	-2.7	-0.5	-4.9
140	**14.0***	**16.8***	**16.9***	17.5	17.9	16.9
% change	**-7.3**	**-8.7**	**-8.6**	-6.9	-3.2	-7.7

[a] On PND 35, animals were given full concentrations of ETU in the diet. From PND 21-35, they received one-half normal dietary concentrations (1.4, 14, 70 ppm)
* Statistically different from control mean by Dunnett's test
Bolded values interpreted to be treatment related.

Test material intake (time weighted average) in Cohort 1A males was 0.238 mg/kg/day at 2.8 ppm, 2.28 mg/kg/day at 28 ppm, and 11.1 mg/kg/day at 140 ppm. The highest intakes of test material in males were recorded for PND 35-42, just after they received their full dietary concentrations and ranged from 0.361 mg/kg/day at 2.8 ppm, 3.40 mg/kg/day at 28 ppm, and 16.8 mg/kg/day at 140 ppm.
Test material intake (time weighted average) in Cohort 1A females was 0.245 mg/kg/day at 2.8 ppm, 2.37 mg/kg/day at 28 ppm, and 11.7 mg/kg/day at 140 ppm. The highest intakes of test material in females were recorded for PND 35-42, ranging from 0.343 mg/kg/day at 2.8 ppm, 3.29 mg/kg/day at 28 ppm, and 16.4 mg/kg/day at 140 ppm.
Despite dietary concentration adjustments prior to PND 35, Cohort 1A animals received 13-20% higher doses of ETU (mg/kg body weight) than pre-mating (male and female) or gestational adults.

4. Sex maturation

There were no significant differences in the interval between vaginal opening and first estrus in any ETU treated group (0.6-1.2 days) relative to the control group (2.2 days). In many animals, first estrus coincided with vaginal opening.
There was no significant difference in mean estrous cycle length in the ETU-treated groups compared with the control group. The mean estrous cycle length ranged from 4.1-4.5 days across all groups. There was no indication of persistent estrus (i.e., greater than 2 consecutive days in estrus) in either the control or ETU-treated animals.

5. Thyroid hormones

Thyroid hormone data for serum levels of T4 and TSH were examined in PND 85 male and female rats. High-dose Cohort 1A males had significant decreases in serum concentrations of T4 (62%) and a coincident significant increase in serum TSH levels (500%; Table 5.8.1-38). At 28 ppm ETU, males had a significant decrease in serum T4 levels (14%), but TSH levels were not significantly altered at this dose level. There were no significant effects on serum T4 or TSH levels in Cohort 1A male rats given 2.8 ppm ETU in the diet. For high-dose Cohort 1A females, there was a significant decrease (78%) in serum T4 levels and a corresponding significant increase (239%) in serum TSH concentrations (Table 5.8.1-38). At 28 ppm ETU, females had a significant decrease in serum T4 levels (32%), but TSH levels were not significantly altered at this dose level. There were no significant changes in serum T4 or TSH in Cohort 1A females given 2.8 ppm ETU in the diet.

Table 5.8.1-38: Serum T4 and TSH Levels in Cohort 1A rats

Dose (ppm)	**Males**				**Females**			
	T4 (µg/dL)	% Change	TSH (ng/mL)	% Change	T4 (µg/dL)	% Change	TSH (ng/mL)	% Change
0	3.85	NA	1.66	NA	2.31	NA	0.95	NA
2.8	3.94	+2.3	2.21	+33.1	2.50	+8.2	0.91	-4.2
28	**3.33***	**-13.5**	2.58	+55.4	**1.58$**	**-31.6**	1.01	+6.3
140	**1.47***	**-61.8**	**9.96$**	**+500**	**0.51$**	**-77.9**	**3.22$**	**+239**

NA = not applicable.
*Statistically different from control mean by Dunnett's test, alpha = 0.05.
$Statistically different from control mean by Wilcoxon's test, alpha = 0.05.
Bold type indicates the effects were interpreted to be treatment related.

6. Hematology

Hematological parameters were examined in 11 Cohort 1A (PND 90) animals/sex/dose level. Males and females given 140 ppm had slightly lower reticulocyte counts (statistically significant in females) as compared to controls (Table 5.8.1-39). The lower reticulocyte counts were interpreted to be reflective of normal variability of this parameter, due to the lack of a clear dose response in males, and because the lower value in Cohort 1A females was in contrast to the statistically significant higher reticulocyte count in P1 females given the same concentration of 140 ppm ETU. All other hematological values, including differential white blood cell counts and prothrombin times, were similar to controls across all dose groups.

Table 5.8.1-39: Hematology Differences – Cohort 1A (PND 90)

Sex	**Males**			
Dose (ppm)	0	2.8	28	140
Reticulocyte Count (E^9/µl)	151.9	130.8	135.3	123.9
Sex	**Females**			
Dose (ppm)	0	2.8	28	140
Reticulocyte Count (E^9/µl)	174.7	178.8	165.1	130.4*

* Statistically different from control mean by Dunnett's test, alpha = 0.05.

7. Clinical Chemistry

Clinical chemistry parameters were examined in 11 Cohort 1A animals/sex/dose group. Statistically identified changes in clinical parameters are summarized in Table 5.8.1-40. The only treatment-related clinical chemistry alteration was an increase in the cholesterol concentration of males given 140 ppm (statistically significant) and females given 140 ppm. Males given 2.8, 28, or 140 ppm had statistically significant lower aspartate aminotransferase (AST) activities and males given 140 ppm had a statistically significant lower urea nitrogen (UN) concentration. Females given 2.8, 28, or 140 ppm had statistically significant lower ALT and AST activities and females given 28 ppm had a statistically significant lower protein concentration. The lower UN, ALT, AST, and total protein parameters were interpreted to be unrelated to treatment because they were of minimal difference from controls, and/or the values did not progress in a clear dose-responsive manner.

Table 5.8.1-40: Clinical Chemistry Differences – Cohort 1A (PND 90)

Sex	**Males**			
Dose (ppm)	0	2.8	28	140
Urea Nitrogen (mg/dL)	14	12	14	12$
Aspartate Aminotransferase (u/L)	95	81*	79*	72*
Cholesterol (mg/dL)	47	53	59	**67***
Sex	**Females**			
Dose (ppm)	0	2.8	28	140
Alanine Aminotransferase (u/L)	35	27*	24*	25*
Aspartate Aminotransferase (u/L)	93	78*	76*	74*
Total Protein (g/dL)	7.2	7.1	6.9*	7.1
Cholesterol (mg/dL)	60	65	61	**74**

* Statistically different from control mean by Dunnett's test, alpha = 0.05.
$Statistically different from control mean by Wilcoxon's test, alpha = 0.05.
Bold type indicates the effects were interpreted to be treatment related.

Urinalysis parameters were examined in 11 Cohort 1A animals/sex/dose group. There were no statistically significant or treatment-related effects on urinalysis parameters for males or females at any dose level.

8. Organ Weights

Males given 140 ppm had a treatment-related, statistically significant 10.3% lower final body weight relative to controls.

Males given 28 or 140 ppm had treatment-related statistically significant lower absolute and relative thymus weights, which was considered treatment related and corresponded with very slight diffuse atrophy of thymic lymphoid tissue in some males given 140 ppm. Males given 140 ppm had treatment-related statistically significant higher absolute and relative thyroid weights, which corresponded with hypertrophy and hyperplasia of thyroid follicular cells. Males given 2.8 ppm had statistically significant lower absolute and relative thyroid weights that may have been related to the decreased amount of colloid that accompanied the very slight follicular cell hypertrophy of the thyroid in males at this dose level. This decrease in thyroid weight due to colloid endocytosis by follicular cells was considered an adaptive response to re-establish thyroid hormone homeostasis in the low-dose Cohort 1A males.

The weights of the following organs were statistically different from controls, but these organ weight changes were deemed unrelated to treatment and/or not considered toxicologically significant. Statistically significant organ weight changes that were interpreted to be reflective of lower body weights and/or were considered not toxicologically significant included lower absolute adrenal, kidney, and spleen weights in males given 140 ppm, because: 1) Weights of the adrenal, kidneys and spleen previously have been shown to be sensitive to body weight changes (OECD, 2001, as cited in the study report [DocID 2013/7002198])). In the current study, relative weights for these organs in the high-dose P1 males were not significantly different from the control group, indicating an impact of body weight on these endpoints. 2) Absolute weights of the adrenals and kidneys were affected in the P1 high-dose males, but relative weights were not significantly different. Neither absolute nor relative weights for these organs were altered in the Cohort 1B high-dose males (PND 120 with a 7.5% decrease in terminal body weight) (see below). 3) High-dose Cohort 1A females did not exhibit any changes in absolute or relative adrenal, kidney, or spleen weights (see below). 4) There was no associated histopathological changes in these organs (see below).
There was a significant decrease in both absolute and relative heart weights in the Cohort 1A males given 140 ppm ETU. Absolute heart weights were significantly decreased in the P1 high-dose males, but relative weights were not significantly altered. Neither absolute nor relative weights for these organs were altered in the Cohort 1B high-dose males (PND 120 with a 7.5% decrease in terminal body weight; see below) 5) High-dose Cohort 1A females did not exhibit any changes in absolute or relative heart weights (see below) and there was no associated histopathological changes in hearts from the high-dose Cohort 1A males (see below).
Relative liver weights were significantly increased (7%) at 140 ppm ETU in Cohort 1A males, despite 4% lower absolute liver weights. Liver weights previously have been shown to be sensitive to body weight changes with relative liver weights typically remaining unchanged or decreasing in feed restriction studies (OECD, 2001; Carney et al., 2004, as cited in the study report [DocID 2013/7002198]). In the current study, the increase in relative liver weight was not present in the high-dose P1 males and Cohort 1B males, but the Cohort 1A high-dose females (PND 90) also had significant increases in relative liver weights. There were no associated histopathological changes in high-dose Cohort 1A male livers; therefore, the toxicological significance of a change in relative liver weights is unclear.

Absolute brain weight was decreased slightly (5%) in high-dose males, but relative weight was increased by the same amount (5%, not statistically significant); therefore, this finding was attributed to the lower terminal body weights in the high-dose animals. Brain weight has previously been shown to be conserved in the presence of body weight changes (OECD, 2001), and as expected, relative brain weights were increased in response to body weight changes in this study. Brain weights were not affected in P1 males or high-dose Cohort 1B males at any dose level (n = 25-27/dose). Absolute brain weights were decreased significantly in high-dose Cohort 2B males, although the sample size was less in this group (n = 12/dose). Furthermore, there were no effects on brain weight in the high-dose Cohort 1A females and no associated histopathological changes in either high-dose P1 males or females.

The absolute epididymal weights were significantly decreased in Cohort 1A males in the 28 and 140 ppm groups; this decrease appeared to be dose-related. Relative epididymal weights were similar to the control group in all ETU-treated Cohort 1A males. Terminal body weights in Cohort 1A males given 28 and 140 ppm ETU were decreased by 4% and 10%, respectively. Previous studies have shown that epididymal weights are often spared in the presence of moderate body weight changes (Carney et al., 2004; Chapin and Gulati 1997, as cited in the study report [DocID 2013/7002198]); however, a feed restriction study by Rehm et al. (2008, as cited in the study report [DocID 2013/7002198]) has shown decreases in absolute epididymal weights with sustained effects on body weight. There were no effects on epididymal weights in the high-dose P1 males, but the high-dose Cohort 1B males also had a significant decrease in absolute epididymal weights (relative weights not altered). There was no effect on epididymal weights in the mid-dose Cohort 1B males. There were no effects on other reproductive parameters in the P1 or Cohort 1A males, including no effects on male reproductive indices, testes or accessory sex tissue weights, sperm motility, sperm morphology and sperm counts (all collected from the epididymis), and no corresponding histopathological changes in either the testes or epididymides in P1, Cohort 1A, or Cohort 1B males. Thus, changes in absolute epididymal weights were judged to be not toxicologically significant.
There were no effects on the absolute or relative weights of the pituitary, prostate, testes, or seminal vesicles at any dose of ETU.

The mean final body weight of females given 140 ppm was 3.6% lower than controls, but was not statistically identified.
Females given 28 or 140 ppm had treatment-related statistically significant lower absolute and relative thymus weights; however, there were no corresponding histopathological changes in the thymus in these Cohort 1A females. Females given 140 ppm had treatment-related statistically significant higher absolute and relative thyroid weights, which corresponded with hypertrophy and hyperplasia of thyroid follicular cells. There were no effects on the absolute or relative weights of the adrenal glands, heart, kidneys, brain, pituitary, spleen, ovaries, or uterus at any dose of ETU.
As seen with the high-dose P1 females and high-dose Cohort 1A males, Cohort 1A females given 140 ppm had a statistically significant increase in relative liver weight (9.7%). Neither absolute nor relative liver weights were altered in the Cohort 1B females at any dose of ETU. There were no histopathological changes in high-dose Cohort 1A female livers; therefore, the toxicological significance of a change in relative liver weights is unclear.

Table 5.8.1-41: Final Body Weights and Selected Organ Weights – Cohort 1A

Sex	Males						
Dose (ppm)	0	2.8	% Change	28	% Change	140	% Change
Final Body Weight (g)	498.4	489.8	-1.7	478.6	-4.0	**447.0***	**-10.3**
Absolute Thymus (g)	0.460	0.433	-5.9	**0.355***	**-22.8**	**0.317***	**-31.1**
Relative Thymus (g/100 g bw)	0.092	0.089	-3.3	**0.074***	**-19.6**	**0.071***	**-22.8**
Absolute Thyroid (g)	0.0207	0.0184$	-11.1	0.0204	-1.4	**0.0290$**	**+40.12**
Relative Thyroid (g/100 g bw)	0.0042	0.0038$	-9.5	0.0043	+2.4	**0.0061$**	**+54.8**
Absolute Adrenals (g)	0.077	0.070	-9.1	0.071	-7.8	*0.061**	*-20.8*
Relative Adrenals (g/100 g bw)	0.015	0.014	-6.7	0.015	0	0.014	-6.7
Absolute Kidneys (g)	3.690	3.518	-4.7	3.522	-4.6	*3.300**	*-10.6*
Relative Kidneys (g/100 g bw)	0.741	0.719	-3.0	0.737	-0.5	0.738	-0.4
Absolute Spleen (g)	0.916	0.846	-7.6	0.845	-7.8	*0.777**	*-15.2*
Relative Spleen (g/100 g bw)	0.184	0.173	-6.0	0.176	-4.3	0.173	-6.0
Absolute Heart (g)	1.577	1.488	-5.6	1.488	-5.6	*1.323**	*-16.1*
Relative Heart (g/100 g bw)	0.317	0.304	-4.1	0.312	-1.6	*0.296**	*-6.6*
Absolute Liver (g)	15.259	14.888	-2.4	14.818	-2.9	14.651	-4.0
Relative Liver (g/100 g bw)	3.054	3.035	-0.6	3.085	+1.0	*3.278**	*+7.3*
Absolute Brain (g)	2.131	2.099	-1.5	2.092	-1.8	*2.017**	*-5.3*
Relative Brain (g/100 g bw)	0.431	0.433	+0.5	0.442	+2.6	0.453	+5.1
Absolute Epididymis (g)	1.283	1.236	-3.7	*1.207**	-5.9	*1.141**	*-11.1*
Relative Epididymis (g/100 g bw)	0.2596	0.254	-1.9	0.255	-1.5	0.256	-1.2
Sex	**Females**						
Final Body Weight (g)	262.1	259.0	-1.2	256.7	-2.1	252.7	-3.6
Absolute Thymus (g)	0.363	0.337	-7.2	**0.301***	**-17.1**	**0.250***	**-31.1**
Relative Thymus (g/100 g bw)	0.138	0.131	-5.1	**0.117***	**-15.2**	**0.099***	**-28.3**
Absolute Thyroid (g)	0.0152	0.0144	-5.3	0.0147	-3.3	**0.0201***	**+32.2**
Relative Thyroid (g/100 g bw)	0.0058	0.0056	-3.4	0.0058	0	**0.0080***	**+37.9**
Absolute Liver (g)	7.635	7.597	-0.5	7.651	+0.2	8.059	+5.6
Relative Liver (g/100 g bw)	2.906	2.937	+1.1	2.987	+2.8	*3.188**	*+9.7*

*Statistically different from control mean by Dunnett's test, alpha = 0.05.
$Statistically different from control mean by Wilcoxon's test, alpha = 0.05.
Bold type indicates the effects were interpreted to be treatment related.
Italics type indicates the values were interpreted to be secondary to lower final body weights.

9. Reproductive Parameters

Sperm Motility:
There were no significant, treatment-related effects on sperm motility or progressive motility when Cohort 1A males were exposed to concentrations of < 140 ppm ETU in the diet through critical windows of development from in utero through adulthood. There was greater variance in sperm motility measurements in the mid-dose and high-dose group males, which were due to a single outlier animal at each dose level. Removal of a single outlier value in the 28 ppm group (animal #814) yielded a percent motile value of 96.7 ± 2.3 and a percent progressive motility value of 87.6 ± 4.3, which were similar to the control values of 96.9 ± 1.9 and 87.7 ± 5.0, respectively. Similarly, when a single outlier value was removed from the 140 ppm group (animal #835), the percent progressive motility value was 86.9 ± 7.36, a value which is similar in magnitude and variance to the control value.

Sperm Counts:
With respect to epididymal sperm counts, there were no significant, treatment-related differences in total sperm count or sperm efficiency (sperm concentration/g cauda epididymis) between control and 140 ppm ETU-treated Cohort 1A males. Sperm counts were not conducted for the lower dose levels due to the lack of effect at the highest dose.

Sperm Morphology:
The proportion of abnormal sperm was not significantly different between control and ETU-treated Cohort 1A males. Values less than 5.0% are considered typical for control male rats (Stump et al., 2012; Linder et al., 1992, as cited in the study report [DocID 2013/7002198]).

Ovarian Follicle Counts:
Females given 140 ppm ETU had statistically significant higher mean small (primordial), growing, and total (small plus growing) ovarian follicle counts, relative to controls (Table 5.8.1-42). The mean corpora lutea count of females given 140 ppm was similar to controls. Generally, reproductive toxicity studies examine ovarian follicle counts because decreases in this endpoint may indicate potential ovotoxicity and/or a potential for shortened reproductive life span. In the current study, a significant increase in ovarian follicle counts (small, growing, and total) was observed in the high-dose group Cohort 1A females. The higher follicle counts in females given 140 ppm were interpreted to be reflective of the inherent variability of the distribution and density of follicles within individual ovaries. Based on the variable follicle distribution in individual ovaries, the lack of a significant difference in corpora lutea number in the high-dose females, and the lack of effects on other related endpoints in the Cohort 1A females (e.g., puberty onset, interval to first estrus, estrous cycle length/pattern, ovarian histopathology), the higher ovarian follicle counts were interpreted to be unrelated to treatment with ETU.

Table 5.8.1-42: Ovarian Follicle Counts (Cohort 1A Females – PND 90)

Dose (ppm)	**0**	**140**
OVARIES (Number examined)	26	26
Small (primordial) Follicles	75	92*
Growing Follicles	37	48*
Total (small and growing) Follicles	112	139*
Corpora Lutea	101	97

*Statistically different from control mean by Dunnett's test, alpha = 0.05

10. Gross Pathology and Histopathological Observations

There were no treatment related gross pathologic observations in males or females from any dose level. All gross pathologic observations were interpreted to be spontaneous alterations, unrelated to dietary administration of ETU.

Treatment-related histopathologic effects are summarized in Table 5.8.1-43. The thyroid gland was the primary target organ in Cohort 1A males administered ETU. Males from all dose groups had treatment-related increases in the incidence of diffuse follicular cell hypertrophy of the thyroid gland, relative to controls. The severity of the hypertrophy increased from very slight in 15/26 males given 2.8 ppm to slight in 22/26 males given 140 ppm. A generalized decrease in the amount of colloid accompanied the hypertrophy of the follicular epithelial cells. However, there were no adverse pathologic observations (i.e., degeneration, necrosis etc.) associated with follicular cell hypertrophy, suggesting that these hypertrophic alterations were adaptive rather than being pathologic and consistent with a more rapid colloid turnover. These thyroid alterations were considered treatment related but not adverse. Males given 28 or 140 ppm had treatment-related diffuse follicular cell hyperplasia of the thyroid gland. There was a dose-responsive increase in the severity of follicular cell hyperplasia, with most affected males at 28 ppm having very slight hyperplasia, and most affected males at 140 ppm having slight hyperplasia. The hyperplasia was characterized by papillary infolding and/or stratification of the follicular epithelial cells. Additional treatment-related thyroid effects consisted of focal slight nodular hyperplasia of follicular cells in one male given 140 ppm, and a follicular cell adenoma in another male given 140 ppm. Thus, follicular cell hyperplasia was considered treatment related and adverse at doses ≥ 28 ppm ETU.
Males from all dose levels had a treatment-related dose-responsive increase in the incidence of very slight or slight hypertrophy of individual cells in the pars distalis of the pituitary gland. The hypertrophic cells were scattered throughout the pars distalis, and had pale eosinophilic cytoplasm with round intracytoplasmic vacuoles of variable size. These hypertrophic cells were interpreted to represent thyrotropes that were responding to low serum thyroxine levels via the hypothalamic–pituitary–thyroid axis, with a resultant increase in the production of thyroid stimulating hormone by the pituitary gland. This hypertrophy in the pars distalis was considered an adaptive response to re-establish thyroid hormone homeostasis.
Treatment-related very slight atrophy of the lymphoid tissue of the thymus was present in 12/26 males given 140 ppm. The atrophy was characterized by generalized thinning of the thymic cortex in affected animals.

The thyroid gland was the primary target organ in Cohort 1A females administered ETU. Females given 140 ppm had treatment-related very slight or slight diffuse follicular cell hyperplasia of the thyroid gland, which was considered treatment related and adverse. Very slight diffuse follicular cell hypertrophy was present in 3/26 females given 28 ppm, and very slight or slight diffuse follicular cell hypertrophy was present in the majority of females given 140 ppm. The hyperplasia and hypertrophy of the thyroid follicular epithelial cells was of the same character as described above in males. As described above, there were no adverse pathologic observations (i.e., degeneration, necrosis etc.) associated with follicular cell hypertrophy, suggesting that these hypertrophic alterations were adaptive rather than being pathologic and consistent with a more rapid colloid turnover. These thyroid alterations were considered treatment related but not adverse.

Table 5.8.1-43: Incidence of selected histopathological lesions in Cohort 1A rats – PND90

Dose [mg/kg]	0	2.8	28	140	0	2.8	28	140
Sex	male				female			
Animals in group	26	26	26	26	26	26	26	26
Thyroid gland # examined	26	26	26	26	26	26	26	26
- hyperplasia, follicular cell, diffuse								
-very slight	0	0	**11**	**4**	0	0	0	**12**
-slight	0	0	**3**	**22**	0	0	0	**9**
- hyperplasia, nodular, follicular cell, focal								
-slight	0	0	0	**1**	0	0	0	0
- hypertrophy, follicular cell, diffuse								
-very slight	4	**15**	**12**	4	0	0	**3**	**12**
-slight	0	0	**3**	**22**	0	0	0	**9**
- adenoma, follicular cell, benign, primary	0	0	0	**1**	0	0	0	0
Pituitary # examined	26	26	26	26	26	26	26	26
- hypertrophy, pars distalis, individual cells								
-very slight	3	**9**	**9**	**6**	0	0	0	0
-slight	0	0	**6**	**13**	0	0	0	0

Bolded values interpreted to be treatment related

K. COHORT 1B – REPRODUCTIVE/ENDOCRINE

This cohort was used for further examinations concerning reproductive and endocrine toxicity. Animals were sacrificed on PND 120.

1. Clinical Observations

Cohort 1B males and females did not exhibit any treatment-related observations throughout the term of the study. Clinical observations recorded during the study were isolated, spontaneous occurrences common to this strain/age of rat and unrelated to exposure. One mid-dose male (animal #1031) died spontaneously on TD 94 due to a urinary tract obstruction (calculi). This animal was observed to have red perinasal soiling prior to death. Also, one high-dose male (animal #1048) was euthanized in moribund condition on TD 105. This animal exhibited the following clinical signs: red perinasal soiling labored respiration, blood in the cage and trauma to its nose and muzzle. This animal fractured its nasal bone and was euthanized due to animal welfare concerns. Similarly, one mid-dose female (animal #1139) from Cohort 1B died spontaneously due to an apparent bacterial infection of the kidneys. This animal had signs of dehydration, labored respiration, soft/limp muscles, decreased feces, and
decreased activity. None of these deaths were attributed to ETU treatment.

2. Body weights

On PND 21, Cohort 1B high-dose males had a 7% decrease in body weight compared to control animals, which was not statistically identified; however, on PND 28, the body weight decrease was 6% and statistically different (Figure 5.8.1-6 and Table 5.8.1-44). High-dose males had significantly decreased body weights throughout the study from PND 28 through PND 112. Body weight gains were significantly decreased in high-dose animals at all intervals from PND 42 through PND 112. From PND 112-118, there were no significant differences in body weight/body weight gains in the high-dose animals relative to controls; the Cohort 1B males were necropsied on PND 120. The effects on body weights/body weight gains in the 140 ppm Cohort 1B males were very similar to the effects seen in the Cohort 1A males at this same dose level. Thus, the effects on body weight/body weight gain in the 140 ppm Cohort 1B males were interpreted to be treatment related. There were no significant differences in body weights/body weight gains in Cohort 1B males given 28 ppm dietary ETU; however, males given 2.8 ppm ETU had significantly decreased body weights during most of the PND 28-63 interval. Body weight gains also were significantly decreased in the low-dose males during PND 21-42 and 21-49. Due to the lack of a dose-response relationship and the inability to reproduce these body weight effects in the Cohort 1A males, the body weight findings in the 2.8 ppm Cohort 1B males were considered incidental and unrelated to ETU treatment. Therefore, it was concluded that ETU did not alter body weights/body weight gains at doses ≤ 28 ppm.

Similar to the Cohort 1A females, there were significant decreases in body weights of high-dose Cohort 1B females at early time points during the postweaning period from PND 28-56; however, from PND 63-118 body weights in the high-dose females did not differ significantly from controls (Figure 5.8.1-6 and Table 5.8.1-44). High-dose Cohort 1B females weighed 9% less than controls on PND 21. This body weight differential was sustained until PND 35, which coincided with the only interval (PND 21-35) during which body weight gains were statistically decreased in the high-dose females. After PND 35, there was a continued diminution in this body weight differential as the study progressed. By PND 56, the last day that body weights were statistically identified in the high-dose females, body weights were decreased by 7% at 140 ppm. As with the Cohort 1A females, the high-dose Cohort 1B females had only a 2% decrease in body weight by PND 77 and had similar body weights to the control group through the end of the monitoring period (PND 118). Body weights were not significantly altered at ETU doses ≤ 28 ppm. With the exception of the PND 21-35 body weight gain value in the high-dose females, body weight gains did not differ at any interval throughout the monitoring period in the Cohort 1A females at any dose level.

Figure 5.8.1-6: Body weights cohort 1B F1

Males Cohort 1B F1

0 ppm
2.8 ppm
28 ppm
140 ppm

Body Weight [g]
0 100 200 300 400 500 600 700
21 28 35 42 49 56 63 70 77 84 91 98 105 112 118
days on test

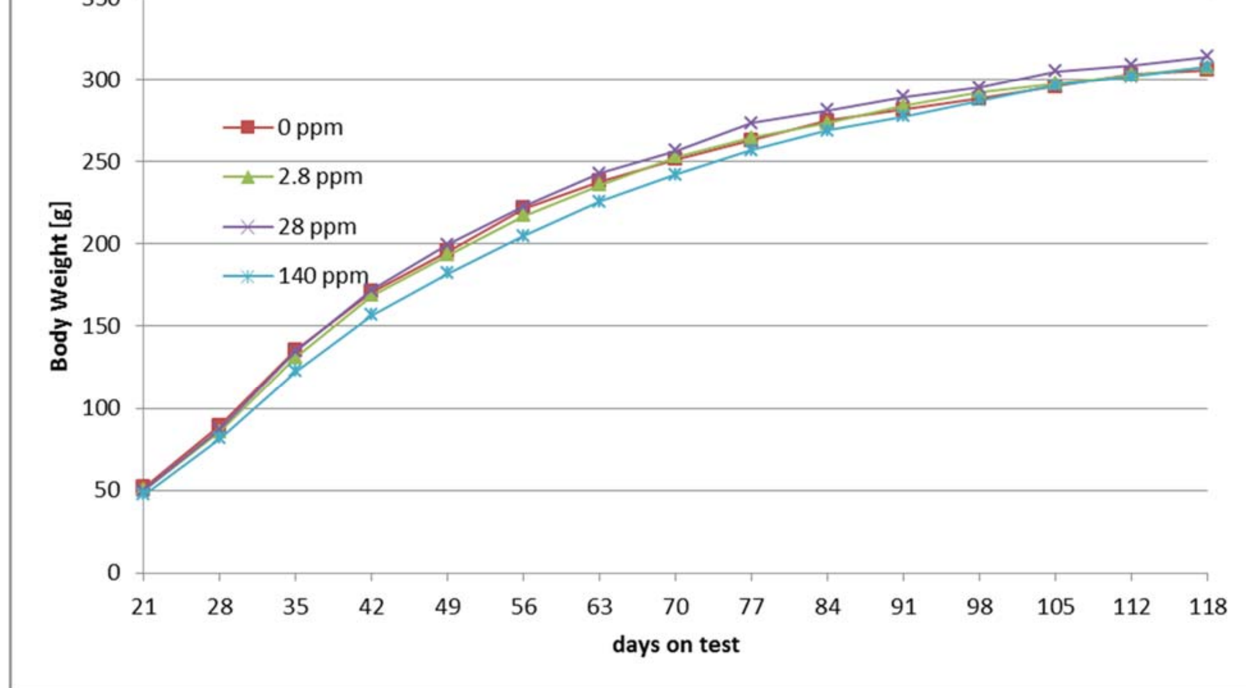


Table 5.8.1-44: Cohort 1B Body weights and body weight gains

Dose (ppm)	BW PND 21	BW PND 28	BW PND 35	BWG PND 21-35[a]	BW PND 49	BWG PND 21-49	BW PND 63	BWG PND 21-63	BW PND 84	BWG PND 21-84	BW PND 118	BWG PND 21-118
Males												
0	53.3	95.4	159.6	106.3	295.8	242.4	406.8	353.5	507.7	454.4	600.5	547.2
2.8	50.6	89.6*	149.9*	99.3	287.2*	227.5*	384.6*	334.0	481.0	430.4	577.2	526.5
% change	-5.1	-6.1	-6.1	-6.6	-6.0	-6.1	-5.5	-5.5	-5.3	-5.3	-3.9	-3.8
28	52.3	93.3	156.7	104.4	285.5	233.2	391.8	391.8	488.4	436.0	583.9	531.6
% change	-1.9	-2.2	-1.8	-1.8	-3.5	-3.8	-3.7	-3.7	-3.8	-4.0	-2.8	-2.9
140	**49.4**	**89.5***	**148.6***	**99.2**	**266.0***	**216.6***	**363.2***	**363.2***	**460.3***	**410.9***	**556.3**	**507.0**
% change	**-7.3**	**-6.2**	**-6.9**	**-6.7**	**-10.1**	**-10.6**	**-10.7**	**-10.7**	**-9.3**	**-9.6**	**-7.4**	**-7.3**
Females												
0	52.0	89.4	135.6	83.6	221.6	169.6	237.9	185.9	263.2	211.2	305.9	253.9
2.8	50.3	85.9	131.1	80.8	217.1	166.8	235.7	185.4	264.5	214.2	307.8	257.5
% change	-3.3	-3.9	-3.3	-3.3	-2.0	-1.7	-0.9	-0.3	+0.5	+1.4	+0.6	+1.4
28	50.1	87.4	135.1	85.1	222.7	172.7	243.1	193.0	273.5	223.5	314.1	264.1
% change	-3.7	-2.2	-0.4	+1.8	-0.5	+1.8	+2.2	+3.8	+3.9	+5.8	+2.7	+4.0
140	**47.5**	**81.6***	**122.8***	**75.2***	**205.1***	**157.5**	225.6	178.1	257.1	209.6	307.8	260.3
% change	**-8.7**	**-8.7**	**-9.4**	**-10.0**	**-7.4**	**-7.1**	-5.2	-4.2	-2.3	-0.8	+0.6	+2.5

BW = Body weight (g); BWG = Body weight gain (g)
[a] On PND 35, animals were given full concentrations of ETU in the diet. From PND 21-35, they received one-half normal dietary concentrations (1.4, 14, 70 ppm)
* Statistically different from control mean by Dunnett's test
Bolded values interpreted to be treatment related.

3. Food consumption

Feed consumption was not measured from PND 21-28 due to group housing.
In high-dose Cohort 1B males, feed consumption was decreased significantly from PND 35-84 and PND 98-118, which coincides with the periods of decreased body weights and body weight gains in these animals (PND 28-112) (Table 5.8.1-45). Feed consumption was not significantly altered in Cohort 1B males given 28 ppm ETU, a dose level that did not affect body weights and body weight gains. At 2.8 ppm, significant decreases in feed consumption were observed on PND 35-56 and PND 77-84. The first decrease in feed consumption corresponds with significant decreases in body weight/body weight gains in low-dose males, whereas the second interval was without effect. The body weight and feed consumption findings in the low-dose males were considered incidental due to the lack of a dose-response relationship and the lack of reproducibility in the low dose Cohort 1A males.

There were significant treatment-related decreases in Cohort 1B female feed consumption during the early post-weaning intervals on PND 28-56 (Table 5.8.1-45). These decreases in feed consumption were consistent with the decreases in body weight/body weight gain seen in the high-dose Cohort 1B females from PND 21 to PND 56. Feed consumption in high-dose females was similar to or greater than the control group for intervals after PND 77, when the body weight decrement between these groups disappeared. Feed consumption was not significantly altered in Cohort 1B females given ≤ 28 ppm ETU at any time interval.

Table 5.8.1-45: Cohort 1B Food consumption (g/day)

Dose (ppm)	PND 35[a]-42	PND 42-49	PND 56-63	PND 77-84	PND 98-105	PND 112-118
Males						
0	24,2	27,1	29,8	30,4	29,1	30,1
2.8	22,4*	25,2*	28,0	27,1*	28,1	28,4
% change	-7,4	-7,0	-6,0	-10,9	-3,4	-5,6
28	23,3	26,0	28,9	28,8	28,7	29,6
% change	-3,7	-4,1	-3,0	-5,3	-1,4	-1,7
140	**21,9***	**23,6***	**26,0***	**26,7***	**26,9***	**26,7***
% change	**-9,5**	**-12,9**	**-12,8**	**-12,2**	**-7,6**	**-11,3**
Females						
0	15,1	17,8	18,1	18,9	18,8	18,2
2.8	14,4	17,3	17,5	17,9	18,6	18,2
% change	-4,6	-2,8	-3,3	-5,3	-1,1	0
28	15,2	18,1	18,3	18,7	19,0	18,9
% change	+0,7	+1,7	+1,1	-1,1	+1,1	+3,8
140	**13,9***	**16,4***	**17,1**	**17,4***	18,8	19,1
% change	**-7,9**	**-7,9**	**-5,5**	**-7,9**	0	+4,9

[a] On PND 35, animals were given full concentrations of ETU in the diet. From PND 21-35, they received one-half normal dietary concentrations (1.4, 14, 70 ppm)
* Statistically different from control mean by Dunnett's test
Bolded values interpreted to be treatment related.

Test material intake (time weighted average) in Cohort 1B males was 0.223 mg/kg/day at 2.8 ppm, 2.15 mg/kg/day at 28 ppm and 10.5 mg/kg/day at 140 ppm. As with the Cohort 1A animals, the highest intakes of test material in males were recorded for PND 35-42, just after they received their full dietary concentrations and ranged from 0.361 mg/kg/day at 2.8 ppm, 3.44 mg/kg/day at 28 ppm, and 17.0 mg/kg/day at 140 ppm. Test material intake (time weighted average) in Cohort 1B females was 0.233 mg/kg/day at 2.8 ppm, 2.25 mg/kg/day at 28 ppm, and 11.5 mg/kg/day at 140 ppm. The highest intakes of test material in females were recorded for PND 35-42, ranging from 0.339 mg/kg/day at 2.8 ppm, 3.30 mg/kg/day at 28 ppm, and 16.4 mg/kg/day at 140 ppm.

With dietary concentration adjustments prior to PND 35, Cohort 1B animals received doses of ETU (mg/kg body weight) that ranged from approximately equal to 8% higher than the doses received by pre-mating (male and female) or gestational adults. The Cohort 1B animals' test material intake was closer to the P1 adults, because these animals were kept on study for a longer period (PND 120) than the Cohort 1A animals (PND 90); this longer study duration allowed a greater period at adult feed consumption levels to offset the increased test material intake seen during growth periods prior to PND 70.

4. Organ Weights

Alterations in final body weights and organ weights are summarized in Table 5.8.1-46. Males given 140 ppm had a 7.5% lower final body weight (not statistically significant), relative to controls, that was interpreted to be treatment related.

Absolute testes weights were significantly lower in males given 2.8 ppm ETU; however, there was no dose-response relationship for this effect. Furthermore, there were no effects on reproductive parameters in the P1 or Cohort 1A males, including no effects on male reproductive indices, testes, or accessory sex tissue weights, sperm motility, sperm morphology and sperm counts, and no corresponding histopathological changes in either the testes or epididymides in P1, Cohort 1A, or 1B males. This finding was judged to be incidental. Males given 140 ppm had a statistically significant lower absolute epididymides weight. Relative weights of the epididymides were similar to controls across all ETU dose levels in the Cohort 1B males. The absolute epididymal weights

also were significantly decreased in Cohort 1A males in the 28 and 140 ppm groups (relative weights not statistically different), but neither absolute nor relative epididymal weights were altered in the P1 males. A feed restriction study by Rehm et al. (2008, as cited in the study report [DocID 2013/7002198]) has shown decreases in absolute epididymal weights with sustained effects on body weight. There were no effects on other reproductive parameters in the P1 or Cohort 1A males, including no effects on male reproductive indices, testes or accessory sex tissue weights, sperm motility, sperm morphology and sperm counts (all collected from the epididymis), and no corresponding histopathological changes in either the testes or epididymides in P1, Cohort 1A, or 1B males. Thus, changes in absolute epididymal weights were judged to be not toxicologically significant.
Males given 28 or 140 ppm had treatment-related statistically significant lower absolute and relative thymus weights, and males given 140 ppm had treatment related statistically significant higher absolute and relative thyroid weights. Males given 2.8 ppm had statistically significant lower absolute and relative thyroid weights. This alteration in low-dose thyroid weights was also noted in the Cohort 1A (PND 90) males, which was possibly related to the decreased amount of colloid that accompanied the very slight follicular cell hypertrophy of the
thyroid in Cohort 1A males given 2.8 ppm.

Alterations in Cohort 1B organ weights are summarized in Table 5.8.1-46. There were no treatment-related effects on final body weights of Cohort 1B females at any dose level. Females given 28 or 140 ppm had treatment-related statistically significant lower absolute and relative thymus weights, and females given 140 ppm had treatment-related statistically significant higher absolute and relative thyroid weights.

Table 5.8.1-46: Final Body Weights and Selected Organ Weights – Cohort 1B

Sex	Males						
Dose (ppm)	0	2.8	% Change	28	% Change	140	% Change
Final Body Weight (g)	570.0	546.8	-4.1	554.8	-2.7	**527.4**	**-7.5**
Absolute Thymus (g)	0.341	0.315	-7.6	**0.256$**	**-24.9**	**0.211$**	**-38.1**
Relative Thymus (g/100 g bw)	0.060	0.058	-3.3	**0.046***	**-23.3**	**0.040***	**-33.3**
Absolute Thyroid (g)	0.0238	0.0199$	-16.4	0.0219	-8.0	**0.0354$**	**+48.7**
Relative Thyroid (g/100 g bw)	0.0042	0.0038$	-11.9	0.0040	-4.8	**0.0067$**	**+59.5**
Absolute Epididymis (g)	1.475	1.393	-5.6	1.381	-6.4	*1.323**	*-10.3*
Relative Epididymis (g/100 g bw)	0.260	0.257	-1.2	0.250	-3.8	0.252	-3.1
Absolute Testes (g)	3.882	3.570$	-8.0	3.586	-7.6	*3.663*	*-5.6*
Relative Testes (g/100 g bw)	0.686	0.658	-4.1	0.652	-5.0	0.698	+1.7
Sex	**Females**						
Final Body Weight (g)	288.5	290.7	+0.8	295.0	+2.3	290.0	+0.5
Absolute Thymus (g)	0.264	0.260	-1.5	**0.212$**	**-19.7**	**0.200$**	**-24.2**
Relative Thymus (g/100 g bw)	0.091	0.090	-1.1	**0.072$**	**-20.9**	**0.070$**	**-23.1**
Absolute Thyroid (g)	0.0162	0.0149	-8.0	0.0156	-3.7	**0.0222$**	**+37.0**
Relative Thyroid (g/100 g bw)	0.0056	0.0051	-8.9	0.0053	-5.4	**0.0077$**	**+37.5**

*Statistically different from control mean by Dunnett's test, alpha = 0.05.
$Statistically different from control mean by Wilcoxon's test, alpha = 0.05.
Bold type indicates the effects were interpreted to be treatment related.
Italics type indicates the values were interpreted to be secondary to lower final body weights.

5. Gross Pathology

There were no treatment-related gross pathologic observations in males or females from any dose level. All gross pathologic observations were interpreted to be spontaneous alterations, unrelated to dietary administration of ETU.

L. COHORT 2A – DEVELOPTMENTAL NEUROTOXICITY

This cohort was used for further examinations concerning developmental neurotoxicity. Animals were sacrificed on PND 78.

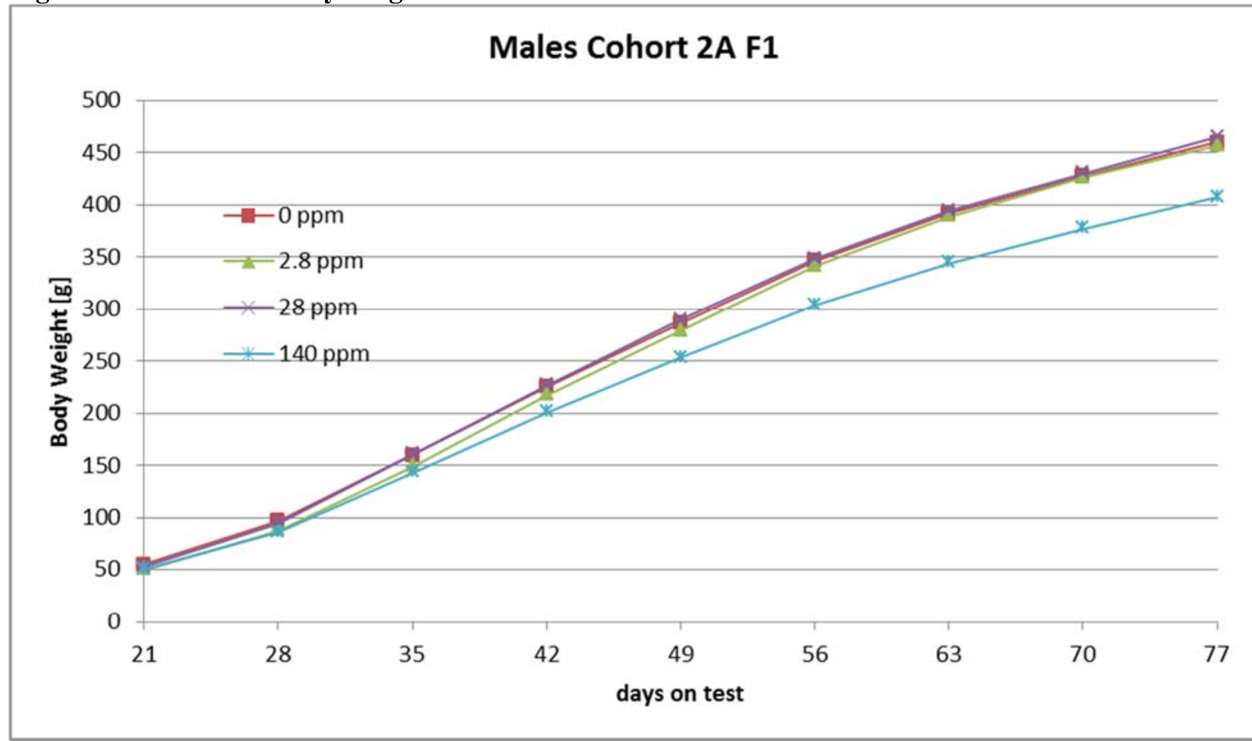
1. Clinical Observations

Cohort 2A males and females did not exhibit any treatment-related observations throughout the term of the study. Clinical observations recorded during the study were isolated, spontaneous occurrences common to this strain/age of rat and unrelated to exposure. One high-dose male (animal #689) had undescended testis (i.e., testicle not in scrotum); this animal was identified as a hermaphrodite at necropsy. This finding was a single occurrence across all F1 offspring and judged to be incidental and unrelated to ETU treatment.
There were no effects of ETU exposure on Cohort 2A male or female offspring survival. All Cohort 2A offspring survived to scheduled necropsy.

2. Body Weights

High-dose Cohort 2A males had a significant decrease (9%) in body weight on PND 21. By PND 28, the decrease in body weight in high-dose males reached 12% relative to control animals (Table 5.8.1-47). The decreases in body weight (11-13%) in these high-dose males were sustained throughout the monitoring period (PND 21-77) and achieved statistical significance at all time points from PND 49-77. Body weight gains were significantly decreased in high-dose animals during two intervals from PND 21-56 and PND 21-63, although body weight gains were decreased by a similar magnitude (12% or more) throughout all study intervals. The effects on body weights/body weight gains in the 140 ppm Cohort 2A males were similar to the effects seen in the Cohort 1A and 1B males at this same dose level. Thus, the effects on body weight/body weight gain in the 140 ppm Cohort 2A males were interpreted to be treatment related. There were no significant differences in body weights/body weight gains in Cohort 2A males given ≤ 28 ppm dietary ETU.
There were no statistically significant decreases in body weights of high-dose Cohort 2A females, although body weights were decreased by 8-10% during the PND 21-35 interval (Table 5.8.1-47). The body weight decrement in the high dose females lessened during the subsequent monitoring periods, reaching a 2% difference in body weight on PND 77. Body weight gains also were most affected in the early post-weaning period, but did not achieve statistical significance. The body weight effects in Cohort 2A high-dose females showed a similar temporal relationship to the Cohort 1A and 1B females in that the most marked effects on body weight occurred early in the post-weaning and diminished as the study progressed. Body weights and body weight gains were not affected at ETU doses ≤ 28 ppm.

Figure 5.8.1-7: Body weights Cohort 2A F1



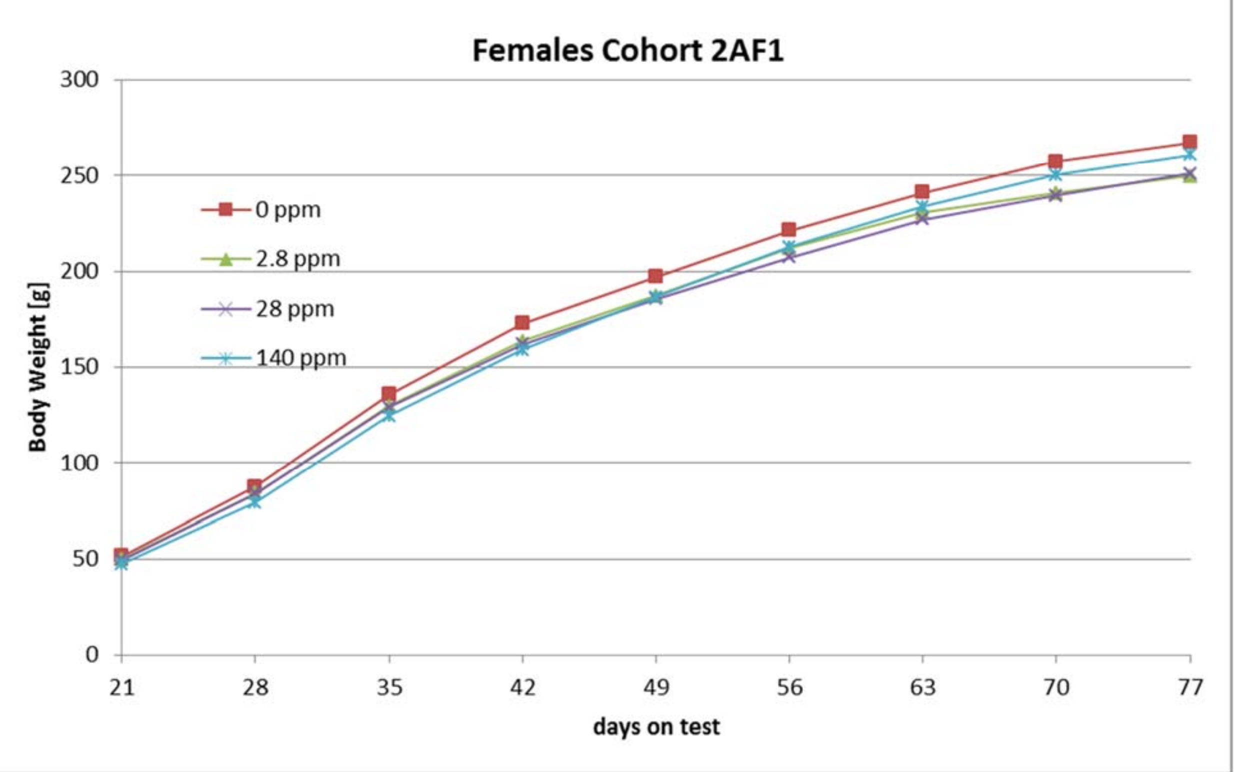


Table 5.8.1-47: Cohort 2A Body weights and body weight gains

Dose (ppm)	BW PND 21	BW PND 28	BWG PND 21-28	BW PND 35[a]	BW PND 49	BWG PND 21-49	BW PND 63	BWG PND 21-63	BW PND 77	BWG PND 21-77
0	55.1	97.1	42.0	160.0	286.8	231.7	392.1	337.0	460.3	405,2
2.8	50.0	87.8	37.8	149.0	279.0	229.0	388.7	338.7	456.5	406,6
% change	-9.3	-9.6	-10.0	-6.9	-2.7	-1.2	-0.9	+0.5	-0.8	+0,3
28	52.9	94.6	41.7	160.4	289.7	236.8	394.4	341.6	464.8	411,9
% change	-4.0	-2.6	-0.7	-0.3	+1.0	+2.2	+0.6	+1.4	+1.0	+1,7
140	**50.3[$]**	**85.8***	**35.5**	**142.8**	**253.1***	**202.8**	**344.3***	**294.0***	**407.4***	**357,1**
% change	**-8.7**	**-11.6**	**-15.5**	**-10.8**	**-11.8**	**-12.5**	**-12.2**	**-12.8**	**-11.5**	**-11,9**
Females										
Dose (ppm)	**BW PND 21**	**BW PND 28**	**BWG PND 21-28**	**BW PND 35[a]**	**BWG PND 21-35**	**BW PND 49**	**BWG PND 21-49**	**BW PND 77**	**BWG PND 21-77**	
0	51.5	87.6	36.0	135.6	84.0	197.1	145.5	267.2	215.6	
2.8	49.85	84.1	34.3	129.6	79.9	187.3	137.6	249.3	199.5	
% change	-3.3	-4.0	-4.7	-4.4	-4.9	-5.0	-5.4	-6.7	-7.5	
28	49.5	84.1	34.6	129.2	79.7	185.4	135.8	251.0	201.4	
% change	-3.9	-4.0	-3.9	-4.7	-5.1	-5.9	-6.7	-6.1	-6.6	
140	47.4	79.1	33.2	124.5	78.8	186.4	140.4	260.9	213.3	
% change	-8.0	-9.7	-7.8	-8.2	-6.2	-5.4	-3.5	-2.4	-1.1	

BW = Body weight (g); BWG = Body weight gain (g)
[a] On PND 35, animals were given full concentrations of ETU in the diet. From PND 21-35, they received one-half normal dietary concentrations (1.4, 14, 70 ppm)
* Statistically different from control mean by Dunnett's test
$Statistically different from control mean by Wilcoxon's test, alpha = 0.05.
Bolded values interpreted to be treatment related.

3. Food consumption

In high-dose Cohort 2A males, feed consumption was decreased significantly from PND 42-70, which coincided with the periods of decreased body weights and body weight gains in these animals (PND 49-77) (Table 5.8.1-48). Feed consumption was not significantly altered in Cohort 2A males given ≤ 28 ppm ETU, dose levels that did not affect body weights and body weight gains.

There were no treatment-related effects on feed consumption in the Cohort 2A females. Feed consumption was not significantly altered at any time interval or any dose level of ETU.

Table 5.8.1-48: Cohort 2A Male Food consumption (g/day)

Dose (ppm)	PND 35[a]-42	PND 42-49	PND 49-56	PND 56-63	PND 63-70	PND 70-77
0	23.3	25.6	28.0	28.7	27.9	28.7
2.8	22.8	25.9	28.0	28.7	27.9	28.1
% change	-2.1	+1.2	0	0	0	-2.1
28	23.6	26.3	28.2	29.1	29.0	29.5
% change	+1.3	+2.7	+0.7	+1.4	+3.9	+2.8
140	21.0	22.4*	23.7*	24.9*	24.9*	25.9
% change	-9.9	-12.5	-15.4	-13.2	-10.8	-9.8

[a] On PND 35, animals were given full concentrations of ETU in the diet. From PND 21-35, they received one-half normal dietary concentrations (1.4, 14, 70 ppm)
* Statistically different from control mean by Dunnett's test

Test material intake (time weighted average) in Cohort 2A males was 0.252 mg/kg/day at 2.8 ppm, 2.39 mg/kg/day at 28 ppm, and 11.7 mg/kg/day at 140 ppm. As with the Cohort 1A animals, the highest intakes of test material in males were recorded for PND 35-42, just after they received their full dietary concentrations and ranged from 0.366 mg/kg/day at 2.8 ppm, 3.42 mg/kg/day at 28 ppm, and 17.1 mg/kg/day at 140 ppm. Test material intake (time weighted average) in Cohort 2A females was 0.255 mg/kg/day at 2.8 ppm, 2.47 mg/kg/day at 28 ppm, and 12.3 mg/kg/day at 140 ppm. The highest intakes of test material in females were recorded for PND 35-42, ranging from 0.349 mg/kg/day at 2.8 ppm, 3.25 mg/kg/day at 28 ppm, and 16.7 mg/kg/day at 140 ppm. Despite dietary concentration adjustments prior to PND 35, young animals received 18-25% higher doses of ETU (mg/kg body weight) than pre-mating (male and female) or gestational adults. The Cohort 2A animals' test material intake differed from the P1 adults by a greater margin than the Cohort 1B animals, because the shorter study period (out to PND 78) did not allowed sufficient time for adult feed consumption patterns to offset the increased test material intake seen during growth periods prior to PND 70.

4. Acoustic Startle Response

There were no treatment-related effects on ASR in male or female Cohort 2A rats (p = 0.7907). The Treatment x Sex interaction was not significant (p = 0.3047), indicating that there was no statistically significant difference in ASR between males and females with treatment. This allowed the male and female data to be analyzed together to increase statistical power. The interaction of Exposure x Block was not statistically significant (p = 0.7663), indicating that the habituation of the ASR was not affected by exposure.

5. Functional Observational Battery (FOB)

Body Weights

There was a significant Treatment x Sex interaction (p = 0.0344), indicating that there was a sex difference in FOB body weight response with treatment; therefore, males and females were analyzed separately. High-dose males had a significant decrease in FOB body weights (12%) compared to control males (p = 0.0221), whereas male FOB body weights were not significantly altered at lower doses of ETU. FOB body weights were not significantly different in Cohort 2A females at any dose level (p = 0.4819).

Hand-Held and Open Field Observations

There were no significant differences across treatment groups in the ETU study. None of the average ranks differed from the controls by ≥ 0.5 or achieved statistical significance. Thus, there were no treatment-related changes in FOB observations in Cohort 2A males and females.

Rectal Temperature

The Treatment x Sex interaction was not significant (p = 0.1751), indicating that there was no statistically significant difference in rectal temperature between males and females with treatment, which allowed both sexes to be analyzed together. There were no treatment-related effects on rectal temperature in male or female Cohort 2A rats (p = 0.1706).

Grip Performance
The Treatment x Sex interaction was not significant for either hindlimb (p = 0.7140) or forelimb (p = 0.3774) grip performance, indicating that there was no statistically significant difference in grip performance between males and females with treatment, which allowed both sexes to be analyzed together. There were no treatment-related effects on hindlimb grip performance (p = 0.6743) or forelimb grip performance (p = 0.7195) in male or female Cohort 2A rats.

Landing Foot Splay
The Treatment x Sex interaction was not significant (p = 0.8823), indicating that there was no statistically significant difference in landing foot splay between males and females with treatment, which allowed both sexes to be analyzed together. There were no treatment-related effects on landing foot splay in male or female Cohort 2A rats (p = 0.0981).

Motor Activity
Generally, motor activity measurements in adult CD rats are monitored for 64 minutes (8 x 8-minute epochs) based on the time for activity to reach an asymptote in this laboratory using this rat strain. In one monitoring session during the ETU study, the motor activity system failed to record epochs 7 and 8; thus, these data are missing for 2-4 rats/sex/dose. To manage this issue, data for motor activity are presented twice: The first presentation is with all Cohort 2A animals evaluated for 6 epochs (n = 96 animals monitored for 48 minutes), whereas the second data presentation is a subset of Cohort 2A animals (8-10 rats/sex/dose) on which motor activity data were collected for all 8 epochs (i.e., n = 75 rats monitored for 64 minute monitoring period). Typically, there is very little motor activity information in the last two epochs; therefore, this failure in data collection is unlikely to have affected data interpretation.

There were no treatment-related effects on motor activity. The Treatment × Sex interaction was not significant when all animals were analyzed across 6 epochs (p = 0.7095) or when a subset of animals was analyzed across 8 epochs (p = 0.4331), i.e., there was no statistically significant difference in motor activity across sexes with treatment. Furthermore, the first analysis (all animals/6 epochs) also revealed no statistically significant treatment interaction (p = 0.1492), i.e., with male and female data considered together, treatment did not affect motor activity. In the second analysis (subset of animals/8 epochs), there was a statistically significant treatment effect on total motor activity (p = 0.0449). The results of the subsequent linear contrasts across both sexes were as follows: Control vs. 2.8 ppm (p = 0.0634), Control vs. 28 ppm (p = 0.3891), and Control vs. 140 ppm (p = 0.8758). None of these comparisons with the control group were significant at alpha = 0.02, which indicated that none of the treated groups were significantly different from the control group. The Treatment × Epoch interaction was not statistically significant in either the first analysis (all animals/6 epochs) (p = 0.5831) or the second analysis (subset of animals/8 epochs) (p = 0.9375), which indicated that the distribution of motor activity counts within each session was not significantly affected by treatment. Together, these data indicate that ETU had no significant effect on motor activity in either males or females at any dose level tested.

6. Brain Weight and Gross Brain Measurements

There were decreases in absolute brain weights in both the Cohort 2A high-dose males and high-dose females on PND 78 (Table 5.8.1-49). High-dose Cohort 2A males had a 12% decrease in terminal body weights and a corresponding 6% decrease in absolute brain weights, but this brain weight difference was not statistically identified. High-dose Cohort 2A females had a minor change in terminal body weight (3%), but a significant decrease in absolute brain weight (7%). There also was a significant decrease in absolute brain weight (6%) in the low-dose females with a 7% decrease in body weight; however, neither the terminal body weight nor the absolute brain weight exhibited a dose-response relationship.

Table 5.8.1-49: Brain weights – Cohort 2A

Dose (ppm)	Males		Females	
	Body Weight (g)	Brain Weight (g)	Body Weight (g)	Brain Weight (g)
0	430.2	2.491	251.5	2.385
2.8	427.6	2.397	234.2	2.243*
28	437.2	2.491	234.0	2.255
140	380.0	**2.352**	243.0	**2.227***

* Statistically different from control mean by Dunnett's test, alpha = 0.05.
Bolded values interpreted to be treatment related.

Given the significant decrease in absolute brain weights in both males and females, a MANOVA was conducted on macroscopic measurements. This analysis was designed to examine whether there was an overall change in brain size rather than an effect on a specific gross brain measurement. When the macroscopic brain measurements (Table 5.8.1-50) were analyzed by MANOVA, there was no significant difference in the Treatment x Sex interaction term; therefore, male and female data were analyzed together. This analysis revealed a significant treatment effect (p = 0.0006), which was localized to the high-dose group (p = 0.0001) based on linear contrasts.

The significant changes in brain weight and gross brain measurements at 140 ppm suggested an overall decrease in brain size. To evaluate this hypothesis further, linear microscopic measurements were examined by MANOVA (see below in Brain Morphometry section). The brain weight and gross measurements were not statistically identified in the low- or mid-dose ETU groups; therefore, the microscopic measurements in these groups were analyzed to look for localized effects on linear microscopic measurements.

Table 5.8.1-50: Gross Brain Measurements – Cohort 2A

Dose (ppm)	**Males**				**Females**			
	Cerebrum Length (mm)	Cerebrum Width (mm)	Cerebellum Length (mm)	Cerbellum Width (mm)	Cerebrum Length (mm)	Cerebrum Width (mm)	Cerebellum Length (mm)	Cerbellum Width (mm)
0	16.59	16.94	7.27	12.80	16.08	16.55	7.48	12.63
2.8	16.49	16.69	7.24	12.61	15.75	16.17	7.26	12.34
28	16.72	16.87	7.37	12.75	15.92	16.04	7.42	12.27
140	**16.42**	**16.45**	**7.13**	**12.26**	**15.69**	**16.02**	**7.33**	**12.09**

*Significantly different by MANOVA analysis and linear contrasts, alpha = 0.05.
Bolded values interpreted to be treatment related.

7. Gross Pathology Observations

There were no treatment-related gross observations attributed to ETU in perfused F1 Cohort 2A male or female offspring. One high-dose male (animal #689) was identified as a hermaphrodite at necropsy as both male and female reproductive organs were present in this animal. This finding was a single occurrence across all F1 offspring and judged to be incidental; therefore, all gross observations were interpreted to be spontaneous alterations, unrelated to dietary administration of ETU.

8. Neuropathology Observations

Males given 28 or 140 ppm had treatment-related dose-dependent increases in the incidence and severity of hypertrophy of individual cells in the pars distalis of the pituitary gland (Table 5.8.1-51). The hypertrophic cells were scattered throughout the pars distalis, and had pale eosinophilic cytoplasm with round intracytoplasmic vacuoles of variable size. These hypertrophic cells were interpreted to represent thyrotropes that were responding to low serum thyroxine levels via the hypothalamic-pituitary-thyroid axis, with a resultant increase in the production of thyroid stimulating hormone by the pituitary gland. This hypertrophy in the pars distalis was considered an adaptive response to re-establish thyroid hormone homeostasis and also was observed in the Cohort 1A offspring at all dose levels.

There were no treatment-related histopathologic effects on the central or peripheral nervous system in males given 2.8 ppm or females at any dose level. All other histopathologic observations were interpreted to be spontaneous alterations, unassociated with dietary administration of ETU.

Table 5.8.1-51: Histopathologic Pituitary Effects – Cohort 2A Males

Sex	**Males**			
Dose (ppm)	**0**	**2.8**	**28**	**140**
Pitutitary Gland (Number Examined)	12	12	12	12
Hypertrophy, pars distalis, individual cells				
-very slight	2	3	**6**	**4**
-slight	0	0	**1**	**6**

Bolded values interpreted to be treatment related.

Brain Morphometrics

As part of the morphometric assessment, 3 structures in the cerebral cortex, 4 structures in the thalamus and hippocampus and 3 structures in the cerebellum were measured. As discussed above, the significant changes in brain weight and gross brain measurements at 140 ppm suggested an overall decrease in brain size. To evaluate this hypothesis further, linear microscopic measurements were examined in the control and high-dose group by MANOVA (linear microscopic measurements were originally collected in the control and high-dose group). The analysis confirmed a significant difference across the linear microscopic measurements between the controls and the high-dose males and females. Thus, it was concluded that the high-dose level of ETU did not target a specific portion of the brain during neurodevelopment, but rather, decreased overall brain size (weight, gross, and microscopic measures).

Overall brain size was decreased in the high-dose animals, but neither brain weight nor brain gross measurements were significantly altered at ≤ 28 ppm ETU. Therefore, linear microscopic measurements were collected from the mid- and low dose ETU groups to examine whether a linear measurement in a specific area of the brain could be affected with ETU treatment. For these analyses, male and female data were analyzed separately by ANOVA. These analyses indicated that some linear microscopic measurements for brain morphometry were statistically significant (Table 5.8.1-52 shows the brain regions identified as having statistical differences via ANOVA). In males, there were five of the brain layers that had no significant differences (i.e., basal nuclei width, frontal cerebral cortex, hippocampus width, cerebellum height and cerebellar lobule thickness). In females, there were eight brain layers that had no significant differences (i.e., basal nuclei width, frontal cerebral cortex, hippocampus height and width, thalamus height and width, cerebellum width, and cerebellar lobule thickness).

The first point of note with the linear microscopic measurements is that the differences between measurements within a given brain region are very small. In this data set, there are two values of the 21 comparisons where the differences were > 10%, whereas eighteen of the 21 comparisons had differences of ≤ 6%. These small differences are within the inherent variance seen with brain morphometry measurements. Secondly, the linear microscopic measurements did not follow a dose-response relationship (Table 5.8.1-52). For example, in the parietal region of the cerebral cortex in males, there was a slight increase in layer thickness at 2.8 ppm (2.2%), a similar increase (0.7%) with a 10-fold higher dose of ETU (28 ppm), then a 5.4% decrease with 5-fold higher dose level (140 ppm). The parietal region of the cerebral cortex in females showed a 4.0% decrease at 2.8 ppm, a 5.5% decrease at 28 ppm, and a 5.2% decrease at 140 ppm, which also does not support a dose-response relationship. Furthermore, males and females did not respond similarly across brain regions. For example, in males, the thickness of the parietal region of the cerebral cortex increased slightly at 2.8 and 28 ppm, whereas in females, this layer decreased at these dose levels. Both males and females showed a decrease in this brain layer at 140 ppm, which was consistent with the overall decrease in brain size that was identified at the high-dose level. For many brain regions, significance was only noted in one sex, despite the lack of gender-related differences in response to ETU in previous toxicity studies.

Together, the small magnitude of these changes, the lack of dose-response relationships, and the lack of consistent responses between males and females indicate that these small changes in brain morphometry are incidental and not treatment related. Interestingly, not all linear microscopic measurements were statistically identified in high-dose samples. The individual ANOVAs for linear brain measurements were not able to detect statistically significant differences in the linear measurements that contributed to an overall decrease in brain size in the high-dose group. Brain weight is a three dimensional measurement, which best describes brain volume; it measures a more global parameter than two-dimensional linear microscopic measurements. The subsequent MANOVA analyses of gross brain measurements and linear microscopic measurements pooled data across multiple endpoints to evaluate the overall trend; hence, the conclusion about overall brain size. However, the individual linear measurements, analyzed by ANOVA, are designed to evaluate whether a particular length or width within the brain has been affected by treatment. Thus, the ANOVA analyses examine each individual measurement for an effect on a specific brain region/layer. The effects on each individual measurement were not sufficient to achieve statistical significance, whereas the overall assessment of brain size detected a treatment-related change.

Table 5.8.1-52: Brain linear measurements – Cohort 2A

Male linear brain measurement[1]	Dose group (ppm)							
	0	HCD	2.8	% change	28	% change	140	% change
Parietal cerebral cortex	1,7700	1,66	1,8089	+2,2	1,7820	+0,7	1,6744	-5,4
Range of values	1.60169-1.86970	1.47602-1.8584	1.64493-2.04725	NA	1.56387-1.91554	NA	1.55884-1.77448	NA
Hippocampus height	1,9125	1,78	2,0348	+6,4	2,1665	+13,3	2,0063	+4,9
Range of values	1.54430-2.11392	1.61541-1.92949	1.67089-2.39241	NA	1.93038-2.44937	NA	1.91139-2.10759	NA
Thalamus Width	7,8550	8,13	8,2241	+4,7	8,2962	+5,6	8,0127	+2,0
Range of values	7.03802-8.50633	7.87284-8.64104	7.50633-8.68354	NA	7.77216-8.63291	NA	7.59494-8.35443	NA
Thalamus height	5,0575	5,21	5,4897	+8,5	5,5684	+10,1	5,1975	+2,8
Range of values	4.69620-5.49367	4.89745-5.41032	4.86076-6.54272	NA	4.92407-6.00000	NA	4.58230-5.83544	NA
Cerebellar width	7,1400	6,40	7,0689	-1,0	7,400	+3,6	6,8756	-3,7
Range of values	6.74667-7.52001	6.01940-6.98709	6.06668-7.66668	NA	7.00001-7.94668	NA	6.26667-7.40000	NA
Female linear brain measurement[1]	**Dose Group (ppm)**							
	0	HCD	2.8	% change	28	% change	140	% change
Parietal cerebral cortex	1,8437	1,67	1,7708	-4,0	1,7420	-5,5	1,7473	-5,2
Range of values	1.73067-2.01618	1.48115-1.92735	1.72866-1.93737	NA	1.66477-1.80722	NA	1.64798-1.85760	NA
Cereballar height	6,1511	5,71	5,8745	-4,5	6,1625	+0,2	5,9389	-3,4
Range of values	5.720002-6.56000	5.48633-6.05808	5.28000-6.48000	NA	5.98667-6.34672	NA	5.41340-6.28000	NA

HCD = Historical control data.
[1]Brain regions identified as having significant differences in measurements based on ANOVA with Benjamini-Hochberg analysis to limit false discovery rate.
Shaded cells show maximum percentage change relative to the control group.
NA = not applicable
n = 10-12/sex/dose

M. COHORT 2B – NEUROPATHOLOGY

This cohort was used for further neuropathological examinations. Weanlings were sacrificed on PND 22.

1. Brain and Brain/Body Weights Summary

There were no statistically significant effects on final body weights in the Cohort 2B weanlings, although body weights were decreased by 8% in high-dose males and females. There were no treatment-related or statistically significant effects on brain weights of Cohort 2B PND 22 male or female weanlings at any dose level.

2. Gross Pathology and Histopathology

There were no gross observations attributed to ETU exposure in either male or female Cohort 2B PND 22 weanlings.
There were no histopathologic observations attributed to ETU exposure in either male or female Cohort 2B PND 22 weanlings. This evaluation, which focused on brain histopathology, did not indicate any structural changes in weanling brains with ETU exposures at any dose level.

N. FINAL ASSESSMENT OF FOUND EFFECTS

1. Liver

Liver function (clinical pathology), weight and histopathology were examined at multiple life stages. The high-dose P1 females had a significant increase in cholesterol concentration, which was considered treatment related. The high-dose males had a 20% increase in cholesterol, but this increase was not statistically identified. In F1 offspring, high-dose Cohort 1A males and females had significant increases in cholesterol levels. Significant increases in relative liver weight were observed at 140 ppm in P1 females, and Cohort 1A males and females. Relative liver weights were not significantly increased in P1 males and absolute liver weights were not significantly increased in any of these groups. P1 males given 190 ppm had an increase in the incidence of slight multifocal vacuolization (consistent with fatty change) of individual hepatocytes. The increase in fatty change of the liver may have been reflective of an alteration in lipid metabolism associated with hypothyroidism in high-dose males. There were no effects on liver histopathology in any of the other high-dose groups (P1 females, Cohort 1A males or females). There were no effects on liver parameters at doses ≤ 28/38 ppm. Together, these data suggest that ETU altered liver parameters at 140/190 ppm; however, it is possible that this result was indirect due to hypothyroid-mediated effects on lipid metabolism. Effects in the liver, including increased hepatic triglycerides, have been reported in previous studies with ETU (e.g., Ugazio et al., 1985, as cited in the study report [DocID 2013/7002198]). Given the minimal magnitude of change in most cases, the absence of an effect on liver weights in the Cohort 1B animals exposed to PND 120, and the lack of associated histopathology (except in the P1 males), the toxicological significance of these results is unclear.

2. Thymus

P1 males and females given 28/38 or 140/190 ppm ETU had significantly decreased absolute and relative thymus weights. There was no associated histopathology in the P1 adults and thymus weights were not significantly altered in the unselected weanling (PND 22) at any dose level of ETU. In the Cohort 1A and Cohort 1B offspring, thymus weights were significantly decreased at the mid- and high-dose. In the high-dose Cohort 1A males, there was a corresponding increase in diffuse atrophy of lymphoid tissue; thymuses from the Cohort 1B animals were not examined histopathologically. These effects on thymus weights and histopathology were considered treatment related. The effects on thymus weight were consistent with a previous report by ████ (2012, as cited in the study report [DocID 2013/7002198]), who reported decreased thymus weights in male Wistar rats (10/group) following dietary exposure to ETU for 4 weeks. Rats exposed to ETU at 50 ppm (4 mg/kg/day) or 250 ppm (4 mg/kg/day) had significant decreases in relative thymus weights; however, there was no associated thymic histopathology in this study. Significantly decreased body weight gains also were seen at 50 and 250 ppm.

3. Reproductive Toxicity

There were no exposure-related and/or toxicologically significant alterations in reproductive indices, time to mating (P1 adults), gestation length (P1 adults), postimplantation loss, reproductive/accessory sex tissue weights (P1 adults, Cohort 1A) or histopathology. Thus, it was concluded that ETU had no effects on reproductive performance in this study.
Triggers: In the extended one generation study, the decision to breed a second generation is triggered based on predetermined criteria. The criteria to produce a second generation and the outcome for these endpoints are presented in Table 5.8.1-53.

The triggers required that a statistically significant, or biologically relevant, dose-related response was demonstrated. As with other toxicological studies, weight of evidence was applied when interpreting the results of the extended one-generation study to determine whether a second generation breeding was required. Based on these results, breeding of a second generation was not triggered in this extended one generation study, an interpretation to which both USEPA and PMRA have agreed ([redacted], 2012, as cited in the study report [DocID 2013/7002198]).

Table 5.8.1-53: Triggers for decision-making on a second generation

Trigger Endpoint	Recommendation	Trigger met?
Fertility endpoints		
P1 Estrous Cycle Evaluation	Mate F1 if biologically relevant, doserelated changes in estrous cycle length without overt toxicity in the dams	No
P1 Fertility (# implantations, pregnancy rate, gestational interval)	Mate F1 in the absence of corresponding, treatment-related reproductive histopatholo	No
F1 Litter parameters (litter size, litter weight)	Mate F1 if significant, treatmentrelated decreases in litter size/pup survival are seen in the absence of severe maternal toxicity or lethality	No
F1 Developmental Landmarks (AGD, nipple retention, puberty onset)	Mate F1 if dose-related effects, in the absence of body weight-mediated changes in these parameters	AGD – No; Nipple Retention – No; Altered puberty onset due to decreased body weight (i.e., growth rate)
F1 Estrous Cycle Evaluation	Mate F1 if biologically relevant, doserelated changes in estrous cycle length without overt toxicity in dams	No
Offspring Endpoints		
↓ Maternal (P) bodyweight (same dose as ↓ F1 pup bodyweight)	Mate F1, only if the F1 pup body weight decrease is at a lower dose than the dose at which maternal body weight decreased	No – significant decreases in maternal body weight (LD 4 and 7) and lactation feed consumption preceded pup body wt effects
↓ Lactation Index (PND 4-21)	Mate F1 in the absence of severe maternal toxicity	No
F1 pup mortality	Mate F1 in the absence of severe maternal toxicity	No
F1 pup malformation	Mate F1 in the absence of severe maternal toxicity	No
↓ F1 pup viability index (PND 0-4)	Mate F1 in the absence of severe maternal toxicity	No
↓ F1 live birth index	Mate F1 in the absence of severe maternal toxicity	No
↓ F1 pup body weight only	Mate F1, if pup body weight decrease is significant and in the absence of maternal body weight decrement	No – significant decreases in maternal body weight (LD 4 and 7) and lactation feed consumption preceded pup body wt effects

4. Endocrine Toxicity

Endocrine - Potential Androgenic or Anti-androgenic Effects

There are two findings that raise questions regarding potential anti-androgenicity of high dose ETU: decreased absolute epididymal weights in F1 males and delayed balano-preputial separation in F1 weanlings.

1) The minimal decreases in absolute epididymal weight in the Cohort 1A and 1B males were judged to be of no toxicological significance based on the weight of evidence across androgen-sensitive endpoints. There were no effects on absolute epididymal weights in the high-dose P1 males. There were no effects on relative epididymal weights in any ETU treated group (P1 males, Cohort 1A, or 1B). Across all groups (P1, Cohort 1A, or 1B males), there were no epididymal histopathological changes and no effect on other reproductive or accessory sex tissue weights or histopathology, including the testes. Other reproductive parameters in the P1 and Cohort 1A males were unaffected, including no effects on male reproductive indices, testes, or accessory sex tissue weights, sperm motility, sperm morphology, and sperm counts (all collected from the epididymis) in P1 or Cohort 1A males. One factor that may have contributed to the statistical significance of absolute epididymal weights are the decreases in body weights/body weight gains seen in the Cohort 1A and 1B offspring. Cohort 1A males in the 28 and 140 ppm groups weighed 4% and 10% less than controls, respectively, whereas Cohort 1B males in the 140 ppm group weighed 7.5% less than controls.

2) Puberty onset was delayed by 1.9 days in 140 ppm Cohort 1A males, but this effect was considered to be related to body weight decrements and slightly delayed growth, not directly due to ETU exposure. Body weight at the time of puberty onset was similar in 140 ppm males and controls (239.5 g compared with 245.4 g in controls). Because high-dose males weighed the same as controls 1.9 days later when puberty onset occurred, these data indicate that 140 ppm ETU had an effect on the rate of growth of peri-pubescent male rats. This result was confirmed by examining F1 juvenile male body weights on PND 28, 35, and 42. Male pups in the high-dose group weighed 7% less than controls at PND 28, and 8% less than controls on PND 35 and 42. These results are consistent with [redacted] (2003), who reported that a similar body weight decrement (10%) in juvenile male rats delays puberty up to 1.8-days. Furthermore, the age at preputial separation in the high-dose males was within the range of historical control values (43.0-45.7 days).

A weight of evidence across androgen-sensitive endpoints leads to the conclusion that there were no exposure-related anti-androgenic effects of ETU:

- There was no evidence of hypospadias, ectopic testes, or exposure-related testicular, prostate, or seminal vesicle histopathology. There was no effect on qualitative testicular staging.
- In P1 males and Cohort 1A males, there were no significant changes in sperm parameters (sperm counts, sperm motility, and sperm morphology).
- In contrast to decreased absolute epididymal weights in F1 males, there were no effects on reproductive or accessory sex gland weights in the P1 generation males and no effect on testicular or accessory sex gland weights in F1 males when assessed at PND 90 (Cohort 1A) or PND 120 (Cohort 1B). Thus, there was no consistent pattern of altered organ weights in androgen-dependent organs.
- There were no exposure-related effects on reproductive organ histopathology (P1, Cohort 1A) at any dose of ETU. This result is consistent with the previous two generation toxicity study ([redacted] 1990, as cited in the study report [DocID 2013/7002198]), which reported no exposure-related alterations in testicular (P1, F1), epididymal (P1, F1), prostate (P1, F1) or seminal vesicle (P1, F1) histopathology with ETU exposure.

- There were no significant, exposure-related changes in reproductive organ weights in P1, Cohort 1A, or 1B females. ETU also had no effects on estrous cycle (P1, Cohort 1A) or reproductive indices, including mating, fertility, time to mating, gestation length, and post-implantation loss.

Thus, there was no consistent pattern of altered androgenicity in male rats treated with ETU. Overall, the data do not support any ETU-mediated anti-androgenic effect.

Endocrine - Potential Estrogenic Effects

There were no exposure-related effects on estrogen-sensitive endpoints at any dose of ETU:

- There were no exposure-related effects on developmental landmarks, including AGD (measured in all pups), nipple retention (measured in all non-culled pups), or age at vaginal opening (measured in all Cohort 1A and 2A animals). Body weight at vaginal opening was significantly decreased in high-dose F1 females; however, the age at vaginal opening was similar to control animals, indicating an effect on growth rate rather than puberty onset. These endpoints are considered sensitive to altered estrogen status (Clark, 1999; Wolf et al., 2002, as cited in the study report [DocID 2013/7002198])
- ETU exposure had no effects on estrous cycle length or estrous cycle pattern, including a lack of persistent estrus, at any dose levels. Estrous cycle was evaluated in all P1 and all Cohort 1A females.
- There were no exposure-related effects on reproductive indices, including mating, fertility, time to mating, gestation length, and post-implantation loss. There were no signs of dystocia in ETU-exposed P1 dams. Litter size and pup survival were not affected by ETU in this study.
- There were no significant effects on female reproductive organ weights (P1, Cohort 1A or 1B animals) at any dose of ETU.
- ETU did not alter reproductive organ histopathology in P1 or Cohort 1A females; Cohort 1A females were exposed to higher ETU doses (mg/kg/day) during critical windows of development. ETU-treated Cohort 1A females had a significant increase in small and growing follicles; however, this result was judged to be an artifact of sampling and unrelated to ETU treatment; corpora lutea counts were not altered in the Cohort 1A females. This result is consistent with the previous two-generation toxicity study ([redacted] 1990, as cited in the study report [DocID 2013/7002198]), which reported no exposure-related alterations in ovarian (P1, F1) or uterine (P1, F1) histopathology with ETU exposure.

Thus, there was no pattern of altered estrogenicity in female rats treated with ETU. Overall, the data do not support any ETU-mediated estrogenic effects.

Endocrine –Thyroid Effects

ETU doses of 28/38 and 140/190 ppm altered thyroid endpoints, including decreased T4, increased TSH, increased thyroid weight and/or produced thyroid histopathology (i.e., follicular cell hypertrophy and/or hyperplasia and decreased colloid). Follicular cell hyperplasia seen at these dose levels was considered treatment-related and adverse. There were indications of thyroid follicular cell hypertrophy and decreased colloid amount at 2.8/3.8 ppm ETU, but there were no signs of follicular cell hyperplasia and no adverse pathological observations (i.e., degeneration, necrosis, etc.). These results indicate that hypertrophic alterations in thyroid follicular cells in the low-dose group were treatment-related, adaptive changes rather than being pathologic and were consistent with a more rapid colloid turnover to re-establish thyroid hormone homeostasis.

Developmental Neurotoxicity (DNT)
There were no treatment-related changes in PND 22 weanling brain weights or neuropathology at ETU doses ≤ 140 ppm (Cohort 2B). Brain weights and/or histopathology were assessed in other non-perfused animals, including P1 adults and Cohort 1A offspring. Absolute brain weights were significantly decreased in high-dose P1 females and high-dose Cohort 1A males, although relative brain weights were not affected in either group. Brain weights were not affected in the P1 males, Cohort 1A females, and Cohort 1B males and females at any dose level. Cohort 1A and 1B animals received the highest exposures to ETU during critical windows of development with the longest duration of exposures in the Cohort 1B animals. There were no exposure-related effects on brain histopathology (non-perfused P1 and Cohort 1A animals) at any age.
There were no exposure-related effects on ASR, FOB parameters, or motor activity in the Cohort 2A animals. Thyroid perturbations during development also can affect motor activity, which would present as incoordination during clinical observations in pups and persistent hyperactivity (increased motor counts) in post-weaning animals during motor activity testing ([redacted], 1995, as cited in the study report [DocID 2013/7002198]); effects not seen in the current extended one generation study. [redacted] (1995, as cited in the study report [DocID 2013/7002198]) also reported that thyroid perturbations in offspring increased startle response amplitude in adults, again suggesting a hyper reactive response. ASR was not affected at any dose of ETU. The absence of exposure-related alterations in DNT-related neurobehavioral endpoints supports the conclusion of no functional neurological changes due thyroid perturbations. However, ETU at 140 ppm was shown to produce an overall decrease in brain size (weight, gross linear measurements, and microscopic measurements). Subsequent comparisons of microscopic linear measurements at the lower dose levels indicated some statistically identified differences; however, these differences in layer thickness were small, did not exhibit a dose-response relationship and were not consistent between male and female Cohort 2A animals in either the brain areas affected or the direction of change (i.e., increased vs. decreased). Thus, the brain differences noted during the ANOVA analysis were considered incidental.

The thyroid-active agent, 6-propyl-2-thiouracil (PTU), has been associated with decreased brain size, altered brain development (i.e., impaired neuronal migration and white matter hypoplasia) (Behnam-Rassoli et al., 1991; Schoonover et al., 2004; Lavado-Autric et al., 2003; Shibutani et al., 2009, as cited in the study report [DocID 2013/7002198]). The decrease in overall brain size is consistent with the effects of ETU at 140 ppm; however, there were no histopathological changes indicating altered neuronal architecture in brain tissues at any dose of ETU.
Thus, it was concluded that ETU did not alter neurodevelopmental endpoints at doses ≤ 28 ppm.

III. CONCLUSIONS

Under the conditions of the present enhanced one-generation reproduction toxicity study the NOAEL for male and female rats for general, systemic toxicity, based on thyroid toxicity, is 2.8 ppm (about 0.2 mg/kg bw/day).
The NOAEL for fertility and reproductive performance for the F_0 parental rats is at least 140 ppm (about 9.7 mg/kg bw/day), the highest dose tested.
The NOAEL for developmental toxicity in the F_1 progeny is at least 140 ppm (about 9.7 mg/kg bw/day), the highest dose tested.

Teratogenicity studies

There is clear evidence that ETU is teratogenic. When administered to pregnant rats it is able to produce severe malfofrmations at the level of brain (hydrocephaly, exencephaly, meningocele), face (cleft lip and palate), limbs, skeleton. The teratogenic effects of ETU are present also at dose levels not maternally toxic. The hypothesis that the teratogenic effects produced by ETU could be mediated by altered thyroid functionality is not supported by the experimental results. This teratogenic effect seems to be species-specific. The rat is the most sensitive species. Similar malformations have been produced in hamsters with higher doses. No teratogenic effects have been produced in mice and rabbits with dose levels able to produce severe maternal toxicity.

There is a number of rat teratogenicity studies available, which have been already summarized in the European Monograph. The most sensitive studies are the studies from Khera, 1973 (BASF DocID 1973/10099) and Chernoff, 1979 (BASF DocID 1979/10167). In the first study (Khera, 1973) pure ETU was administered orally at doses of 0, 5, 10, 20, 40, and 80 mg/kg bw in distilled water to nulliparous Wistar rats. Treatments were from days 21 to 42 days before mating up to day 15 of gestation, from day 6 to 15 of gstation, or from day 7 to day 20 of gestation.
No signs of toxicity were seen at 40 mg/kg bw. The highest doses testes of 80 mg/kg bw was letzal to 9 out of 11 rats. Mean foetal weight was reduced at the two highest dose levels. ETU induced dose-related increased incidence of meningo-encephalocele, hydrocephaly, obstructed neural tube at 10 mg/kg bw and above in all phases of the experiment. No malformation was noted in the low dose group, however, a retarded ossification was observed in the parietal bone when compared to control.
The $NOAEL_{maternal}$ was 40 mg/kg bw based on mortality, the $NOAEL_{development}$ was 5 mg/kg bw based on central nervous system malformations.

In the second study (Chernoff, 1979) ETU was administered in water by oral gavage to pregnant rats at doses of 0, 5, 10, 20, 30, 40 or 80 mg/kg bw from day 7 through day 21 of gestation.
At the high dose, there was 25% maternal mortality and reduced weight gain. Foetal toxicity, including mortality was also noted at this dose level. At 40 mg/kg bw, hydrocephaly, encephalocele and kyphosis were observed. Hydrocephaly was also seen at 20 mg/kg bw, Decreased foetal weight was recorded at 10 mg/kg bw. No defects were seen at 5 and 10 mg/kg bw, when dosing up to gestation day 21.
This study provides confirmation of ETU teratogenicity on the brain. The $NOAEL_{maternal}$ is 40 mg/kg bw based on mortality and the $NOAEL_{development}$ is 5 mg/kg bw based on fetal effects including teratogenicity.

A summary of the reproduction toxicity studies (reproduction and teratogenicity studies) performed with ETU and peer-reviewed is given in Table 5.8.1-54.

Table 5.8.1-54: Summary of already per reviewed reproductive toxicity studies on ETU

Type of test Test species	Test substance purity	Dose tested	NOAEL	Reference
2-generation rat	98%	0, 2.5, 5, 25, 125 ppm	Reproductive NOAEL: > 125 ppm (>4.3 – 21 mg/kg bw) Overall NOAEL: 2.5 ppm, equivalent to 0.11-0.43 mg/kg bw based on thyroid effects	1992/5000
Perinatal rat	-	0, 8, 25, 83, 250 ppm	8 ppm, equivalent to 0.4 mg/kg bw based on follicular cell hyperplasia in males	1992/12022 1992/1004097
Perinatal mouse	-	0, 33, 110, 330, 1000 ppm	330 ppm, equivalent to 50 mg/kg bw based on reduced neonatal survival and growth	1992/12022 1992/1004097
Teratology/rat		0, 5, 10,20, 40, 80 mg/kg bw	Maternal toxicity: 40 mg/kg bw Developmental: 5 mg/kg bw based on central nervous system malformations	1973/10099
Teratology/rat		0, 40 mg/kg bw	Not determined	1978/10173
Teratology/rat		0, 10, 20, 30, 40, 50 mg/kg bw	Maternal: > 50 mg/kg bw Developmental: 10 mg/kg bw based on e.g. brain malformations	1978/10175
Teratology/rat		0, 5, 10, 20, 30, 40, 80 mg/kg bw	Maternal: 40 mg/kg bw Developmental: 5 mg/kg bw based on e.g. brain malformations 5	1979/10167
Teratology/rat		0, 15, 25, 35 mg/kg bw	Maternal: >35 mg/kg bw Developmental: 15 mg/kg bw based on e.g. brain malformations	1991/11550
Teratology/mouse		0, 100, 400, 800 mg/kg bw	>800 mg/kg bw	1978/10175
Teratology/mouse		0, 100, 200 mg/kg bw	Maternal: <100 mg/kg bw Foetal: 100 mg/kg bw	1979/10167
Teratology/rabbit		0, 10, 20, 40, 80 mg/kg bw	Maternal: >80 mg/kg bw Foetal: 40 mg/kg bw	1973/10099
Teratology/Hamster		0, 90, 270, 810 mg/kg bw	Maternal: >810 mg/kg bw Developmental: 90 mg/kg bw (cleft palate, skeletal anomalies)	1978/10175

Type of test Test species	**Test substance purity**	**Dose tested**	**NOAEL**	**Reference**
Teratology/Guinea pig		0, 50, 100 mg/kg bw	Maternal and developmental: >100 mg/kg bw	1979/10167
Additional Information: Teratology/Cat		0, 5, 10, 30, 60 mg/kg bw	Maternal: 10 mg/kg bw Fetal: >120 mg/kg bw	1978/10161

Two new teratogenicity studies had been conducted with ETU since the European Peer Review. The rabbit teratogenicity study has been required during the US-EPA Data Call in process and is summarized in detail below. The Mancozeb Consortium has also conducted a new rat teratogenicity study, which is described in the Mancozeb Dossier. The NOAEL$_{development}$ of 5 mg/kg bw has been confirmed in this new rat teratogenicity study.

Report: CA 5.8.1/17
[redacted] 2010a
Ethylenethiourea (ETU): A 14-day tolerability study in non-pregnant rabbits
2010/1050667
Guidelines: none
GLP: no

Report: CA 5.8.1/18
[redacted] 2010b
Ethylenethiourea (ETU): Developmental toxicity study in rabbits
2010/1050668
Guidelines: EPA 870.3700, OECD 414, EEC 87/302, JMAFF No 12 Nosan No 8147
GLP: yes
(certified by United States Environmental Protection Agency)

Report: CA 5.8.1/19
[redacted] 2010c
Ethylenethiourea (ETU): Pilot developmental toxicity study in rabbits
2010/1050669
Guidelines: none
GLP: no

Executive Summary

In a developmental toxicity study, ethylene thiourea (ETU; lot: 00721KH; purity 99.4%) formulated in 0.5% aqueous methylcellulose was administered by gavage to groups of 22 time-mated female New Zealand White rabbits at dose levels of 0, 5, 15 and 50 mg ETU/kg bw/day. Animals were dosed on days 7 through 28 of gestation.

There were test substance-related effects on maternal body weight parameters at 15 and 50 mg/kg/day. Cumulative weight gains from GD 7 to 29 were 17 and 30% lower than the control group mean, respectively. Cumulative mean food consumption was also reduced 7 and 14% relative to controls at 15 and 50 mg/kg/day, respectively. There were statistically significant reductions in mean fetal weight at 15 and 50 mg/kg/day, which were 3 and 9% lower than the control mean, respectively, and associated with maternal effects, notably the 17 and 30% reductions in body weight gains at these dose levels. At 15 mg/kg/day, the fetal weight was within the laboratories historical control range and therefore, was considered not to be an adverse effect. There were no test substance-related increases in the incidences of fetal malformations or variations at any dose level.
Therefore, under the conditions of this study, the maternal no-observed-adverse-effect level (NOAEL) is 5 mg/kg/day based on evidence of maternal toxicity produced at 15 and 50 mg/kg/day. The fetal NOAEL is 15 mg/kg/day based on reduced fetal weight at 50 mg/kg/day. The NOAEL for fetal malformations is 50 mg/kg/day based on the lack of increased incidences of fetal malformations or variations at any dose level. The results of this study indicate that the test substance is not selectively toxic to the rabbit conceptus.
(BASF DocID 2010/1050668)

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** Ethylene thiourea (ETU)

Description:	white powder with lumps
Lot #:	00721KH
Purity:	99.4%
Stability of test compound:	The test substance was stable over the study period

2. **Vehicle and/or positive control:** 0.5% aqueous methylcellulose

3. **Test animals:**

Species:	New Zealand White rabbit
Strain:	Hra: (NZW) SPF
Sex:	female (nulliparous, timed mated, GD 0 = day mating confirmed)
Age:	5.5 – 6 months
Weight at dosing:	2709 to 3662 g
Source:	[redacted]
Acclimation period:	at least 3 days
Diet:	Certified rabbit LabDiet® 5322 (PMI® Nutrition International, LLC), approximately 125 g
Water:	Tap water ad libitum
Housing:	individual housing in stainless steel cages suspended above cage boards
Environmental conditions:	
Temperature:	16 - 22°C
Humidity:	30 - 70%
Air changes:	not indicated in the report
Photo period:	12 h light / 12 h dark

B. STUDY DESIGN AND METHODS

The study was conducted at E.I. du Pont de memours and Company, DuPont Haskell Global Centers for Health & Environmental Sciences, P.O. Box50, Newark, Delaware 19714, USA.

1. **Dates of experimental work**: 19-Oct-2008 - 22-Dec-2008
(In life dates: 14-Oct-2008 to 20-Nov-2008)

2. Selection of Dose Levels

At first a range-finding study in rabbits (DocID 2010/10506679 was done with three female non-pregnant rabbits per dose. These animals received by gavage daily either 100, 200 or 400 mg/kg bw/day for 14 consecutive days. At 100 mg/kg/day, all 3 rabbits survived to scheduled sacrifice. The animals at 100 mg/kg/day ate their daily ration of food, gained weight, and were free of any adverse clinical or gross postmortem observations. At 200 mg/kg/day, one of 3 rabbits was sacrificed on test day 10 after several days of not eating and subsequent marked weight loss; gross postmortem examination revealed a hairball in the stomach. The remaining 2 animals survived to scheduled sacrifice in good condition. At 400 mg/kg/day, 2 of 3 rabbits were sacrificed on test day 6. All 3 rabbits stopped eating early in the dosing period and subsequently lost weight; one of the 3, however, did resume eating towards the end of the first week of dosing and survived to scheduled sacrifice. Based on these data, the dose levels selected for the pilot developmental toxicity study were 0, 50, 100, and 200 mg/kg/day.
In a pilot developmental toxicity study in rabbits conducted to prepare for this study (DocID 2010/1050669) doses of 0, 50, 100, 150, or 200 mg/kg/day were evaluated. Doses were prepared as suspensions in 0.5% aqueous methylcellulose and administered once daily at 5 mL/kg. At 150 and 200 mg/kg/day, dosing was terminated and the animals were euthanized prior to scheduled sacrifice due to excessive toxicity characterized by marked reductions in food consumption, body weight losses, and clinical observations suggestive of impending abortion. The remaining groups dosed at 50 and 100 mg/kg/day survived to scheduled termination of gestation day 29. Test substance related and adverse maternal and fetal effects occurred at 50 and 100 mg/kg/day. Regarding maternal toxicity, means for cumulative body weight gain were 47 and 65% lower than controls at 50 and 100 mg/kg/day, respectively; means for cumulative maternal food consumption were 16 and 20% lower than controls at the same respective dose levels. Regarding fetal effects, mean fetal weight was 22 and 29% lower than controls at 50 and 100 mg/kg/day. Based on these data, the dose levels selected for the current study were 0, 5, 15, and 50 mg/kg/day.

3. Animal assignment and treatment:

Animals were pre-mated. Gestation day (GD) 0 corresponds to day where mating was confirmed. Animals arrived at GD 1 or 2. Ethylene Thiourea was administered to groups of 22 pregnant rabbits by gavage at dose levels of 0, 5, 15 and 50 mg/kg bw during days 7 to 28 of gestation. The animals were assigned to the treatment groups based on a randomization plan as well as body weight.

4. Test substance preparation and analysis:

Prior to study initiation the test substance was shown to be stable in the vehicle for up to 5 days at room temperature. Thus, dosing formulations of the test substance in the vehicle were prepared and used within the period of established stability.

Analyses to verify concentration and stability of the dose formulations were conducted near the beginning of the dosing period; an additional check for concentration verification was conducted at the end of the dosing period. Formulation homogeneity was verified in the previous pilot study (DocID 2010/1050669).The results of these analyses are shown below:

Analysis of diet preparations				
Dose level [mg/kg bw/day]	Sampling	Concentration[$] [mg/mL]	Relative standard deviation[$] [%]	% of Nominal
5	22.10.2008 14.11.2008	1.08 ±0.03 1.04 ±0.05	3 5	108.0 104.0
15	22.10.2008 14.11.2008	3.16 ±0.06 3.08 ±0.04	2 1	105.3 102.7
50	22.10.2008 14.11.2008	10.80 ± 0.2 10.40 ± 0.1	2 1	108.0 104.0

[$] Concentration for each dose level is the average of the duplicate analysis results

The results of the analytical chemistry evaluations confirmed that the dose formulations were at targeted concentrations and stable under the conditions of use for the current study. Measured concentrations ranged from 102.3 to 109.8% of nominal concentrations.

5. Statistics:

Significance was judged at $p < 0.05$.

For litter parameters, the proportion of affected fetuses per litter or the litter mean was used as the experimental unit for statistical evaluation.

Statistics

		Method of statistical analysis	
Parameter	Preliminary test	If preliminary test is not significant	If preliminary test is significant
Maternal Weight Maternal Weight Change Maternal Food Consumption *Corpora Lutea* Implantations per Litter Live Fetuses per Litter Dead Fetuses per Litter Resorptions per Litter	Levene’s test for homogeneity and Shapiro-Wilk test for normality	One-way analysis of variance followed by Dunnett's test	Kruskal-Wallis test followed by Dunn's test
Incidence of Pregnancy Maternal Mortality Females with Total Resorptions Abortions	None	Sequential application of the Cochran-Armitage test [a]	
Incidence of Fetal Alterations	None	Exact Mann-Whitney with a Bonferroni-Holm adjustment	
Fetal Weight (Covariates: litter size, sex ratio) Sex Ratio (Covariate: litter size)	Levene’s test for homogeneity and Shapiro-Wilk test for normality[b]	Analysis of covariance and Dunnett-Hsu	Non-parametric analysis of covariance

[a] If the incidence was not significant, but a significant lack of fit occurred, then Fisher’s Exact test with a Bonferroni correction was used.

[b] A normalizing, variance stabilizing transformation was used as needed.

C. Methods

1. Treatment:

The test substance was administered by gavage once a day from gestation day 7 to 28. The volume administered each day was 5 mL/kg body weight. The calculation of the volume administered was based on the most recent individual body weight.

2. Observation:

The animals were examined for mortality twice daily. Examination for clinical signs of toxicity were performed twice daily on GD 7 to 28 during weighing and at least two hours post-dosing

3. Body weight:

All animals were weighed daily on GD 7 to 29.

4. Food consumption:

The consumption of food was determined daily during gestation days 7 – 29.

5. Sacrifice:

Females were euthanized by intravenous injection of an approved commercial euthanasia agent (B-euthanasia D) followed by exsanguination; fetuses were euthanized by an intraperitoneal injection of the same agent after the external examination was complete.

Immediately after sacrifice the does were subjected to a gross external and visceral examination
For each doe, the thyroid was examined, removed, weighed, and retained in 10% neutral buffered formalin for possible future histopathologic examination.
Gross lesions were retained for possible histologic examinations; lesions for which a microscopic diagnosis would not be additive were not saved.
A terminal blood sample was collected; serum for possible future hormonal analyses was prepared and stored frozen, (approximately -80°C).
Uteri with no visible implantation sites were placed in a 10% aqueous solution of ammonium sulfide to detect very early resorptions.
The gravid uterus of each female having at least one viable fetus was weighed to permit calculation of maternal body weight adjusted to exclude the products of conception. The corpora lutea count for each ovary of females with visible implantation sites was recorded. For each female with visible implantation sites, the types of implantations (live and dead fetuses, early and late resorptions) and their relative positions in the uterus were recorded.
The types of implantations were classified as follows:

- live fetus: fully formed and responds to stimuli
- dead fetus: fully formed with little or no evidence of maceration
- late resorption: identifiable structures (i.e. digital rays)
- early resorption: no visible fetal structures

6. Examination of fetuses:

The body weight of each live fetus was recorded. Each fetus was underwent external, visceral, head, and skeletal examinations.
Each fetus was examined for gross external alterations. The external examination included inspection of all externally observable structures as well as the fetal palate.
The visceral examination included inspection of all viscera for presence, size, shape, color, location, and texture. Fetal sex was recorded during the visceral examination. The fetal head examination included inspection of the eyes and internal brain structures. The eyelids of each fetus were removed to permit examination of each eye and surrounding structures. Following removal and inspection of the eyes, a transverse cut between the frontal and parietal bones permitted examination of the brain hemispheres and ventricles for proper size and evidence of hydrocephaly.
All fetuses were fixed in alcohol, processed, and the skeletons stained with alizarin-red. The skeletal bodies and heads of all the fetuses were examined for alterations. The skeletal examination included inspection of the individual bones and cartilage of the axial and appendicular skeleton for overall structure, relationship, and stage of ossification.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Mortality

There was no test substance-related mortality at any dose level tested. One female at 5 mg/kg/day aborted on gestation day 20 and was subsequently euthanized prior to scheduled euthanasia. One female at 15 mg/kg/day was euthanized on GD 20 following observations of head tilt; this observation was first recorded on GD 16 and was observed daily until euthanasia on GD 20. Neither of these unscheduled sacrificed were considered test substance-related. Otherwise, all remaining does survived until scheduled euthanasia on GD 29.

2. Clinical signs of toxicity

There were no test substance-related clinical observations at any level tested. The signs observed were unremarkable and seen in only one or 2 animals from any given dose group with incidences that were not dose-dependent.

B. BODY WEIGHT AND FOOD CONSUMPTION

1. Body weight

There were test substance-related effects on maternal body weight parameters at 15 and 50 mg/kg/day. The cumulative weight gain means calculated from GD 7 to 29 were 17 and 30% lower than the control group mean at 15 and 50 mg/kg/day, respectively; these changes were statistically significant. Figure 5.8.1-8 illustrates that at 50 mg/kg/day, mean body weights were lower soon after the onset of the dosing period and that the lower weights persisted for the remainder of the study. At 15 mg/kg/day, the reduction in gain relative to controls is most evident during the last week of the dosing period. When the mean weight gains are calculated using the final weight adjusted gravid uterine weight, there was a mean loss of 77 and 136 grams at 15 and 50 mg/kg/day, respectively, compared with a mean loss of 66 grams in the control group. There were no test substance-related effects on maternal body weight parameters at 5 mg/kg/day.

2. Food consumption

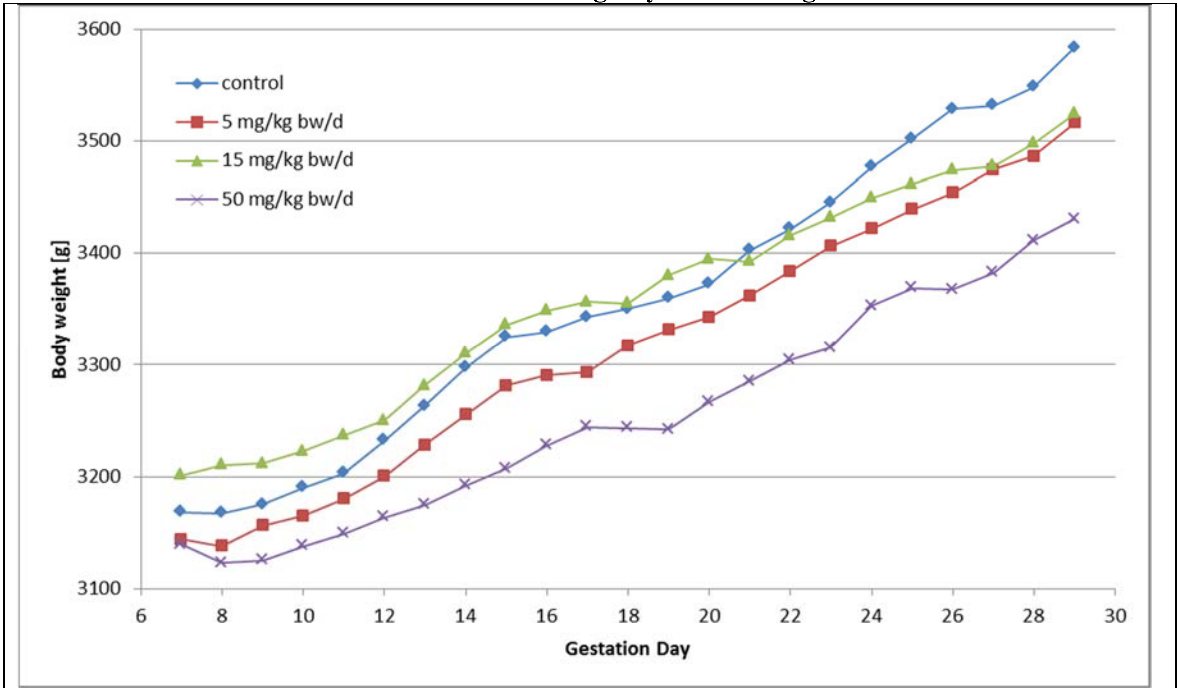
There were test substance-related reductions in maternal food consumption at 15 and 50 mg/kg/day that were consistent with the decreased maternal body weight gains described previously. Mean food consumed from GD 7 to 29 was 7 and 14% lower than controls at 15 and 50 mg/kg/day, respectively; these reductions were statistically significant. There were no test substance-related effects on maternal food consumption at 5 mg/kg/day. The cumulative mean (GD 7 to 29) was 4% lower than the control mean and this difference was not statistically significant.

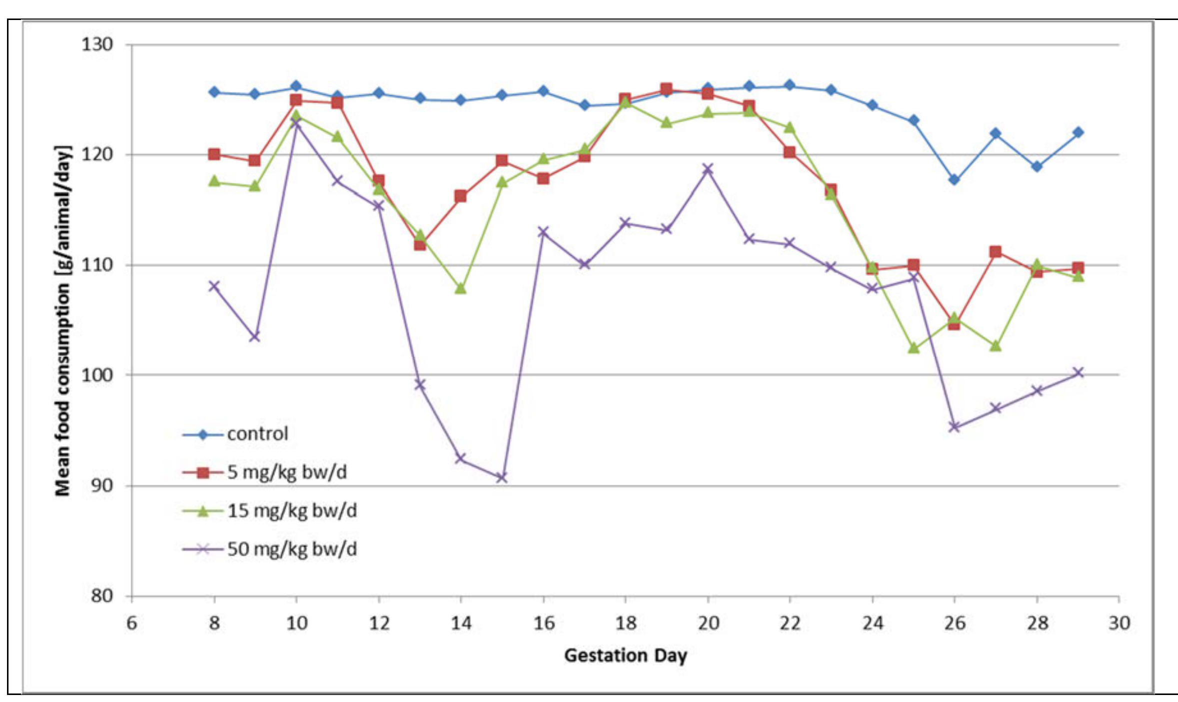
Table 5.8.1-55: Body weight and food consumption development in rabbits administered ETU during days 7 to 28 of gestation

Dose level [mg/kg]	0	5	15	50
Body weight gain [g]				
Day 7 to 29 C BWG:day29	415.0 -66.08	390.7 -69.63	344.9* -77.08	291.1* -136.12
□□		-6%	-17%	-30%
Food consumption [g/animal/day]				
Day 7 to 29 □□	124.3	118.9 -4%	115.6& -7%	107.2& -14%

C_BWG:day29 = Net body weight change on day 29 = Net body weight on day 29 minus the body weight on day 7.
* Parametric comparison to control (Dunnett/Tamhane-Dunnett) significant
& Nonparametric comparison to control (Dunn's) significant

Figure 5.8.1-8: Body weight and food consumption development in rabbits administered ETU during days 7 to 28 of gestation





C. NECROPSY OBSERVATIONS

1. Maternal Gross Postmortem and Organ Weight Data

Discolored/darkened thyroids were observed at necropsy in 2 and 3 does at 15 and 50 mg/kg/day, respectively. These observations were considered possibly related to the test substance (see Table 5.8.1-56).

There were no effects on mean thyroid weights at any dose level tested; data were generally comparable across all groups on study (see Table 5.8.1-57).

Table 5.8.1-56: Incidences of Gross observations in female rabbits administered ETU during days 7 to 28 of gestation

Dose Level [mg/kg]	0	5	15	50
Females examined	22	22	22	22
Stomach: discoloration; red; granular	0	0	1	0
Thyroid gland: discoloration; dark	0	0	2	3
Thyroid gland: large	0	0	1	0

Table 5.8.1-57: Mean final body weight and thyroid gland weight for maternal rabbits

Dose level [mg/kg]	0	5	15	50
Body weight [g]	3583.5.3 ±266.4	3516.0 ±196.1	3511.0 ±266.8	3415.1 ±276.4
Thyroid gland weight [g]	0.223 ±0.053	0.243 ±0.054	0.252 ±0.062	0.242 ±0.059
Thyroid gland weight relative to final body weight x 100	0.0062 ±0.0014	0.0069 ±0.0014	0.0072 ±0.0020	0.0071 ±0.0016

2. Cesarean section data (Table 5.8.1-58)

There were statistically significant reductions in mean fetal weight at 15 and 50 mg/kg/day. The means for fetal weight were 3 and 9% lower than the control mean at these respective doses. These reductions were associated with significant dose-related maternal effects, most notably on cumulative body weight gains from GD 7-29, which were 17 and 30% lower than the control group mean. In addition, at 15 mg/kg/day, the fetal weight was within the testing laboratories historical control range (39.77 to 43.53 g). For these reasons, although the marginal difference may be treatment-related, it was considered not to be an adverse effect as it was considered to have no biological significance. At 5 mg/kg/day, mean fetal weight was similar to the control group mean.

Otherwise, there were no test substance-related effects on reproductive outcome and quantitative litter data. The mean number of implantation sites, resorptions, live fetuses, as well as mean litter sex ratio was comparable across all groups tested. At 15 mg/kg/day, the incidence of resorptions/early resorptions (0.5/0.3 per litter on average) was statistically higher than for controls. This change, however, is considered spurious and unrelated to the test substance because it is not dose dependent, minimal in magnitude, and well within the range reported in the test facility historical control data (0.0 to 0.8).

Table 5.8.1-58: Cesarean section data of rabbits administered ETU during days 7 to 28 of gestation

Dose level [mg/kg bw/d]		0	5	15	50
Pregnancy status					
- pregnant	[n]	22	22	22	19
- conception rate	[%]	100	100	100	86.3
- aborting	[n]	0	1	0	0
- permature birth	[n]	0	0	0	0
- dams with viable fetuses	[n]	2322	21	20	19
- dams with total resorption	[n]	0	0	0	0
Cesarean section data[a]					
- Corpora lutea	[n]	8.8 ± 1.2	8.4 ± 1.9	8.0 ± 2.3	8.8 ± 1.8
- Implants	[n]	8.5 ± 1.3	8.3 ± 1.9	8.1 ± 2.2	8.4 ± 1.6
- total Resorptions	[n]	0.09 ± 0.29	0.10 ± 0.30	0.50[&] ± 0.69	0.47 ± 0.77
- Early resorptions		0.00 ± 0.00	0.05 ± 0.22	0.30[&] ± 0.57	0.26 ± 0.65
- Late resorptions		0.09 ± 0.29	0.05 ± 0.22	0.20 ± 0.52	0.21 ± 0.54
- Dead fetuses	[n]	0	0	0	0
- Live fetuses		8.4 ± 1.1	8.2 ± 1.9	7.6 ± 2.2	7.9 ± 1.7
- female fetuses	[n]	4.0 ± 1.3	4.4 ± 1.1	3.6 ± 1.6	3.7 ± 1.6
- male fetuses	[n]	4.4 ± 1.0	3.8 ± 1.5	4.0 ± 1.5	4.2 ± 1.9
- Sex Ratio		0.52 ± 0.13	0.46 ± 0.11	0.53 ± 0.18	0.52 ± 0.18
Mean fetal weight	[g]	41.1 ± 3.8	40.7 ± 3.0	39.9! ± 5.9	37.3! ± 4.2
- males	[g]	41.4 ± 3.8	40.9 ± 2.9	39.9 ± 6.0	38.0 ± 4.2
- females	[g]	40.8 ± 4.4	40.6 ± 3.9	39.1 ± 5.4	36.2 ± 4.9

Sex Ratio = Number male foetuses/total number foetuses per litter
[&] Nonparametric comparison to control (Dunn's) significant
! Analysis of Covariance and Dunnett-Hsu

D. EXTERNAL, VISCERAL AND SKELETAL EXAMINATION OF FETUSES

1. Malformations and Variations (Table 5.8.1-59)

There were no test substance-related increases in the incidences of fetal malformations or variations at any level tested. The findings were generally unremarkable and the incidences of any fetal findings were low and not statistically different across groups. There were no dose-dependent increases in any individual finding.

At 50 mg/kg/day, 2 fetuses from 2 litters were observed with domed heads. The head examination for only one of these fetuses revealed internal hydrocephaly. This single incidence is not considered test substance related. For the thirteen studies for which historical control data were previously cited, there is one study conducted in 2001, which include one control fetus with hydrocephaly. In addition, there are 2 additional studies conducted in 1996 and 1998 in which there were 3 fetuses from 2 control litters and 5 fetuses from 5 control litters reported with distended brain lateral ventricles, which is considered to be a slightly less severe manifestation of internal hydrocephaly.

Table 5.8.1-59: Incidence of fetal malformations and variations

Dose level [mg/kg]	0	5	15	50
Litters Evaluated	22	20	20	19
Fetuses Evaluated	185	165	151	150
External Defects				
- Limb, Paw hyperflexion (V)				
- Fetal incidence	0 (0.0)[a]	0 (0.0)	1 (0.7)	1 (0.7)
- Litter incidence	0 (0.0)	0 (0.0)	1 (5.0)	1 (5.2)
- Limb, Malrotated (M)				
- Fetal incidence	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)
- Litter incidence	0 (0.0)	0 (0.0)	1 (5.0)	0 (0.0)
- Limb, Hyperextension (V)				
- Fetal incidence	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)
- Litter incidence	0 (0.0)	0 (0.0)	1 (5.0)	0 (0.0)
- Head, Cranium, Anecephaly (M)				
- Fetal incidence	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
- Litter incidence	1 (4.5)	0 (0.0)	0 (0.0)	0 (0.0)
- Head, Cranium, Domed head (GF)				
- Fetal incidence	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.3)
- Litter incidence	0 (0.0)	0 (0.0)	0 (0.0)	2 (10.5)
Head Defects				
- Eye, Hemorrhagic (V)				
- Fetal incidence	3 (1.6)	1 (0.6)	0 (0.0)	1 (0.7)
- Litter incidenc	3 (13.6)	1 (4.7)	0 (0.0)	1 (5.2)
- Eye, Enlarged (M)				
- Fetal incidence	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
- Litter incidence	1 (4.5)	0 (0.0)	0 (0.0)	0 (0.0)
- Eye, Microphtalmia (M)				
- Fetal incidence	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)
- Litter incidence	0 (0.0)	0 (0.0)	1 (5.0)	0 (0.0)
- Brain, Hydrocephaly (M)				
- Fetal incidence	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)
- Litter incidence	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.2)
Visceral Defects				
- Heart; Pulmonary trunk, dilated (M)				
- Fetal incidence	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)
- Litter incidence	0 (0.0)	1 (4.7)	0 (0.0)	0 (0.0)
- Heart, Microcardia (M)				
- Fetal incidence	1 (0.5)	1 (0.6)	0 (0.0)	2 (1.3)
- Litter incidence	1 (4.5)	1 (4.7)	0 (0.0)	1 (5.2)
- Heart, Enlarged (M)				
- Fetal incidence	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)
- Litter incidence	0 (0.0)	1 (4.7)	0 (0.0)	0 (0.0)

Table 5.8.1-59: Incidence of fetal malformations and variations

Dose level [mg/kg]	0	5	15	50
- Heart, Pale (V)				
- Fetal incidence	0 (0.0)	0 (0.0)	9 (6.0)	0 (0.0)
- Litter incidence	0 (0.0)	0 (0.0)	1 (5.0)	0 (0.0)
- Heart; A-V septum, Septum Defect (M)				
- Fetal incidence	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)
- Litter incidence	0 (0.0)	1 (4.7)	0 (0.0)	0 (0.0)
- Heart; Aortic valve, absent (M)				
- Fetal incidence	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)
- Litter incidence	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.2)
- Heart; Aortic arch, dilated (M)				
- Fetal incidence	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)
- Litter incidence	0 (0.0)	1 (4.7)	0 (0.0)	0 (0.0)
- Thorax; Thymus, discolored (V)				
- Fetal incidence	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)
- Litter incidence	0 (0.0)	1 (4.7)	0 (0.0)	0 (0.0)
- Abdomen;Ovary enlarged (V)				
- Fetal incidence	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)
- Litter incidence	0 (0.0)	0 (0.0)	1 (5.0)	0 (0.0)
- Abdomen; Bladder distended (M)				
- Fetal incidence	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)
- Litter incidence	0 (0.0)	1 (4.7)	0 (0.0)	0 (0.0)
- Abdomen; Bladder enlarged (V)				
- Fetal incidence	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)
- Litter incidence	0 (0.0)	1 (4.7)	0 (0.0)	0 (0.0)
- Abdomen; Stomach distended (M)				
- Fetal incidence	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)
- Litter incidence	0 (0.0)	1 (4.7)	0 (0.0)	0 (0.0)
- Abdomen; Spleen discolored (V)				
- Fetal incidence	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)
- Litter incidence	0 (0.0)	0 (0.0)	1 (5.0)	0 (0.0)
- Abdomen; Gallbladder small (V)				
- Fetal incidence	1 (0.5)	1 (0.6)	4 (2.6)	3 (2.0)
- Litter incidence	1 (4.5)	1 (4.7)	3(15.0)	2(10.5)
- Abdomen; Gallbladder absent (V)				
- Fetal incidence	0 (0.0)	0 (0.0)	2 (1.3)	0 (0.0)
- Litter incidence	0 (0.0)	0 (0.0)	1 (5.0)	0 (0.0)
- Abdomen; Liver discolored (V)				
- Fetal incidence	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)
- Litter incidence	0 (0.0)	0 (0.0)	1 (5.0)	0 (0.0)
- Abdomen; Kidney misshapen (V)				
- Fetal incidence	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)
- Litter incidence	0 (0.0)	0 (0.0)	1 (5.0)	0 (0.0)
- Abdomen; Kidney enlarged (M)				
- Fetal incidence	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)
- Litter incidence	0 (0.0)	1 (4.7)	0 (0.0)	0 (0.0)
- Abdomen; Kidney discolored (V)				
- Fetal incidence	0 (0.0)	0 (0.0)	6 (4.0)	0 (0.0)
- Litter incidence	0 (0.0)	0 (0.0)	1 (5.0)	0 (0.0)
- Abdomen; Renal papilla, misshapen (M)				
- Fetal incidence	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)
- Litter incidence	0 (0.0)	0 (0.0)	1 (5.0)	0 (0.0)
Skeletal – Head Defects				
- Skull; Hyoid, bent (M)				
- Fetal incidence	2 (1.1)	2 (1.2)	1 (0.7)	1 (0.7)
- Litter incidence	2 (9.0)	2 (9.5)	1 (5.0)	1 (5.2)
- Skull; general, craniofenestria (M)				
- Fetal incidence	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)
- Litter incidence	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.2)
- Skull; general, acrania (M)				
- Fetal incidence	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
- Litter incidence	1 (4.5)	0 (0.0)	0 (0.0)	0 (0.0)

Table 5.8.1-59: Incidence of fetal malformations and variations

Dose level [mg/kg]	0	5	15	50
Skeletal – Body Defects				
- Pelvic girdle; Pubis, incomplete ossificiation (V)				
- Fetal incidence	0 (0.0)	0 (0.0)	1 (0.7)	2 (1.3)
- Litter incidence	0 (0.0)	0 (0.0)	1 (5.0)	1 (5.2)
- Vertebrae; lumbar vertebra, hemivertebra (M)				
- Fetal incidence	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)
- Litter incidence	0 (0.0)	1 (4.7)	0 (0.0)	0 (0.0)
- Vertebrae; thoracic arch, fused (M)				
- Fetal incidence	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)
- Litter incidence	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.2)
- Vertebrae; lumbar centrum, fused (M)				
- Fetal incidence	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)
- Litter incidence	0 (0.0)	1 (4.7)	0 (0.0)	0 (0.0)
- Vertebrae; thoracic centrum, bipartite ossification (V)				
- Fetal incidence	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)
- Litter incidence	0 (0.0)	1 (4.7)	0 (0.0)	0 (0.0)
- Ribs; cervical rib (V)				
- Fetal incidence	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
- Litter incidence	1 (4.5)	0 (0.0)	0 (0.0)	0 (0.0)
- Ribs; fused (M)				
- Fetal incidence	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)
- Litter incidence	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.2)
- Sternebrae; unossified (V)				
- Fetal incidence	17 (9.2)	14 (8.1)	19 (12.6)	19 (12.7)
- Litter incidence	9 (40.9)	10 (47.6)	8 (40.0)	8 (42.1)
- Sternebrae; fused (M)				
- Fetal incidence	1 (0.5)	0 (0.0)	2 (1.3)	1 (0.7)
- Litter incidence	1 (4.5)	0 (0.0)	2 (10.0)	1 (5.2)
- Sternebrae; bipartite ossification (V)				
- Fetal incidence	0 (0.0)	1 (0.6)	1 (0.7)	1 (0.7)
- Litter incidence	0 (0.0)	1 (4.7)	1 (5.0)	1 (5.2)

[a] values in brackets give % incidence
V = Variation, M = Malformation, GF = gross finding

E. CONCLUSIONS

Under the conditions of this study, the **maternal** no-observed-adverse-effect level (NOAEL) is 5 mg/kg bw/day based on evidence of maternal toxicity produced at 15 and 50 mg/kg/day. The **fetal** NOAEL is 15 mg/kg bw/day based on reduced fetal weight at 50 mg/kg/day. The NOAEL for **fetal malformations** is 50 mg/kg/day based on the lack of increased incidences of fetal malformations or variations at any dose level.

Further published studies investigating the endpoint of reproduction/developmental toxicity of ETU were found and are listed in the following:

Report:	CA 5.8.1/20 Maranghi F. et al., 2013a Reproductive toxicity and thyroid effects in Sprague Dawley rats exposed to low doses of Ethylenethiourea 2013/1419083
Guidelines:	none
GLP:	no

Executive summary:
Ethylenethiourea (ETU) is a metabolite of mancozeb. It is an Endocrine Disruptor as it interferes with thyroid hormone biosynthesis by inhibiting thyroid peroxidase activity. This study has been performed to assess potential effects of ETU exposure at low dose levels, i.e. below the established LOAEL and NOAEL, during critical phases of development. The study aimed to verify the short- and long-term effects on thyroid function and on reproduction and development after oral exposure to ETU levels comparable to and lower than LOAEL/NOAEL in rats. Groups of 11-20 pregnant Sprague Dawley rats were treated daily by gavage from day 7 to 20 of pregnancy (GD) and from post natal days (PND) 1 to 22 with 0, 0.1, 0.3 or 1.0 mg/kg bw per day of ETU. At least 10/sex/group F1 generation pups were selected and dosed from weaning to sexual maturity (day 60 for males and day 75 for females). Thyroid biomarkers were analysed in dams and in F1 offspring. Reproductive biomarkers were analysed in F1 rats.
The number of total litter resorptions was 0, 1, 1 and 4 in the control, 0.1, 0.3 and 1.0 mm/kg/day groups respectively. Although not statistically significant, 20% of dams in the 1.0 mg/kg bw/day group showed a markedly reduced number of live pups (1-2 per litter) and, at necropsy, early resorptions were evidenced by the presence of corpora lutea. Relative thyroid weight was significantly higher in the 0.1 mg/kg bw/day groups dams but not in the higher dose groups. Effects in the thyroid were seen histologically in the thyroid of ETU treated dams (small follicles with less colloid and increased epithelium height).
In F1 generation pups there were no significant effects on viability or pup body weight gain were observed in treated groups. The timing of incisor eruption was significantly earlier in all treated groups than in the control group. Timing of ear pinna detachment and eye opening were significantly earlier in the 0.1 and 0.3 mg/kg bw/day groups than in controls. Monitoring of the estrous cycle revealed a significant increase in the mean number of days per cycle ($P<0.05$) and a significant decrease in the mean number of cycles completed by the 17-day evaluation period in all treated groups when compared with control. T3 value was significantly higher in the 0.1 and 0.3 mg/kg bw/day groups than in control.
Morphological and histological effects on the thyroid were seen in both sexes.
The authors believe this study has demonstrated effects on reproduction and the thyroid at doses lower than LOAEL dose exposure in pregnant dams and F1 generation pups. They suggest that even low doses of ETU can interfere with thyroid homeostasis and reproductive hormone profile if exposure starts in critical stages of development.

Objective:
To verify the short- and long-term effects on thyroid function, reproduction and development of oral exposure to ETU levels comparable to and lower than LOAEL/NOAEL in rats.

Materials and methods:

Test material	Ethylenethiourea
CAS No.	96-45-7
Source	Sigma-Aldrich, Milan, Italy
Batch	No data
Purity	98%
Stability	No data

Vehicle: The test substance was administered in distilled water. Dose formulation s were prepared fresh each day. Dose formulations were not analysed.

Test Animals	
Species	Rat
Strain	Sprague Dawley
Age/weight requested from supplier	230-250 g, nulliparous, pregnant
Source	Harlan, Italy.
Acclimatisation period	5 days
Diet	4RF25 GLP Top Certificate supplied by Mucedola, Milan, Italy, ad libitum
Water	No data, ad libitum
Environmental conditions	Temperature: 20±0.5°C Humidity: 50-60% Air changes: 12-14/hour Photoperiod: 12 hours light / 12 hours dark

Groups of 11-20 females were dosed by oral gavage from days 7 to 20 of gestation and from PND 1 to 22 at dose levels of 0, 0.01, 0.30 or 1.0 mg/kg bw/d ethylenethioiurea. The amount of ETU dosed during gestation was based on GD 7 body weights. F1 pups were dosed from weaning to sexual maturity at the same dose levels as their dams. Dose volume was 2 ml/100 g body weight.

Parental observations during gestation: Observations, bodyweights and food consumption daily;

Litters: Pups counted and checked for abnormalities.

Observations during lactation: Dam and pup bodyweights and food consumption: every 4 days; developmental parameters: pinna detachment, eye and ear opening and incisor eruption; Sexual development: vaginal opening from PND35 and preputial separation from PND42.

Blood sampling for thyroid biomarkers:

PND 1 and 23 dams bled by intracardiac puncture under isoflurane anaesthesia. Samples analysed for triiodothyronone (T3), thyroxine (T4) and thyroid stimulating hormone (TSH).

Day 23 blood samples taken from orbital sinus of pups after local anaesthesia with oxybuprocaine.

Termination: PND 23 dams and half pups killed by i.p. injection of pentothal. Thyroid and reproductive organs excised and weighed.

F1 selection: At least 10/sex/group selected. Dosed each day by oral gavage based on individual body weight to day 60 (males) or day 70 (females).

F1 observations: Body weight and food consumption every 2 days. PND 55-70: vaginal smears taken and examined from females to determine oestrus cycle.
F1 Blood sampling for thyroid and reproductive biomarkers:
PND 42 blood samples taken from orbital sinus after local anaesthesia with oxybuprocaine. Samples analysed for T3, T4 and TSH.
At full sexual maturity blood samples were taken by intracardiac puncture under isoflurane anaesthesia. Samples analysed for T3, T4 and TSH, testosterone (T, males only), dihyroxytestosterone (DHT) and oestradiol (E2, females only).
F1 Termination: Rats were killed by i.p. injection of pentothal and thyroid and reproductive organs excised and weighed. The thyroid was examined histologically.

Results
Effects in dams: The number of total litter resorptions was 0/11, 1/15 (6.7%), 1/14 (7%) and 4/20 (20%) in the control, 0.1, 0.3 and 1.0 mg/kg bw/day groups respectively. 1 female in the 1.0 mg/kg bw/day group had dystocia. In all treated groups >10% of dams had a gestation length of 20-21 days, whereas all the control dams had gestation length of 22-23 days.
Body weight gain during gestation (GD 7-21) was significantly higher than control in dams dosed with 0.1 and 0.3 mg/kg bw/day ETU. There were no effects on food consumption.
There were no significant effects of treatment on litter size. There were no stillborn pups in the control or 1.0 mg/kg bw/day groups but stillborn pups were found in the 0.1 and 0.3 mg/kg bw/day groups. Although not statistically significant, 20% of dams in the 1.0 mg/kg bw/day group showed a markedly reduced number of live pups (1-2 per litter) and, at necropsy, early resorptions were evidenced by the presence of corpora lutea.
During lactation body weight gain was significantly reduced, whereas food consumption was significantly increased, in in dams dosed with 0.1 and 0.3 mg/kg bw/day ETU which was considered to reflect the higher number of pups in these groups.
Relative thyroid weight was significantly higher in the 0.1 mg/kg bw/day group dams compared to control.

Table 5.8.1-60: General toxicity, pregnancy and lactation data for dams

Parameter	ETU (mg/kg bw/day)			
	0	**0.1**	**0.3**	**1.0**
Number of females	11	14	13	15
Number of deliveries before GD22	0%	**64%##**	**46%#**	13%
Weight gain GD 7-21 (g)	100±13	**127±10+**	**130±13+**	92±36
Weight gain GD 1-23 (g)	28±8	**9±14+**	**11±11+**	17±17
Food consumption GD 7-21 (g/day)	22±1	22±1	23±1	21±2
Food consumption GD 1-23 (g/day)	59±11	**70±6+**	**69±5+**	64±8
% Dams with resorptions	0%	0%	0%	20%
% Dams with still born pups	0%	21%	15%	0%
Litter size	11.5±2.8	13.6±2.8	13.1±2.7	10.7±5.5
PND 23 Thyroid weight absolute (g)	0.051±0.013	0.068±0.016	0.064±0.012	0.053±0.016
PND 23 Thyroid weight relative (x10^3)	0.18±0.05	**0.24±0.04+**	0.23±0.05	0.19±0.06

Mean ± SD. The table excludes dams with resorptions or dystocia from treated groups.
Statistically significant difference from control group, p<0.05 Fisher's Exact test
Statistically significant difference from control group, p<0.01 Fisher's Exact test
+ Statistically significant difference from control group, p<0.05, ANOVA and Bonferroni's correction

Dams histological and histomorphometrical analysis: Thyroid tissue for histological examination of dams with total litter resorptions or dystocia (from all treatment groups combined) was considered separately. These dams were killed on expected PND1, showed a significant increased frequency of reduced follicular lumen size and of reduced and/or absent colloid within the same follicles when compared with control dams (killed on PND 23).
For dams killed on PND 23, there was a statistically significant dose-dependent increase of vacuolisation of follicular epithelial cells, reduction in follicular lumen size, and reduction and/or absence of colloid in follicles. Quantitative analysis showed significant differences among treatment groups in the ratio between follicle area and colloid, colloid area, and follicular epithelium height. The ratio of follicle area and colloid was significantly higher in the1.0 mg/kg bw/day group than the controls. Follicular epithelium height was significantly in the 0.1 and 0.3 mg/kg bw/day ETU groups.
Biochemical evaluations: Significant differences in serum TSH, T3 and T4 values are shown in Table 5.8.1-62**.**

F I generation males: Thyroid histology showed a reduction in follicular lumen size, reduction and/or absence of colloid within thyroid follicles, and increased pyknotic nuclei PND 23. No significant differences were observed in body weight, thyroid and testis weight (relative and absolute) among treatment groups.
Histomorphometrical analysis showed a dose-dependent decrease in follicular density (P<0.0I). In addition, follicular maturation (expressed as the ratio between follicular epithelium area and number of nuclei) was significantly increased in the 0.3 and 1.0 mg/kg bw/day groups when compared with control.

F l male treated pups did not show any significant difference in serum T3 and T4 mean levels, whereas TSH mean levels were significantly higher in the 1.0 mg/kg bw/day group. PND 42. No significant effect was observed on body weight and timing of preputial separation. There was no significant difference in serum TSH concentrations. However, Serum T4 mean levels were significantly lower than control in the 0.3 mg/kg bw/day group.
PND 60. No effects on food consumption, body weight, thyroid and testis weight or quantitative histomorphometrical analysis. T3 values were significantly lower in the 0.1 and 0.3 mg/kg bw/ day groups than control. DHT was reduced in males treated with 0.3 mg/kg bw/day when compared with control.
F1 generation females: Histopathology of the thyroid showed a reduction in follicular lumen size, and reduction and/or absence of colloid within same thyroid follicles were found in treated females.
PND 23. No effects on body weight, thyroid or uterus weights Relative ovary weight significantly higher in low dose group than control. Female Fl pups showed no significant differences in serum T3 and TSH significantly lower in 0.3 mg/kg bw/day groups than in control.
PND 42. No effect on timing of vaginal patency or body weight, T3 or TSH. T4 concentration was significantly lower 0.3 mg/kg bw/day groups than in control.
PND 75. No effects on food consumption, body weight, thyroid, uterus, or ovary weights.
Monitoring of the oestrous cycle revealed a significant increase in the mean number of days per cycle (P<0.05) and a significant decrease in the mean number of cycles completed by the 17-day evaluation period in all treated groups when compared with control.
T3 value was significantly higher in the 0.1 and 0.3 mg/kg bw/day groups than in control.
Follicular epithelium height was significantly reduced in 0.1 mg/kg bw/day compared to control.

Table 5.8.1-61: Intergroup comparison of oestrus cycle – F1 generation

	ETU (mg/kg bw/day)			
	0	**0.1**	**0.3**	**1.0**
Number of females	**12**	**14**	**13**	**13**
Cycles per female	3.1±0.3	**1.4±0.7****	**1.0±0.7****	**0.8±0.7****
Oestrus cycle length (days)	4.8±0.4	10.0 ±3.9*	**8.7 ±3.8***	**8.4 ±3.9***
Days in proestrus	3.9±1.0	**2.8 ±1.1***	3.6±1.9	**1.5±1.1****
Days in oestrus	5.3±0.8	4.6±2.7	**3.1±1.9***	**7.8±2.5***
Days in metoestrus	4.0±0.6	5.9±2.7	**6.7±2.0***	**5.5±1.6****
Days in dioestrus	3.8±0.7	3.8±1.7	3.5±2.6	**2.1±1.4***

Mean ± SD
* Statistically significant difference from control group, p<0.05, ANOVA and Bonferroni's correction
** Statistically significant difference from control group, p<0.01, ANOVA and Bonferroni's correction

Table 5.8.1-62: Thyroid biomarkers and reproductive hormones – F1 generation

		ETU (mg/kg bw/day)							
		Males				Females			
Day	**Parameter**	**0**	**0.1**	**0.3**	**1.0**	**0**	**0.1**	**0.3**	**1.0**
PND 23	**Samples**	27	41	53	22	27	39	27	23
	T3	1 54±0.25	1.50±0.23	1.58±0.23	1.50±0.20	1.57±0.21	1.47±	1.52±0.23	1.72±0.27
	T4	43.0±4.7	41.7±5.3	39.1±7.6	44.6±7.6	44.5±6.3	40.4±5.3	**36.6±4.4***	44.3±5.7
	TSH	1.7±0.4	**2.9±1.0****	2 1±0.7	2.1±0.4	2.1±0.8	2.6±1.1	2.0±0.9	1.9±0.4
PND 42	**Samples**	14	15	15	19	15	15	15	13
	T3	1.75±0.07	**1.35±0.15****	**1.26±0.14****	1.62±0.16	1.50±0.08	1.57±0.17	1.48±0.18	1.53±0.15
	T4	58.8±5.4	57.8±6.3	**48.9±4.6****	59.7±5.8	44.2±8.5	36.6±6.7	**31.6±4 5****	41.0±8.3
	TSH	4.8±1.1	5.7±1.8	4.3±1.5	6.4±2.0	3.1±0.6	3.2±0.9	2.9±1 1	3.0±0.5
Males PND 60 Females PND 75	**Samples**	14	15	15	19	15	15	15	13
	T3	1 54±0.15	1.31±0.13*	1 34±0.15*	1.46±0.21	0.87±0.16	**1.63±0.19****	**1.38±0.22****	0.98±0.22
	T4	51.9±5.4	58.7±6.7	44.8±4.4	54.6±6.8	49.3±7.2	49.1±10.6	**33.5±4 9****	47.1±5.8
	TSH	5.9±2.0	8.3±3.0	7.5±2.0	8.3±3.4	2.7±0.8	**5.8±1.5****	3.6±2.0	3.0±0.5
	T	1 17±0.75	0.87±0.61	1.04±0.72	1.40±0.99	0.036±0.027	0.036±0.043	0.04±0.036	0.018±0.02
	DHT	0.60±0.13	0.52±0.12	**0.43±0.16****	0.55±0.07	-	-	-	-
	E2	-	-	-	-	0.039±0.013	0.032±0.010	**0.029±0.007***	**0.054±0.007****

* Statistically significant difference from control group, p<0.05
** Statistically significant difference from control group, p<0.01

Conclusion:
The authors believe this study has demonstrated effects on reproduction and the thyroid at doses lower than LOAEL dose exposure in pregnant dams and F1 generation pups. They suggest that even low doses of ETU can interfere with thyroid homeostasis and reproductive hormone profile if exposure starts in critical stages of development.

Applicant´s assessment:
This study contradicts the findings of (1992) (BASF DocID 1992/5000) and (2013) (BASF DocID 2013/7002198). In this study ETU was administered to pregnant rats from GD 7 to PND 22 by gavage at doses from 0.1 to 1.0 mg/kg bw/d. The authors reported that ETU caused total litter resorption or dystocia at all doses. Other effects, such as altered estrous cyclicity in offspring were also described. These results are not supported by the more extensive studies of (1992) and (2013). The adverse pregnancy outcomes described are also not supported by the prenatal developmental toxicity studies published by Khera, 1973 (1973/10099) and Chernoff, 1979 (BASF DocID 1979/10167). Here doses encompassed those used by Maranghi et al (2013) and the gavage route of exposure was also employed but no litter resorption or problems with pregnancies occurred at any dose. It is suggested that the finding described by Maranghi are spurious in nature.

Report: CA 5.8.1/21
Lemos P.V.R.B. et al., 2012a
Hepatic damage in newborns from female rats exposed to the pesticide derivative Ethylenethiourea
2012/1368183
Guidelines: none
GLP: no

Executive Summary:

In order to evaluate the hepatic morphological abnormalities in newborns from four female Wistar rats exposed to ethylenethiourea, Wistar rats were exposed to 1% ETU (125 mg/kg bw) via gavage on the 11th day of gestation. Control animals received 0.9% physiological solution. Caesarean section was performed on the 20th day of gestation. The newborns' livers were examined and any morphological-histological abnormalities were registered. The presence of megakaryocytes was quantified in 50 microscope fields, as the total number of these cells per mm². Megakaryocytes are cells that present a multilobulated nucleus without a nucleolus, with eosinophilic homogenous cytoplasm. They histologically characterize hematopoiesis well.
The entire treated group (34 animals) presented a variety of muscle and skeletal abnormalities, in association with abnormalities of the digestive tract. All of them also presented characteristic fluid accumulations in extracellular tissues and in the abdominal cavity, skin edema and an enlarged barrel-shaped abdomen, thus characterizing a condition of fetal hydrops. No macroscopic abnormalities were observed in any of the 21 newborns in the control group. In the control group, the surgical findings showed that the organs were complete, and the livers of the newborns presented anatomically conserved lobe structures, with a firm consistency and characteristic coloration. In the group exposed to ethylenethiourea, the hepatic abnormalities were manifested as distorted morphology, edema and enlarged volume in 74%, and as altered consistency, presenting friability, without any increase in volume in 26% of the newborns, along with a change in coloration, to wine red. The histopathological analysis showed that morphological-histological hepatic destruction had occurred in all entire experimental with removal of the hepatic trabeculae and severe hepatic megakaryocytosis. The mean megakaryocyte density ranged from 107.9 to 114.2 per mm², and it was eight times greater than in the control group, thus characterizing a situation of extramedullary hematopoiesis.

Conclusion of the author:

Exposure of pregnant rats to the pesticide derivative ETU gave rise to fetal hepatic damage, with congestion, hepatic friability and morphological-histological hepatic destructuring in all the newborns exposed to ethylenethiourea, with removal of the hepatic trabeculae and severe hepatic megakaryocytosis, thus characterizing extramedullary hematopoiesis. The latter was shown to be an important predisposing factor for hydrops and low fetal weight.

Conclusion of the applicant:

After single treatment with 125 mg/kg bw ETU of pregnant female Wistar rats on gestation day 11 the newborns show clear hepatic damage. No data was found about the maternal toxicity. Since the developmental NOAEL of ETU is defined to be at 15 mg/kg bw/day these data show no new aspects because the rats were treated with 125 mg/kg bw and no data about maternal toxicity was given. Therefore, this study is considered to be not relevant for human risk assessment but is supplementary information on ETU effects.
Classification of the study: Supplementary information

Conclusion on developmental toxicity

ETU was found to be teratogenic in rats. Also in the new rabbit developmental toxicity study, no teratogenicity was observed. ETU is teratogenic in rats (Khera, 1987), causing primarily, neural tube abnormalities, but the mechanism of teratogenicity appears to be unrelated to its effects on thyroid hormones. Experiments in rats where thyroid hormones were adjusted did not alter the teratogenicity of ETU (Lu and Staples, 1978) and direct addition of ETU to rat embryos in vitro gave similar malformations to those observed in vivo (Daston, 1990). Khera (1987) concluded that ETU-induced teratogenicity was not related to reduction of thyroid hormones. This is further supported by the absence of teratogenicity induced by treatment of pregnant rats with potent antithyroid drugs propylthiouracil and methimazole (Mallela et al (2014), BASF DocID 2014/1324212, Shibutani, 2009, BASF DocID 2009/1131863, Axelstad, 2008, BASF DocID 2008/1102636)

Ethylene thiourea is registered as a REACH Tier 1 substance, it is covered by Index number 613-039-00-9 in Regulation (EC) No. 1272/2008 and classified in Annex VI, part 3, Table 3.1 as harmful if swallowed (Acute Tox. 4, H 302) and toxic for reproduction, Repr. 1B (H360D: "May damage the unborn child"). The corresponding classification in Annex VI to this regulation, part 3, Table 3.2 (the list of harmonized classification and labelling of hazardous substances from Annex I to Council Directive 67/548/EEC) is Xn, R22 and Repr.Cat. 2, R61 ("May cause harm to the unborn child"). Ethylene thiourea is not classified for carcinogenicity. An Annex XV Dossier has been submitted, proposing to consider ethylene thiourea as a SVHC substance, based on CMR properties.

Mechanistic studies conducted with ETU

A number of mechanistic studies has been conducted with ETU. Some of them were already included in the existing Monograph. The additional studies (either already discussed in the Monograph, publically available or newly conducted) are sorted by type of toxicity:

1. Studies are summarized investigating the thyroid mode of action
2. Other endocrine mode of action
3. Liver mode of action
4. Immunotoxicity

ETU is well known to inhibit the thyroid peroxidase, however the inhibition reaction does not involve a covalent binding to the thyroid peroxidase, as well as there is evidence for a reversibility of the reaction.

Supplementary studies on ETU as extracted from Monograph

Thyroid effects

Groups of 50 male and 50 female Sprague-Dawley rats were fed diets containing 0, 75, 100 or 150 ppm ETU (purity not stated) for 7 weeks. Ten rats from each group were killed at 7 weeks and at 2, 3 and 4 weeks (20 rats) post-dosing on control diets in order to assess the extent of effect on the thyroid and the subsequent reversibility of these effects. Thyroid weight, brain weight, serum T3 and T4 levels (0 and 150 ppm groups only) were measured at term. All animals were necropsied and thyroids examined histologically. No apparent clinical signs of toxicity were observed. At 7 weeks food consumption was decreased in all treated groups and body weights decreased with increasing dose, while thyroid weights (absolute and relative) increased in both sexes. T3 levels were somewhat variable, while T4 levels were significantly decreased at 150 ppm in both sexes. The magnitude of the changes in body weight, thyroid weight, and T4 blood levels were decreased after ETU was removed from the diet indicating at least partial reversibility of the ETU-induced effects. Histopathological findings included a consistently reduced colloid content of thyroid acini in the 150 ppm groups after 7 and 9 weeks on test. Acinar epithelial cell size and height were not different from control. Two tumours were identified in the 150 ppm male group after 7 weeks on test: a follicular cell adenoma and a medullary carcinoma. The authors conclude that the relationship between the duration of exposure to ETU and the possible reversibility of various thyroid lesions requires further study.
(Arnold et al, 1983, BASF DocID 1983/10162).

In two 90 day feeding trials, Sprague-Dawley rats (12/sex/group) were given 75 or 100 ppm ETU, and parameters of thyroid function, serum T4, T3, and TSH, T3 uptake in vitro, 131I uptake, and thyroid to body weight ratios were measured at days 46 and 91. Additionally, the fate of the incorporated 131I was traced in thyroid fractions (100 ppm level). Groups of both sexes at the lower feeding level and the females at 100 ppm were functionally euthyroid whereas males at the 100 ppm level were somewhat hypothyroid despite elevated serum T3, TSH, and T3/T4 ratios. Results showed that the inhibitory effects of ETU are similar to those for methimazole. ETU inhibited monoiodotyrosine (MIT) utilization, and the coupling of diiodotyrosine (DIT) residues to form T4, resulting in significantly reduced active synthesis of T3 and T4 prohormones (males, 100 ppm trial). The capacity of the serum to bind T3 was reduced; however, in contrast to propylthiouracil, there was no evidence for inhibition by ETU of T4 to T3 monodeiodination or interference with the normal feedback mechanisms of thyroid hormones on TSH secretion.
(O'Neil and Marshall, 1984, BASF DocID 1984/10115).

The mechanism of thyroid peroxidase inhibition by ETU was studied in vitro using purified thyroid peroxidise obtained from dog thyroid. ETU inhibited iodination reactions catalysed by thyroid peroxidase. Inhibition occurred only in the presence of iodide ion and proceeded with concomitant oxidative metabolism of ETU to imidazoline and bisulfite ion. The inhibition ceased upon consumption of ETU, with no loss of enzymatic activity and negligible covalent binding of ETU to the enzyme. This reversible thyroid peroxidase inhibition contrasts with the activity of the therapeutic antithyroid drugs such as methimazole which act as suicide inhibitors via covalent binding to the prosthetic heme group.
(Doerge and Takazawa, 1990, BASF DocID 1990/10618).

During the literature search, other papers were identified, which also had tested ETU for its thyroid peroxidase inhibiting properties:

Report: CA 5.8.1/22
Freyberger A.,Ahr H.-J., 2005a
Studies on the goitrogenic mechanism of action of N,N,N,N-Tetramethylthiourea
2006/1051537
Guidelines: none
GLP: no

Executive Summary:
Interactions of .ethylenethiourea (ETU) with thyroid peroxidase (TPO) were investigated using a partially purified fraction from hog thyroids or solubilized hog thyroid microsomes and 10,000g supernatant from rat liver homogenate as enzyme sources. ETU only marginally affected TPO-catalyzed oxidation of guaiacol but concentration-dependently suppressed TPO-catalyzed iodine formation with concomitant oxidative metabolism. Suppression ceased upon consumption of thiourea derivatives and the rate of reappearing iodine formation was similar to that of controls. ETU also suppressed non-enzymatic and TPO-catalyzed monoiodination of L-tyrosine with a stoichiometry of 2:1 i.e. one molecule of ETU suppressed two times monoiodination. ETU was unable to irreversibly inhibit TPO.

Objective:
To investigate the goitrogenic mechanism of action of N,N,N',N'-tetramethylthiourea (TMTU). Ethylenethiourea (ETU), propylthiouracil (PTU) and amitrole were used for comparison.

Materials and Methods:
ETU (98%) was obtained from Aldrich (Taufkirchen, Germany). Partially purified TPO and preparations of solubilized microsomes were prepared from hog thyroids. Guaiacol oxidation was used as a measure for peroxidase activity. The following determinations were performed: TPO-catalyzed iodine formation; TPO-catalyzed and non-enzymatic L-tyrosine monoiodination; TPO-catalyzed in vitro metabolism of thiourea derivatives; irreversible inhibition of TPO by thionamides.

Results:
ETU gave only a marginal inhibition of the oxidation of guaiacol at the highest concentration tested (1mM) whilst PTU effectively inhibited guaiacol oxidation in a concentration-dependent manner. When iodine was used as the substrate, ETU concentration-dependently suppressed TPO-catalyzed iodine formation for a time and then iodine formation reappeared with a rate similar to that of controls.

ETU concentration-dependently suppressed TPO-catalyzed and non-enzymatic monoidination of L-tyrosine to monoiodotyrosine (MIT) (see Table below)

Table 5.8.1-63: Suppression of TPO-catalysed and non-enzymic iodination by ETU

Assay condition	Test compound	MIT formed (µM)	MIT suppressed (µM)
Enzymatic iodination (Experiment 1)	Control ETU 10 (µM) ETU 30 (µM)	37.7 ± 4.0 14.9 (15.4, 14.3) Not detectable	- 22.8 37.7
Enzymatic iodination (Experiment 1)	Control ETU 5 (µM) ETU 10 (µM) ETU 50 (µM)	44.7 ± 0.4 36.6 (36.5, 36.6) 26.2 (26.4, 25.9) 17.0 (17.4, 16.6)	- 8.1 18.5 27.7
Non-enzymatic iodination	Control ETU 5 (µM) ETU 10 (µM) ETU 50 (µM)	28.4 ± 0.7 18.0 (17.9, 18.0) 8.2 (8.1, 8.2) Not detectable	- 10.6 20.2 28.4

Values are means ± SD. If the means are from duplicate incubations then individual values are in brackets.

ETU was shown to be a substrate for TPO and was completely metabolised in complete incubations with TPO. In the presence of a TPO inhibitor (amitrole) or absence of enzyme, H2O2 or KI then ETU was not metabolised. As long as ETU was present in the incubations hardly any iodine formation occurred, but once ETU was metabolised iodine formation reappeared.

To test for irreversible TPO inhibition, ETU was incubated with TPO in the presence of H_2O_2 and then TPO isolated from the assay system and activity tested using guiacol as substrate. ETU did not affect TPO activity under these conditions, unlike amitrole that caused irreversible inhibition.

Conclusion:
The data provide evidence with that ETU interferes with thyroid hormone synthesis at the level of iodination and demonstrate a metabolic route for the oxidative detoxification of ETU in the thyroid suggesting that low-level or intermittent exposure to ETU would have only minimal effects on thyroid hormone synthesis.

There are further mechanistic studies in eleuterembryos and in amphibian available studying the interference of ETU with the thyroid (Thienpont et al, 2011 – BASF DocID 2011/1297251, Thienpont, 2013 – BASF DocID 2013/1419082, Opitz et al., 2005 – BASF DocID 2005/1043721, Opitz et al., 2006 – BASF Doc ID 2006/1051535, Opitz et al., 2006 – BASF DocID 2006/1051534). While effects on thyroid hormone system were seen in the studies a direct correlation between the aquatic/amphibian species and mammals remains to be established.

Report: CA 5.8.1/23
Paul K.B. et al., 2013a
Cross-species analysis of Thyroperoxidase inhibition by xenobiotics demonstrates conservation of response between pig and rat
2013/1419086

Guidelines: none

GLP: no

Summary:
TPO inhibition was studied in rat and porcine microsomes in order to investigate species differences. Twelve chemicals were tested in a guaiacol oxidation assay, where the time-course of the reaction was monitored (ETU was not included). All chemicals that inhibited TPO in porcine microsomes also inhibited TPO in rat microsomes. PTU was more potent on rat TPO compared with porcine TPO. Results show that rat TPO shows greater sensitivity to inhibition by a thiourea compared to pig TPO. This indicates that rat may more responsive than pig to this class of chemicals.

Report: CA 5.8.1/24
Paul K.B. et al., 2014a
Development of a Thyroperoxidase inhibition assay for high-throughput screening
2014/1323274
Guidelines: none
GLP: no

Executive Summary:
The paper describes the development of a high throughput screen (HTS) to detect inhibitors of thyroperoxidase (TPO). Ethleylene thiourea (ETU) was used as a positive control TPO inhibitor. ETU was positive in the Amplex UltraRed TPO inhibition assay.

Objective:
To test the hypothesis that a commercial fluorescent horse-radish peroxidase substrate, Amplex UltraRed, could be used to detect TPO activity in thyroid microsomes obtained from rats to generate an in vitro 384-well format assay that can be used for HTS.

Materials and Methods:
Thyroid microsomes were prepared from thyroids removed from male Long Evans rats. ETU was obtained from TCI (no information on purity). Fifteen concentrations were tested (final concentrations: 0, 0.000053, 0.00016, 0.00048, 0.0014, 0.013, 0.039, 0.13, 0.35, 1.0, 3.1, 9.4, 28, 84 and 253 µM). The assay was run on three separate days, with freshly prepared reagents each day, and each with three separate pooled lots of thyroid microsomes for 3 biological replicates or n = 3. The Amplex UltraRed TPO inhibition assay was used to detect peroxidase activity in the presence of excess H2O2. Fluorescence was measured using 10 flashes per well at 544 nm/590 nm excitation/emission with the fluorometer in the top position.

Results:
ETU, a known TPO inhibitor, was positive in the Amplex UltraRed TPO inhibition assay.

Conclusions:
ETU was positive in the Amplex UltraRed TPO inhibition assay consistent with its activity as an inhibitor of TPO.

Report: CA 5.8.1/25
Thienpont B. et al., 2011a
Zebrafish Eleutheroembryos provide a suitable vertebrate model for screening chemicals that impair thyroid hormone synthesis
2011/1297251
Guidelines: none
GLP: no

Executive Summary:

This study evaluated the suitability of zebrafish eleutheroembryos as a potential generalized vertebrate model in Tier 1 screening batteries for detecting molecules that impair thyroid hormone synthesis. The taxonomic similarity of the screening model organism to humans is not a primary concern for a Tier 1 screening assay, so zebrafish eleutheroembryos, commonly used in biomedical research, represent a logical candidate. Recently, the authors developed a simple, rapid zebrafish eleutheroembryo bioassay for assessing the potential of chemical pollutants and drugs to disrupt thyroid gland function. This bioassay used a T4 immunofluorescence quantitative disruption test (TIQDT) to measure impairment of the thyroid function as a decrease in the intrafollicular T4-content (IT4C). For evaluation of this test several chemicals with known thyroid concerning effects and no thyroid effects were used. ETU was one of them. Since ETU is relevant for this dossier the results of ETU are presented in the following. IT4C in eleutheroembryos exposed to ETU was significantly lower than in controls ($p<0.05$), therefore this compound was classified as thyroid gland function disruptor (TGFD) on zebrafish eleutheroembryos which corresponds to the known data.

Conclusion of the author:

TIQDT performed on zebrafish eleutheroembryos is an alternative whole-organism screening assay that provides relevant information for environmental and human risk assessments.

Conclusion of the applicant:

In this study an alternative method for the evaluation of the impairment of thyroid hormone synthesis is described. In order to verify this method ETU, a known thyroid gland function disruptor, was one of the positive controls. That ETU effects thyroid function is known and discussed in this dossier. Since this is no new information but supports the known information this study is considered to be not relevant for human risk assessment but is supplementary information on ETU effects.
Classification of the study: Supplementary information

Report: CA 5.8.1/26
Thienpont B., 2013a
Modeling mixtures of thyroid gland function disruptors in a vertebrate alternative model, the zebrafish eleutheroembryo
2013/1419082
Guidelines: none
GLP: no

Executive Summary:

The present study used the intrafollicular T4-content (IT4C) of zebrafish eleutheroembryos as integrative endpoint for testing the hypotheses that the effect of mixtures of thyroid gland function disruptors (TGFDs) with a similar mode of action [inhibition of thyroid peroxidase (TPO)] was well predicted by a concentration addition concept (CA) model, whereas the response addition concept (RA) model predicted better the effect of dissimilarly acting binary mixtures of TGFDs [TPO-inhibitors and sodium-iodide symporter (NIS)-inhibitors]. MMI, PTU, BP2, phloroglucinol, SMX, amitrole, and ETU were selected as representative of TPO-inhibitors. Potassium perchlorate ($KClO_4$) and potassium thiocyanate (KSCN) were selected as NIS-inhibitors. However, CA model provided better prediction of joint effects than RA in five out of the six tested mixtures. The exception being the mixture MMI (TPO-inhibitor)-$KClO_4$ (NIS-inhibitor) dosed at a fixed ratio of EC10 that provided similar CA and RA predictions and hence it was difficult to get any conclusive result.

Conclusion of the author:

The results support the phenomenological similarity criterion stating that the concept of concentration addition could be extended to mixture constituents having common apical endpoints or common adverse outcomes.

Conclusion of the applicant:

Zebrafish model is increasingly used as an intermediate step between in vitro cellular system and mammalian models. A concordance between TIQDT on zebrafish and mammalian data was demonstrated. However, the direct translatablity is not straightforward. Thus currently it is difficult to compare dietary dose levels (mg/kg/day) in humans or mammalian models with aquatic test concentrations (mg/L). Therefore, this study is considered to be not relevant for human risk assessment but is supplementary information on ETU effects.
Classification of the study: Supplementary information

Report: CA 5.8.1/27
Opitz R. etal., 2004a
Description and initial evaluation of a Xenopus metamorphosis assay for detection of thyroid system-disrupting activities of environmental compounds
2005/1043780
Guidelines: none
GLP: no

Executive Summary:

The general aim of the present study was to develop the conceptual and methodological framework of a standardized amphibian testing approach for detection of thyroid system–disrupting effects by environmental compounds. This issue was addressed by exploiting anuran postembryonic development as a biological model. The underlying idea of this amphibian metamorphosis assay is that potential thyroid system–disrupting effects become evident in *X. laevis* tadpoles as morphological alterations during metamorphosis due to the obligatory dependence of normal metamorphic development on undisturbed function of the thyroid system. A test protocol for a *Xenopus* metamorphosis assay (XEMA) was developed and its interlaboratory transferability was evaluated in an informal ring test with six laboratories participating. In the XEMA test, exposure of *Xenopus laevis* tadpoles was initiated at stages 48 to 50 and continued for 28 d. Development and growth of tadpoles were assessed by means of developmental stage and whole body length determinations, respectively. For initial test protocol evaluation, thyroxine (T4), and propylthiouracil (PTU) were used as positive controls for thyroid system–modulating activity. Exposure of tadpoles to 1 mg/L T4 produced a significant acceleration of metamorphosis whereas PTU concentrations of 75 and 100 mg/L completely inhibited metamorphosis. Ethylenethiourea was used as a test substance during the initial evaluation of the XEMA test because of its reported antithyroidal activity in mammals. To the best of our knowledge, this is the first study reporting antithyroidal activity of ETU in amphibian tadpoles. A main target organ of ETU is the thyroid gland. In the XEMA test, a concentration-dependent inhibition of metamorphic development was observed for ETU. The highest test concentrations of ETU (50 and 100 mg/L) completely inhibited development at stages 53/54, indicating that these concentrations were sufficient to cause an almost complete inhibition of TH synthesis in *X. laevis* tadpoles. Lower concentrations of ETU (10 and 25 mg/L) also showed partial inhibitory effects on development. Although it is difficult to relate observations made in this study with results from mammalian exposure studies, the data indicate elevated sensitivity of the testing approach used in XEMA to detect the antithyroidal activity of ETU. Exposure of male Wistar rats for 28 d to 100 to 300 mg/L ETU in drinking water caused significant reductions in serum T4 and T3 concentrations that were accompanied by elevated plasma thyroid-stimulating hormone levels, but these changes were not reflected in thyroid gland morphology when assessed by light microscopy. Preliminary results from histological analyses of thyroid glands dissected from *X. laevis* tadpoles after ETU exposure during XEMA revealed morphological alterations at ETU concentrations in the lower mg/L range (R. Opitz, unpublished data). None of the compounds affected tadpole survival, and only PTU caused a slight retardation in tadpole growth.

Conclusion of the author:
This study demonstrates that the XEMA test provides a sensitive, robust, and practical testing approach for detection of compounds with both agonistic and antagonistic effects on the thyroid system in *Xenopus* tadpoles.

Conclusion of the applicant:
In this paper a new method for detection of compounds with agonistic and antagonistic effects on the thyroid system was developed. This test system is done in Xenopus and seems to be sensitive and robust. A correlation to mammalians could has to be further observed. Therefore, this study is considered to be not relevant for human risk assessment but is supplementary information on ETU effects.
Classification of the study: Supplementary information

Report: CA 5.8.1/28
Opitz R. et al., 2005a
Evaluation of histological and molecular endpoints for enhanced detection of thyroid system disruption in Xenopus laevis tadpoles
2006/1051535
Guidelines: none
GLP: no

Executive Summary:

Amphibian metamorphosis represents a promising model for the identification of thyroid system–disrupting chemicals due to the pivotal role played by thyroid hormones for the initiation and regulation of metamorphosis. An important aspect of bioassay development is the identification and evaluation of sensitive and diagnostic endpoints. In this study, several morphological, histological, and molecular endpoints were evaluated for their utility to detect alterations in thyroid system function after exposure of stage 51 *Xenopus laevis* tadpoles to various concentrations (1.0, 2.5, 10, 25, and 50 mg/L) of the anti-thyroidal compound ethylenethiourea (ETU). Analysis of developmental stages on exposure day 20 and monitoring of time to fore limb emergence (FLE) revealed retardation and complete arrest of tadpole development at 25 mg/L and 50 mg/L ETU, respectively. Development was not affected by 1.0, 2.5, and 10 mg/L ETU. Histological alterations in the thyroid gland were observed in FLE-displaying tadpoles after exposure to 2.5, 10, and 25 mg/l ETU, as well as in developmentally arrested tadpoles exposed to 50 mg/L ETU. Prevalence and severity of histological changes increased in a concentration dependent manner. Semiquantitative reverse transcriptionpolymerase chain reaction (RT-PCR) showed increased mRNA expression of the α- and β-subunits of thyroid-stimulating hormone (TSHα, TSHβ) in pituitary tissue of tadpoles exposed to 25 and 50 mg/L ETU. This RT-PCR was done because in mammalian studies, measurements of plasma concentrations of T3 , T4 and thyroid-stimulating hormone (TSH), combined with histopathological and morphometric analyses of the thyroid gland, represents a common approach to detect anti-thyroidal effects of ETU. In tadpoles it is difficult to get blood samples therefore analysis of TH-dependent gene expression in peripheral tissue might provide an alternative means of assessing thyroidal status indirectly in X. laevis tadpoles.

Conclusion of the author:

Results of this publication demonstrate the successful detection of anti-thyroidal effects of ETU in *Xenopus laevis* tadpoles using various endpoints and highlight the particular sensitivity of thyroid gland histology to detect thyroid system disruption in tadpoles. The authors showed that metamorphic retardation caused by ETU is associated with concentration-dependent histological changes in the thyroid gland and increased mRNA expression levels of TSHβ in the pituitary.

Conclusion of the applicant:

In this publication the authors present the anti-thyroidal effects of ETU in *Xenopus laevis* tadpols. This is a new fundamental research concerning the molecular effects of ETU on thyroid hormones. Since this study is done in amphibians and a correlation to the mammalian system is not evaluated this study is considered to be not relevant for human risk assessment but is supplementary information on ETU effects.
Classification of the study: Supplementary information

Report: CA 5.8.1/29
Opitz R. et al., 2006a
Expression of sodium-iodide symporter mRNA in the thyroid gland of Xenopus laevis tadpoles: Developmental expression, effects of antihyroidal compounds, and regulation by TSH
2006/1051534
Guidelines: none
GLP: no

Executive Summary:

The uptake of iodide represents the first step in thyroid hormone synthesis by thyroid follicular cells and is mediated by the sodium-iodide symporter (NIS). In mammals, expression of NIS is stimulated by TSH and transcription of the NIS gene involves regulation by the thyroid-specific transcription factors Pax8 and Nkx2·1. In this study, the authors examined the mRNA expression of NIS, Pax8 and Nkx2·1 in the thyroid gland of Xenopus laevis tadpoles by semiquantitative reverse transcriptase (RT)-PCR. During spontaneous metamorphosis, NIS mRNA expression was low in premetamorphic tadpoles, increased throughout prometamorphosis, and peaked at climax stage 60. Analysis of TSH β–subunit (TSHβ) mRNA in the pituitary of the same tadpoles revealed a close temporal relationship in the expression of the two genes during metamorphosis, suggesting a regulatory role of TSH in the developmental expression of NIS. Treatment of tadpoles with goitrogenic compounds (sodium perchlorate and ethylenethiourea) increased TSHβ mRNA expression (approximately twofold) and caused thyroid gland hyperplasia, confirming that feedback along the pituitary–thyroid axis was operative. Analysis of gene expression in the thyroid gland revealed that goitrogen treatment was correlated with increased expression of NIS mRNA (≈20-fold). In the thyroid gland organ culture experiments, bovine TSH (bTSH; 1 mU/mL) strongly induced NIS mRNA expression. This effect was mimicked by co-culture of thyroid glands with pituitaries from stage 58 tadpoles and by agents that increase intracellular cAMP (forskolin, dibutyryl-cAMP). In addition, it could be shown that thyroid glands of X. laevis tadpoles express Pax8 and Nkx2·1 mRNA in a developmentally regulated manner and that ex vivo treatment of thyroid glands with bTSH, forskolin, and cAMP analogs increased the expression of Pax8 and Nkx2·1 mRNA.

Conclusion of the author:

This is the first report on developmental profiles and hormonal regulation of thyroid gland gene expression in amphibian tadpoles and, together, results reveal a critical role of TSH in the regulation of NIS mRNA expression in the thyroid gland of *X. laevis* tadpoles. The developmental NIS mRNA expression profile in the thyroid gland was closely related to previously reported profiles of other well-established markers of thyroid gland activity such as thyroidal iodide uptake, T4 secretion, and thyroid gland histology. This suggests that NIS mRNA expression could represent a valuable molecular marker of thyroid gland activity in metamorphosing X. laevis tadpoles.

Conclusion of the applicant:

In this publication the authors present the first characterization of the developmental profile and hormonal regulation of NIS, Pax8 and NKx2·1 gene expression in the amphibian thyroid gland. This is a new fundamental research concerning the molecular effects of ETU on thyroid hormones. Since this study is done in amphibians this study is considered to be not relevant for human risk assessment but is supplementary information on ETU effects.
Classification of the study: Supplementary information

Comparison of ETU with other TPO inhibitors
Several other direct acting TPO inhibitors are known. These include the pharmaceuticals Propylthiouracil and methimazole and the herbicide amitrole (Howdeshell, 2002, BASF DocID 2002/1027618, Freyburger & Ahr BASF DocID 2006/1051537). However these compounds are generally more potent than ETU and the mode of action between ETU and the others is somewhat different. Doerge and Takazawa, 1990 (BASF DocID 1990/10618) and Freyberger and Ahr, 2006 (BASF DocID 2006/1051537) showed that ETU is a reversible inhibitor of TPO that is itself metabolized by TPO. Once ETU has been consumed the enzyme resumes oxidation of iodide. ETU did not covalently bind to TPO (Doerge and Takazawa, 1990, BASF DocID: 1990/10618). This is unlike the MoA for propylthiouracil and methimazole, which are suicide substrates of TPO (Taurog, 1989, BASF DocID 1989/1003743). Freyburger and Ahr, 2006 (BASF DocID 2006/1051537) also showed that amitrole – in contrast to ETU - is an irreversible inhibitor or TPO that is not metabolised by TPO (see above). In vivo, the reversibility of the action of ETU has been shown 4 weeks after a 7-week exposure of rats to ETU (Arnold et al., 1983, BASF DocID 1983/10162).

In order to compare the potency of ETU and propylthiouracil to induce thyroid(-related) toxicity in rats, two studies conducted with propylthiouracil are summarized in more detail in this chapter (one is a developmental neurotoxicity study conducted with a BASF herbicide, where propylthiouracil was used as a positive control, and the other is a published study by Axelstad, 2008, who investigates the thyroid and neurodevelopmental effects of propylthiouracil).

Report:	CA 5.8.1/30 [redacted] 2011b BAS 455 H (Pendimethalin) - Developmental thyroid study in the Sprague-Dawley rat - Oral administration (diet) 2011/1276730
Guidelines:	EPA Guidance for thyroid assays in pregnant animals fetuses and postnatal animals and adult animals (24 October 2005)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

For the purpose of this dossier only the data on the positive control substance (propylthiouracil) are summarized. In this extensive study as positive control data comparative developmental thyroid studies with 6-propyl-2-thiouracil (PTU) was performed. In this study summary the focus lies on these developmental thyroid studies with (PTU).
In two separate developmental thyroid studies, the relative sensitivity and potential differences in response to treatment of the Sprague-Dawley and Wistar rat strains were investigated using 6-propyl-2-thiouracil (PTU), a known thyroid modulator. For both studies, time-mated females were dosed by gavage, once daily, from day 6 post coitum (GD 6) through to day 21 post partum (PND 21), at dosages of 0.0, 0.1 or 2.5 mg/kg/day. Thyroid hormones (T3, T4 and TSH) were measured in dams on GD 20 and PND 21 and in fetuses/pups on GD 20, PND 4 and PND 21. At termination, thyroid weights were determined for the dams and histopathology of the thyroids was evaluated in both dams and fetuses/pups.
The results showed a clear dose response for the thyroid hormones in both rat strains. T4 was decreased and TSH increased for dams, fetuses and pups at all time points measured. T3 values were reduced at the high dose in dams on GD 20 and pups on PND 4 and 21. The dams, fetuses and pups of both rat strains showed dose-dependent and comparable incidences and severity of diffuse hypertrophy/ hyperplasia of the thyroid gland follicular cells and the morphological effects observed correlated well with the hormone effects. The results obtained in these two studies showed no obvious differences between the Wistar and Sprague-Dawley rat strains. The results demonstrated that the methods used had sufficient sensitivity to detect treatment-related changes in thyroid hormones in dams, fetuses and pups at multiple time points during gestation and lactation.

(DocID 2011/1276730)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	6-propyl-2-thiouracil (PTU)
Description:	no data
Lot/Batch #:	no data
Purity:	no data
Stability of test compound:	The test substance formulations were stable over 4 hours at room temperature

2. Vehicle control:	highly deinonized water
3. Test animals:	
Species:	Sprague-Dawley and Wistar rats
Strain:	Crl:CD(SD) and Crl:WI(Han)
Sex:	Time mated female
Age:	10 to 12 weeks
Weight at dosing:	153.1 – 280.2 g (Sprague-Dawley) 139.7 – 188.9 g (Wistar)
Source:	[redacted]
Acclimation period:	6 days
Diet:	Maintenance diet for mouse/rat, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water ad libitum
Housing:	individual housing in Makrolon type MIII (Becker & Co, Castrop-Rauxel, Germany) for Sprague Dawley rats; Wistar rats were housed individually in wire cages (Type DK III, Becker & Co., Castrop-Rauxel, Germany) up to GD18 and were then transferred to Macrolon cages (Type M III, BECKER & Co., Castrop-Rauxel, Germany) for the birth and rearing of their litters For enrichment wooden gnawing blocks (Typ NGM E-22; Abedd® Lab. and Vet. Service GmbH, Vienna, Austria) were provided for Sprague-Dawley rats.
Environmental conditions:	
Temperature:	20 - 24°C
Humidity:	30 - 70%
Air changes:	no data
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN AND METHODS

The study was conducted at BASF Aktiengesellschaft, Experimental Toxicology and Ecology, 67056 Ludwigshafen, Germany.

1. Dates of experimental work: no data

2. Animal assignment and treatment:

PTU was administered to 35 presumed pregnant female Sprague-Dawley rats or Wistar rats by gavage at doses of 0.1 and 2.5 mg/kg bw/day from gestation day 6 (GD) through post natal day 21 (PND). Control animals were similarly dosed with the vehicle, highly deionized water. Ten dams per group were killed on GD 20 and the remaining dams were allowed to litter and rear their pups to PND 4 or PND 21 Animals were randomly distributed to the test groups.

3. Test substance preparation and analysis:
For both studies, 6-propyl-2-thiouracil was obtained from Sigma-Aldrich, Taufkirchen, Germany and was formulated with highly deionized water to provide solutions containing either 1 mg/100 mL or 25 mg/100 mL. The dose formulations were prepared on one occasion at the beginning of each study, divided into daily portions and deep-frozen until use. The stability of the test substance formulations at room temperature, over a period of 4 hours, was verified in a separate study and an analysis of the concentration of the test substance preparations was performed using samples taken from each concentration at the beginning and end of the administration periods.

4. Statistics:
Means and standard deviations were calculated. In addition, the following statistical analyses were performed:
Dunnett test (two-sided, $^{**}p<1\%$, $^{*}p<5\%$): food consumption, body weights and body weight change (dams and pups); number of pups delivered per litter; duration of gestation.
Wilcoxon test: number of live and dead pups; viability index and lactation index. Fisher's exact test: thyroid hormone data.
Kruskal-Wallis and Wilcoxon tests: body weights for anesthetized animals, absolute and relative thyroid weights.

C. Methods

1. Insemination:
The animals were paired by the breeder ("time mated") and supplied on GD 0.

2. Treatment:
The pregnant animals and their litters continuously received the test substance at the appropriate concentrations by gavage from GD 6 to PND 21.

3. Observation:
All animals were checked at least once daily for any signs of morbidity, pertinent behavioral changes and signs of overt toxicity. Littering and lactation behavior were observed but only abnormal behaviors were noted.

4. Body weight:
Body weight was recorded at regular intervals throughout pregnancy and lactation.

5. Food consumption:
Food consumption were recorded at regular intervals throughout pregnancy and lactation.

6. Clinical pathology
Dams: For the dams, blood samples were collected on GD20 prior to sacrifice by puncturing the retro-orbital venous plexus, in conscious animals. On PND 21, blood samples were withdrawn from at least 20 surviving dams.
Triiodothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH) levels were measured in the serum samples obtained.

Offspring: For the PND 21 pups (from one male and one female pup per litter), blood samples were collected by puncturing the retro-orbital venous plexus, in conscious animals. For the GD 20 fetuses and PND 4 pups, blood samples were collected from the vena cava cranialis, after decapitation. Triiodothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH) levels were measured in the serum samples obtained.
On PND 4, where possible, litter size was standardized to 4 male and 4 female pups per litter. Surplus pups were sacrificed and blood samples for thyroid hormone determinations (pooled samples on a litter basis) were obtained from a maximum of 10 litters per group.

7. Pathology

After blood sampling for thyroid hormone measurements, the GD 20 and PND 21 dams were anesthetized with isoflurane, weighed, sacrificed by decapitation and examined macroscopically. The thyroid/parathyroid glands were removed, trimmed, weighed and preserved for histopathological examination. In addition, for the dams killed on GD 20, the following reproductive parameters were recorded - weight of the unopened uterus, the number of corpora lutea, the number of implantations (differentiated as to live and dead fetuses and early and late resorptions) and the position of the implantations in the uterus. The fetuses were examined externally and individual fetal weights and sex were recorded. For the pups culled on PND 4, one randomly selected male and female pup from each of 10 litters were sacrificed by decapitation and the thyroid glands/parathyroid glands were removed and preserved for histopathological examination. On PND 21, the pups selected for thyroid hormone measurements were bled before being anesthetized with isoflurane, sacrificed by decapitation and the thyroid glands/parathyroid glands were removed and preserved for histopathological examination. Surplus pups on PND 4 and PND 21 were sacrificed with CO_2 and discarded without any further examination. All pups that died or were stillborn were discarded without further examination. Adult females which did not litter were necropsied and discarded after the uterus had been stained for evidence of early resorptions. The thyroid/parathyroid glands removed at necropsy were fixed in neutral buffered 4% formaldehyde. Weights were determined after fixation and trimming, for the adult animals only. After fixation, the tissues were embedded in paraplast, sectioned and stained with hematoxylin-eosin, prior to histopathological evaluation.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Mortality

For both rat strains, there were no treatment-related deaths during the study.

2. Clinical signs of toxicity

For both rat strains, there were no treatment-related clinical signs noted during either the gestation or lactation periods.

B. BODY WEIGHT AND FOOD CONSUMPTION

1. Food consumption

At 2.5 mg/kg/day, for both rat strains, maternal food consumption was reduced during the last week of the gestation period and throughout the lactation period.

2. Body weight

At 2.5 mg/kg/day, for both rat strains, maternal body weight gain was retarded during the last week of the gestation period and overall body weight gain was increased during the lactation period.

C. NECROPSY OBSERVATIONS

1. Histopathology of the thyroid glands

For the GD 20 fetuses, PND 4 and PND 21 pups, the thyroid glands were removed together with adjacent tissue at necropsy in order to maintain optimal glandular architecture for histopathological evaluation. This precluded measurement of organ weights in these animals. For the GD 20 and PND 21 dams at 2.5 mg/kg/day, both absolute and relative (to body weight) thyroid weights were increased and significantly different from controls.
At macroscopic examination, enlarged thyroid glands were noted at 2.5 mg/kg/day.
For both rat strains, there was a clear, dose-related and statistically significant increase in the incidence and severity of hypertrophy/hyperplasia of the thyroid follicular cells in the GD 20 and PND 21 dams, in the GD 20 fetuses and in the PND 4 and 21 pups [see Table 5.8.1-64, Table 5.8.1-65 and Table 5.8.1-66].

Table 5.8.1-64: Necropsy findings in dams administered PTU from GD 6 to PND 21

	GD 20 dams						PD 21 dams					
	Wistar rats			Sprague Dawley			Wistar rats			Sprague Dawley		
Dose Level [mg/kg]	0	0.1	2.5	0	0.1	2.5	0	0.1	2.5	0	0.1	2.5
Females examined					10	10	20	23	19	22	23	25
Hypertrophy/hyperplasia, follicular cells												
No finding					6		20	15		16	7	
Grade 1					4			8			10	
Grade 2											6	
Grade 3									7			
Grade 4						10			12			18
Grade 5												

Table 5.8.1-65: Necropsy findings in fetuses administered PTU from GD 6 to PND 21

	GD 20 males fetuses						GD 20 female fetuses					
	Wistar rats			Sprague Dawley			Wistar rats			Sprague Dawley		
Dose Level [mg/kg]	0	0.1	2.5	0	0.1	2.5	0	0.1	2.5	0	0.1	2.5
Females examined	10		10	10	9	8	10	10	10	10		8
Hypertrophy/hyperplasia, follicular cells												
No finding	10			10	9	4	10	2		10		
Grade 1								8			2	
Grade 2			4			2			9		4	
Grade 3			6									2
Grade 4												

Table 5.8.1-66: Necropsy findings in pups administered PTU from GD 6 to PND 21

	PD 4 male pups						PD 4 female pups					
	Wistar rats			Sprague Dawley			Wistar rats			Sprague Dawley		
Dose Level [mg/kg]	0	0.1	2.5	0	0.1	2.5	0	0.1	2.5	0	0.1	2.5
Females examined	10	10		10	10	10	10	10	10	10	10	10
Hypertrophy/hyperplasia, follicular cells												
No finding	10	2		10	2		10	2		10		
Grade 1		8			6	4					3	
Grade 2					2	2					6	8
Grade 3						4						
Grade 4												

2. Thyroid hormone measurements (see Figure 5.8.1-9 and Figure 5.8.1-10**)**

For both rat strains, TSH values were increased significantly at 2.5 mg/kg/day in dams at GD 20 and PND 21, in fetuses at GD 20 and in pups at PND 4 and 21. At the low dose, 0.1 mg/kg/day, TSH values were also significantly increased for the GD 20 fetuses and the pups on PND 4 and 21, but values for the dams on GD 20 and PND 21 were only minimally increased.

For both rat strains, there was a dose-related and statistically significant reduction in the T4 values for the dams on GD 20. For the dams on PND 21 and the GD 20 fetuses, T4 values showed a clear reduction at 2.5 mg/kg/day only. For the PND 4 pups, there was a dose-related reduction in T4 values for both rat strains and for the PND 21 pups, T4 values were significantly reduced at 2.5 mg/kg/day (both rat strains) and at 0.1 mg/kg/day (Wistar strain only).

For both rat strains, T3 values were significantly reduced in the dams on GD 20 and the pups on PND 4 and 21 at 2.5 mg/kg/day, but not in the dams on PND 21. Sample volumes were insufficient to measure T3 values in the GD 20 fetuses.

Figure 5.8.1-9: Thyroid hormone levels in dams administered PTU from GD 6 to PND 21

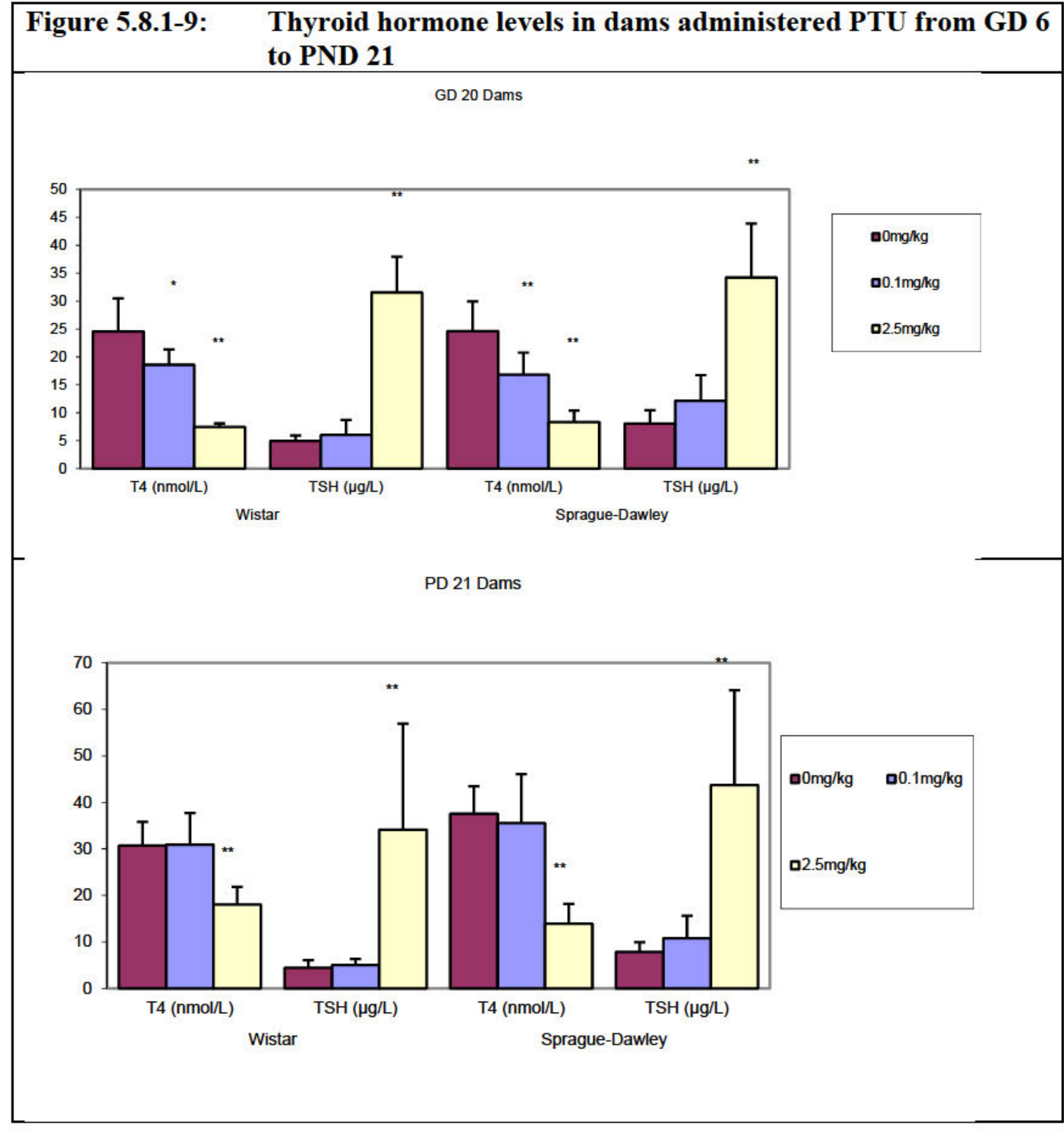
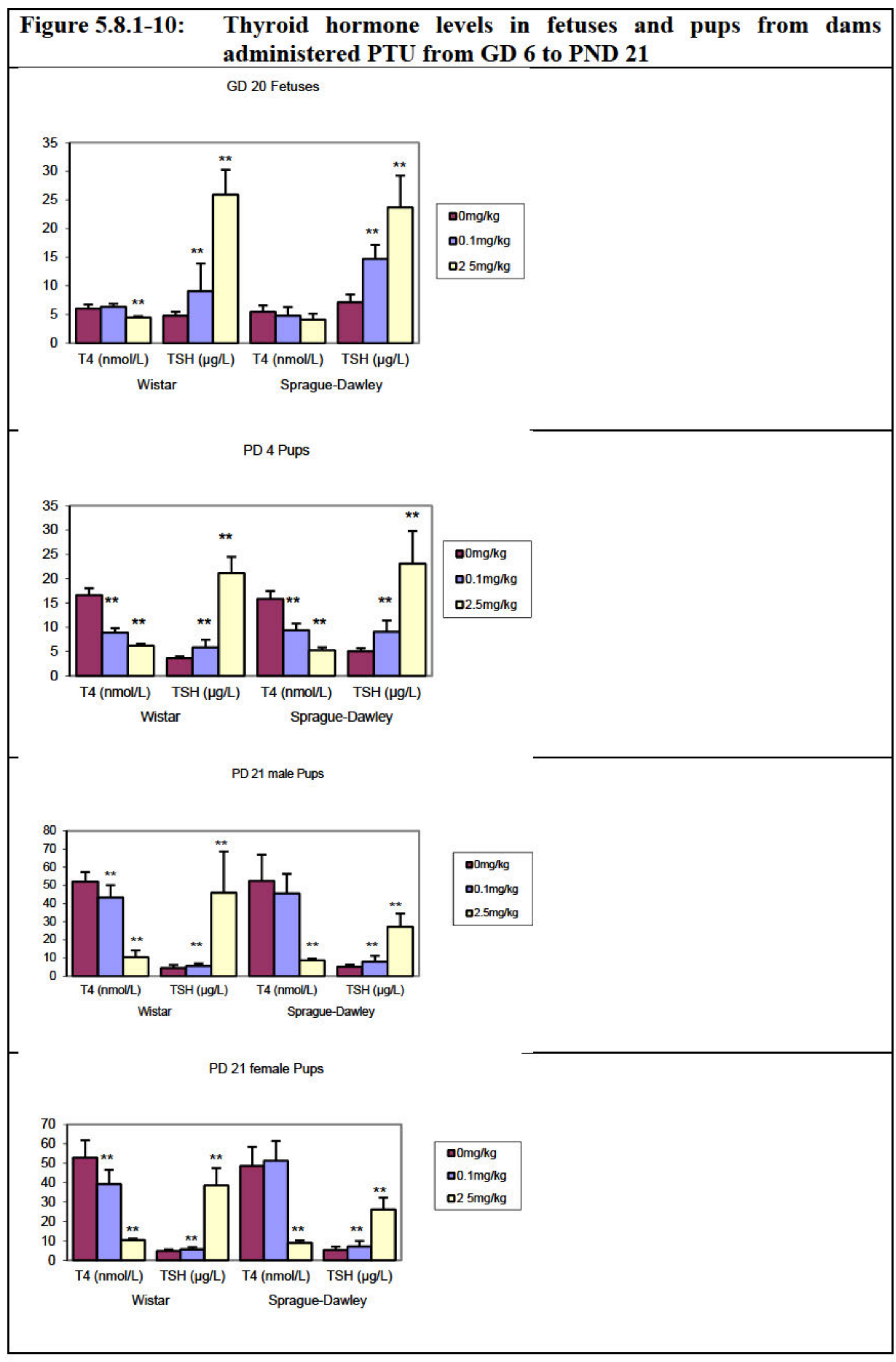


Figure 5.8.1-10: Thyroid hormone levels in fetuses and pups from dams administered PTU from GD 6 to PND 21



D. SUMMARY AND CONCLUSION

Results indicate that PTU-treated rats of Wistar and Sprague-Dawley strains, demonstrate clear dose response relationships for thyroid hormones. Evident effects were decreased T4 and increased TSH levels for dams (GD 20, PD 21), fetuses (GD 20) and pups (PD 4 & 21). T3 responded with less sensitivity to treatment. Average variability of the data was acceptable.
At these time points, dams, fetuses and pups of both rat strains showed comparable signs of diffuse hypertrophy and hyperplasia of the thyroid gland follicular cells of dose-dependent severity. A treatment-free period of 10 days (PD 11 – 21) did not result in any recovery. Effects on morphology are in good correlation to hormone effects.
Furthermore, results indicate no apparent significant differences between the Wistar and Sprague-Dawley strains when tested in this study design. The results indicate both strains may be used to test for potential effects on thyroid hormone and light microscopy in fetuses/pups to ascertain NOAELs/LOAELs following exposure to dams during pregnancy and lactation.
EPA Guidance (October 2005) was modified in some respects. Pooling of blood samples per litter was necessary for thyroid hormone evaluation in GD 20 fetuses and PD 4 pups. No thyroid organ weights were taken (GD 20 fetuses and PND 4 and PND 21 pups) in order to best preserve integrity of thyroid tissue sections for microscopic examination, which was considered to be a more informative endpoint than thyroid weights.

Report: CA 5.8.1/31
Axelstad M. et al., 2008a
Development neurotoxicity of Propylthiouracil (PTU) in rats; Relationship between transient hypothyroxinemia during development and long-lasting behavioural and functional changes
2008/1102636
Guidelines: none
GLP: no

Executive Summary

6-propyl-2-thiouracil (PTU) is an antithyroid drug which inhibits both the synthesis of thyroid hormones in the thyroid gland, and the conversion of thyroxine (T4) to its active form, thiiodothyronine (T3). In this publication a study was performed with pregnant Wistar rats which were dosed with 0, 0.8, 1.6 or 2.4 mg/kg bw/day PTU from gestation day (GD) 7 to postnatal day (PND) 17 in order to assess the physiological and behavioural development of rat offsprings.
For this dossier the main focus lies on the effects of PTU on the thyroid. In this publication it was shown that both dams and pups in the higher dose groups had markedly decreased thyroxine (T4) levels during the dosing period, and the weight and histology of the thyroid glands were severely affected.
It was shown that in the adult offspring behavioural deficitis in learning and memory and impaird auditory function was observed. Generally, the results showed that PTU-induced hypothyroxinemia influenced the developing rat brain, and that all effects on behaviour and loss of hearing in the adult offspring were significantly correlated to reductions in T4 during development.

(DocID 2008/1102636)

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** 6-propyl-2-thiouracil (PTU)
Description: no data
Lot/Batch #: no data
Purity: >99.0%
Stability of test compound: no data

2. **Vehicle control:** corn oil

3. Test animals:

Species:	Wistar rats
Strain:	HanTac:WH
Sex:	Time mated female
Age:	young adult
Weight at dosing:	no reported
Source:	Taconic Europe, Ejby, Denmark
	Acclimation period: 4 days
	Diet: Altromin standard diet 1314, ad libitum
Water:	acidified tap water, ad libitum
	Housing: in pairs until GD17 and individually thereafter, housing in semitransparent plastic cages (15x27x43 cm) with Aspen bedding (Tapvei, Gentofte, Denmark)
Environmental conditions:	
Temperature:	21 - 23°C
Humidity:	50 - 60%
Air changes:	10 air changes per hour
Photo period:	12 h light / 12 h dark (reversed dark- light cyles) (21:00 - 09:00 / 09:00 - 21:00)

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: no data

2. Animal assignment and treatment:
The animals were randomly assigned to four groups of 22 animals, with similar weight distribution. Dams were gavaged once a day at approximately the same time, from GD7 to postnatal day (PND) 17 with 0, 0.8, 1.6 or 2.4 mg/kg bw PTU. The dams were treated at a constant volume of 2 mL/kg bw/day. The study was run in three blocks, with 2 weeks in between each block and an equal representation of each dose group in each block.

3. Test substance preparation and analysis:
The vehicle control and the PTU solutions were continuously stirred during the dosing period, and prepared anew for each of the three study blocks.

4. Statistics:
For all analyses, the alpha level was set at 0.05. Data were examined for normal distribution and homogeneity of variance, and if relevant, transformed. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a non-parametric Kruskall-Wallis test was used, followed by Wilcoxon's test for pair wise comparisons. Statistical analyses of the effects on macroscopic lesions and histopathology, were done using Fisher's Exact Test.

Data with normal distribution and homogeneity of variance were analyzed using analysis of variance (ANOVA). Litter size was included as a covariate in the analyses of body weight and body weight gain of the pups. Body weight was included as a covariate in the analyses when relevant, e.g. when testing terminal organ weights. When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor in ANOVA (i.e. for data on body and organ weights, activity levels, radial arm maze performance and Morris maze performance). Where an overall significant treatment effect was observed, two-tailed comparison was performed using least square means.
For correlation analysis between T4 and behavioural and auditory endpoints, simple linear regression analysis was run using letter means. Additionally for the auditory endpoints curvilinear regressions were included, assuming an exponential fit. For acitivty levels at PND 14, 17 and 23, and for the auditory endpoints, results from male and female offspring were combined into litter means, wen analyzing the correletiaon with T4 levels. For endpoints were data ware analysed seperately for males and females (errors in the RAM, and activity levels in adult animals) litter means were also used, when more than one pup per litter per sex had been tested. Asterisks in tables and figures, indicate a statistically significant difference compared to controls *: $p \leq 0.05$; ** $p < 0.01$. All analysis were performed using SAS Enterprise Guide 3 (2004), SAS Institute Inc., Cary, NC, USA.

C. Methods

1. Insemination:

The animals were paired by the breeder (“time mated”) and supplied on GD 3.

2. Treatment:

The pregnant animals and their litters continuously received the test substance at the appropriate concentrations by gavage from GD 7 to PND 17.

3. Observation:

All animals were observed daily for signs of toxicity.

4. Body weight:

Body weight was recorded at GD 4 and during the entire dosing period.

5. Delivery and postnatal development

After delivery, weights of dams and individual pups were recorded. The pups were counted, sexed, and checked for anomalies. Pups found dead were macroscopically investigated for changes when possible. The expected day of delivery, GD 22, was designated PND0 for the pups. Thereby, the age of the pups related to the time of conception, but was rather similar to postnatal age as the animals gave birth on GD22-23. Body weight of offspring was recoreded on PND 66 and at the age of 4, 5 and 7 months.
At PND 16, litter size was standardized to 3 males and 3 females, when possible. From these offspring, 1-3 males and 1-2 females from each litter were weaned on PND27, and kept for later behavioural testing. The weaned offspring was housed in pairs of the same sex and exposure status. After weaning each of the four dose groups consisted of two subgroups of animals. One had 18-20 male and 18-20 female pups from 16-17 different litters per group. These animals were used for motor activity tests. Morris water maze test and assessment of hearing. The other subgroup consisted of 18-20 males rats per dose group (from 17 litters per group), and these animals were tested in the radial arm maze.

6. Thyroid weights and histopathology

On PND16, 1-9 pups in each litter were sacrificed depending on the original size of the litter. All sacrificed pups were weighed and decapitated, and trunk blood was collected for T4 analysis (pooled for all males and all females within each litter). Thyroid glands from two males and two females per litter were used for histopathological investigations.
Dams and offspring (one male and one or two females in each litter) that were not kept for behavioural and functional studies, were sacrificed on PND 27. All animals were weighted and decapitated after CO_2/O_2 anesthesia, and trunk blood was collected for measurements of thyroxine in serum. The uteri of the dams were excised, and the number of implantation scars was registered. The thyroid glands were dissected, weighted, and used for histopathological investigation. In the offspring, the thyroid gland from one male and one female per litter were excised, weighed, and used for histopathological investigations. In the offspring, the thyroid gland from on male and one female per litter were excised, weighed, and used for histopathology. The glands were fixed in formalin, embedded in paraffin, and examined by light microscopy after staining with haematoxylin and eosin.
The histological evaluation included scoring of thyroids as being “normal” or having “moderated” or “marked” effects. In PND 16 pups, thyroids were classified as having “marked” effects. In PND 16 pups, thyroids were classified as having “marked” effects, when hyperplasia and hypertrophy of epithelial cells were observed along with papillary projections into the follicle lumen. A score for “moderated” changes required one of the following: slightly irregular follicular lumen, increased cellularity, and few papillary structures in the follicle lumen. In PND 27 pups, histological changes were generally milder, and a score for “moderate” changes required the presence of enlarged colloid-filled follicles, an increased cellularity and/or pseudostratified epithelium projecting into the lumen. In dams, thyroid effects at PND27 were generally more marked than in the PND27 pups, and a score for “marked” changes required the presence of irregular follicles, pseudostratified epithelium, and papillary projections into the follicle lumen. In adulthood, effects were mild, and a score for “moderate” effects was given when increased number of large follicles with flattened epithelium were observed.
Seven months old male and female offspring (18-20 of each sex per group) were anesthetized by CO_2/O_2 and decapitated. Thyroid glands from all animals, were excised, weighed, fixed in formalin, embedded in paraffin and examined by light microscopy after staining with haematoxylin and eosin.

7. Thyroid hormone analysis

On GD15 dams were anesthesized and blood was drawn from the tail vein. From PND16 pups, PND 27 pups, dams on day 27 and from 7 months old offspring, trunk blood was used for the hormone analysis. The plasma level for the thyroid hormone thyroxine (T4) was analyzed.
Seven months old male and female offspring (18-20 of each sex per group) were anesthetized by CO_2/O_2 and decapitated. Trunk blood was collected and analysed for T4.

8. Behavioural testing

The investigation were performed between 9 a.m. and 4 p.m. during the animals’ dark cycle, i.e. their active period.

Motor acitivity and habituation cabability: Motor acitivity in the rat offspring was measured four times in the study, using the same animals in all tests. One to two male and female pups were randomly selected from each litter, and tested for the first time on PND14, again on PND 17 and 23. All animals that were tested in pre-puberty were kept for the activity testing in adulthood, and tested again at 16 weeks of age. At testing, the animals were placed individually in clean plastic cages without bedding and the cages were placed in activity boxes with photocells, which measured horizontal activity for 30 minutes. The total activity during the 30 minute observation period was used as a measure of general activity. In order to assess habituation, the 30 minutes were divided into three periods, period 1 (1-9 min), period 2 (10-21 min), and period 3 (22-30 min). As there were no sex differences in motor activity levels in the prepubescent animals, litter means were calculated, and used for calculation of group mean values.

Learning and memory (Morris water maze): All animals that had previously been tested for activity in pre-puberty, were tested at the age of 8 and 9 weeks in a Morris water maze. The pool had a diameter of 220 cm, and a circular transparent platform was situated on a solid support and submerged 1 cm below the water surface, and thus invisible from water level. The animals were tested in four daily trials using four different starting points. When the rats swam to and climbed onto the platform, the trial was completed. If the animal failed to locate the platform within 60 sec., it was led to the platform. A video-tracking device tracked the route of the animals, and the latencies to find the platform, the path lengths, and swimming speeds were used as endpoints. The following scheme was used:
Learning: With the platform situated at the same place, the animals were trained for 5+2 days until a stable performance was established.
New platform position (reversal learning): The day after the last learning test, the animals were tested in a reversal procedure with the platform placed opposite the original location. The animals were tested for two consecutive days.

Radial arm maze: At the age of 5-6 months, the second subset of weaned animals (not previously tested males), were tested in a standard 8-arm Radial Arm Maze (RAM) with photoelectric cells. The maze was elevated 90 cm above the floor, and built of transparent Plexiglas with 8 arms (55 cm long) radiating from an octagonal central area (50 cm wide). Two photocells were mounted on each maze-arm, one close to the central area, for registration of the rat's entry to the arm, and one at the end of each arm, for registration of movement at the end of the arm, where the reward was placed. The animals were tested in one daily session in a total of 15 sessions during 3 consecutive weeks (5 trials per week). All eight arms in the maze were baited with small pieces of peanut (therefore the animals got restriction of food one week before testing). In the daily test sessions, each rat was placed in the central maze area in a plexiglass tube. The tube was elevated, giving the rats access to the arms of the maze. The rats were allowed to explore the maze until all arms were visited, or 10 minutes had elapsed. Latency to pass all the distally placed photoelectric cells and the choice of arms was registered by computer. The number of errors, defined as visiting an arm that had already been visited, was calculated from the data.

9. Test of hearing

Hearing was assessed with the animals in general anesthesia by measuring distortion product oto-acoustic emissions (DPOAE). Using the same stimulus source (probe assembly), hearing thresholds (HT) at 4kHz were also assessed by auditory brain stem response (ABR). Tweleve males and twelve females per group, from the subset of animals earlier tested in the Morris Water Maze, fulfilled the experimental protocol.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS AND BODY WEIGHT

Maternal body weight gain during pregnancy, gestation length, litter size, postimplantation loss, neonatal death, and pup birth weight were similar in the four groups (see Table 5.8.1-67). During lactation (PND 1-17) dams receiving 2.4 mg/kg PTU gained more weight than controls. Pup body weights were unaffected by PTU treatment during this period, and no significant differences in body weights between controls and dosed offspring were seen on PND 17 or earlier. When measured on PND 23 and PND 27 both female and male pup body weights were significantly decreased in the 2.4 mg/kg group (PND 23; p = 0.002, p = 0.004 – PND 27; p = 0.002, p = 0.001). On PND 27, male body weights were also significantly affected in the 1.6 mg/kg group (p = 0.019). Similar results were seen when body weight gains PND 17-27 were calculated (Table 5.8.1-67). The analyses of body weights PND 27 were based on data from all offspring, both those that were kept for weaning, and those sacrificed on PND 27. After weaning body weights were recorded on PND 66, and at this point there were no longer any differences in body weight or weight gains among groups (Table 5.8.1-67). This was also the case during the remaining period of the study.

Table 5.8.1-67: Pregnancy and litter data, including body weight (BW) of dams and offspring exposed to 0, 0.8, 1.6 or 2.4 mg/kg bw/day PTU from GD7 to PND17

	Control	0.8 mg PTU	1.6 mg PTU	2.4 mg PTU
Dams and litters				
No. of dams (litters)	n=22 (17)	n=22 (16)	n=22 (16)	n=22 (17)
Dam BW-gain, GD7–GD21	83.2±4.3	78.9±3.8	83.7±3.9	76.5±3.2
Dam BW-gain, GD7–PND 1	17.4±1.6	20.6±1.8	17.1±1.5	14.8±2.3
Dam BW-gain PND 1–PND 17	34.6±2.6	31.6±4.0	36.9±2.4	44.9±3.3 *
Dam BW-gain PND 17-PND 27	−20.1±2.7	−19.8±2.4	−22.0±3.3	−20.7±3.1
Gestation length	23.0±0.2	22.8±0.1	22.7±0.1	23.0±0.1
% postimplantation loss	10.2±3.0	15.5±6.6	13.9±3.3	8.6±3.5
% perinatal loss	14.8±5.7	18.2±6.6	15.0±3.4	10.4±3.4
Litter size	10.8±0.8	9.6±0.8	11.1±0.7	10.9±0.8
% perinatal deaths	0.8±0.5	3.4±1.4	1.3±0.9	1.8±0.9
% males	48.1±4.7	51.8±4.8	50.4±2.6	48.6±3.9
Offspring				
Mean birth weight	5.9±0.1	6.2±0.1	5.9±0.1	5.9±0.1
Mean BW-gain 1–6	6.7±0.3	7.4±0.3	7.1±6.5	6.5±0.3
Mean BW-gain 6–17	21.0±0.4	22.6±0.8	21.2±0.8	21.0±0.7
Mean BW day 17	33.7±0.8	36.8±1.3	34.1±1.1	33.6±1.1
Mean female BW day 23	53.7±1.1	56.7±1.3	52.9±1.0	50.0±1.2**
Mean male BW day 23	56.5±1.3	59.1±1.2	54.9±1.2	53.4±1.6**
Mean female BW day 27	71.9±1.4	75.2±1.5	70.3±1.2	67.2±1.4**
Mean male BW day 27	78.0±1.6	79.8±1.4	74.3±1.4*	73.5±2.1**
Female BW-gain 17–27	38.7±0.8	39.4±0.7	36.7±0.7	34.0±0.5**
Male BW-gain 17–27	43.8±0.9	43.2±0.7	39.8±0.6**	39.7±1.4**
Female BW-gain 27–66	120.0±2.6	128.8±6.5	116.6±2.6	120.6±2.8
Male BW-gain 27–66	221.6±4.4	221.3±5.7	208.0±3.3	221.6±3.5

Data represent group means based on litter means ± SE. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; **: $p<0.01$.

B. NECROPSY OBSERVATIONS

1. Autopsy, thyroid weight and histopathology PND 16 and PND 27

On PND 16, pup body weights of the sacrificed animals did not differ between males and females, and were unaffected by PTU exposure. The weight of the thyroid gland in offspring of both sexes, was significantly higher in all PTU-dosed groups (p<0.0001), and displayed a clear dose effect relationship (Table 5.8.1-68). In PTU-dosed offspring, diffuse hyperplasia and hypertrophy of thyroid follicular cells were observed in all treatment groups, along with a reduced lumen size and papillary enfolding of the epithelium (Table 5.8.1-69).
On PND 27, body weights of the dams were unaffected but weights of the thyroid glands were significantly elevated in the two highest dose groups (p=0.0024 and p<0.0001 respectively). In the offspring sacrificed at PND 27, female body weights were not significantly affectd in any of the treatment groups, whereas male body weights in the high dose offspring were significantly reduced (p=0.0027). The thyroid gland weights on PND 27 were clearly elevated compared to controls. This was statistically significant for all three PTU-dosed groups (p=0.016 for the 0.8 mg/kg group, and p<0.0001 for the two high dosed groups). Histological examination of the thyroid glands from dams and pups on PND 27 showed irregular follicles and increased cellularity in PTU-dosed animals, although the effects were less prominent that on PND 16 (Table 5.8.1-69).

Table 5.8.1-68: Terminal body weights, thyroid gland weights, and T4 levels in rat offspring sacrificed at PND 16, PND 27 or in adulthood, and from dams sacrificed PND 27, after exposure to 0, 0.8, 1.6 or 2.4 mg/kg bw/day PTU from GD 7 to PND 17

	Control	0.8 mg PTU	1.6 mg PTU	2.4 mg PTU
Pups PND 16				
No. of litters (male/female pups)	17 (18/21)	13 (18/18)	15 (20/19)	15 (20/20)
Body weight (g)	29.80±0.56	31.34±0.55	29.12±0.63	28.87±0.44
Thyroid gland weight	4.38±0.13	6.43±0.22**	7.97±0.23**	9.41±0.38**
T_4 levels (nM)	30.33±0.8	15.22±0.8**	6.89±0.5**	4.79±0.4**
Pups PND 27				
No. of litters (male/female pups)	18 (10/17)	16 (12/15)	17 (13/17)	15 (13/15)
Female body weight (g)	71.5±1.5	73.9±2.1	69.1±1.0	68.2±1.5
Male body weight (g)	78.3±2.3	80.3±1.3	73.6±1.7	69.6±1.9**
Thyroid gland weight	8.08±0.3	10.1±0.4*	11.1±0.3**	11.9±0.3**
T_4 levels (nM)	18.28±1.8	21.11±1.3	19.71±1.1	16.20±1.1
Dams				
No. of dams	17	16	17	17
T_4 levels in dams GD 15 (nM)	28.05±1.6	26.24±1.1	15.38±1.3**	11.38±1.4**
T_4 levels in dams PND 27 (nM)	17.66±2.1	14.56±1.7	18.24±1.6	24.04±2.2
Body weight PND 27 (g)	259.9±3.6	255.6±6.1	257.7±5.3	261.4±3.9
Thyroid gland weight PND 27 (mg)	15.32±0.54	16.18±0.75	18.14±0.57**	20.24±0.7**
Adult female offspring				
No. of females	20	18	20	16
Body weight (g)	251.8±5.3	261.67±6.4	249.5±4.3	249.94±4.7
Thyroid gland weight	17.23±1.05	18.81±1.31	21.24±1.81	21.84±1.98
T_4 level (nM)	36.49±2.6	32.97±2.9	39.20±2.6	27.90±2.0**
Adult male offspring				
No. of males	20	18	20	18
Body weight (g)	429.15±9.4	35.39±11.5	421.42±9.6	442.56±12.1
Thyroid gland weight (mg)	15.25±0.92	18.88±1.44	24.23±2.05**	23.62±1.37**
T_4 level (nM)	25.18±1.1	21.50±1.7	21.52±1.0	23.15±1.3

Data represent group means based on litter means ± SE. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; **: $p<0.01$.

2. Hormone levels GD 15, PND 16 and PND 27

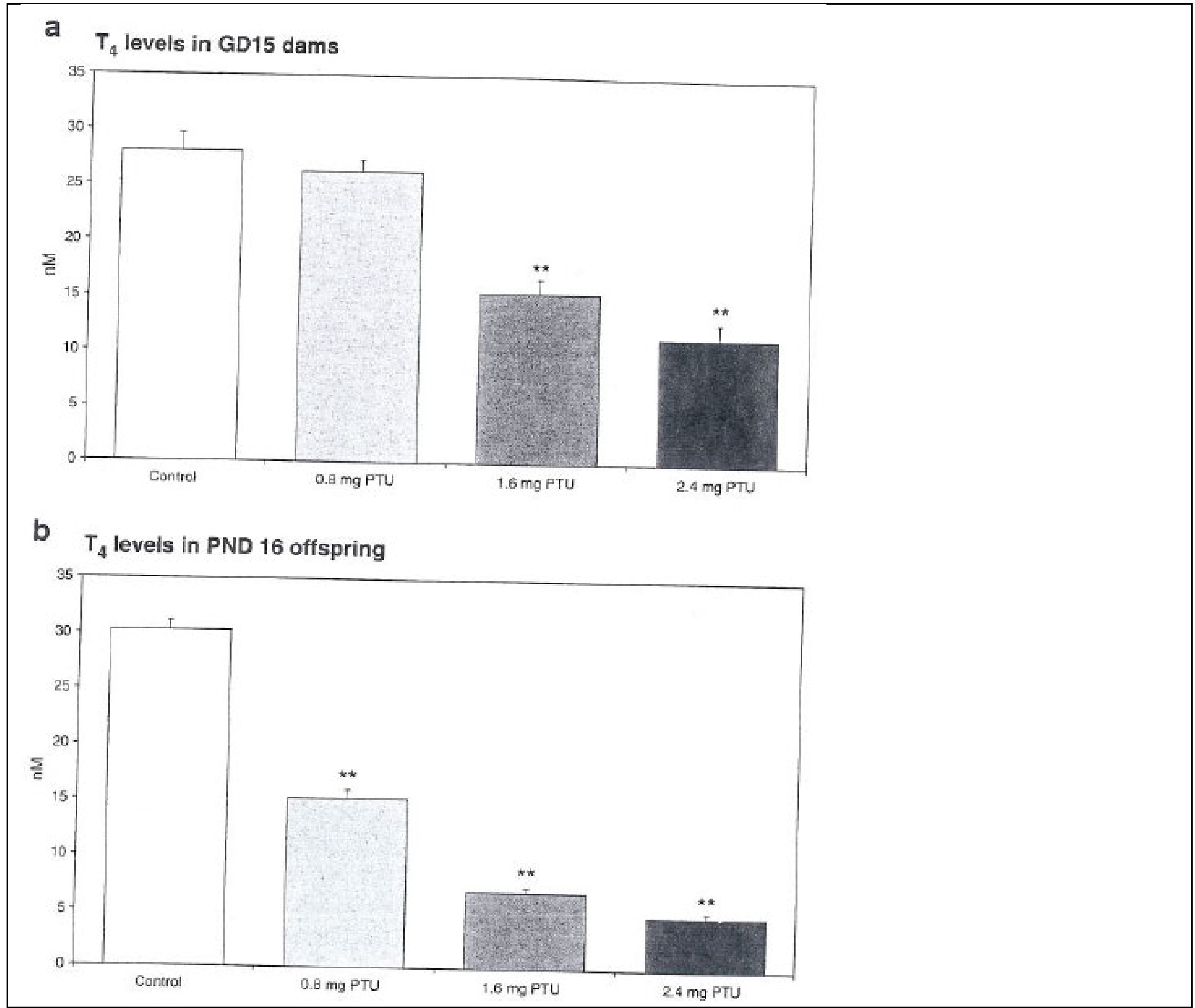
Thyroxine (T4) levels were measured in blood from dams on GD 15 and PND 27, and from the offsprings on PND 16 and 27. Results are shown in Figure 5.8.1-11 and Table 5.8.1-68. The T4 levels decreased with higher doses of PTU in both dams on GD 15 and pups on PND 16, as expected. The difference was significant for the two highest dose in GD 15 dams, and for all three PTU doses in pups on PND 16 (p<0.0001). On PND 27, 10 days after dosing had stopped, the T4 levels in exposed dams and pups had normalized, and compared to values in control animals (Table 5.8.1-68).

Table 5.8.1-69: Histopathological changes in the thyroid gland of rats exposed perinatally to 0, 0.8, 1.6 or 2.4 mg/kg bw/day PTU from GD 7 to PND 17

	Control	0.8 mg PTU	1.6 mg PTU	2.4 mg PTU
Male and female pups PND 16				
Normal	38/39	18/33**	2/36**	0/ 34**
Moderate	1/39	14/33**	6/36	0/ 34
Marked	0/39	1/33	28/36**	34/ 34**
Male and female pups PND 27				
Normal	26/26	21/24	13/26**	4/26**
Moderate	0/26	3/24	13/26**	22/26**
Marked	0/26	0/24	0/26	0/26
Dams PND 27				
Normal	13/17	10/16	2/16**	2/17**
Moderate	4/17	4/16	5/16	0/17
Marked	0/17	2/16	9/16**	15/17**
Adult offspring				
Normal	31/34	21/34**	12/34**	15/33**
Moderate	3/34	13/34**	22/34**	18/33**
Marked	0/34	0/34	0/34	0/33

The cellular changes have been categorized into 3 groups, normal, moderate, and marked. See Materials and methods section for further description of the histological alterations, as the character and severity of the changes varies between the age groups. Asterisks indicate a statistically significant difference compared to controls *: $p < 0.05$; **: $p < 0.01$.

Figure 5.8.1-11: Thyroxine (T4) levels (nM) in dams on GD 15 (a) and offspring on PND 16 (b) after exposure to 0, 0.8, 1.6 or 2.4 mg/kg bw/day PTU from GD 7 to PND 17.



Data represent group means, based on litter means + SE, n = 16-17. Asterisks indicate a statistically significant difference compared to control. *: p<0.05, **: p<0.01

C. BEHAVIOURAL OBSERVATIONS

Since the main focus in this dossier lies on the thyroidal effects of ETU, the behavioural alterations due to hypothyroxinemia are only shortly summarized here.

PTU exposure caused motor acitivity levels to decrease on PND 14, and to increase on PND 23 and in adulthood. In the adult offspring, learning and memory was impaired in the two highest dose groups when tested in the radial arm maze, and auditory function was impaired in the highest dose group.

D. SUMMARY AND CONCLUSION

Human data indicate that even small reductions in maternal thyroxine levels during critical periods in early pregnancy, can have severe consequences for the neurological development of the child. The objective of this study was to investigate this relationship in an animal model, and thereby contribute to a clearer characterization of the relationship between disruption of thyroid hormones and adverse effects on the brain.
For this dossier the main interest lies on the effects of PTU on the thyroid. Taken this into account the following data can be summarized. The pregnant dams were dosed with PTU in order to induce transient developmental hyptohyroxinemia without causing general maternal toxicity. When the T4 levels were measured in dams after 7 days of dosing (GD 15), T4 levels were measured in the two highest dose groups were significantly reduced compared to controls, while pups from all three dose groups had significantly lowered T4 levels at the end of the 30-day dosing period (PND 16). This marked decrease was however only temporary since T4 levels were not signicifantly affected in dams and offspring when measured 10 days after dosing had stopped. Unlike T4 levels, thyroid gland size and histology had not normalized with within 10 days of cessation of treatment. Thyroid weights were still significantly elevated on PND 27 in offspring from all dose groups, and the histopathological examination showed marked effects, including hyperplasia, hypertrophy and the presence of papillary structures. In adulthood, the weight of the thyroid gland was still elevated in PTU animals, although the difference was only statistically significant in the males. T4 levels in adulthood were significantly lowered in the high dose female offspring, the thyroids were thus large compared to their T4 production, indicating persistent dysfunction and goiter. This slight dysfunction was also evident histologically, as follicles were enlarged and displayed flattened epithelium in PTU exposed animals compared to controls. These effects were accomplished with no general maternal toxicity or any effects on litter size or pup mortality.
Due to this results the NOAEL for maternal toxicity can be set to 0.8 mg/kg bw/day. A NOAEL for fetal toxicity could not be determined.

Table 5.8.1-70: Overview on the NOAELs and effects at LOAELs regarding thyroid (-related) toxicity comparing ETU and propylthiouracil

Effects	ETU			Propylthiouracil		
	Effect at LOAEL	NOAEL	Reference	Effect at LOAEL	NOAEL	Reference
Hormone levels in adults	T4↓, TSH ↑	25 ppm (≈ 2 mg/kg bw)	DocID 1977/10341 (30-day time point of 90-day study)	T4 ↓	< 0.01 mg/kg bw	Yamasaki, 2002 DocID 2002/1027697
Hormone levels in offspring	T4 ↓	0.2 mg/kg bw	Ext.1-Gen study DocID 2013/7002198	T4 ↓	< 0.8 mg/kg bw	Axelstad et al., 2008 DocID 2008/1102636
Hormone levels in offspring	T4 ↓ TSH ↑ (PND 4, PND 22)	2 mg/kg bw		TSH ↑ (PND 4)	< 0.1 mg/kg bw	DocID 2011/1276730
Hormone levels in dams	T4 ↓	0.2 mg/kg bw		T4 ↓	< 0.1 mg/kg bw	
Brain histopathology	No effects (PND 22)	>10 mg/kg bw		Permanent structural abnormalities in brain	Not given	Zoeller and Crofton, 2005 (DocID 2005/1043980) DocID 2005/1043980
Brain size / weight	Size ↓*	2 mg/kg bw		Size of corpus callosum, cerebellum, hippocampus**	Not given	
Behavioural effects in offspring	No effects	>10 mg/kg bw		Motor activity ↓, learning and memory ability ↓, auditory function ↓	0.8 mg/kg bw	Axelstad et al., 2008 DocID 2008/1102636

*no specific portion of the brain during neurodevelopment was targeted.
**together with changes in brain structure

Conclusion:

Propylthiouracil is significantly more potent to induce thyroid toxicity compared to ETU, as shown by changes in thyroid hormone levels in adults and in offspring from dams treated with the test substances. Furthermore, propylthiouracil induces changes in brain structure and behaviour of offspring, when the mothers had been treated with propylthiouracil during pregnancy. There is no evidence for neurodevelopmental changes in ETU studies. The only effects were decreased brain sizes, which were not specific to individual brain portions, in contrast to the findings seen after propylthiouracil exposure. A NOAEL for these changes was determined at 2 mg ETU/kg bw in the extended-1-generation toxicity study. Taking into account the fact that propylthioracil is covalently binding to the thyroid peroxidase (whereas ETU shows a **reversible** binding), there is clear evidence, that

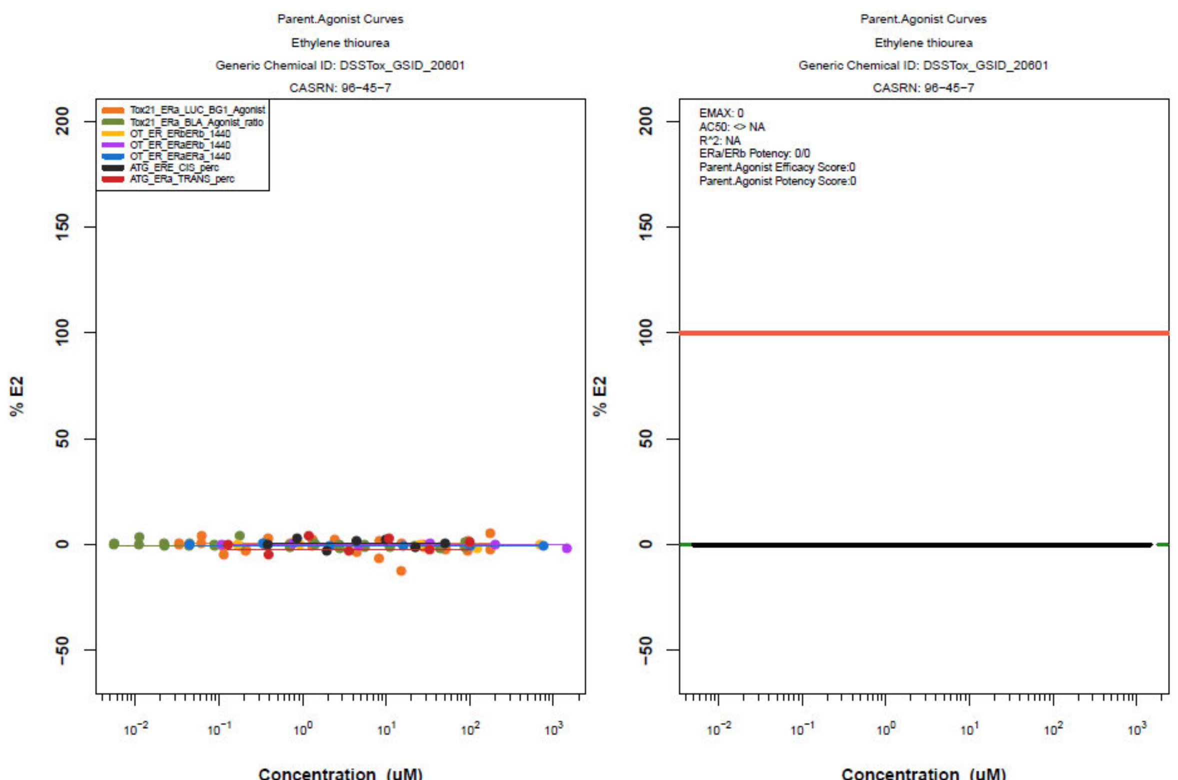
1. the thyroid effects caused after direct adult exposure and
2. the thyroid-related effects after in-utero exposure

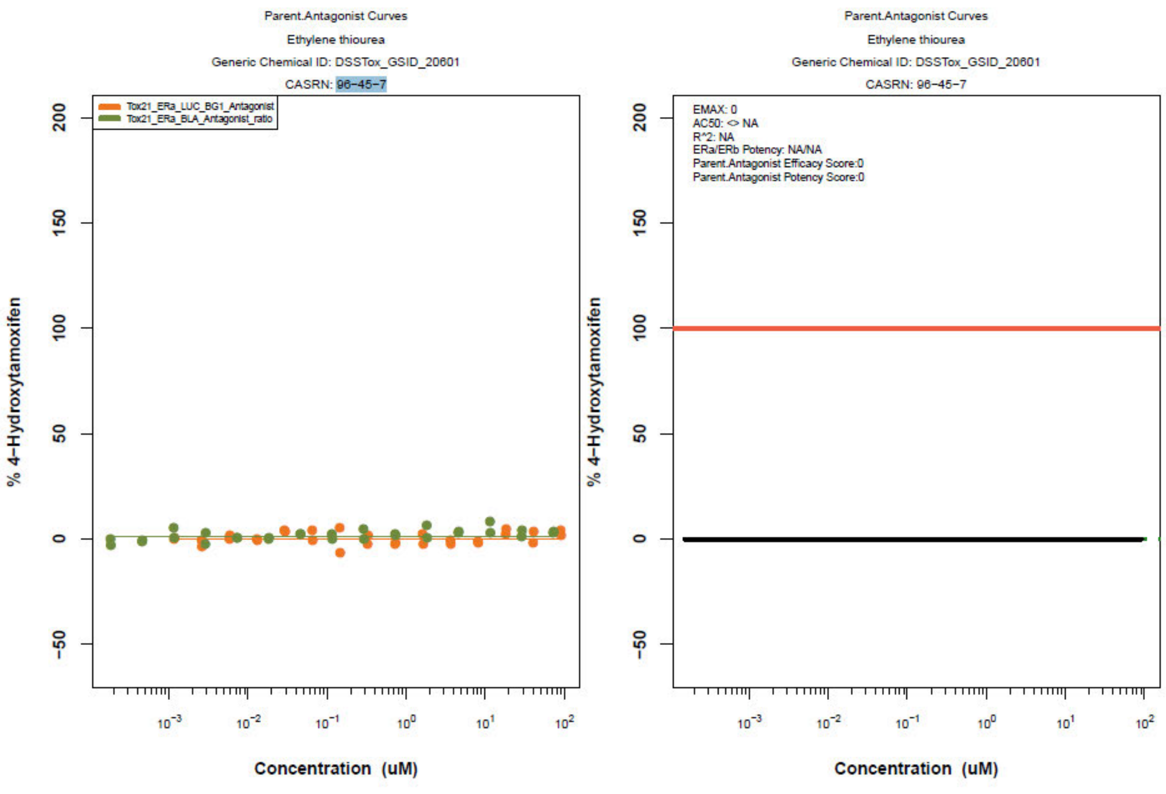
of rats to propylthiouracil are in quality and potency not comparable to ETU-related effects. Rats dosed with ETU do not show any behavioural changes or learning difficulties, as well as there is no pathological changes of brain structures seen. Other thyroid-related effects (hormone levels) caused by propylthiouracil occur at lower doses, compared to respective ETU studies.

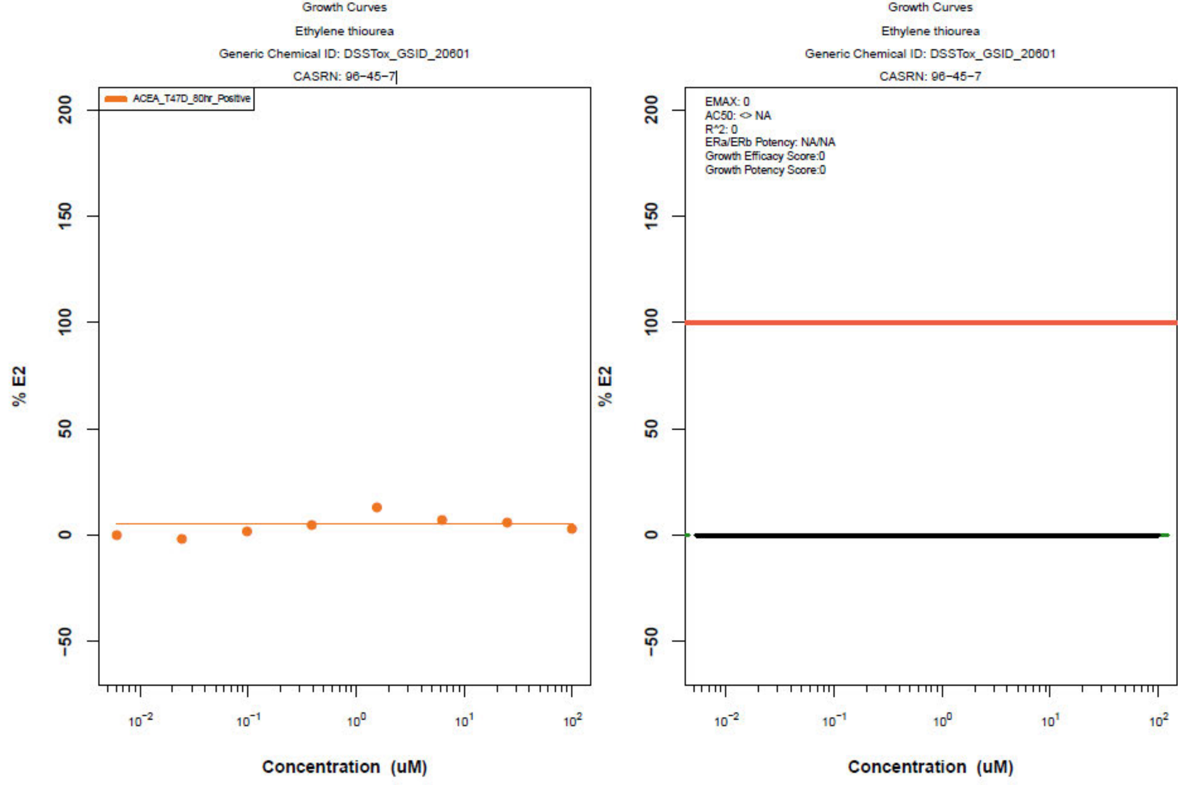
Report: CA 5.8.1/32
Rotroff D.M. et al., 2014a
Predictive endocrine testing in the 21st century using in vitro assays of estrogen receptor signaling responses
2014/1323273
Guidelines: none
GLP: no

Executive Summary:

Thousands of environmental chemicals are subject to regulatory review for their potential to be endocrine disruptors (ED). In vitro high-throughput screening (HTS) assays have emerged as a potential tool for prioritizing chemicals for ED-related whole-animal tests. In this study, 1814 chemicals including pesticide active and inert ingredients, industrial chemicals, food additives, and pharmaceuticals were evaluated in a panel of 13 in vitro HTS assays. The panel of in vitro assays interrogated multiple end points related to estrogen receptor (ER) signalling, namely binding, agonist, antagonist, and cell growth responses. The results from the in vitro assays were used to create an ER Interaction Score. For the ~1,800 chemicals evaluated in this study, 82% did not display indications of interacting with the ER signalling pathway and would be low priorities for additional ER testing. If maximum sensitivity is desired, the model can be run with narrower confidence intervals around the composite curves. This would result in an increased false positive rate and a decreased false negative rate.
Since for this dossier the metabolite ETU is relevant the results for ETU are described in the following figures. In summary, it is seen that ETU is for all estrogen receptor (ER) signalling endpoints, namely binding, agonist, antagonist and cell growth responses negative. For ETU the ER Interaction Score was found to be 0. Taking together, ETU is one of the 82% chemicals which did not display indications of interacting with the ER signalling pathway.




Parent.Antagonist Curves
Ethylene thiourea
Generic Chemical ID: DSSTox_GSID_20601
CASRN: 96-45-7
Tox21_ERa_LUC_BG1_Antagonist
Tox21_ERa_BLA_Antagonist_ratio
% 4-Hydroxytamoxifen
Concentration (uM)
EMAX: 0
AC50: <> NA
R^2: NA
ERa/ERb Potency: NA/NA
Parent.Antagonist Efficacy Score:0
Parent.Antagonist Potency Score:0


Growth Curves
Ethylene thiourea
Generic Chemical ID: DSSTox_GSID_20601
CASRN: 96-45-7
ACEA_T47D_80hr_Positive
% E2
Concentration (uM)
EMAX: 0
AC50: <> NA
R^2: 0
ERa/ERb Potency: NA/NA
Growth Efficacy Score:0
Growth Potency Score:0

Conclusion of the author:
An ER Interaction Score was developed by aggregating data from 13 different in vitro ER assays based on the known cellular ER signalling pathways. This model produced scores for an overall likelihood of a chemical being estrogenic, and these scores were highly correlated with in vivo data and ER reference chemical classifications, indicating that the model is capable of predicting estrogenic likelihood with a high degree of accuracy.

Conclusion of the applicant:
This study analyses around 1800 substances concerning their potential to be an endocrine disruptor. ETU was one of them and found to be zero for the binding group, agonist group, growth group and the ER Interaction Score and would be therefore of low priority for additional ER testing. This study is considered to be relevant for human risk assessment.

Classification of the study: relevant

Report: CA 5.8.1/33
Kjeldsen L.S. et al., 2013a
Currently used pesticides and their mixtures affect the function of sex hormone receptors and aromatase enzyme activity
2013/1415820
Guidelines: none
GLP: no

Executive Summary:

The in vitro effects of pesticides on estrogen receptor (ER) and androgen receptor (AR) transactivity, and aromatase enzyme activity of 14 pesticides including mancozeb, and its metabolite ethylene thiourea (ETU) were investigated. The pesticides were analysed separately and in selected mixtures. Mancozeb was not included in the mixtures investigated. Effects of the pesticides on ER and AR function were assessed in human breast carcinoma MVLN cells and hamster ovary CHO-K1 cells, respectively, using luciferase reporter gene assays. Effects on aromatase enzyme activity were analysed in human choriocarcinoma JEG-3 cells, employing the classical [(3)H](2)O method.
Neither mancozeb nor ETU induced ER transactivaton and had no effect on aromatase activity. Mancozeb antagonised AR activity in a concentration-dependent manner, ETU had no effect.
The authors conclude that currently used pesticides possess endocrine-disrupting potential *in vitro* which can be mediated via ER, AR and aromatase activities. The observed mixture effects, which did not include mancozeb, emphasize the importance of considering the combined action of pesticides in order to assure proper estimations of related health effect risks.

Objective:

To investigate the in vitro effects on estrogen receptor (ER) and androgen receptor (AR) transactivity, and aromatase enzyme activity of pesticides, including mancozeb, ETU and pesticide mixtures

Materials and Methods:

Test material 1	Mancozeb
CAS No.	8018-01-7
Source	Sigma-Aldrich, Denmark
Batch	No data
Purity	> 96%
Stability	No data
Test material 2	Ethylene thiourea (ETU) metabolite of mancozeb
CAS No.	96-45-7
Source	Sigma-Aldrich, Denmark
Batch	No data
Purity	> 99%
Stability	No data

Methods:

Estrogen receptor transactivation assay: The stably transfected MVLN cell line (carrying an estrogen response element luciferase reporter vector) was used to determine estrogenic and anti-estrogenic activities. The cells were cultured at 37°C in a humidified atmosphere of 5% CO^2 in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) with supplements [4 mM L.-glutamine, 6 g/l insulin, 64 mg/l hexamycin, 1 mM sodium pyruvate, 20 mM HEPES and 5% fetal calf serum (FCS). MVLN cells were seeded in 96-well microtitre plates (approx. 4 x 10^4 cells/well) in DMEM with supplements and 1% charcoal/dextran-treated fetal calf serum, cultured for 24 h and then exposed to serial dilutions of pesticides for 24 h. The measured luciferase data were corrected for cell density

Test compounds were assessed within the range of 1 x 10^{-10} to 1 x 10^{-4} M. The assay was repeated after co-exposure with 25 pM E2 (the concentration inducing 65% of the maximum E2 effect) at concentration of 1 x 10-^{10} to 1 x 10^{-5} M. As positive control was analysed in parallel in each assay

Androgen receptor transactivation assay. The Chinese hamster ovary cell line CHO-KI was used to assess androgenic and anti- androgenic activities. The cells were transiently co-transfected with the MMTV-LUC reporter vector and the human AR expression plasmid pSVARO. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO^2 in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 with phenol red (DMEM/F-12, no glutamine) with supplements 12 mM L-glutamine, 64 mg/L hexamycin and 10% FCS. 24 h before transfection, CHO-K1 cells were seeded in 96-well microtitre plates (approximately 8000 cells/ well) in DMEM/F-12 with supplements and 10% CD-FCS. The transfection was carried out for 5 h, using 0.3 µL per well of the transfection reagent and 150 ng cDNA per well of the AR expression plasmid pSVARO and the MMTV-LUC reporter vector in a ratio of 1:100. Cells were then exposed to serial dilutions of pesticides and incubated for 20 h. Concentrations within the range of 1 x 10^{-10} to 1 x 10^{-5} M, without removal of the transfection reagent and cDNA. The measured luciferase data were corrected for cell density. The pesticides were also tested with co-exposure with 25 pM of one of the AR agonists R1881 or DHT (corresponding to approximately the half maximum effect concentration).

Aromatase enzyme activity assay. Aromatase activity was assessed using the human choriocarcinoma JEG-3 cell line. The cells were cultured at 37°C in a humidified atmosphere of 5% CO^2 in Minimum Essential Medium with phenol red (MEM, NEAR, no glutamine) with supplements 12 mM L-glutamine, 64 mg,/L hexamycin, 1 mM sodium pyruvate and 10% FCS. JEG-3 cells were seeded in 24-well microtitre plates (approx., 4 x 10^4 cells/well in MEM with supplements and 10% FCS. The cells were cultured for 48 h at 37°C. During this period the medium was changed once. The culture medium was then aspirated, and the cells were incubated with pesticide for 18 h. The aromatization process was performed with serum-free MEM (500 µL/well) containing 0.2 µCi [1β-^{3}H] 4-androstene-3,17-dione and 10 mM unlabelled 4-androstene-3,17-dione. After termination of the 2 h aromatization process, 300 µL of the culture medium was extracted with 750 µL chloroform), and 150 µL of the aqueous phase was treated with 150 µL dextran-charcoal in PBS (5%). Finally, an aliquot of 150 µL of the treated water phase was mixed with 4 ml Hionic Fluor scintillation vial and assayed for radioactivity. The measured aromatase activities were corrected for background radiation and cell protein concentration, and related to the solvent control (set to 100%). Pesticide concentrations were within the range of l x 10^{-9} to 1 x 10^{-4} M. In each assay, the aromatase inhibitor 4-AOD was analysed in parallel at two concentrations, 1 x 10^{-8} M and 1 x 10 ' M, corresponding to approximately 4-AOD-EC50 and 4-AOD-EC100, respectively.

Cytotoxicity. Cytotoxicity was assessed for all pesticides. As a positive control, cells in triplicate were lysed to give a maximum release of lactate dehydrogenase (LDH). Culture medium from cells exposed to solvent control was the negative control. For each test compound, only results obtained at non-cytotoxic concentrations were included in the statistical analysis

Statistics: Nonparametric statistics were used. The Kruskal-Wallis test was used to compare differences between concentrations and the Jonckheere-Terpstra test (two-tailed) was used to analyse for a linear trend between concentration and response. If one or both tests showed a significant difference (p 0.05), the Mann-Whitney test was used to compare each concentration with the control. Concentration-response curves and calculations of EC50 and IC50 (the concentration that induces half of the maximum inhibitory response).were performed in SigmaPlot.

Results:

Estrogen receptor transactivation: Mancozeb was cytotoxic to the MVLN cells at concentrations $\geq 1 \times 10^{-4}$ M. Mancozeb did not affect ER transactivity. ETU had no effect.

Androgen receptor transactivation assay. Mancozeb did not elicit agonistic AR effects but did cause a down-regulating effect at 1×10^{-5} M. Upon co-treatment with 25 pM of a potent AR agonist (R1881 or DHT), mancozeb elicited significant ($p < 0.05$) and concentration-dependent antagonistic effects on the agonist-induced AR transactivity. At the lowest tested concentration causing the maximum effect (MOEC), the agonist-induced response (set to 100% for control) was 50%. Since the down-regulating effect of mancozeb, alone and upon co-exposure with R1881 or DHT, was only seen at the highest test concentration (1×10^{-5} M), it cannot be ruled out that this effect was due to an incipient toxicity of the pesticide towards the CHO-KI cells. ETU had no effect.

Aromatase enzyme activity

Neither mancozeb nor ETU induced or inhibited aromatase activity

Conclusions:

The authors conclude that currently used pesticides possess endocrine-disrupting potential *in vitro* which can be mediated via ER, AR and aromatase activities. The observed mixture effects, which did not include mancozeb or ETU, emphasise the importance of considering the combined action of pesticides in order to assure proper estimations of related health effect risks.

Liver microsomal metabolism and related studies

Flavin monooxygenase (FMO) enzyme purified from dog liver catalyses NADPH and oxygen-dependent sequential S-oxidation of ETU, proceeding through an intermediate imidazolinyl sulfenic acid to the corresponding sulfinic acid. Further oxidation to the sulfonic acid was partly enzymic and partly due to autooxidation. The FMO-oxidative pathway predominated over P-450 pathways in dog and hamster liver microsomes .
(Poulsen et al, 1979, BASF DocID 1979/10118).

Hepatic RNA synthesis, as measured by incorporation of 3H-orotic acid into nuclear or cytoplasmic RNA fractions, was not inhibited in rats (male Sprague-Dawley) treated with ETU.
(Austin and Moyer, 1979, BASF DocID 1979/10166).

The microsomal enzymes (aminopyrine, N-demethylase, aniline hydroxylase, and cytochrome P-450 content) were inhibited in rat whereas in mouse they were stimulated or unaffected. This suggests that ETU is metabolized by different enzymatic pathways in the two species.
(Lewerenz and Plass, 1984, BASF DocID 1984/10114)

In vitro studies of metabolism in liver microsomes isolated from male and female Swiss-Webster mice indicate that the mouse preferentially metabolizes ETU by the flavin-dependent monooxygenase (FMO) pathway over the cytochrome P-450 enzyme system. FMO activity decreases as males, but not females, increase in age from 3 to 30 weeks, and this difference is reflected in decreased overall metabolism of ETU and decreased FMO-mediated binding to microsomal protein. Rapid FMO-mediated metabolism of ETU may contribute to the relative lack of acute toxicity and teratogenicity in the mouse relative to the rat, and to the increased hepatotoxicity in the mouse.
(Hui et al., 1988, BASF DocID 1988/10481)

In microsomes prepared from Sprague-Dawley rat liver extracts, ETU is preferentially metabolized by the FMO pathway over the P-450 pathway. Metabolism proceeds via initial oxidation of ETU to imidazoline-2-sulfenic acid, followed by reaction with glutathione (GSH) or protein sulfhydryls under conditions of GSH depletion, resulting in regeneration of ETU (redox cycling). ETU inhibited P-450, cytochrome P-450 reductase, and FMO activity, and was covalently bound to microsomal protein (primarily P-450), but not to DNA. At physiologically relevant concentrations, GSH prevented both inhibition and protein binding, and was capable of regenerating P-450 activity with release of ETU and glutathione disulfide (GSSG). Under normal physiological conditions, such as after consumption of low levels typically present in foods, reactive metabolites generated from ETU by either FMO or P-450 are trapped by endogenous GSH and do not interact with cellular targets.
(Decker and Doerge, 1991, BASF DocID 1991/11523).

Report: CA 5.8.1/34
[redacted] 2011c
Ethylenethiourea - Test study in Wistar rats - Administration via the diet for 2 weeks
2011/1293971
Guidelines: EPA 870.7800
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Report: CA 5.8.1/35
[redacted] 2012b
Ethylenethiourea - Immunotoxicity study in male Wistar rats - Administration via the diet for 4 weeks
2012/1362403
Guidelines: EPA 870.7800
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

Ethylene thiourea (ETU, batch 07202TD, purity 100%) was administered via the diet to male Wistar rats at dietary dose levels of 0, 10, 50 and 250 ppm, respective 0, 1, 4 and 19 mg/kg bw/day for 4 weeks. Cyclophosphamide monohydrate (4.5 mg/kg bw/day) was used as a positive control.
SRBC IgM antibody titers were similar between control and ETU treated animals. No immunopathological findings were observed after treatment of animals with ETU. Animals in the highest dose group revealed a decreased body weight and food consumption as well as decreased T_4 levels and increased TSH levels. Thyroid gland weights were increased and moderate to severe follicular hypertrophy/hyperplasia in thyroid glands were observed. These effects diminished in groups with lower ETU dosage but in the lowest dose group (10 ppm) a decreased T_4 level was still observed.
The administration of the positive control cyclophosphamide monohydrate (4.5 mg/kg bw/d) led to effects indicative of immunotoxicity, i.e. reduced absolute and relative spleen and thymus weight. Furthermore, SRBC IgM antibody titers were significantly lower compared to the control group. In addition, an impaired body weight development was observed after treatment of animals with cyclophosphamide.

Based on the results of the study the NOAEL was identified at 250 ppm (19 mg/kg bw/day) for immunotoxicity and below 10 ppm (< 1 mg/kg bw/day) for general toxicity for ethylene thiourea.

(DocID 2012/1362403)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: Ethylene thiourea (ETU)

Description:	solid, white
Lot/Batch #:	07202TD
Purity:	100%
Stability of test compound:	The test substance was stable over the study period (Expiry date 23-Jun-2013).

2. Positive control: Cyclophosphamide Monohydrate (CAS No.: 6055-19-2)

Description:	solid, white
Lot/Batch #:	1362353
Purity:	100%
Stability of test compound:	The test substance was stable over the study period (Expiry date 22-Sep-2012).

3. Test animals:

Species:	Rat
Strain:	Crl:WI (Han)
Sex:	Male
Age:	33 ± 1 days at delivery; 42 ± 1 at the beginning of the administration period
Weight at dosing:	mean: 141.4 g
Source:	[redacted]
Acclimation period:	9 days
Diet:	Kliba maintenance diet rat/mouse Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water ad libitum
Housing:	Group housing (5 animals per cage) in H-Temp (PSU, TECNIPLAST, Hohenpeißenberg, Germany) cages; floor area about 2065 cm^2 with environmental enrichment
Environmental conditions:	
Temperature:	20 - 24°C
Humidity:	30 - 70%
Air changes:	Fully air-conditioned rooms; 15 of air-changes per hour
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN AND METHODS

The study was conducted at BASF SE, Experimental Toxicology and Ecology, 67056 Ludwigshafen, Germany.

1. In life dates: 20-Sep-2011 - 28-Oct-2011)
(Dates of experimental work:20-Sep-2011 to 24-Jan-2012)

2. Dose selection:
In a range-finding study (DocID 2011/1293971) ethylene thiourea was administered via the diet to groups of 4 male Wistar rats at dose levels of 0 ppm (vehicle control), 50 ppm, 300 ppm and 600 ppm over a period of 2 weeks. In conclusion, ethylene thiourea revealed adverse effects in male Wistar rats at concentrations of 50 ppm (5.2 mg/kg bw/d) and above. Assuming that the decrease of thymus weight at 50 ppm is correlated with a functional or morphological adverse effect the no observed adverse effect level (NOAEL) was below 50 ppm (5.2 mg/kg bw/d).Based on the results of this range-finding study, the following ETU concentrations in the diet were selected:

250 ppm: as high concentration (target dose: 20 mg/kg bw/d)
50 ppm: as mid concentration (target dose: 4 mg /kg bw/d)
10 ppm: as low concentration (target dose: 1 mg/kg bw/d)

3. Animal assignment and treatment:
ETU was administered to groups of 10 male rats at dietary concentrations of 0, 10, 50, and 250 ppm for 4 weeks via the diet. Cyclophosphamide monohydrate (4.5 mg/kg bw/d) was administered by gavage as a solution in drinking water.
All animals were immunized 6 days before blood sampling and necropsy using 0.5 mL sheep red blood cells ($4x10^8$ SRBC/ml) administered intraperitoneally. The animals were assigned to the treatment groups by means of a computer generated randomization lists based on body weights.

4. Test substance preparation and analysis:
Previous tests indicated that ETU was stable in the diet at room temperature for at least 10 days at a concentration of 50 ppm. The stability of the test substance in the diet at a concentration of 10 ppm was demonstrated close to the start of the administration period. The stability of cyclophosphamide monohydrate (positive control substance) in the vehicle (drinking water) over a period of 7 days at room temperature and 32 days (in the freezer) was proven prior to the start of the study.
Homogeneity analyses were performed at all concentrations at the start of the study (see Table 5.8.1-71). Samples also served for concentration control.

Table 5.8.1-71: Analysis of diet preparations for homogeneity and test-item content

Dose level [ppm]	Sampling	Concentration [ppm] Mean ± SD	Relative standard deviation [%]	Mean / of nominal concentration
10 ppm	29.09.2011	10.20	2.0	102.0
50 ppm	29.09.2011	52.08	1.5	104.2
250 ppm	29.09.2011	269.2	0.9	107.7

Relative standard deviations of the homogeneity samples in the range of 0.9 to 2.0% indicate the homogenous distribution of ETU in the diet preparations. The actual test-item concentrations were in the range of 102.0 to 107.7% of the nominal concentrations.
For cyclophosphamide monohydrate the 100.9% of the nominal concentration was achieved.

5. Statistics:

Means and standard deviations of each test group were calculated for several parameters. Further statistical analyses were performed according to following tables:

Statistics of clinical examinations

Parameter	Statistical test
Body weight, body weight gains (control and test substance treatment)	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means
Body weight, body weight gains (control and Cyclophosphamide monohydrate treatment)	A comparison of the dose group with the control group was performed using the t-test (two-sided) for the hypothesis of equal means

Statistics of clinical pathology

Parameter	Statistical test
Clinical pathology parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair-wise comparison of each dose group with the control group was performed using Wilcoxon-test (two-sided) for the equal medians

Statistics of pathology

Parameter	Statistical test
Organ weights	Non-parametric one-way analysis using KRUSKALWALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using the WILCOXON test for the hypothesis of equal medians

C. METHODS

1. Observations:

The animals were examined for evident signs of toxicity or mortality twice daily on working days and once daily on weekends and public holidays.
The clinical condition of the test animals were recorded individually.
Detailed clinical observations of all animals were performed in a standard arena prior to the administration period and weekly thereafter. The standard arena had a size of 50 x 37.5 cm with walls of 25 cm height. If applicable the findings were ranked according to the degree of severity. The following parameters were examined:

1. abnormal behavior during handling
2. fur
3. skin
4. posture
5. salivation
6. respiration
7. activity/arousal level
8. tremors
9. convulsions
10. abnormal movements
11. impairment of gait
12. lacrimation
13. palpebral closure
14. exophthalmus
15. feces (appearance/consistency)
16. urine
17. pupil size

2. Body weight:
The body weight of the animals was determined before the start of the administration period in order to randomize the animals, at the start of the exposure period and thereafter twice weekly. The difference between the body weight on the respective day of weighing and the body weight on day 0 was calculated as body weight change.

3. Food consumption, food efficiency and compound intake:
Group food consumption was determined weekly (as representative value over 1 day) for each cage. The average food consumption was used to estimate the mean food consumption in grams per animal and day.
Water consumption was observed daily by visual inspection of the water bottles for any overt changes in volume.

The mean daily intake of test substance (group means) was calculated based upon individual values for body weight and food consumption.

$$\text{Substance intake for day x} = \frac{FC_x \times C}{BW_x}$$

BW_x = body weight on study day x [g]
FC_x= mean daily food consumption on study day x [g]
C = concentration in the food on study day x [mg/day]

4. Clinical pathology:
Immunotoxicological examinations:
Primary T-cell dependent antibody response (anti-SRBC IgM ELISA) was performed at a dilution of 1:500.

Hormones:
The concentration of thyroid stimulating hormone (TSH), total triiodothyronine (T_3) and total thyroxine (T_4) were determined.

5. Sacrifice and pathology:
All animals were sacrificed by decapitation under isoflurane anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology. The following organs were sampled, weighed and examined histopathologically:

Pathology:
The following organs were collected (column C), weighed (W) and examined histopathologically (H, ✓: all groups, #: control and top dose).

C	W	H	
✓	✓		adrenals
✓			gross lesions
✓	✓	✓	liver
✓	✓	✓	spleen
✓	✓	✓	thymus
✓	✓	✓	Thyroid gland
	✓		body (anesthethized)

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity

No test substance-related clinical signs were observed in all animals treated with ETU and in animals which received cyclophosphamide monohydrate as positive control.

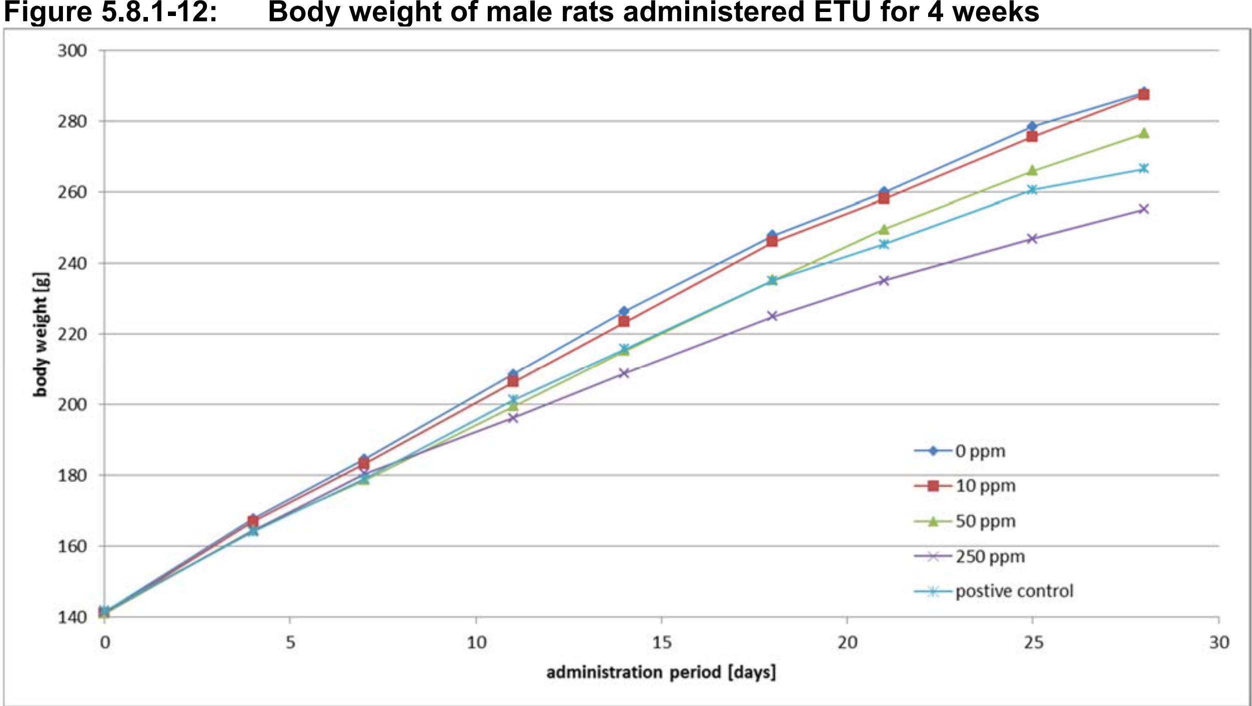
2. Mortality

No mortality was observed throughout the study.

B. BODY WEIGHT AND BODY WEIGHT GAIN

For male animals of test group 3 (250 ppm), mean body weight was significantly reduced from study day 11 onwards until study day 28 and reached a maximum of 11% less on study days 25 and 28. Mean body weight change values of this test group were significantly reduced over the entire study period (except study day 7) with a maximum of -23% on study day 25. These findings were considered to be treatment-related, direct adverse systemic effects of ETU.
At dietary concentrations of 10 and 50 ppm, there was no significance decrease in mean body weight. However, body weight change values of test group 2 (50 ppm) was significantly decreased on study days 7, 11, 14, 18 and 25 with a maximum of -13% on study day 7. The finding was assessed as being related to treatment.
For positive control animals receiving cyclophosphamide monohydrate (test group 4) mean body weights were significantly decreased from study day 14 onwards with a maximum of -8% on study day 28. The body weight change values of test group 4 (4.5 mg/kg bw/d cyclophosphamide monohydrate) were significantly decreased over the entire study period with a maximum approximating -16% on study day 4. [see Figure 5.8.1-12 and Table 5.8.1-72].

Figure 5.8.1-12: Body weight of male rats administered ETU for 4 weeks



C. FOOD CONSUMPTION AND COMPOUND INTAKE

When compared to control group 0, no relevant changes in food consumption were observed for test groups 1 and 2 (10 ppm and 50 ppm) treated with ethylene thiourea as well as for test group 4 treated with cyclophosphamide monohydrate. Deviated values were considered to be unrelated to treatment as they were still within the range typical for male rats of this age and strain. In test group 3 (250 ppm) food consumption was decreased over the entire study period with a maximum of 35% on study day 28 (see Table 5.8.1-72).
The mean daily test substance intake was determined to be 1, 4 and 19 mg/kg bw/day for the 10, 50 and 250 ppm group, respectively.

Table 5.8.1-72: Body weight and food consumption data of rats administered ETU for 4 weeks

	Males				
Dose level [ppm]	**0**	**10**	**50**	**250**	**Positive control**
Body weight [g]					
- Day 0	141.4	141.1	141.1	141.4	141.8
- Day 28	288.0	287.3	276.4	**255.1****	**266.5****
% (compared to control)		-0.3	-4.0	**-11.4**	**-7.5**
Overall body weight gain [g]	146.6	146.2	135.3	**113.7****	**124.7****
% (compared to control)		-0.3	-7.7	**-22.5**	**-14.9**
Food consumption [g/day]					
- d27 -> d28	25.5	23.0	24.4	16.5	25.1
% (compared to control)		-9.8	-4.5	-35.3	-1.6

** $p \leq 0.01$ (Student's test, two sided); $ calculated from the weekly means

D. BLOOD ANALYSIS

1. Primary T-cell dependent antibody response (anti-SRBC IgM ELISA)

Six days after immunization no difference between control and substance treated animals was observed. SRBC titers were significantly lower in animals treated with cyclophosphamide monohydrate [see Table 5.8.1-73].

Table 5.8.1-73: Blood analysis findings of rats administered ETU for 4 weeks

Sex	**Males**				
Dose [ppm]	**Values**				
	0	**10**	**50**	**250**	**Pos. control**
SRBC [U/mL]	11073±11775	15624±7866	23761±19808	12938±6208	1774±860**

** p ≤ 0.01 (Wilcoxon-test, two sided)

2. Hormones

At the end of the study T4 levels were dose-dependently decreased in male rats of all dose groups with the test substance (10, 50 and 250 ppm), whereas no change of T4 levels was observed in the positive control group (test group 4; 4.5 mg/kg bw/d cyclophosphamide monohydrate). TSH levels were only increased in the high-dose group 3 (250 ppm). T3 levels were not altered in any dose group (see Table 5.8.1-74).

Table 5.8.1-74: Hormone levels in rats administered ETU for 4 weeks

Group	0 ppm	10 ppm	50 ppm	250 ppm	Positive control
T3 [nmol/L]	1.54±0.25 k	1.34±0.19	1.30±0.17	1.63±0.34	1.56±0.31
T4 [nmol/L]	64.72±8.90 v	**53.42**±5.23**	**41.33**±4.73**	**26.70**±6.77**	59.04±4.29
TSH [µg/L]	2.61±1.36 v	3.99±1.93	4.30±2.25	**91.07**±60.84**	2.20±1.59

k = Kruskal-Wallis; v = Kruskal-Wallis-Wilcox, ** p≤ 0.01

D. NECROPSY

1. Organ weight

Organs which were significantly decreased or increased are shown in Table 5.8.1-75. All other mean weight parameters did not show significant differences when compared to the control group 0.

Absolute weights:

The decrease of terminal body weight and the increase of thyroid gland weights in animals of test group 3 (250 ppm) were considered to be treatment-related.

The thymus weights were decreased in animals of test groups 2 (50 ppm) and 3 (250 ppm). Histopathological examinations showed no histopathological findings in the thymus. Because a histomorphological correlate was missing, a treatment-related decrease is unlikely. Also, in the absence of a clear dose response, and the clustering of these absolute organ weights within a 10-year historical control range in performing laboratory (thymus absolute and relative weights: 280.9 mg – 614.4 mg (mean: 492.945 mg) / 0.1% - 0.218% (mean: 0.182%)), a relationship to treatment was not established.

The decrease of absolute spleen weight in animals of test group 3 (250 ppm) was considered secondary to decreased terminal body weights. Histopathological examinations showed no histopathological findings in the spleen. In addition, a dose-response relationship (it increased at lower dose levels) as well as a histomorphological correlate was missing, and the decrease was within the 10-year historical control range of male Wistar rats for the performing laboratory (spleen absolute and relative weights: 0.442 g – 0.620 g (mean: 0.534 g) / 0.172% - 0.245% (mean: 0.198%)). The totality of this evidence demonstrates a lack of relationship to treatment for these top dose changes in absolute weights.
The positive control group (test group 4; cyclophosphamide monohydrate) revealed a significant decrease of terminal body weight (-8%), spleen (-27%) and thymus (-33%) weights, which were the expected results.

Table 5.8.1-75: Terminal body and organ weights of rats administered ETU for 4 weeks

Sex	Males								
Dose [ppm]	Values					% (compared to control)			
	0	10	50	250	PC	10	50	250	PC
Terminal body weight abs. [g]	264.9	264.0	254.0	**235.5****	244.4**	100	96	**89****	92**
Liver abs. [g]	7.173	7.32	7.292	6.92	6.921	102	102	95	96
rel. [%]	2.71	2.774	2.871*	**2.892***	2.831	102	106*	**107***	104
Spleen abs. [g]	0.525	0.58	0.583	0.432**	0.383**	110	111	82**	73**
rel. [%]	0.198	0.22	0.229*	0.184	0.157**	111	116*	93	79**
Thymus abs. [mg]	525.2	514.4	415.1**	403.5**	349.3**	98	79**	77**	67**
rel. [%]	0.199	0.195	0.164*	0.172*	0.142**	98	82*	86*	72**
Thyroid glands abs. [mg]	18.7	20.0	19.4	**41.5****	20.7	107	104	**222****	111
rel. [%]	0.007	0.008	0.008	**0.018****	0.008	107	108	**248****	120

* < 0.05; ** p ≤ 0.01; Kruskal-Wallis H and Wilcoxon test, two sided
PC = positive control
Bolded values are considered to be treatment related.

Relative weights:
The increase of liver and thyroid gland weights in animals of test group 3 (250 ppm) was considered treatment-related based on histopathological correlation with a hypertrophy in the respective organs.
The relative thymus weights were decreased in animals of test groups 2 (50 ppm) and 3 (250 ppm). Because a histomorphological correlate was missing, coupled with lack of a clear dose response and data magnitudes within 10-year historical controls for male Wistar rats in the performing laboratory, a treatment-related decrease was not established.
The increase of relative spleen weight in animals of test group 2 (50 ppm) did not follow dose response and was hence considered incidental.
The positive control group (test group 4; cyclophosphamide monohydrate) revealed a significant decrease of spleen (-21%) and thymus (-28%) weights, which were the expected results.

2. Gross and histopathology

The thyroid glands were enlarged in 9 animals of test group 3 (250 ppm), which was assessed as being a treatment-related effect.
No other gross lesions were observed.

The thyroid glands revealed a moderate to severe follicular hypertrophy/hyperplasia in all animals of test group 3 (250 ppm). A minimal to slight centrilobular hepatocellular hypertrophy in the liver was seen in 8 animals of test group 3 (250 ppm).
All other findings occurred either individually or were biologically equally distributed over the control group and the treatment groups. They were considered incidental or spontaneous in origin and without any relation to treatment.
No histopathological findings were recorded in the thymus and spleen, which could explain the weight decrease. Also no findings pointing to an immunotoxic effect were observed.

III. CONCLUSIONS

Under the conditions of the study ethylene thiourea did not reveal any signs of immunotoxicity when administered via the diet over a period of 4 weeks to male Wistar rats. The NOAEL for the immunotoxicologically relevant endpoints was set to 250 ppm (19 mg/kg bw/day), the highest dose tested. With regard to systemic toxicity excluding immunological aspects, the NOAEL for this test compound in male Wistar rats was below 10 ppm taking decreased thyroid hormone levels into account. The oral administration of the positive control substance cyclophosphamide monohydrate (4.5 mg/kg bw/day) led to severe findings indicative of immunotoxicity. This was represented by significantly lower SRBC IgM antibody titres as well as reduced spleen and thymus weights. Thus, assay sensitivity was verified in the present immunotoxicity study performed in male Wistar rats.

Mode of action and human relevance of ETU toxicity
Similarly to the thionamide drugs, the primary toxicological finding with ETU in laboratory animals is inhibition of the synthesis of thyroid hormones, thyroxine (T4) and triiodothyronine (T3), leading to elevated serum levels of thyroid stimulating hormone (TSH) via feedback stimulation of the hypothalamus and pituitary. Prolonged and continuous elevation of serum TSH levels results in hypertrophy and hyperplasia of the thyroid follicular cells in rats, mice, monkeys and dogs, and ultimately in the development of nodular hyperplasia, adenoma, and/or carcinoma in rats and mice, but not hamsters. There is evidence for reversibility. Direct evidence for inhibition of thyroid hormone synthesis by ETU has been obtained in rats in vivo. ETU also reversibly inhibited thyroid peroxidase-catalysed iodination reactions in vitro.

Similarly, the long-term stimulation of the pituitary via hypothalamic thyrotropin-releasing hormone (TRH) also results in morphologic changes in the pituitary of rats in all four species tested, culminating in adenomas of the pars distalis after two years exposure in mice.

The sequence of events relating thyroid hormone inhibition via hormonal imbalance to the onset of pituitary and thyroid follicular neoplasia in rodents is well-characterized, with the inference that the threshold for the early steps in the sequence, particularly the key elevation of TSH levels, is necessarily a threshold for the remaining steps in the process, including carcinogenesis. Thus, for purposes of human oncogenic risk assessment, the principle of the existence of a threshold for thyroid and pituitary neoplasia resulting from thyroid inhibition has been accepted. In addition, in comparison to laboratory animals humans are expected to exhibit a lesser degree of sensitivity to thyroid inhibitors. The reasons for this are twofold:

1. Humans have substantial reserve supply of thyroid hormone, much of which is carried in thyroxine-binding globulin, a serum protein that is missing in laboratory rodents.
2. Under conditions of prolonged thyroid insufficiency, caused for example by dietary iodine deficiency, the primary human response is goiter rather than neoplasia. Thus, a large uncertainty factor is not needed to insure adequate protection of the human population from effects of dietary exposure to ETU. (Hurt SS 1992, BASF DocID 1992/11595)

In order to better quantify the differences between humans and rats with regard to thyroid toxicity, the following table compares the relevant factors:

Table 5.8.1-76: Thyroid-relevant parameter compared between rats and humans

Parameter	Humans	Rats	Reference
Thyroxin-binding globulin (TBG)	Present (68% is bound to TBG)	Essentially absent; low affinity binding to albumin	Kaptein et al., 1994 (DocID 1994/1005509) Lewandowski et al., 2004 (DocID 2004/1040994)
T4 half life	5 – 9 days*	0.5 – 1 day	Jahnke et al., 2004 (DocID 2004/1040995)
T3 half life	1 day	0.25 days	Lewandowski et al., 2004 (DocID 2004/1040994)
T4 production rate/kg bw	1x	10x compared to humans	Lewandowski et al., 2004 (DocID 2004/1040994); Dohler et al., 1979 (DocID 1979/1001743)
TSH	1x	6 – 60 x compared to humans	Lewandowski et al., 2004 (DocID 2004/1040994), Hill et al., 1989 (Doc ID 1989/1003744)
Sex differences Serum TSH	Sexed equal	Adult males > adult females	Jahnke et al., 2004 (DocID 2004/1040995)
Thyroid cancer frequency	Rare	Dependant of rat strain frequent in old aged rats	Hill et al., 1989 (DocID 1989/1003744) McClain et al., 1992 (DocID 1992/1005417)

*Lewandowski have reported a T4 half life of 5-6 days

To further prove the lower sensitivity of humans to show thyroid hormone imbalance after inhibition of the thyroid peroxidase, the blood levels of rats and humans after oral doses (leading to an efficient TPO inhibition) of propylthiouracil have been compared.

1. Humans: Serum concentrations above 4-5 µg/L 1 h after an oral dose of 400 mg of propylthiouracil (corresponds to a dose of 7 mg/kg bw) will secure a sufficient and rapid antithyroid effect during continuous therapie (Kampmann and Hansen, 1981, DocID 1981/1001481)
2. Rats: At serum concentrations between 0.09 and 0.18 µg/L propylthiouracil in rats the thyroid hormone system was completely blocked (Francis and Rennert, 1981)

This comparison shows, that rats are considerably more sensitive towards propylthiouracil with regard to effects on thyroid hormone synthesis caused via TPO inhibition. A factor of roughly 30 can be calculated. Thus the rat as an animal species is considered to be overly susceptible towards thyroid-related toxicity.

Derivation of reference values for ETU

ARfD

An acute reference dose has already been agreed upon during the registration process of metiram and is included in the list of endpoints.

The ARfD is 0.05 mg/kg bw, based on the NOAEL of 5 mg/kg bw determined in the developmental toxicity study in rats (Khera et al., 1979)

AOEL and ADI (as discussed in the Monograph)

In the Monograph of Metiram a proposal for AOEL and ADI setting for ETU was made by the rapporteur: "In light of the effects on the thyroid observed in the long-term studies, the RMS proposes an AOEL for ETU of 0.004 mg/kg bw/day, established by applying a Safety Factor of 100 to the NOAEL of 0.37 mg/kg bw/day (2-year study, rat)."

"The overall lowest NOEL is derived from a long term study on rat and it is equivalent to 0.37 mg/kg bw/day. The compound has no genotoxic carcinogenic potential but it is a potent teratogen (rat) a safety factor of 200 is considered appropriate to determine the ADI. The proposed ADI is therefore: 0.002 mg/kg bw/day based on the most sensitive animal study."

AOEL and ADI proposal

The following studies conducted with ETU might be considered relevant starting points to derive an AOEL:

Table 5.8.1-77: Summary of relevant subchronic and chronic studies with ETU

Type of test Test species	Test substance purity	Dose tested	NOAEL	Reference
Rat 90-day feeding study	96.8%	0, 1.0, 5.0, 25, 125, 625 ppm	25 ppm, equivalent to **1.44 mg/kg bw** based on altered thyroid fuction and follicular hyperplasia	1977/10212
Mouse 90-day feeding study	98%	0, 125, 250, 500, 1000, 2000 ppm	250 ppm, equivalent to **38 mg/kg bw** based on thyroid follicular hyperplasia and liver hepatocellular cytomegalies	1992/12022 1992/1004097
Dog 13-week feeding study	98%	0, 10, 150, 2000 ppm	150 ppm, equivalent to **6.02 mg/kg bw** based on deaths, thyroid effects and related anemia	1991/5117
Dog 1-year feeding study	98%	0, 5, 50, 500 ppm	5 ppm, equivalent to 0.18 mg/kg bw based on increased thyroid weight, hypertrophy with colloid retention at 50 ppm	1992/5082
Rat 2-year feeding study	96.2%	0, 0.5, 2.5, 5.0, 125 ppm	5 ppm, equivalent to **0.37 mg/kg bw** based on decreased body weight in males and thyroid, pituitary, and liver effects in both sexes	1992/11621
Rat extended One-generation toxicity study	100%	0, 2.8, 28, 140 ppm	2.8 ppm, equivalent to 0.2 mg/kg bw based on thyroid follicular hyperplasia seen in parental generations	2011/7009688

Any endpoints taken from the rat studies are considered to be overly sensitive in comparison to humans, based on the clearly lower half-lifes of T4, T3 and TSH, the lower binding affinity to a binding protein, and to the reported significantly increased production rates of T4 and TSH. This assessment is further supported by a rat/human factor of 30 identified for internal blood levels after an effective dosage of propylthiouracil. Thus it is proposed to use the dog studies for relevant endpoint derivation for AOEL and ADI
The AOEL is 0.06 mg/kg bw based on the NOAEL of 6.02 mg/kg bw identified in the 13 week dog study. No correction would have to be made for oral absorption, as ETU is rapidly and completely absorbed after oral exposure [redacted] 1985; BASF DocID 1985/0470).
The ADI is 0.002 mg/kg bw based on the NOAEL of 0.2 mg/kg bw identified in the 1 year dog study.

Metabolite: Ethylene Urea

QSAR predictions on Ethylene Urea:

OASIS TIMES (V.2.27.16.8; Mutagenicity S-9 activated v09.09) [see molecule 10 of report DocID 2015/1094106]
There were **no** Ames mutagenicity alerts for ethylene urea and no structural alerts were received. The parent substance was in the model applicability domain.

VEGA: Mutagenicity model (CAESAR, version 2.1.12) [see molecule 11 of report DocID 2015/1094107]
Ethylene urea could be out of the model applicability domain. The prediction is **'non-mutagen'** with a **moderate** overall reliability. Deficiencies result from:
- some similar molecules found in the training set have experimental values that disagree with the predicted value
- accuracy of prediction for similar molecules found in the training set is not optimal

VEGA: Mutagenicity model (SarPy; version 1.0.6-DEV) [see molecule 11 of report DocID 2015/1094107]
Ethylene urea could be out of the model applicability domain. The prediction is **'non-mutagen'** with a **moderate** overall reliability. Deficiencies result from:
- some similar molecules found in the training set have experimental values that disagree with the predicted value
- accuracy of prediction for similar molecules found in the training set is not optimal

VEGA: Mutagenicity model (Toxtree; version 1.0.0-DEV) [see molecule 11 of report DocID 2015/1094107]
Ethylene urea could be out of the model applicability domain. The prediction is **'non-mutagen'** with a **moderate** overall reliability. Deficiencies result from:
- accuracy of prediction for similar molecules found in the training set is not optimal

Toxicological information on Ethylene Urea:
Ethylene urea (CAS No. 120-93-4) is a REACH Tier 2 chemical and a registration has been submitted to ECHA. The dossier can be found on the ECHA homepage. The study summaries are included in this dossier for the convenience of the evaluators.

Acute Toxicity (EU)

Report: CA 5.8.1/36
[redacted] 2011a
Translation - Report on the study of the acute oral toxicity of Ethyleneurea in rats
2011/1293133
Guidelines: none
GLP: no

Executive Summary
In an acute oral toxicity study, originally performed 1958, rats were dosed once orally with ethyleneurea in a 10% aqueous solution. The approximative median lethal dose after 24 h and 8 days was greater than 5 g/kg.

(BASF DocID 2011/1293133)

Report: CA 5.8.1/37
[redacted] 2012a
Ethylene Urea - Acute dermal toxicity study in rats
2012/1360603

Guidelines: OECD 402 (1987), EPA 870.1200, JMAFF No 12 Nosan No 8147, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. L 142

GLP: yes
(certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

Executive Summary

In an acute dermal toxicity study 10 Wistar rats (5/sex) were dermally exposed to a single dose of 'Ethylene Urea' (batch 11-0041, purity: 99.1%) as suspension in deinodized water at a dose level of 2000 mg/kg bw. The treated skin was clipped and exposed under semi-occlusive dressing for 24 hours.

No mortality occurred. Accordingly, the oral LD50 was found to be greater than 2000 mg/kg bw for rats.

Rat, dermal: LD50 > 2,000 mg/kg bw

No signs of systemic toxicity or skin effects were observed in the animals during the 14 day observation period. The expected body weight gain was observed in the course of the study. No abnormalities were noted at necropsy of animals sacrificed at the end of the study.

(BASF DocID 2012/1360603)

II. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: Ethylene Urea
Description: solid, white
Lot/Batch #: 1-0041
Purity: 90.1 g / 100 g
Stability of test compound: The stability of the test substance was guaranteed by the sponsor.
CAS No.: 120-93-4

2. Vehicle: deionized water

3. Test animals:

Species:	Rat
Strain:	Wistar (Crl:Wl (Han) SPF)
Sex:	males and females
Age:	young adults
Weight at dosing (mean):	mean female: 207 g; mean male: 243 g
Source:	
Acclimation period:	at least five days
Diet:	VRF1 (P); SDS Special Diets Services, 67122 Altrip, Germany
Water:	tap water ad libitum
Housing:	Single housing in Makrolon cage, type III with H 15005-29 Bedding (Ssniff, Spezialitäten GmbH, Experimental Animal Diets Inc., 59494 Soest Germany) and NGM E-022 enrichment (ABEDD® LAB & VET Services GmbH, Hasnerstraße 84/6, 1160 Wien, Austria)
Environmental conditions:	
Temperature:	19 – 25°C
Humidity:	30 – 70%
Air changes:	fully air-conditioned
Photo period:	12-hour light-dark cycle (06:00 – 18:00, 18:00 – 06:00)

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 25-Oct-2011 to 09-Nov-2011 (day of first administration and day of last observation)

2. Animal assignment and treatment:

Twenty four hours before treatment the fur was clipped. Ten rats (5 per sex per group) received a single dose of 2000 mg/kg bw of test substance in deionized water to the clipped epidermis (dorsal and dorsolateral parts of the trunk). Test substance was given to the animals at a volume of 4 mL/kg bw and was covered with a semi-occlusive dressing (4 layers of absorbent gauze and stretch bandage) for 24 hours. Afterwards the semi-occlusive dressing was removed and the application site was rinsed with warm water. The application area was about 40 cm² which corresponds to at least 10% of the body surface. The observation period lasted 14 days. Individual body weights were recorded shortly before application (day 0) and weekly thereafter and at the end of the study. Clinical signs and symptoms were recorded several times on the day of administration, at least once each workday for the individual animals. The Scoring of skin findings were evaluated 30 to 60 minutes after removal of the semi-occlusive dressing (day 1), weekly thereafter and on the last day of observation. The evaluation of skin reactions was performed according to Draize (1959): Appraisal of the safety of chemicals in food, drugs and cosmetics. The association of food and drug officials of the United States Austin, Texas. After the observation period a necropsy of all animals with grosspathology followed.

II. RESULTS AND DISCUSSION

A. MORTALITY

No deaths occurred after dermal administration of 2000 mg/kg bw of the test substance.

B. CLINICAL OBSERVATIONS

No systemic clinical signs or no local effects were observed during clinical examination.

C. BODY WEIGHT

Normal body weight gain was observed during the 14 days of the observation period.

D. NECROPSY

No macroscopic anomaly was noted in the organs examined after dermal treatment.

III. CONCLUSION

Under the experimental conditions of this study the dermal LD50 of ‘Ethylene Urea’ in rats was determined to be greater than 2000 mg/kg bw for males and females.

Irritation/corrosion

Report: CA 5.8.1/38
[redacted] 2011b
Translation - Report on the study of the primary irritation/corrosion of Ethyleneurea to the intact skin of rabbits
2012/1360608
Guidelines: none
GLP: no

Executive Summary
In a primary irritation/corrosion study, originally performed 1958, rabbits were percutaneously with ethyleneurea (90% in water in the form of a paste). Slight erythema with individual hemorrhages were observed after 24 h. After 8 days circumscribed crusts (lentil-sized; fallen off) were observed

(BASF DocID 2012/1360608)

Report: CA 5.8.1/39
[redacted] 2011c
Translation - Report on the study of the primary irritation of Ethyleneurea to the eye of rabbits
2011/1293135
Guidelines: none
GLP: no

Executive Summary

In a primary irritation study, originally performed 1958, rabbits were treated with ethyleneurea (powder). The application was performed once with 0.1 g of the test substance into the conjunctival say of the eyelid. One hour after treatment inflammation was observed and was still present after 24 h. In addition, lentil-sized opacity of the cornea was observed after 24 h. No abnormalities were reported after 8 days.

(BASF DocID 2011/1293135)

Skin sensitisation

Report: CA 5.8.1/40
[redacted] 2009a
1,3-Ethyleneurea - Murine local lymph node assay (LLNA)
2009/1129202
Guidelines: OECD 429 (2002), EEC 2004/73 B.42, EPA 870.2600
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

For the determination of potential sensitizing properties of 1,3-Ethyleneurea (batch: 08-0034, purity: 92.1%) a mouse local lymph node assay (LLNA) was conducted.
Topical application of the formulation to the ears of groups of 5 female mice at 3 different concentrations for three consecutive days did not result in a biologically significant increase of the stimulation indices (SI) for lymph node cell count, ^{3}H-thymidine incorporation into the lymph node cells and lymph node weights when assessed approximately 3 days after the last application. No increase in ear weights was observed for the test substance preparations.

In conclusion, based on the results of this study 1,3-Ethyleneurea does not display skin sensitizing properties under the conditions of the test.
(DocID 2009/1129202)

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** 1,3-Ethyleneurea (CAS 120-93-4)
   Description: not provided in the report
   Lot/Batch #: 08-0034
   Purity/content: 92.1 g / 100 g
   Stability of test compound: The stability under storage conditions over the study period was guaranteed by the manufacturer, and the manufacturer holds this responsibility.

2. **Vehicle and/or positive control:** 1% Pluronic® L92 in bi-distilled water

3. Test animals:

Species:	Mouse
Strain:	CBA/J
Sex:	females
Age:	6 to 12 weeks
Weight at dosing:	17.5 – 22.2 g
Source:	
Acclimation period:	at least 14 days
Diet:	Kliba-Labordiät (Maus / Ratte Haltung "GLP"), Provimi Kliba SA, Kaiseraugst, Basel, Switzerland, ad libitum
Water:	tap water ad libitum
Housing:	individual housing in Makrolon Type II cages with Lignocel FS 14 (SSNIFF) bedding and environmental enrichment: Nest-building material (wood wool) (Typ NBF E-011); Abedd® Lab. and Vet. Service GmbH Vienna, Austria
Environmental conditions:	
Temperature:	20 - 24°C
Humidity:	30 - 70%
Air changes:	not indicated in the report
Photo period:	12 h light / 12 h dark (06:00 - 18:00/18:00 – 06:00)

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 23-May-2008 - 06-Oct-2008
(in life work: 06-Aug-2008 – 25-Aug-2008)

2. Animal assignment and treatment:
The skin sensitizing potential of 1,3-Ethyleneurea was assessed using the radioactive Murine Local Lymph Node Assay. For this, female mice were allocated to groups of 5 animals according to the randomization instructions of „Nijenhuis, A. and Wilf, H.S.: Combinatorial Algorithms, Academic Press, New York, San Francisco, London, 1978, pp. 62 – 64".
The groups were treated either with

- the vehicle (1% aqueous Pluronic®),
- a 10% (w/w) dilution of the test article in 1% aqueous Pluronic®,
- a 30% (w/w) dilution of the test article in 1% aqueous Pluronic®, or
- a 50% (w/w) dilution of the test article in 1% aqueous Pluronic®.

3. Analysis of treatment solutions:
The stability of the test substance in the vehicle was confirmed and determined indirectly by the concentration control analysis. For this purpose, the samples taken were stored at room temperature over the maximum duration of the application period and were subsequently deep-frozen. Afterwards, these samples were analyzed. The homogeneity of the test substance preparation in the vehicle was not determined by analysis but was visually homogenous (solution). The actual test substance concentration was determined once for the 10, 30% and 50% dilution. The correctness of the concentration of the test-substance preparations (10%, 30% and 50%) for the first application was confirmed by analysis. For details see raw data.

4. Statistics:
Not performed in this study

5. Clinical observation:
Mortality was checked twice daily on working days and one on weekends and public holidays. No detailed clinical examination of the individual animals was performed but any obvious signs of systemic toxicity and/or local inflammation at the application sites were recorded

6. Body weights:
Individual body weights were determined on day 0 prior to the first application and on day 5 prior to the sacrifice of the animals.

7. Treatment of animals:
The dosing solutions were applied daily to the dorsal part of the ears at a volume of 25 µL per ear for 3 consecutive days. On study day five, i.e. 66 to 72 hours after the last application, 20 µCi of ^{3}H-thymidine in 250 µL sterile saline was injected into the tail vein of each mouse.

8. Terminal procedures:
Approximately 5 hours after ^{3}H-thymidine injection the animals were killed by cervical dislocation.
Immediately after the death of each animal a circular piece of tissue (diameter 0.8 cm) was punched out of the apical part of each ear of all animals. The weight of the pooled punches was determined for each test group. These measurements served for the detection of a potential inflammatory ear swelling. Immediately after removal of the ear punches the left and right auricular lymph nodes were dissected. The weight of the pooled lymph nodes from both sides was determined for each animal. After weight determination, a single cell suspension was prepared per test group from the pooled lymph nodes by carefully passing all lymph nodes through an iron mesh (mesh size 200 µm) into 40 mL of phosphate-buffered physiological saline. Subsequently the cell counts were determined with an aliquot of each suspension using a Casy®-Counter. The remaining cell suspensions were washed twice with phosphate buffered saline (PBS) and precipitated with 5% trichloro-acetic acid. Each precipitate was transferred to scintillation fluid and incorporation of ^{3}H-thymidine into the cells was measured in a □-scintillation counter.

9. Data evaluation and interpretation
The stimulation indices (SI) of cell count, 3H-thymidine incorporation, lymph node weight and ear weight were calculated as the ratio of the test group values for these parameters divided by those of the vehicle control group.

The lymph node cell count and the ^{3}H-thymidine incorporation into the lymph node cells as well as to a certain extent lymph node weight are used to determine the potential sensitizing properties of a test article. Because not only sensitization induction but also irritation of the ear skin by the test substance may induce lymph node responses, the weight of ear punches taken from the area of test-substance application is determined as a parameter for inflammatory ear swelling as an indicator for the irritant action of the test substance.

Stimulation indices of >1.5 for cell count and/or of ≥ 3 for ^{3}H-thymidine incorporation are generally considered as indicative for a sensitizing potential of a test substance. If applicable, the EC (estimated concentration) leading to the respective SI values were calculated by linear or semi-logarithmical regression.

If the increase in cell count, ^{3}H-thymidine incorporation and/or lymph node weight is accompanied by a biologically relevant increase in ear weights it cannot be ruled out that the lymph node response was caused by irritation and not by skin sensitization. Depending on the magnitude of lymph node response, based on expert judgment, the evaluation of the sensitizing potential may be modified or additional studies might be necessary.

If a test substance does not elicit a biological relevant increase in cell count, 3H-thymidine incorporation but shows a clear concentration related increase in response, further investigation of the sensitization potential at higher concentrations should be considered.

10. Positive controls
A concurrent positive control (reliability check) with a known sensitizer was not included into this study. Studies using the positive control substance alpha-hexylcinnamaldehyde are performed twice a year in the laboratory in order to show that the test system is able to detect sensitizing compounds under the test conditions chosen.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

No clinical observations or mortality were observed.

B. BODY WEIGHTS

There were no effects on body weight development. The increase of body weights during the study was within the expected range.

C. STIMULATION INDICES

The stimulation indices (SI) for lymph node cell counts, ^{3}H-thymidine incorporation and lymph node and ear weights are given in Table 5.8.1-78.

Table 5.8.1-78: Stimulation indices for cell counts, ^{3}H-thymidine incorporation, lymph node and ear weight in mice after treatment with 1,3-Ethyleneurea

Test Group	Treatment	Parameter evaluated	Stimulation index[1]
		Cell count [counts/lymph node pair]	
1	vehicle 1% aqueous Pluronic®	6236000	1.00
2	10% in 1% aqueous Pluronic®	5501333	0.88
3	30% in 1% aqueous Pluronic®	6056000	0.97
4	50% in 1% aqueous Pluronic®	6156000	0.99
		^{3}H-Thymidine incorporation [DPM/lymph node pair]	
1	vehicle 1% aqueous Pluronic®	588.2	1.00
2	10% in 1% aqueous Pluronic®	1101.5	1.87
3	30% in 1% aqueous Pluronic®	998.0	1.70
4	50% in 1% aqueous Pluronic®	1135.7	1.93
		Lymph node weight [mg/lymph node pair]	
1	vehicle 1% aqueous Pluronic®	4.4	1.00
2	10% in 1% aqueous Pluronic®	4.6	1.87
3	30% in 1% aqueous Pluronic®	5.3	1.70
4	50% in 1% aqueous Pluronic®	5.4	1.93
		Ear weight [mg/animal]	
1	vehicle 1% aqueous Pluronic®	32.2	1.00
2	10% in 1% aqueous Pluronic®	32.0	0.99
3	30% in 1% aqueous Pluronic®	31.9	0.99
4	50% in 1% aqueous Pluronic®	32.7	1.02

[1] test group x / test group 1 (vehicle control)

At any concentration tested, topical treatment of mouse ears with 1,3-Ethyleneurea did not result in a biologically significant increase of the stimulation index for auricular lymph node cell counts, ^{3}H-thymidine incorporation and lymph node weight.

None of the test-substance preparations caused increases in ear weights.

D. POSITIVE CONTROL

The sensitivity of mice (CBA/CaOlaHsd, Harlan Winkelmann GmbH, Borchen, Germany or CBA/J, Charles River Laboratories, Research Models and Services, Germany GmbH, Sandhofer Weg 7, 97633 Sulzfeld) and the reliability of experimental techniques is assessed regularly using a known sensitizer. Positive results were consistently obtained over the years using several variations of the methods and different vehicles. The results of 6 control studies are presented in Table 5.8.1-79.

Table 5.8.1-79: Positive control LLNA studies performed

Project No.	58H0288/ 982068#	58H0288/ 982075#	58H0508/ 062114#	58H0288/ 982082&	58H0288/ 982085&	58H0288/ 982086#
Strain used	CBA/CaOlaHsd	CBA/CaOlaHsd	CBA/CaOlaHsd	CBA/J	CBA/J	CBA/J
Date of performance	Aug 05	Feb 06	Aug 06	Jun 07	Oct 07	Jan 08
Name of test substance	Alpha-Hexylcinnam-aldehyde, techn. 85%	Alpha-Hexylcinnam-aldehyde, techn. 85%	Alpha-hexylcinnam-aldehyde, 95+%	Alpha-Hexylcinnam-aldehyde, techn. 85%	Alpha-Hexylcinnam-aldehyde, techn. 85%	Alpha-Hexylcinnam-aldehyde, techn. 85%
Concentrations tested	2.5%, 5%, 10%	3%, 10%, 30%	3%, 10%, 30%	1%, 3%, 10%	1%, 3%, 10%	10%, 30%, 50%
Vehicle	AOO 4:1 (acetone : olive oil 4:1 v/v)	acetone	1% Pluronic® L92 Surfactant in bi-distilled water	acetone	acetone	acetone
Stimulation index Cell counts[a]	1.13, 1.30, 1.83	1.75, 2.36, 2.98	1.13, 2.20, 3.38	1.42, 1.97, 2.75	1.10, 1.34, 1.77	1.77, 2.49, 2.79
Stimulation index 3H-thymidine incorporation[b]	1.12, 1.19, 2.84[1]	4.56, 6.63, 9.86	1.16, 4.64, 17.98	1.81, 3.24, 3.74	1.84, 2.35, 3.26	4.06, 7.77, 22.42
Evaluation of study results	Positive	Positive	Positive	Positive	Positive	Positive

[a] = Ratio of test group values to control group values (Stimulation index) greater than 1.5 indicates a positive result
[b] = Ratio of test group values to control group values (Stimulation index) greater than 3.0 indicates a positive result
[#] = Individual lymph nodes
[&] = Pooled lymph nodes
[1] = Borderline SI at 10% in accordance with published results using AOO as the vehicle

III. CONCLUSION

Based on the results of this study it is concluded that 1,3-Ethyleneurea has no sensitizing properties under the test conditions chosen.

Short term toxicity

Report:	CA 5.8.1/41 [redacted] 2002a Ethylenharnstoff - Repeated dose oral toxicity study in Wistar rats - Administration in drinking water for 4 weeks 2002/1026377
Guidelines:	EEC 96/54 B, OECD 407
GLP:	yes (certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)

Executive Summary

Ethylenharnstoff (batch: 63-9380; purity: 89%) was administered by drinking water to five male and female Wistar rats each at concentrations of 0, 1000, 4000 and 12000 ppm for four weeks. At the high and mid dose level, food and water consumption as well as body weights were impaired. Clinical signs of toxicity (observed at the high dose level only) were anogenital region smeared with urine, piloerection and reduced general state. These were substance-related, non-specific signs of toxicity but do not indicate a neurotoxic potential of the test substance.
Regarding clinical pathology findings, decrease in red blood cell counts, hemoglobin concentrations and hematocrit values and increases in microcytosis and anisochromia were measured in the high dose animals of either sex. These changes are assessed as being test substance-related and are indicative of a mild anemic process.
Serum examinations revealed decreased alkaline phosphatase activities and triglyceride concentrations in the high dose animals of either sex. These findings are considered not to be directly related to a toxic effect of the test compound but are consequences of the reduced food consumption of the animals. The increases in chloride concentrations and the decreases in inorganic phosphate and calcium levels are probably also caused by nutritional effects.

Thus, toxic effects were seen at all dose levels. Target organs were thyroid glands, liver, testes, epididymides, duodenum, urinary bladder, spleen, and lymph nodes. The no observed adverse effect level under the conditions of this study was below 1000 ppm (76 mg/kg bw/day in males, 91 mg/kg bw/day in females).

(BASF DocID 2002/1026377)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Ethylenharnstoff
Description:	crystalline / slight yellowish
Lot/Batch #:	63-9380
Purity:	89% (study no. 97L00097)
Stability of test compound:	proven by reanalysis after the in-life phase of the study (study no. 98L00039)

2. **Vehicle:** drinking water

3. **Test animals:**

Species:	Rat
Strain:	Wistar Chbb: THOM
Sex:	Male and female
Age:	49 days
Weight at dosing start:	♂: 213 – 253 g ♀: 135 – 174 g
Source:	
Acclimation period:	9 - 10 days
Diet:	Kliba maintenance diet for rat/mouse/hamster, meal supplied by Klingentalmühle AG, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water in bottles, ad libitum
Housing:	Single housing in type DK III stainless steel wire mesh cages supplied by Becker & Co., Castrop-Rauxel, Germany (floor area about 800 cm^2). Motor activity measurements were conducted in new clean polycarbonate cages with a small amount of bedding for the duration of the measurement.
Environmental conditions:	
Temperature:	20 - 24°C
Humidity:	30 - 70%
Air changes:	no data
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN AND METHODS

1. **Dates of experimental work:** 08-Dez-1997 - 11-Oct-2002
(In life dates: 17-Dec-1997 (start of administration) to 19-Jan-1998 (necropsy))

2. Animal assignment and treatment:

Ethylharnstoff was administered daily in the drinking water to groups of 5 male and 5 female rats at concentrations of 0, 1000 (low dose), 4000 (mid dose) and 12000 ppm (high dose) over a period of about 4 weeks. The animals were assigned to the treatment groups by means of a computer generated randomization list based on body weights.

3. Test substance preparation, preparation frequency and analyses of the test-substance preparations:
The test substance was administered as a solution in drinking water. The test substance was weighed in depending on the dose group, then drinking water was filled up to the desired weight, and mixed with a magnetic stirrer for about 5 minutes. The drinking water solutions were prepared at least twice a week.

The stability of Ehtylenharnstoff in drinking water was tested over a period of 4 days at room temperature.

As the preparations were real solutions, no homogeneity analyses of the test substance preparations were performed. Additionally, concentration control was verified in all concentrations at the beginning of the study. The mean values were 99.5% - 102.1% of the target concentrations.

4. Statistics:
Means and standard deviations of each test group were calculated for several parameters. Further statistical analyses were performed according to following tables:

Statistics of clinical examinations

Parameters	Statistical test	Markers in the tables
Food consumption, water consumption, body weight, body weight change, food efficiency	Parametric one-way analysis using the F-test (ANOVA) (two-sided). If the resulting p-value was equal or less 0.05, a comparison of each group with the control group using the DUNNETT's test (two-sided) was performed for the hypothesis of equal means	* for $p \leq 0.05$ ** for $p \leq 0.01$
Feces, rearing, grip strength forelimbs, grip strength hindlimbs, foot-splay test, motor activity	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using MANN-WHITNEY U-test (two-sided) for the equal medians	* for $p \leq 0.05$ ** for $p \leq 0.02$ *** for $p \leq 0.002$

Statistics of clinical pathology

Parameter	Statistical test	Markers in the tables
Clinical pathology paramters, except differential blood count	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using MANN-WHITNEY U-test (two sided) for the equal medians.	* for $p \leq 0.05$ ** for $p \leq 0.02$ *** for $p \leq 0.002$
Urinalysis, except volume, color, turbidity and specific gravity	Pairwise comparison of each dose group with the control group using the FISHER'excact test for the hypothesis of equal proportions	* for $p \leq 0.05$ ** for $p \leq 0.01$

Statistics of pathology

Parameter	Statistical test	Markers in the tables
Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test for the hypothesis of equal medians.	* for $p \leq 0.05$ ** for $p \leq 0.01$

C. METHODS

1. Observations:

A check for moribund and dead animals and signs of toxicity was made twice daily on working days and once daily on Saturdays, Sundays and public holidays. If animals were in a moribund state, they were sacrificed and necropsied. Detailed clinical observations (DCO) were performed in all animals prior to the administration period and thereafter at weekly intervals. The findings were ranked according to the degree of severity, if applicable. The animals were transferred to a standard arena (50 × 50 cm with sides of 25 cm height). The following parameters were examined:

1. abnormal behavior during handling
2. fur
3. skin
4. body posture
5. salivation
6. respiration
7. activity/arousal level
8. tremors
9. convulsions
10. abnormal movements
11. impairment of gait
12. lacrimation
13. palpebral closure
14. exophthalmus
15. feces (appearance/consistency)
16. urine
17. pupil size

2. Body weight:
Body weight was determined before the start of the administration period in order to randomize the animals. During the administration period the body weight was determined on day 0 (start of the administration period) and thereafter at weekly intervals. The difference between the body weight on the respective day of weighing and the body weight on day 0 was calculated as body weight change.

3. Food consumption:
Food consumption was determined weekly over a period of 7 days and calculated as mean food consumption in grams per animal and day.

4. Water consumption:
Water consumption was determined weekly over a period of 3 or 4 days and calculated as mean water consumption in grams per animal and day.

5. Ophthalmoscopy:
Not performed in this study.

6. Functional observation battery (FOB):

A functional observational battery was performed in all animals at the end of the administration period starting at about 10:00 a.m. The FOB started with passive observations without disturbing the animals (home cage observations), followed by removal from the home cage and open field observations in a standard arena. Thereafter, sensorimotor tests and reflex tests were conducted. The findings were ranked according to the degree of severity, if applicable. The observations were performed at random.

During the home cage observation special attention was paid to posture, tremors, convulsions, abnormal movements, impairment of gait and general observations (all other abnormal findings).

For open field observation the animals were transferred to a standard arena (50 x 50 cm with sides of 25 cm height) and observed for at least 2 minutes. The following parameters were assessed:

1. behavior when removed from cage	10. respiration
2. fur	11. tremors
3. skin	12. convulsions
4. salivation	13. abnormal movements / stereotypes
5. exophthalmus	14. impairment of gait
6. lacrimation	15. feces (number of fecal pellets/appearance/ consistency) within two minutes 15.
7. posture	16. urine (appearance/quantity) within two minutes
8. palpebral closure	17. number of rearings within two minutes
9. activity/arousal level	

For sensorimotor tests and reflexes the animals were removed from the open field. The following tests were performed:

1. approach response	9. examination of catalepsy (“descending from the box”)
2. touch response	10. coordination of movements ("righting response")
3. vision ("visual placing response")	11. behavior during "handling"
4. pupillary reflex	12. vocalization
5. winking reflex	13. pain perception ("tail pinch")
6. pinna reflex	14. grip strength of forelimbs
7. audition ("startle response")	15. grip strength of hind limbs
8. olfaction	16. landing foot-splay test

7. Motor activity measurement:

Motor activity was measured at the same day when the FOB was performed. The examinations were performed in the dark using the Multi-Varimex-System (Columbus Instruments Int. Corp., Ohio, USA) with 4 infrared beams per cage. For this purpose, the rats were placed in new clean polycarbonate cages with a small amount of bedding for the duration of the measurement. The number of beam interrupts was counted over 12 intervals for 5 minutes per interval. The sequence in which the rats were placed in the cages was selected at random. On account of the time needed to place the rats in the cages, the starting time was "staggered" for each animal. The measurement period began when the 1st beam was interrupted and finished exactly 1 hour later. No food or water was offered to the rats during these measurements.

8. Hematology and clinical chemistry:

Blood was withdrawn in the morning from non-fasted, unanesthetized animals from the retro-orbital venous plexus. The blood sampling procedure and the subsequent analysis of the blood and serum samples were carried out in a randomized sequence.
The following hematological and clinical chemistry parameters were determined for all animals:

Hematology:					
	Red blood cells		*White blood cells*		*Clotting Potential*
✓	Erythrocyte count (RBC)	✓	leukocyte count (WBC)	✓	Prothrombin time
✓	Hemoglobin (HGB)	✓	Differential blood count		
✓	Hematocrit (Hct)	✓	Platelet count (PLT)		
✓	Mean corp. volume (MCV)				
✓	Mean corp. hemoglobin (MCH)				
✓	Mean corp. Hb. conc. (MCHC)				

Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Chloride	✓	Bilirubin (total)	✓	Aspartate aminotransferase (AST)
✓	Phosphorus (inorganic)	✓	Cholesterol	✓	Alkaline phosphatase (ALP)
✓	Potassium	✓	Creatinine	✓	γ-glutamyltransferase (GGT)
✓	Sodium	✓	Globulin (by calculation)		
✓	magnesium	✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

9. Urinalysis:

For urinalysis the individual animals were transferred to metabolism cages and urine was collected overnight. No food or water was supplied during urine collection. The samples were analyzed in a randomized order.

The following parameters were determined for all animals:

Urinalysis					
	Quantitative parameters:		*Semi quantitative parameters*		
✓	Urine volume	✓	Bilirubin	✓	Protein
✓	Specific gravity	✓	Blood	✓	pH-value
✓	Color and turbidity	✓	nitrite	✓	Urobilinogen
		✓	Glucose	✓	Sediment (microscopical exam.)
		✓	Ketones		

10. Sacrifice and pathology:

The animals were sacrificed by decapitation under CO_2 anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology. The following organs were sampled, weighed and examined histopathologically:

Pathology:											
The following organs were collected (column C), weighed (W) and examined histopathologically (H, ✓: all groups, #: control and top dose).											
C	W	H		C	W	H		C	W	H	
✓	✓	#	Adrenal glands	✓	✓	✓	liver	✓	✓	✓	thymus
				✓		#	lung	✓		✓	thyroid gland
✓		#	bone marrow[§]	✓		✓	lymph nodes[#]	✓		#	trachea
✓	✓	#	brain	✓	✓	✓	ovaries and oviduct**	✓		✓	urinary bladder[m]
✓		#	caecum	✓		#	Parathyroid gland	✓		#	uterus
✓		#	colon	✓		#	pituitary gland	✓		#	vagina
✓		✓	duodenum	✓		#	Prostate gland				
✓	✓	✓	epididymides	✓		#	rectum		✓		body (anesthetized animals)
✓			eyes	✓		#	Sciatic nerve				
✓		✓	gross lesions	✓		#	seminal vesicles				
✓	✓	#	Heart[+]	✓		#	spinal cord (3 levels)[@]				
✓		#	ileum	✓	✓	✓	spleen				
✓		#	jejunum (w. Payer's plaque)	✓		#	stomach (non-glandular & glandular)				
✓	✓	✓	kidneys	✓	✓	✓	testes				
[§] from femur; [#] mandibular and mesenteric; [@] cervical, thoracic, lumbar; ** oviduct not weighed and only in control and top dose; [+] also all females from mid dose; [m] from low and mid dose group only male animals											

The organs or tissues were fixed in 4% formaldehyde. Testes and epididymides were fixed in Bouin's solution. Parts of the liver were fixed in Carnoy's solution.

The immunorelevant organs and tissues were evaluated according to the following parameters:

Thymus:	
•	Increased/decreased grade of cortico-medullar ratio (related only to area)
•	Increase of starry sky cells
•	Changes of cellular density in the cortex
•	Changes of cellular density in the medulla
Spleen:	
•	Changes of the cellularity of PALS, lymphoid follicles, marginal zone, red pulp
•	Altered cellular composition of follicles
•	Altered number of germinal centers
Lymph nodes (mesenteric and mandibular lymph nodes):	
•	Changes in the cellularity of follicles, interfollicular area, paracortical area, medulla
•	Altered cellular composition of paracortex
•	Altered number of germinal centers
•	changes of the cellularity of sinus
Peyer's patches (of the jejunum):	
•	Changes of the cellularity of follicles (including mantle zone and germinal centers)
•	Changes of the cellularity of interfollicular area

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

See Section B 3. above

B. OBSERVATIONS

1. Clinical signs of toxicity

Towards the end of the study, the following abnormal clinical signs were obtained at the high dose level: anogenital region smeared with urine (3 males), piloerection (5 males, 1 female), reduced general state (1 female), crust formation at the nose (1 female), skin lesion (1 male) and alopecia (1 male). Anogenital region smeared with urine, piloerection and reduced general state were assessed as being treatment-related, whereas the other findings were most probably incidental in nature. These effects are non-specific signs of toxicity, but do not indicate a neurotoxic potential of the test substance.
During the open field observations in the detailed clinical observations no abnormal findings were obtained.

2. Mortality

No mortality was observed throughout the study.

3. Ophthalmoscopy

Not performed in this study.

4. FOB and Motor Activity

Home cage observation revealed no indication of treatment-related effects. Open field observations showed deviations from the “zero value” in the high dose group (see Table 5.8.1-80). Anogenital region smeared with urine and piloerection were assessed as being treatment-related, whereas the other findings were most probably incidental in nature.
Sensorimotor tests/Reflexes showed in the touch response deviations from “zero value” as shown in Table 5.8.1-80. All deviations from "zero values" were equally distributed between treated groups and controls or occurred in single animals only and thus were considered to be incidental.
Quantitative observations (feces, rearing, grip strength, landing foot-splay test) showed no substance-related effects.
Regarding the overall motor activity (summation of all intervals), statistically significantly increased values were seen in low dose females on day 28. This was assessed as being incidental, due to the lack of dose-response relationship. The comparison of the single intervals with the control group resulted in some statistically significantly deviations in low and mid dose females. Due to the isolated occurrence and the lack of a dose-response relationship, this was assessed as being incidental.

Table 5.8.1-80: Functional observational battery – deviations from “zero values”

Finding	Ranking	Sex	No. of affected animals			
			0 ppm	1000 ppm	4000 ppm	12000 ppm
Open field observations						
Alopecia	Fur rank 5	male	0	0	0	1
Skin lesion hindlimb ,right	Skin, Lre HE	male	0	0	0	1
Anogenital region smeared with urine	Fur, rank 3	male	0	0	0	4
Piloerection	Fur, rank 4	male	0	0	0	1
Piloerection	Fur, rank 4	female	0	0	0	1
Sensorimotor tests/Refelexes						
No reaction	Touch response, rank 1	male	3	3	4	5
No reaction	Touch response, rank 1	female	3	4	5	3

C. BODY WEIGHT AND BODY WEIGHT GAIN

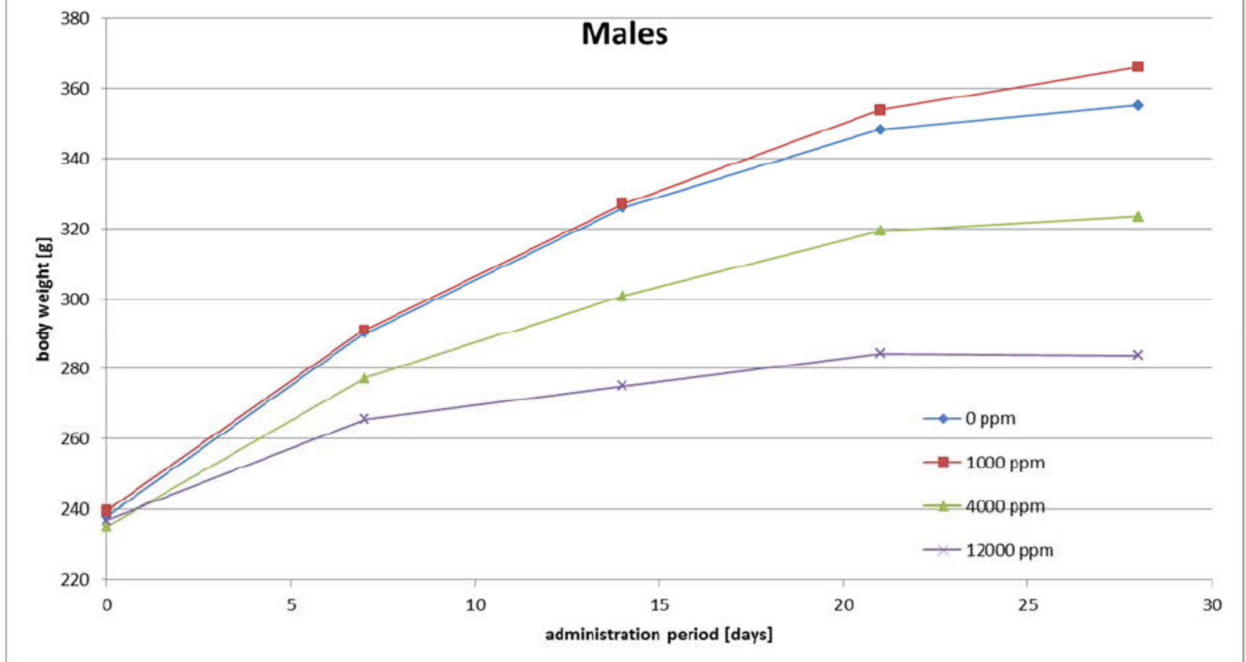
In high dose males and females, body weight was statistically significantly impaired from days 7 – 28 (except females day 7), the values on day 28 being 20.1%/19.3% below control. Corresponding body weight values were 60.1%/77.2% below control. Also in the mid dose males statistically significantly lower values were seen (body weight days 14- 28, body weight change days 7 – 28). The impairment of body weight on day 28 was 8.9% and of body weight change 24.6%. This was assessed as being treatment-related. In mid dose females, only a minor impairment was seen (body weight 6.8%, body weight change 21.3%) without statistical significance. Nevertheless, a substance-relationship cannot be excluded with certainty [see Table 5.8.1-81, Figure 5.8.1-13].

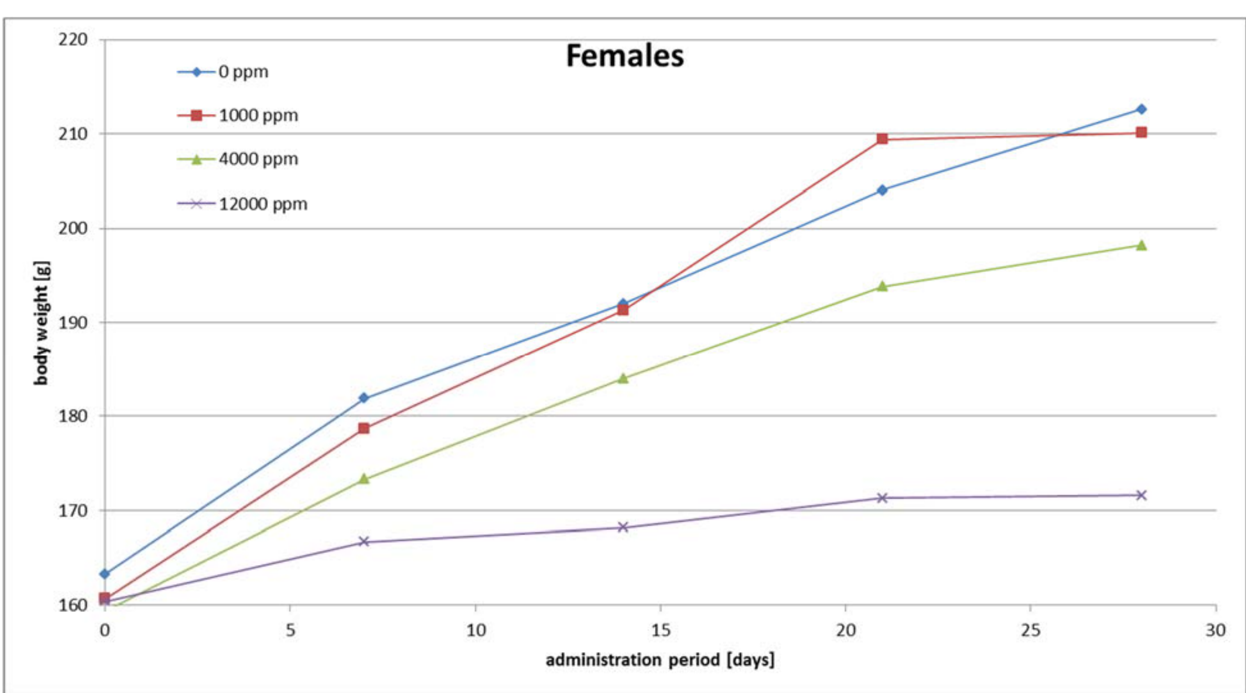
Table 5.8.1-81: Mean body weight in rats administered Ethyleneharnstoff for 28 days

	Males				Females			
Dose level [ppm]	0	1000	4000	12000	0	1000	4000	12000
Body weight [g]								
- Day 0	238.0	239.6	235.3	236.9	163.3	160.7	159.4	160.4
- Day 28	355.0	365.9	**323.5***	**283.6****	212.6	210.1	**198.2**	**171.6****
Δ% (compared to control)		+3.1	-8.9	-20.1		-1.2	-6.8	-19.3
Overall body weight gain [g]	117.0	126.3	**88.2***	**46.7****	49.3	49.4	**38.8**	**11.2****
Δ% (compared to control)		+7.9	-24.6	-60.1		+0.3	-21.3	-77.2

* p<= 0.05, ** p<= 0.01 – Anova + Dunnett's test (two-sided)

Figure 5.8.1-13: Body weight development in rats administered Ethyleneharnstoff for 28 days





D. FOOD CONSUMPTION AND COMPOUND INTAKE

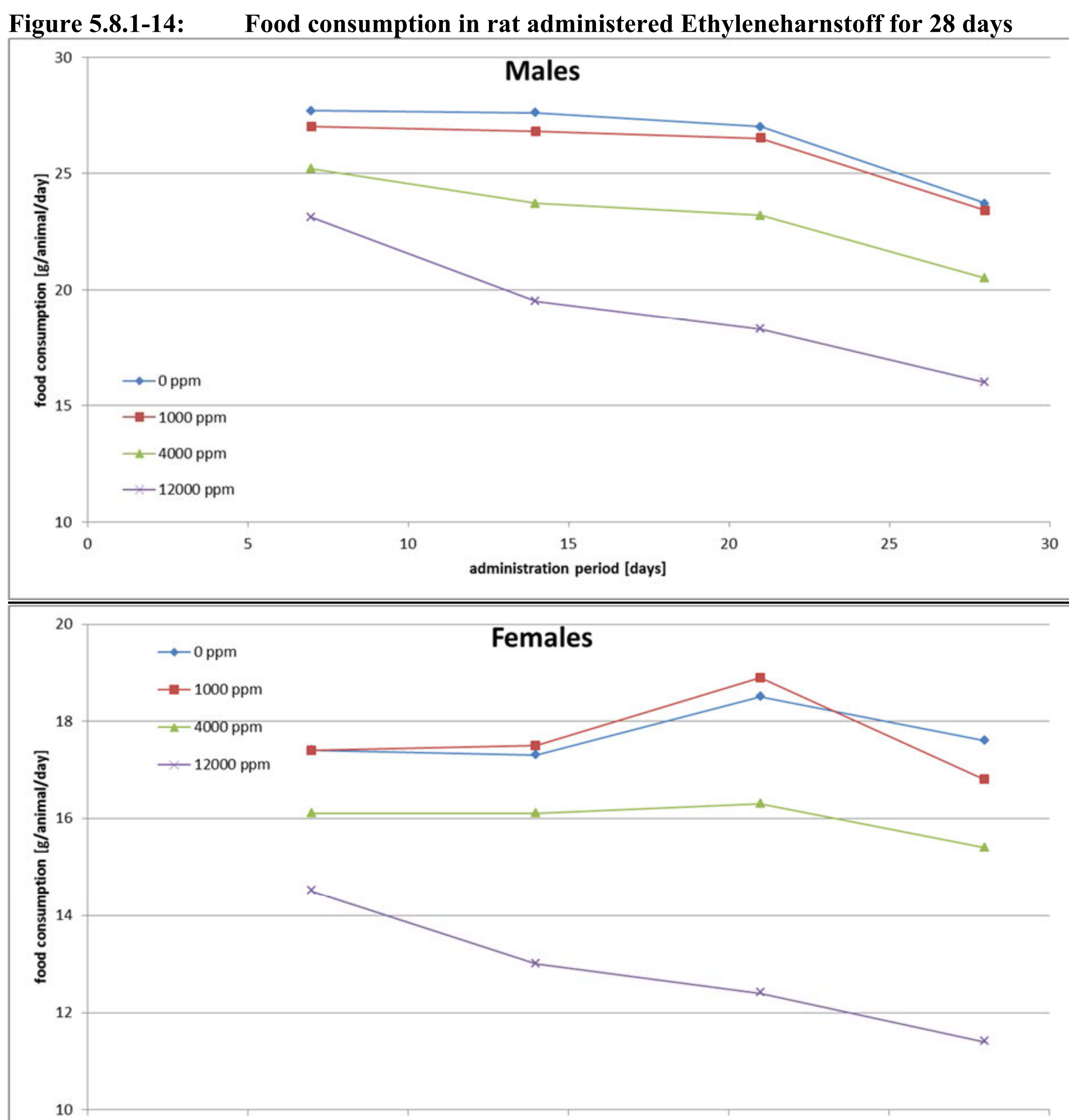
Food consumption was statistically significantly decreased in high and mid dose males as well as in high dose females during the treatment period. The values were up to 32.4% (high dose males), 35.4% (high dose females), and 13.5% (mid dose males) below controls. This was assessed as being related to treatment. ln mid dose females, decreased values were seen, being up to 12.5% below control. Although lacking statistical significance, a substance-relationship cannot be excluded with certainty (see Figure 5.8.1-14).

The mean daily test substance intake is found in the following table:

Table 5.8.1-82: Intake of test substance

Test group	Concentration in drinking water (ppm)	Mean daily test substance intake in mg/kg bw	
		males	females
1	1000	76	92
2	4000	288	362
3	12000	724	1002

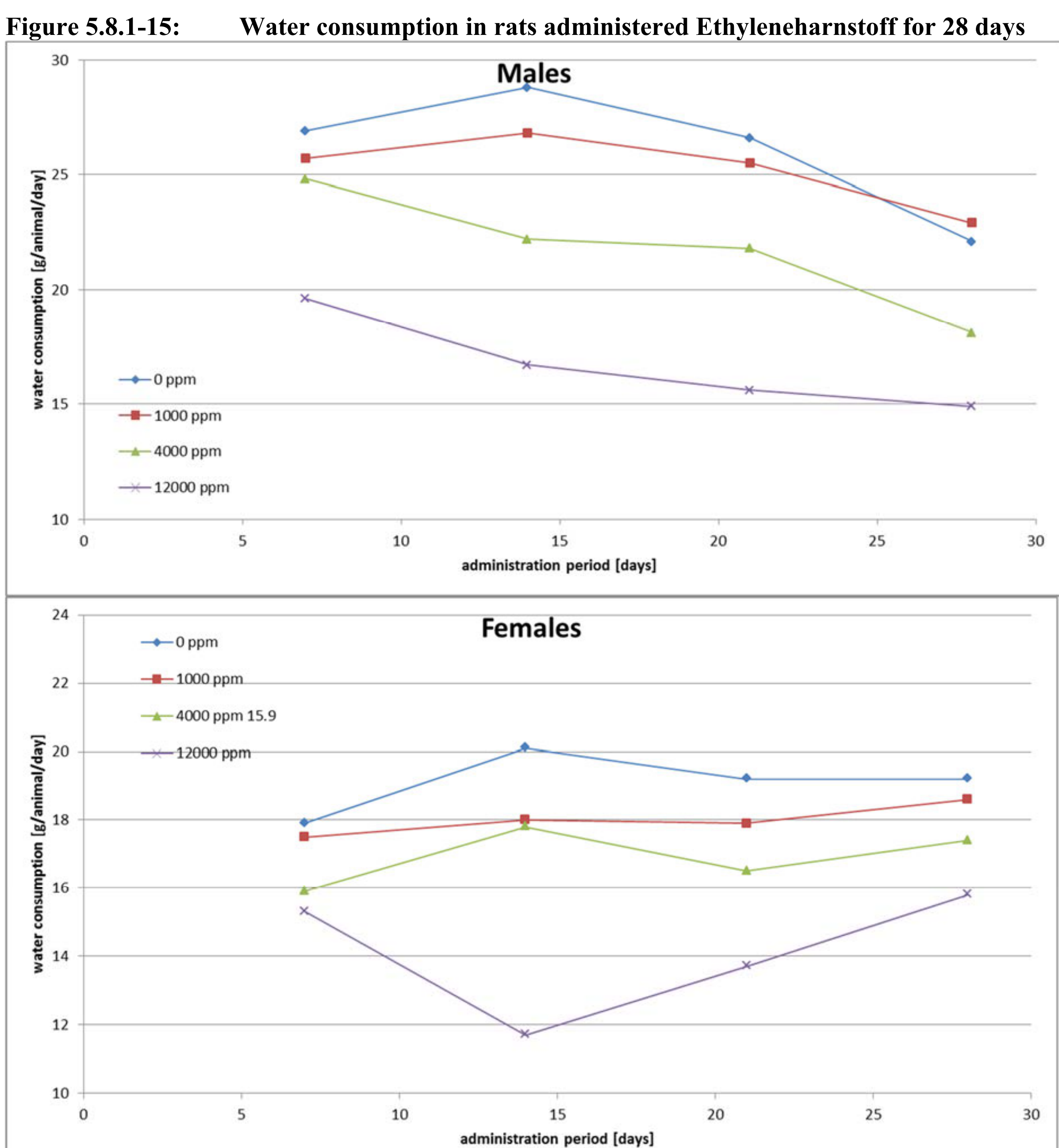
Figure 5.8.1-14: Food consumption in rat administered Ethyleneharnstoff for 28 days



E. WATER CONSUMPTION

Water consumption was statistically significantly decreased in high dose males and females. The values were up to 41.9% (males) and 41.6% (females) lower then controls. Also in mid dose males statistically significantly decreased values were seen on day14 (23%) and day 21 (17.9%). This was assessed as being related to treatment. In mid dose females decreased values were seen being up to 14% below control. Although lacking statistical significance a substance-relationship cannot be excluded with certainty (see).

Figure 5.8.1-15: Water consumption in rats administered Ethyleneharnstoff for 28 days



F. BLOOD ANALYSIS

1. Hematological findings

At the end of the study significantly decreased red blood cell counts, hemoglobin concentrations and hematocrit values were found in the peripheral blood of the high dose females. In the high dose males decrease in erythrocytes, hemoglobin and hematocrit were also observed. However, in the males these finding occurred only as a trend toward reduced values. Increased microcytosis and anisochromia were measured in the differential blood count of the animals of either sex. The other hematology examinations revealed no treatment-related changes.

2. Clinical chemistry findings

Decreased alkaline phosphatase activities were detected in the sera of the high dose males and females. However, in the males this finding was not statistically significantly different to the respective control. The other serum enzyme examinations revealed no test substance-related changes.
Blood chemistry examinations exhibited a dose dependent increase in serum creatinine level in the mid and high dose males and in all treated females. Reduced triglyceride and increased chloride concentrations were also measured in the sera of the high dose animals of either sex. However, there was only a trend toward increased chloride concentrations in the high dose females. Moreover, in the males inorganic phosphate concentrations were decreased in the sera of the mid and high dose animals and calcium level was reduced in the high dose males only. No treatment-related changes were found in the other blood chemistry parameters examined.

3. Urinalysis

At the end of the study increased ketones occurred in the urine specimens of the mid and high dose animals of either sex. In addition, mid and high dose males produced decreased amounts of urine with increased specific gravity and most of the urine specimens of the high dose males varied the color from yellow to dark yellow and appeared cloudy.

G. NECROPSY

1. Organ weight (see Table 5.8.1-83)

The mean terminal body weight was significantly decreased in males and females of group the high and mid dose. This was regarded as treatment related. In males of the mid and high dose group and in females of the high dose group the mean weights of liver and thymus were significantly decreased. In males of the high dose group also the kidney mean weights were significantly decreased.
Related to the decreased mean terminal body weight, the mean heart weight was decreased in males of the high dose group; however, this was not significant. In males of the mid dose group, the mean heart weight was significantly decreased. This was also regarded to be related to the decreased mean terminal body weight.
As a consequence of the significantly decreased mean terminal body weight, in females of the high dose group, the mean weights of the ovaries, heart, and spleen were also significantly decreased. The other mean absolute weight parameters did not show significant differences when compared with the control group.

In males of the high dose group, the mean relative liver weight was significantly decreased. Due to the significantly decreased mean terminal body weight, the mean relative weights of testes (high dose), kidney (females, high dose), and brain (males high and mid dose, females high dose) were significantly increased. Hence, this was not regarded treatment related. In females of the high dose group, the mean thymus weight was slightly but significantly decreased. The other mean relative weight parameters did not show significant differences when compared with the control group.

Table 5.8.1-83: Selected mean absolute and relative organ weights of rats administered Ethyleneharnstoff for at least 28 days

Sex		Males		Females	
Organ weight	Dose [ppm]	Absolute weight	Relative weight [%]	Absolute	Relative weight [%]
Terminal body weight [g]	0	341.56	100	200.08	100
	1000	343.86	100	196.26	100
	4000	**307.84****	100	184.52	100
	12000	**274.26****	100	**155.02****	100
Liver [g]	0	12.51	3.66	6.39	3.19
	1000	11.92	3.46	6.43	3.27
	4000	**10.17****	3.30	6.20	3.36
	12000	**8.48****	**3.08***	**5.33***	3.45
Kidney [g]	0	2.60	0.76	1.55	0.78
	1000	2.59	0.75	1.60	0.82
	4000	2.36	0.77	1.62	0.88
	12000	**2.16***	0.79	1.51	0.98*
Testes [g]	0	3.21	0.94	na	na
	1000	3.30	0.96		
	4000	3.02	0.98		
	12000	3.05	1.11**		
Ovaries [mg]	0	na	na	84.4	0.042
	1000			83.6	0.043
	4000			84.8	0.046
	12000			58.0*	0.037
Heart [g]	0	1.26	0.37	0.86	0.43
	1000	1.23	0.36	0.83	0.42
	4000	1.11*	0.36	0.82	0.44
	12000	1.05	0.38	0.65**	0.42
Spleen [g]	0	0.79	0.23	0.55	0.28
	1000	0.84	0.24	0.49	0.25
	4000	0.77	0.25	0.52	0.28
	12000	0.59	0.22	0.40*	0.26
Thymus [mg]	0	470.2	0.14	275.8	0.14
	1000	493.0	0.14	283.0	0.14
	4000	**370.4***	0.12	245.8	0.13
	12000	**349.2***	0.13	**157.6***	**0.10***
Brain [g]	0	1.96	0.58	1.81	0.91
	1000	2.03	0.59	1.80	0.92
	4000	1.95	0.63*	1.79	0.97
	12000	1.89	0.69*	1.78	1.16**

* $p \leq 0.05$; ** $p \leq 0.01$: Kruskal-Wallis-H + Wilcoxon-Test
na = not applicable

2. Gross and histopathology

At necropsy incidental gross lesions were noted in the uterus (dilation in a mid dose group female). This finding was confirmed by histopathology. Sparse hair in the skin in a high dose male was found at necropsy but this finding lacked a microscopic correlate. A relationship to treatment can be denied for both gross lesions and/or microscopic findings.

Histopathology revealed treatment related findings in the thyroid glands, thymus, liver, spleen, testes epididymides, duodenum, urinary bladder, mesenteric and mandibular lymph nodes and bone marrow.

In the thyroid glands hypertrophy of the follicular cells was noted in male and female rats. Hypertrophy affected the whole organs and its severity was graded minimal, slight or moderate (see Table 5.8.1-84). In addition to hypertrophy, slight follicular cell hyperplasia with multilayering and budding of epithelial cells was a consisting finding in the mid dose group.

In the thymus individual animals of both sexes revealed a slight increase in starry sky cells in control and treatment groups with the exception of high dose group females in which this finding was not obvious. This observation was regarded to be within the limits of biologic variability and of spontaneous nature. However, the female animal No. 36 of the high dose group exhibited a moderate depletion of lymphocyte tissue which was regarded to be most likely treatment related. This animal revealed slight hypocellularity in the bone marrow.

In the liver of top dose animals, focal necrosis and bile duct proliferation were noted as treatment related findings. In addition, one female animal of the high dose group revealed many altered liver cell foci of the clear cell type. This finding was also regarded to be related to treatment.

In the spleen the most obvious finding deviating from the controls was the presence of germinal centers (“secondary follicles”) in the white pulp. In addition, in the graded severity of animals affected with hemosiderin deposition was increased in both sexes in the high dose group. In males, all top dose animals showed slight hemosiderosis, whereas in females of the high dose group, two animals had a moderate grade of hemosiderin deposition.

In the testes, tubular giant cells were noted in all groups including the control. The graded severity ranged from very few to many, with a grade 3 or 4 only in animals of the mid and high dose group. Those animals with only very few giant cells, exhibited this finding always only unilateral. In addition to the presence of tubular giant cells, a few males coincidently showed tubular hyperplasia (i.e. a decreased amount of spermatogenetic cells). As a consequence of an altered spermatogenesis in the testes, the epididymides revealed an increased amount of debris – mainly consisting of residual bodies, intermingled with tubular giant cells, and necrotic cells of spermatogenesis – in the lumen. All males of the high dose group showed this finding, either minimal or slight or moderate. In the mid dose group one male rat showed slight amounts of debris in the tubular lumina.

Thickening of the mucosa of the duodenum – measured morphometrically as the elongation of the villi – was recorded in treated male and female rats after histometric evaluation of the mucosa. As a consistent parameter for the grading of this finding was not recognized in all groups, the finding was noted as being present for those animals exceeding the highest individual value of the controls. In male rats, the critical value would have been 0.63 mm, and in females 0.74 mm, respectively.

In the urinary bladder of three male rats of the high dose group a slight diffuse hyperplasia of the urothelium was recorded, accompanied by a moderate focal metaplasia to squamous epithelium in one of the three affected males.

Germinal centers were more often noted in the high dose group of male and female rats in the mesenteric lymph nodes and in the mandibular lymph nodes. For the evaluation of this finding in the mesenteric lymph nodes, only the cross sections were used.
No reasonable morphologic correlate was obtained for the significantly decreased mean absolute weights of liver and thymus, kidneys, heart, ovaries and spleen, the decreased relative weights of the liver and thymus or the increased mean relative weights of testes, kidneys and brain.
In none of the other organs investigated remarkable microscopic finding were noted. All microscopic findings recorded in these organs and all other findings in the target organs were either single observations or they occurred in control animals only, or they were recorded at comparable incidence and graded severity in control and treated males and/or females.

Table 5.8.1-84: Incidence of selected histopathological lesions in rats administered Ethyleneharnstoff for at least 28 days

Test group (ppm)	Male animals				Female animals			
	0	1000	4000	2000	0	1000	4000	12000
No. of animals	5	5	5	5	5	5	5	5
Thyroid glands (hypertrophy of follicular cells		2	5	5		1	5	5
• Grade 1						1		
• Grade 2		2	5	5			4	
• Grade 3							1	5
Thyroid glands (hyperplasia of follicular cells			2	5			4	5
• Grade 2			2	5			4	5
Thymus (starry sky cells)	2	1	2	2	2	2	1	0
• Grade 2	2	1	2	2	2	2	1	
Liver (focal necrosis)				3				2
• Grade 1				2				1
• Grade 3				1				1
Liver (bile duct proliferation)				3				3
• Grade 1				1				
• Grade 2				1				3
• Grade 3				1				
Liver (focus, cellular alt.)								1
• Grade 4								1
Spleen (germinal centers)	1	1	3	5	0	1	2	4
Spleen (hemosiderin deposition)	5	5	5	5	5	5	5	5
• Grade 1	2	3	3		1	1		
• Grade 2	3	2	2	5	4	4	5	3
• Grade 3								2
Testes (tubular giant cells)	2	1	2	2				
• Grade 1	2			1				
• Grade 2		1	1					
• Grade 3			1					
• Grade 4				1				
Testes (hypoplasia, tubular)			1	2				
• Grade 1				1				
• Grade 2			1					
• Grade 3				1				
Epididymides (debris in lumen)			1	5				
• Grade 1				1				
• Grade 2			1	1				
• Grade 3				3				
Duodenum (thickening of mucosa)	0	5	5	5	0	1	2	4
Urinary bladder (hyperplasia, urothel.)				3				
• Grade 2				3				

Test group (ppm)	Male animals				Female animals			
	0	1000	4000	2000	0	1000	4000	12000
No. of animals	5	5	5	5	5	5	5	5
Urinary bladder (metaplasia, squam. c)				1				
• Grade 3				1				
Mesentric lymph node (germinal centers)	2	1	2	5	1	2	1	3
Mandibular lymph node (germinal centers)		1	1	4	1	1	2	4
Mandibular lymph node (pigment deposition)	1		4		1	4	4	3
• Grade 1	1		4			3	4	
• Grade 2					1	1		3

The following codes were used for a grading system that takes into consideration either the severity or the number or the size of a microscopic finding:

	Severity	Number	Size
Grade 1	Minimal	Very few	Very small
Grade 2	Slight	Few	Small
Grade 3	Moderate	Moderate number	Moderate size
Grade 4	Marked; severe	Many	Large
Grade 5	Massive; extreme	Extensive number	Extensive size

All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

III. CONCLUSIONS

Thus toxic effects were seen at all dose levels. Target organs were thyroid glands, liver, testes, epididymides, duodenum, urinary bladder, spleen and lymph nodes. The no observed adverse effect level under the conditions of this study was below 1000 ppm (76 mg/kg bw/day in males and 92 mg/kg bw/day in females).

Genotoxicity

Report: CA 5.8.1/42
Engelhardt G., 1991a
Report on the study of Ethylenharnstoff in the AMES test (Salmonella/mammalian-microsome mutagenicity test - Standard plate test and preincubation test)
1991/1005168
Guidelines: OECD 471, EEC 84/449 B 14
GLP: no

Executive Summary

S. typhimurium strains TA98, TA100, TA1535 and TA1537 were exposed to ethylene urea (batch: 74-7718, purity: ca. 88%) using aqua dest. as a solvent in the presence and absence of metabolic activation in two independent experiments. Triplicate plates were used per dose and per test condition. Vehicle and positive controls were included in each experiment.

In the Ames standard plate test (SPT) ethylene urea was tested in concentrations of 20 to 5000 µg/plate with and without S9 mix (Aroclor-induced rat liver S9 mix). In the preincubation assay the test item was tested in concentrations of 4 to 5000 µg/plate also with and without metabolic activation.

No precipitation of the test substance was found up to the highest concentration. A bacteriotoxic effect was not observed.

An increase in the number of his^+ revertants was not observed both in the standard plate test and in the pre-incubation test either without S-9 mix or after the addition of a metabolizing system. The positive controls induced the appropriate response in the corresponding strains, thus demonstrating the sensitivity of the test system.

According to the results of the study, the test substance ethylene urea is not mutagenic in the Ames standard plate test under the experimental conditions of the study.

(BASF DocID 1991/1005168)

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material** ethylene urea

Description:	not given in the report
Lot/Batch #:	74-7718
Purity:	about 88%
Stability of test compound:	No data concerning stability of the test compound was presented in the study report.
Solvent used:	aqua dest.

2. Control Materials:

Negative control: In parallel to each experiment, negative control experiments were carried out in order to check for possible contaminants (sterility control) and to determine the spontaneous mutation rate (vehicle control).

Vehicle control: The vehicle control with and without S9 mix only contained the vehicle used for the test substance at the same concentration and volume for all tester strains.

Solvent/final concentration: 100 µL/plate

Positive control compounds tested without addition of metabolic activation system:

Strain	Mutagen	Solvent	Concentration
TA 100	N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)	DMSO	5 µg/plate
TA 1535	N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)	DMSO	5 µg/plate
TA 1537	9-Aminoacridine (AAC)	DMSO	100 µg/plate
TA 98	4-nitro-o-phenylendiamine (NOPD)	DMSO	10 µg/plate

Positive control compounds tested with addition of rat metabolic activation system:

Strain	Mutagen	Solvent	Concentration
TA 100	2-aminoanthracene	DMSO	10 µg/plate
TA 1535	2-aminoanthracene	DMSO	10 µg/plate
TA 1537	2-aminoanthracene	DMSO	10 µg/plate
TA 98	2-aminoanthracene	DMSO	10 µg/plate

To demonstrate the efficacy of the rat liver S9 mix in this assay, the S9 batch was only characterized with 2-aminoanthracene and not as recommended in the current guideline a second substance as i.e.benzo(a)pyrene.

3. Activation: S9 was produced from the livers of induced male Sprague-Dawley rats. The rats received a single intraperitoneal injection of 500 mg/kg bw Aroclor 1254. Five days after administration the animals were sacrificed and the livers are prepared. Aliquots of the S9-mix are deep frozen and stored at -70°C to -80°C. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction is thawed at room temperature and 1 volume of S9-fraction is mixed with 9 volumes of S9-supplement (cofactors). This preparation, the so-called S9-mix, was kept on ice until used.

The rat liver S9-mix was prepared immediately before use and had the following composition:

Component	Concentration
Phosphate buffer (pH 7.4)	100 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM
S9	10%

4. Test organisms:

S. typhimurium strains: TA98, TA100, TA1535, TA1537
Salmonella typhimurium:
The Salmonella strains are checked for the following characteristics at regular intervals: deep rough character (rfa); UV sensitivity (uvrB); ampicillin resistance (R factor plasmid).

Histidine auxotrophy is automatically proven in each experiment via the spontaneous rate.

According to the current guideline a fifth strain is obligatory, i.e. E. coli or TA102.

5. Test concentrations:

Plate incorporation assay:

In the first experiment triplicate plates were prepared for each concentration (neg. control; 20, 100, 500, 2500 and 5000 µg/plate and positive controls at the concentrations indicated above) and conditions (i.e. with and without rat liver S9 mix) for all tester strains indicated above. In a second experiment only the TA98 strain was tested with 0, 200, 400, 600, 800 and 1000 µg/plate with and without S9 mix.

Preincubation assay:

In the third experiment the test article / vehicle / positive control substance, S9 mix were incubated at 37°C for the duration of about 20 minutes. Triplicate plates were prepared for each concentration (neg. control; 4, 20, 100, 500 and 2500 µg/plate and positive controls at the concentrations indicated above) for all tester strains indicated above. In a fourth experiment only the TA98 strain was tested with 0, 4, 20, 100, 500 and 2500 µg/plate with and without S9 mix.

B. TEST PERFORMANCE:

1. Dates of experimental work: not given, finalization date: 22-April-1991

2. Plate incorporation assay:
To test tubes containing 2-mL portions of warm soft agar (containing 0.5 mM histidine + 0.5 mM biotin), 0.1 mL test solution or vehicle, 0.1 mL fresh bacterial culture, 0.5 mL S9-mix (in tests with metabolic activation) or 0.5 mL phosphate buffer (in tests without metabolic activation) were added in the given order. After mixing, the samples are poured onto Vogel-Bonner agar plates (minimal glucose agar plates). After incubation at 37°C for 48 - 72 hours in the dark, the bacterial colonies (his^+ revertants) are counted.

3. Preincubation assay:
100 µL of test solution or vehicle, 0.1 mL bacterial suspension and 0.5 mL S9 mix were incubated at 37°C for about 20 minutes. Subsequently, 2 mL of soft agar was added and, after mixing, the samples are poured onto the Vogel-Bonner agar plates.

After incubation in the dark for 48 hours at 37°C revertant colonies were counted.

4. Titer determination:
In order to assess bacteriotoxic effects, the titer was additionally determined in experimental parts with S9 mix using the vehicle control and the two highest doses. The procedure followed in both experiments (plate incorporation and pre-incubation) was the same as described above for the individual experiments with the exception that the soft agar used contained maximal amino acid solution (5 mM histidine + 0.5 mM biotin). After the incubation in the dark for 48 hours at 37°C the number of bacterial colonies was determined.

5. Statistics:
No special statistical tests were performed.

6. Evaluation criteria:
The test chemical is considered positive in this assay if the following criteria are met:

- A dose-related and reproducible increase in the number of revertant colonies, i.e. about doubling of the spontaneous mutation rate in at least one tester strain either without S9 mix or after adding a metabolizing system.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

The stability of the test substance at room temperature in the vehicle water was not verified analytically.

B. TOXICITY

A preliminary cytotoxicity assay was not performed in this laboratory. Cytotoxicity was determined in the course of the mutation assay.

No bacteriotoxic effect was observed in the Ames standard plate test or the preincubtion assay.

C. MUTATION ASSAYS

Neither in the plate incorporation nor in the preincubation experiment with and without metabolic activation was a biologically relevant increase in number of revertants observed in any strain tested [see Table 5.8.1-85 and Table 5.8.1-86]. The positive controls yielded revertant numbers in a range expected for the respective strains and thus demonstrated the sensitivity of the test system.

No test item precipitation was observed with and without S9 mix.

Table 5.8.1-85: Standard plate assay with ethylene urea - Mean number of revertants

Experiment 1: Standard plate test								
Strain	TA 98		TA 100		TA 1535		TA 1537	
Metabol. activation	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Neg.control (aqua dest.)	28+2	41+5	140+5	122+13	19+6	20+11	15+4	14+2
Test item								
20 µg/plate	33+5	57+5	129+8	139+6	24+3	21+4	10+4	15+5
100 µg/plate	33+3	54+12	131+27	126+4	19+1	18+2	15+4	15+7
500 µg/plate	38+11	62+23	99+8	137+16	22+5	20+4	13+5	21+1
2500 µg/plate	26+6	36+9	114+6	140+45	22+4	19+4	10+5	20+8
5000 µg/plate	23+7	36+10	139+5	117+30	23+4	18+1	10+3	16+3
Pos. control§	922+88	785+62	1347+43	334+47	1349+273	379+53	430+91	96+15

Experiment 2: Standard plate test		
Strain	TA 98	
Metabol. activation	-S9	+S9
Neg.control (aqua dest.)	28+5	44+4
Test item		
200 µg/plate	33+12	38+4
400 µg/plate	26+4	40+3
600 µg/plate	24+5	36+1
800 µg/plate	20+2	39+3
1000 µg/plate	26+4	30+11
Pos. control§	1118+125	1850+26

§ = Compound and concentrations see Material and Methods (I.A.2.) above

Table 5.8.1-86: Preincubation assay with ethylene urea - Mean number of revertants

Experiment 3: preincubation test								
Strain	TA 98		TA 100		TA 1535		TA 1537	
Metabol. activation	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Neg.control (aqua dest.)	24+4	35+3	104+11	113+19	14+3	17+6	12+2	12+1
Test item								
20 µg/plate	29+2	59+20	117+16	126+12	15+3	18+2	12+2	14+2
100 µg/plate	36+3	51+10	95+15	148+18	16+2	19+1	14+1	11+1
500 µg/plate	38+6	49+4	95+5	129+18	16+6	14+2	9+4	14+5
2500 µg/plate	39+9	48+5	115+13	126+21	19+3	17+2	13+2	20+3
5000 µg/plate	31+2	46+3	118+11	135+10	14+2	17+2	11+2	19+1
Pos. control§	1320+131	865+241	1343+81	886+63	1317+6	167+11	313+36	141+22

Experiment 4: preincubation test		
Strain	TA 98	
Metabol. activation	-S9	+S9
Neg.control (aqua dest.)	26+2	41+4
Test item		
4 µg/plate	23+2	39+6
20 µg/plate	26+2	34+13
100 µg/plate	21+2	35+13
500 µg/plate	27+1	38+6
2500 µg/plate	26+2	33+4
Pos. control§	1120+98	1247+87

§ = Compound and concentrations see Material and Methods (I.A.2.) above

III. CONCLUSION

According to the results of the present study, the test substance ethylene urea is not mutagenic in the Ames standard plate test and in the preincubation test under the experimental conditions chosen here.

Report: CA 5.8.1/43
Wollny H.-E., 2012a
Gene mutation assay in chinese hamster V79 cells in vitro (V79/HPRT) with Ethylene Urea
2012/1360606

Guidelines: OECD 476, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.17, EPA 870.5300

GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Executive Summary

Ethylene Urea (batch: 11-0041, purity: 90.1%) was tested in vitro for its ability to induce forward mutations in mammalian cells by assessing the mutation of the HPRT locus in Chinese Hamster CHO cells. Two independent experiments were conducted in the presence and absence of metabolic activation at concentrations of up to 990 µg/mL. The treatment intervals in the absence of metabolic activation were 4 and 24 h in the first and second experiment, respectively. With metabolic activation treatment was 4 h for the original and confirmatory experiment. Ethylmethanesulfonate (EMS) and 7,12-dimethylbenz(a)anthracene (DMBA) served as positive controls in the experiments without and with metabolic activation, respectively.

Cytotoxic effects indicated by reduced cloning efficiencies of below 20% of the respective vehicle control were not observed up to the highest concentrations with and without metabolic activation following 4 and 24 hours treatment.

Neither in the original nor in the confirmatory experiments a relevant increase in the mutant frequency was observed. The positive control substances, however, induced a marked increase in mutant frequency.

Based on the results of the study it is concluded that under the conditions of the test Ethylene Urea does not induce forward mutations in mammalian cells in-vitro.

(BASF DocID 2012/1360606)

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material** Ethylene Urea
   Description: Solid, white
   Lot/Batch #: 11-0041
   Purity: 90.1% (tolerance ± 1.0%)
   Stability of test compound: Not indicated in the report
   Solvent used: deionized water

2. Control Materials:

Negative control: A negative control was not employed in this study
Solvent control: deionized water
Positive control -S9: Ethyl methanesulfonate (EMS) 150 µg/mL
Positive control +S9: 7,12-dimethylbenz(a)anthracene (DMBA) 1.1 µg/mL

3. Activation:

S9 was produced from the livers of induced male Wistar [Hsd Cpb:WU] rats. The rats were administered injections of phenobarbital (80 mg/kg; i.p.) and ß-napthtoflavone (80 mg/kg; per gavage) on three consecutive days. 24 h after the last administration the animals were sacrificed.

The S9-mix was prepared immediately before use and had the following composition:

Component	Concentration
Phosphate buffer (pH 7.4)	100 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM
S9	10%

4. Test organism:

Chines hamster V79 cells. Stocks of the V79 cell line were maintained at -196°C in liquid nitrogen. Each batch used for mutagenicity testing was checked for mycoplasma contamination and checked for karyotype stability and spontaneous mutant frequency. For the selection of mutant cells the complete medium was supplemented with 11 µg/mL 6-thioguanine. All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO_2 (see below).

5. Culture media:

Culture medium: MEM (minimal essential medium) containing Hank's salts, neomycin (5 µg/mL) and amphotericin B (1 %).

Selection medium: During pulse exposure (4 hours) to the test substance, MEM was used without FCS supplementation. In the case of continuous treatment (24 hours) MEM with FCS supplementation was used.

6. Locus examined:

hypoxanthine-guanine-phosphoribosyl transferase (H(G)PRT)

7. Test concentrations:

a) Preliminary toxicity assay: Nine concentrations ranging from 7.7 to 990 µg/mL

b) Mutation assay:
1st experiment: 30.9, 61.9, 123.8, 247.5, 495.0 and 990.0 µg/mL with and without metabolic activation
2nd experiment: 30.9, 61.9, 123.8, 247.5, 495.0 and 990.0 µg/mL with and without metabolic activation

B. TEST PERFORMANCE:

1. Dates of experimental work: 01-Nov-2011 to 28-Feb-2012

2. Preliminary cytotoxicity assay:

Cytotoxicity was assessed by determination of the cloning efficiency. About 500 cells were incubated with 8 test substance concentrations in MEM for about 4 hours (with and without metabolic activation) or 24 hours (only without metabolic activation). The general culture conditions and experimental conditions in this pre-test were the same as described for the mutagenicity experiment below. After the incubation period of approximately 7 days the colonies were fixed, stained and counted. In addition to the cloning efficiency the following parameters were measured: pH, osmolarity and the determination of precipitates (solubility).

3. Mutation Assay:

Seeding of Cells: Two to three days after sub-cultivation stock cultures were trypsinized at 37°C for 5 minutes. Then the enzymatic digestion was stopped by adding complete culture medium with 10% FBS and a single cell suspension was prepared. The trypsin concentration for all sub-culturing steps was 0.2% in PBS. Prior to the trypsin treatment the cells were rinsed with Ca-Mg-free salt solution containing 200 mg/L EDTA (ethylene diamine tetraacetic acid). Approximately 1.5 x 10^6 (single culture) and 5 x 10^2 cells (in duplicate) were seeded in plastic culture flasks. The cells were grown for 24 hours prior to treatment.

Cell treatment: After 24 hours the medium was replaced with serum-free medium containing the test item, either without S9 mix or with 50 µL/mL S9 mix. Concurrent solvent and positive controls were treated in parallel. After 4 hours this medium was replaced with complete medium following two washing steps with "saline G". In the second experiment the cells were exposed to the test item for 24 hours in complete medium, supplemented with 10% FBS, in the absence of metabolic activation.

Expression: Three or four days after treatment 1.5 x 10^6 cells per experimental point were subcultivated in 175 cm² flasks containing 30 mL medium for 7 days.

Selection: After 7 days five 80 cm² cell culture flasks were seeded with about 3 – 5x 10^5 cells each in medium containing 6-TG. Two additional 25 cm² flasks were seeded with approx. 500 cells each in non-selective medium to determine the viability. The cultures were incubated at 37°C in a humidified atmosphere with 1.5 % CO_2 for about 8 days. The colonies were stained with 10% methylene blue in 0.01% KOH solution.

Determination of Cytotoxicity: Cloning efficiency 1 (survival):

The survival (cloning efficiency 1; CE_1) was determined in parallel to the mutagenicity test. After cell treatment the colonies were fixed and stained approximately 7 days after treatment as described above.

Cloning efficiency 2 (viability):

The viability (cloning efficiency 2; CE_2) was determined after the expression period. About 500 cells were separated during the transfer into selection medium and seeded in two flasks (25 cm²) containing non-selective medium. After seeding of the cells, the flasks were incubated for about 8 days to form colonies. These colonies were fixed, stained and counted.

Calculations: Cloning efficiency I (survival): cloning efficiency determined immediately after treatment to measure toxicity.

Cloning efficiency II (viability): cloning efficiency determined after the expression period to measure viability of the cells without selective agent.

Cloning efficiency I (survival, absolute): mean number of colonies per flask divided by the number of cells seeded

Cloning efficiency I (survival, relative): (mean number of colonies per flask divided by the mean number of colonies per flask of the corresponding control) x 100

Cell density % of control: (cell density at 1st subcultivation divided by the cell density at 1st subcultivation of the corresponding control) x 100

Cloning efficiency II (viability, absolute): mean number of colonies per flask divided by the number of cells seeded

Cloning efficiency II (viability, relative): cloning efficiency II absolute divided by the cloning efficiency II absolute of the corresponding control x 100

Cells survived (after plating in TG containing medium): number of cells seeded x cloning efficiency II absolute

Mutant colonies / 10^6 cells: mean number of mutant colonies per flask found after plating in TG medium x 10^6 divided by the number of cells survived

Induction factor: mutant colonies per 10^6 cells / mutant colonies per 10^6 cells of the corresponding solvent control

4. Statistics:

A linear regression (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. The number of mutant colonies obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05. However, both, biological and statistical significance was considered together.

5. Evaluation criteria:

The test chemical is considered positive in this assay if the following criteria are met:

- A test item is classified as mutagenic if it reproducibly induces a mutation frequency that is three times above the spontaneous mutation frequency at least at one of the concentrations in the experiment.
- The test item is classified as mutagenic if there is a reproducible concentration-related increase of the mutation frequency. Such evaluation may be considered also in the case that a threefold increase of the mutant frequency is not observed.

However, in a case by case evaluation this decision depends on the level of the corresponding solvent control data. If there is by chance a low spontaneous mutation rate within the laboratory´s historical control data range, a concentration-related increase of the mutations within this range has to be discussed. The variability of the mutation rates of solvent controls within all experiments of this study was also taken into consideration.

A test substance is generally considered negative in this test system if:

- A test item producing neither a concentration-related increase of the mutant frequency nor a reproducible positive response at any of the test points is considered non-mutagenic in this system.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Not indicated by sponsor.

B. PRELIMINARY CYTOTOXICITY ASSAY

The range finding pre-experiment was performed using a concentration range of 7.7 to 990 µg/mL (≈10 mM) with respect to the molecular weight and the purity (≥87% to ≤90%, preliminary information at the start of the experiment) to evaluate toxicity in the presence (4 hours treatment) and absence (4 hours and 24 hours treatment) of metabolic activation. No relevant toxic effect occurred up to the maximum concentration with and without metabolic activation following 4 and 24 hours treatment.

No precipitation occurred up to the highest concentration with and without metabolic activation following 4 and 24 hours treatment. There was no relevant shift of pH and osmolarity of the medium even at the maximum concentration of the test item.

Based on the results of the pre-experiment, the maximum concentration of the main experiments was again, 990 µg/mL or 10 mM. The lower concentrations were spaced by a factor of 2.

C. MUTAGENICITY ASSAYS

No relevant and reproducible increase of mutant colony numbers/10^6 cells was observed in the main experiments up to the maximum concentration (see
Table 5.8.1-87 and Table 5.8.1-88). The mutant frequency remained well within the historical range of solvent controls. An increase of the induction factor reaching or exceeding the threshold of three times the mutation frequency of the corresponding solvent control was observed in the first culture of the first experiment without metabolic activation at 61.9 and 123.8 µg/mL and in the first culture of the second experiment 123.8 and 247.5 µg/mL. However, the increases were judged as biologically irrelevant fluctuations since they were based on a rather low mutation frequency of the solvent controls of just 5.9 (experiment I) and 7.7 colonies per 10^6 cells. Furthermore, the effects were not reproduced in the parallel cultures under identical experimental conditions and not dose dependent as indicated by the lacking statistical significance. Statistical analysis revealed no dose dependent trend of the mutation frequency in any of the experimental groups.

Treatment with the positive controls EMS and DMBA resulted in a marked increase in the number of mutant colonies in all experiments, thus demonstrating the sensitivity of the test.

No relevant toxic effects occurred up to the maximum concentration of 990 µg/mL [see
Table 5.8.1-87 and Table 5.8.1-88].

The pH and osmolarity of the tested concentrations were not altered at the concentrations tested. No test substance precipitation was observed up to the maximum concentration with and without metabolic activation.

III. CONCLUSION

Based on the results of the study it is concluded that under the conditions of the test ethylene urea does not induce gene mutations in the HPRT locus in V79 cells in vitro.

Table 5.8.1-87: Gene mutation in mammalian cells - 1st experiment

Test group	Number of mutant colonies[a]	Mutant colonies (per 10^6 cells)	Induction factor	CE_1 (survival) (4h after treatment; approx. 500 cells/flask seeded	CE_2 (viability) (at the end of the expression period; approx. 500 cells/flask seeded)	Number of mutant colonies[a]	Mutant colonies (per 10^6 cells)	Induction factor	CE_1 (survival) (4h after treatment; approx. 500 cells/flask seeded	CE_2 (viability) (at the end of the expression period; approx. 500 cells/flask seeded)
				Cloning efficiency (%)					Cloning efficiency (%)	
				relative					relative	
	Culture I					Culture II				
Without metabolic activation; 4-hour exposure period										
Vehicle (water)	2.0	5.9	1.0	100.0	100.0	7.2	20.2	1.0	100.0	100.0
30.9 µg/mL	n.c.[1]	n.c.[1]	n.c.[1]	95.6	n.c.[1]	n.c.[1]	n.c.[1]	n.c.[1]	106.9	n.c.[1]
61.9 µg/mL	6.4	21.7	3.7	99.6	98.4	3.0	9.1	0.4	96.3	94.6
123.8 µg/mL	5.6	19.4	3.3	96.4	91.7	5.0	16.5	0.8	105.1	93.1
247.5 µg/mL	1.2	3.9	0.7	99.0	92.9	2.8	8.2	0.4	110.5	92.2
495.0 µg/mL	2.2	6.1	1.0	97.4	97.7	3.2	10.4	0.5	106.5	90.1
990.0 µg/mL	4.8	14.6	2.5	99.2	87.0	2.4	8.3	0.4	98.4	91.5
Positive control EMS										
150.0 µg/mL	24.2	72.6	12.4	97.4	95.8	42.8	118.2	5.8	106.0	98.5
With metabolic activation; 4-hour exposure period										
Vehicle (water)	1.8	5.4	1.0	100.0	100.0	2.0	8.5	1.0	100.0	100.0
Ethylene urea										
30.9 µg/mL	n.c.[1]	n.c.[1]	n.c.[1]	101.8	n.c.[1]	n.c.[1]	n.c.[1]	n.c.[1]	98.6	n.c.[1]
61.9 µg/mL	2.4	7.5	1.4	95.8	99.5	3.2	9.5	1.9	100.4	90.6
123.8 µg/mL	2.8	8.4	1.5	98.2	89.6	4.6	10.9	2.5	93.0	98.6
247.5 µg/mL	3.4	12.0	2.2	97.0	84.7	3.8	6.0	2.2	92.4	89.6
495.0 µg/mL	2.2	6.8	1.3	97.7	89.0	2.4	17.8	1.3	93.9	96.6
990.0 µg/mL	3.0	8.2	1.5	95.6	100.8	5.0	9.0	2.6	94.7	92.9
Positive control DMBA										
1.1 µg/mL	230.2	537.8	99.1	27.9	99.8	282.0	188.8	203.7	35.9	74.2

[a] number of colonies 7 days after seeding 3 x 10^5 cells/flask into selection medium (sum of 5 plates)
n.c.[1] culture was not continued since a minimum of only four analyzable concentrations is required

Table 5.8.1-88: Gene mutation in mammalian cells – 2nd experiment

Test group	Number of mutant colonies[a]	Mutant colonies (per 10^6 cells)	Induction factor	CE1 (survival) (4h after treatment; approx. 500 cells/flask seeded	CE2 (viability) (at the end of the expression period; approx. 500 cells/flask seeded)	Number of mutant colonies[a]	Mutant colonies (per 10^6 cells)	Induction factor	CE1 (survival) (4h after treatment; approx. 500 cells/flask seeded	CE2 (viability) (at the end of the expression period; approx. 500 cells/flask seeded)
				Cloning efficiency (%)					Cloning efficiency (%)	
				relative					relative	
	Culture I					Culture II				
Without metabolic activation; 24-hour exposure period										
Vehicle (water)	2.0	7.7	1.0	100.0	100.0	2.8	8.5	1.0	100.0	100.0
30.9 µg/mL	n.c.[1]	n.c.[1]	n.c.[1]	98.2	n.c.[1]	n.c.[1]	n.c.[1]	n.c.[1]	96.9	n.c.[1]
61.9 µg/mL	2.4	13.7	1.8	99.4	80.3	2.2	9.5	1.1	97.2	89.4
123.8 µg/mL	7.4	33.1	4.3	100.0	89.2	3.0	10.9	1.3	97.6	99.8
247.5 µg/mL	5.6	23.5	3.0	98.0	95.0	2.4	6.0	0.7	97.0	100.8
495.0 µg/mL	3.0	13.4	1.7	98.6	89.7	4.8	17.8	2.1	100.5	92.5
990.0 µg/mL	2.8	9.3	1.2	101.9	103.2	2.4	9.0	1.0	97.6	87.5
Positive control EMS										
150.0 µg/mL	45.6	151.6	19.6	101.5	93.8	51.0	188.8	22.1	99.2	84.8
With metabolic activation; 4-hour exposure period										
Vehicle (water)	3.4	9.2	1.0	100.0	100.0	4.2	14.3	1.0	100.0	100.0
Ethylene urea										
30.9 µg/mL	n.c.[1]	n.c.[1]	n.c.[1]	97.7	n.c.[1]	n.c.[1]	n.c.[1]	n.c.[1]	96.4	n.c.[1]
61.9 µg/mL	3.2	8.0	0.9	93.5	112.1	6.2	20.9	1.5	93.7	87.4
123.8 µg/mL	3.0	9.0	1.0	97.0	103.4	3.0	11.5	0.8	95.4	89.1
247.5 µg/mL	1.8	5.6	0.6	95.0	97.0	2.6	9.6	0.7	86.3	93.2
495.0 µg/mL	3.2.	9.1	1.0	91.8	96.4	3.4	15.6	1.1	90.0	98.0
990.0 µg/mL	3.2	10.0	1.1	90.4	92.3	4.8	13.7	1.0	96.2	105.3
Positive control DMBA										
1.1 µg/mL	151.8	530.1	57.6	43.1	83.8	232.2	676.7	47.4	34.6	94.2

[a] number of colonies 7 days after seeding 3 x 10^5 cells/flask into selection medium (sum of 5 plates)
n.c.[1] culture was not continued since a minimum of only four analyzable concentrations is required

Read across

~~**Report:**~~	~~CA 5.8.1/44~~ ~~Anonymous, 2013a~~ ~~Reporting format for the analogue approach of 2 Imidazolidone With comparison to 1 (2 Hydroxyethyl)imidazolidin 2 one~~ ~~2013/1065851~~
~~**Guidelines:**~~	~~none~~
~~**GLP:**~~	~~no~~

Report:	CA 5.8.1/44 Naumann S., 2016 a 2-Imidazolidone: Micronucleus test in human lymphocytes in vitro 2016/1321628
Guidelines:	OECD 487 (2014), Commission Regulation (EU) No 640/2012, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.49
GLP:	yes (certified by Hessisches Ministerium fuer Umwelt, Klimaschutz, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Hypothesis for the analogue approach

This report addresses the analogue approach of 2-imidazolidone with its structurally similar analogue 1-(2-hydroxyethyl)imidazolidin-2-one. These two substances are structurally very close to each other. Both substances are substituted ureas, as can be seen from the structural formulas below.
Experimental data of 2-imidazolidone is lacking for the following toxicological endpoint: In vitro chromosome aberrations.

Source chemicals

Some physical and chemical properties of the target chemical 2-imidazolidone and the source chemical 1-(2-hydroxyethyl)imidazolidin-2-one are shown below.
Although the impurities of the two substances are currently not known, it is not expected that any of the impurities will affect the physico-chemical parameters, environmental fate and (eco)toxicological properties. It is therefore concluded that the impurities will not affect the read-across.

Chemical name CAS number	2-imidazolidone 120-93-4	1-(2-hydroxyethyl)imidazolidin-2-one 3699-54-5
PHYSICO-CHEMICAL DATA		
Melting Point	**68.2°C (measured)**	**45°C (measured)**
Boiling Point	**132.6°C (measured)**	**113°C (measured)**
Density	**1.34 at 20°C (measured)**	**1.22 at 20°C (measured)**
Vapour Pressure	**249 hPa at 89.6°C (measured) 0.017 hPa at 25°C (calculated)**	**0.0000332 hPa at 25°C (calculated)**
Partition Coefficient (log Kow)	**-1.16 at 20°C (measured)**	**-3.07 at 25°C (calculated)**
Water Solubility	**596 g/L at 20°C (measured)**	**1240 g/L at 25°C (calculated)**
MAMMALIAN TOXICITY		
Acute oral toxicity (LD50 in mg/kg bw)	**≥5010**	**≥2000**
Inhalation toxicity (LC50 in mg/m^3)	**no data**	**no data**
Acute dermal toxicity (LD50 in mg/kg bw)	**≥2000**	**≥5000**
Skin irritation	**not irritating**	**not irritating**
Eye irritation	**irritating**	**not irritating**
Skin sensitisation	**not sensitising (LLNA)**	**not sensitising (maximisation)**
In vitro gene mutation (in bacteria - Ames test)	**negative**	**negative**
In vitro gene mutation (mammalian cells)	**negative**	**no data**
In vitro chromosome aberrations (in mammalian cells)	***read across***	**negative**
Repeated dose toxicity, oral; 28-day study	**NOAEL: 37 mg/kg bw/day (male rats) 57 mg/kg bw/day (female rats)**	**NOAEL = 1000 mg/kg bw/day (male and female rat)**
Carcinogenicity	**not carcinogenic**	**no data**
Reproduction/developmental toxicity screening test (NOAEL in mg/kg bw/day)	**Reproduction and fertility: 2000 ppm (155 mg/kg bw/day in parental males, 214 mg/kg bw/day in parental females) Developmental: F1 pups 500 ppm ie: 37 mg/kg bw/day (Parental male rats) 57 mg/kg bw/day (Parental female rats)**	**1000**

Analogue approach justification
Read-across from 1-(2-hydroxyethyl)imidazolidin-2-one to 2-imidazolidone is based on
1) structural similarity of the two compounds,
2) similarities in physico-chemical data, and
3) the available toxicological data which are also comparable and strengthen the category approach (acute toxicity data, skin-irritation/corrosion and mutagenicity).

Therefore, read-across to 2-imidazolidone from 1-(2-hydroxyethyl)imidazolidin-2-one for the following endpoints is considered acceptable: *In vitro* chromosome aberrations.

Conclusions per endpoint for C&L and dose descriptor
Both substances show a low potential for acute oral and dermal toxicity (all LD50 values are ≥2000 mg/kg bw/day). 2-imidazolidone shows eye irritating properties, while 1-(2-hydroxyethyl)imidazolidin-2-one does not show eye irritating properties. Both substances do not show skin irritating or -sensitising properties.
In a 28-day oral repeated dose toxicity study, no effects were observed for 1-(2-hydroxyethyl)imidazolidin-2-one resulting in a NOAEL of 1000 mg/kg bw/day. The NOAEL for 2-imidazolidone is 37 mg/kg bw/day based on a 28 repeated dose study including reproductive and developmental screening. Although there is a difference in toxicity between the compounds after sub-acute exposure, this is considered not relevant for the read-across of the genotoxicity endpoint of chromosome aberration. An Ames test was negative for both substances, a HPRT assay with 2-imidazolidone was negative and a chromosome aberration assay with 1-(2-hydroxyethyl)imidazolidin-2-one was also negative. Furthermore, 2-imidazolidone is not-carcinogenic, strengthening the argument that this substance will also be negative in the in vitro chromosome aberration test.
Comparing all available experimental results on toxicity of 1-(2-hydroxyethyl)imidazolidin-2-one and 2-imidazolidone, it could be that 2-imidazolidone is more toxic than 1-(2-hydroxyethyl)imidazolidin-2-one. This is based on the results of the eye irritation and 28-day oral repeated dose toxicity test. However, the acute toxicity, skin irritation, skin sensitiation and genotoxicity studies show comparable results.

Reproductive toxicity

Report:	CA 5.8.1/45
	[redacted] 2013a
	Ethylene Urea - Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test in Wistar rats - Administration via the drinking water
	2013/1089127
Guidelines:	EPA 870.3650, OECD 422 (1996)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

Ethylene Urea was administered orally via drinking water to groups of 10 male and 10 female Wistar rats (F0 animals) at dose levels of 100, 500, and 2000 ppm during a pre-mating and mating period (male and female) and during gestation and lactation (females). The daily administered doses were calculated as 7/12, 37/57, and 155/214 mg/kg bw/day for males/females at the three dose levels. Clinical observations included decreased body weight gains in males and female animals and decreased food consumption in females. Clinical pathology revealed no treatment-related effects. Pathology findings included hypertrophy/hyperplasia of follicular epithelium in animals of the high dose group and were evaluated as treatment-related and adverse. Increased numbers of secondary follicles with germinal centers in axillary lymph nodes and spleen were regarded non-adverse. No effect on fertility and reproductive performance were observed. Regarding developmental toxicity, signs of toxicity were observed in the decreased pup viability index in test group 3 (2000 ppm) and in one litter loss, wherefore a relationship to the treatment cannot be excluded. However, at necropsy four pups of the dam with complete litter loss had an empty stomach indicating that this pup mortality was likely to be subsequent to insufficient maternal care.
In conclusion, the **NOAEL** (no observed adverse effect level) for **general, systemic toxicity** was 500 ppm (37 mg/kg bw/day in parental males, 57 mg/kg bw/day in parental females), based on the decreased body weight/body weight gain and decreased food consumption. The **NOAEL** for **reproductive performance** and **fertility** was 2000 ppm (155 mg/kg bw/day in parental males, 214 mg/kg bw/day in parental females) for the F0 parental rats. The **NOAEL** for developmental toxicity in the F1 progeny was found to be 500 ppm (37 mg/kg bw/day in parental males, 57 mg/kg bw/day in parental females), based on the decreased pup viability which was likely to be subsequent to insufficient maternal care.

(DocID 2013/1089127)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Ethylene Urea
Description:	solid / white
Lot/Batch #:	11-0041
Purity:	90.1%
Stability of test compound:	The stability of the test substance under storage conditions over the test period was guaranteed by the sponsor, and the sponsor holds this responsibility.
2. Vehicle:	Drinking water
3. Test animals:	
Species:	Rats
Strain:	Crl:WI(Han)
Sex:	Male and female
Age:	11-12 (start of administration)
Weight at dosing (mean):	males: 293 - 345 g females: 187 - 226 g
Source:	
Acclimation period:	7 days
Diet:	ground Kliba maintenance diet mouse-rat "GLP" (Provimi Kliba SA, Kaiseraugst, Switzerland)
Water:	water, ad libitum
Housing:	Single housing in Makrolon type M III cages (Becker & Co., Castrop-Rauxel, Germany) (floor area of about 800 cm^2). Exceptions: - During overnight mating, male and female mating partners were housed together (Makrolon type M III) - Pregnant animals and their litters were housed together until PND 4. - For motor activity measurements the animals were hosed individually in polycarbonate cages (TECNIPLAST, Hohenpeißenberg, Germany)
Environmental conditions:	
Temperature:	20 - 24°C
Humidity:	30 - 70%
Air changes:	15 per hour
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 12/20/2011 - 11/14/2012

2. Animal assignment and treatment:

F0 generation parental animals and their progeny
After the acclimatization period, the test substance was administered orally via drinking water to the F0 generation parental animals daily. The animals of the control group received vehicle (drinking water) only. Fourteen days after the beginning of treatment, males and females from the same test group were mated overnight in a ratio of 1:1.

Mating of F0 generation parental animals
In general, each of the male and female animals was mated overnight in a 1:1 ratio for a maximum of 2 weeks. Throughout the mating period, each female animal was paired with a predetermined male animal from the same test group. The animals were paired by placing the female in the cage of the male mating partner from about 16.00 h until 07.00 - 09.00 h of the following morning. Deviations from the specified times were possible on weekends and public holidays and were reported in the raw data. A vaginal smear was prepared after each mating and examined for the presence of sperm. If sperm was detected, pairing of the animals was discontinued. The day on which sperm was detected was denoted gestation day (GD) 0 and the following day "GD 1".

3. Test substance preparation and analysis:

Ethylene Urea was applied as a solution. To prepare this solution, the appropriate amount of test substance was weighed out depending on the desired concentration. Then, drinking water was filled up to the desired weight, subsequently released with a magnetic stirrer. The test substance preparations were produced at least twice a week.
The stability of the test substance in drinking water for a period of 7 days at room temperature was proven before the start of the study at a concentration of 5000 ppm (Analytical Report: 08B0060/976010). To verify the stability for the lowest concentration in the current study (100 ppm) a stability analysis was carried out during the study period (No. 01Y0054/08Y010).
Concentration control analysis of the test substance preparations were performed in samples of all concentrations at the start and towards the end of the administration period. Given that the test substance is completely miscible with corn oil, solutions were considered to be homogenous without further analysis.

4. Statistics:

Means and standard deviations of each test group were calculated for several parameters. Further statistical analyses were performed according to following tables:

Statistics of clinical examinations

Parameters	Statistical test	Markers in the tables	References
Food consumption (parental animals), body weight and body weight change (parental animals and pups; for the pup weights, the litter means were used), duration of gestation, number of implantation sites, number of pups delivered per litter, viability index	Simultaneous comparison of all dose groups with the control group using the DUNNETT-test (two-sided) for the hypothesis of equal means	* for p ≤ 0.05 ** for p ≤ 0.01	Dunnett, C.W. (1955): A multiple comparison procedure for comparing several treatments with a control. JASA, Vol. 50, 1096–1121 Dunnett, C.W. (1964): New tables for multiple comparisons with a control. Biometrics, Vol. 20, 482-491
Male and female mating indices, male and female fertility indices, gestation index, females with liveborn pups, females with stillborn pups, females with all stillborn pups, live birth index, pups stillborn, pups died, pups cannibalized	Pairwise comparison of each dose group with the control group using FISHER'S EXACT test for the hypothesis of equal proportions	* for p ≤ 0.05 ** for p ≤ 0.01	Siegel, S. (1956): Non-parametric statistics for behavioral sciences. McGraw-Hill New York
Number of mating days	Pairwise comparison of the dose group with the control group using the WILCOXON-test (one-sided) with Bonferoni-Holm-Adjustment for the hypothesis of equal medians	* for p ≤ 0.05 ** for p ≤ 0.01	Siegel S. (1956): Non-parametric statistics for behavioral sciences. McGraw-Hill New York Holm (1979): A Simple Sequentially Rejective Multiple Test Procedure. Scand. J. Statist, 6, 65-70
Feces, rearing, grip strength of forelimbs and hindlimbs, landing foot-splay test, motor activity	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians	* for p ≤ 0.05 ** for p ≤ 0.01	Siegel, S. (1956): Non-parametric statistics for the behavioural sciences. McGraw-Hill New York

Statistics of clinical pathology

Parameters	Statistical test	Markers in the tables	References
Clinical pathology parameters (except for urine color and turbidity)	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the negative control group was performed using WILCOXON-test (two-sided) for the equal medians.	* for p ≤ 0.05 ** for p ≤ 0.01	SIEGEL, S. (1956): Nonparametric statistics for the behavioural sciences. McGraw-Hill New York

Statistics of pathology

Parameter	Statistical test	Markers in the tables	References
Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each test group with the control group was performed using the WILCOXON test for the hypothesis of equal medians	* for $p \leq 0.05$ ** for $p \leq 0.01$	HETTMANNSPERGER, T.P. (1984): Statistical Inference based on Ranks, John Wiley & Sons New York, 132-140. International Mathematical and Statistical Libraries, Inc., 2500 Park West Tower One, Houston, Texas 77042-3020, USA, nakl-1 - nakl-3 MILLER, R.G. (1981): Simultaneous Statistical Inference Springer-Verlag New York Inc., 165-167 NIJENHUIS, A. and S.W. WILF (1978): Combinatorial Algorithms, Academic Press, New York, 32-33

C. METHODS

1. Clinical examinations:

<u>**Parental animals**</u>

Mortality

A check for moribund and dead animals was made twice daily on working days and once daily on Saturdays, Sundays and public holidays. If animals were in a moribund state, they were sacrificed and necropsied. The examinations of these animals were carried out according to the methods established at the pathology laboratory.

Clinical observations

A cage side examination was conducted at least once daily for any signs of morbidity, pertinent behavioral changes and signs of overt toxicity. Abnormalities and changes were documented daily for each affected animal.

The littering and lactation behavior of the dams was generally evaluated in the mornings in combination with the daily clinical inspection of the dams. Only particular findings (e.g. inability to deliver) were documented on an individual dam basis. On weekdays (except public holidays) the parturition behavior of the dams was inspected in the afternoons in addition to the evaluations in the mornings. The day of littering was considered the 24-hour period from about 15.00 h of one day until about 15.00 h of the following day.

Detailed clinical observations

Detailed clinical observations (DCO) were performed in all animals prior to the administration period and thereafter at weekly intervals. The findings were ranked according to the degree of severity, if applicable. The animals were transferred to a standard arena (50 × 37.5 cm with sides of 25 cm high). The following parameters were examined:

1. abnormal behavior during "handling"	10. abnormal movements
2. fur	11. impairment of gait
3. skin	12. lacrimation
4. posture	13. palpebral closure
5. salivation	14. exophthalmus
6. respiration	15. feces (appearance/consistency)
7. activity/arousal level	16. urine
8. tremors	17. pupil size
9. convulsions	

2. Water consumption and test substance intake

Water consumption

Generally, water consumption was determined once a week (over a period of max. 4 days) for male and female parental animals, with the following exceptions:

- Water consumption was not determined during the mating period (male and female F0 animals)
- Water consumption of the F0 females with evidence of sperm was determined on GD 6 - 7, 13 - 14 and 19 - 20.
- Water consumption of F0 females, which gave birth to a litter was determined for PND 3 - 4.

Water consumption was not determined in females without positive evidence of sperm (during the mating period of dams used in parallel) and females without litter (during the lactation period of dams used in parallel) and in males after the premating period

Intake of test substance

The intake of test substance was calculated from the amount of water consumed and expressed in mg/kg body weight per day.

The calculation of the group values/day was carried out according to the following formula:

$$IT_x = \frac{WCx}{BWx}\ x\ C$$

IT_x = intake of test substance on day x in mg/kg body weight/day
BW_x = body weight on study day x [g]
IT_x= intake of test substance on study day x [mg/kg bw/day]
C = concentration [ppm]

3. Food consumption

Generally, food consumption was determined once a week for male and female parental animals, with the following exceptions:

- Food consumption was not determined during the mating period (male and female F0 animals).
- Food consumption of the F0 females with evidence of sperm was determined on GD 0 - 7, 7 - 14 and 14 - 20.
- Food consumption of F0 females, which gave birth to a litter was determined for PND 1-4.

Food consumption was not determined in females without positive evidence of sperm (during the mating period of dams used in parallel) and females without litter (during the lactation period of dams used in parallel) and in males after the premating period.

4. Body weight data

Body weight was determined before the start of the administration period in order to randomize the animals. During the administration period body weight was determined on study day 0 (start of the administration period) and thereafter once a week at the same time of the day (in the morning). The body weight change of the animals was calculated from these results.
The following exceptions are notable for the female animals:

- During the mating period the parental females were weighed on the day of positive evidence of sperm (GD 0) and on GD 7, 14 and 20.
- Females with litter were weighed on the day of parturition (PND 1) and on PND 4.
- Females without a litter and without positive evidence of sperm in the vaginal smear were weighed weekly. These body weight data were solely used for the calculations of the dose volume.

5. Functional observational battery

A functional observational battery (FOB) was performed in the first five parental males and females (with litter) per group at the end of the administration period starting at about 10:00 h. The FOB started with passive observations without disturbing the animals, followed by removal from the home cage, open field observations in a standard arena and sensorimotor tests as well as reflex tests. The findings were ranked according to the degree of severity, if applicable. The observations were performed at random.

Home cage observations

The animals were observed in their closed home cages; any disturbing activities (touching the cage or rack, noise) were avoided during these examinations in order not to influence the behavior of the animals. Attention was paid to:
1. posture
2. tremor
3. convulsions
4. abnormal movements
5. impairment of gait
6. other findings

Open field observations
For open field observation the animals were transferred to a standard arena (50 x 50 cm with sides of 25 cm height) and observed for at least 2 minutes. The following parameters were assessed:

1.	behavior when removed from cage	10.	respiration
2.	fur	11.	tremors
3.	skin	12.	convulsions
4.	salivation	13.	abnormal movements / stereotypes
5.	nose discharge	14.	impairment of gait
6.	lacrimation	15.	feces (number of fecal pellets/appearance/ consistency) within two minutes
7.	posture	16.	urine (appearance/quantity) within two minutes
8.	palpebral closure	17.	number of rearings within two minutes
9.	activity/arousal level	18.	eyes/pupil size

For sensorimotor tests and reflexes the animals were removed from the open field. The following tests were performed:

1.	approach response	8.	behavior during "handling"
2.	touch response	9.	vocalization
3.	vision ("visual placing response")	10.	pain perception ("tail pinch")
4.	pupillary reflex	11.	grip strength of forelimbs
5.	pinna reflex	12.	grip strength of hind limbs
6.	audition ("startle response")	13.	landing foot-splay test
7.	coordination of movements ("righting response")	14.	other findings

6. Motor activity measurement:
The motor activity assessment (MA) was carried out in the first five parental males and females (with litter) per group at the end of the administration period. Motor activity was measured on the same day as FOB was performed. The examinations were performed using the TSE Labmaster System supplied by TSE Systems GmbH, Bad Homburg, Germany. For this purpose, the animals were placed in new clean polycarbonate cages for the time of measurement. Eighteen beams were allocated per cage. The numbers of beam interrupts were counted over 12 intervals of 5 minutes. The sequence at which the animals were placed in the polycarbonate cages was selected at random. Motor activity measurements were carried out starting at 14:00 h. On account of the measuring variant "staggered", the starting time varied according to the time needed to place the animals in the cages. For each animal, measurement started individually when the 1st beam was interrupted and was finished exactly 1 hour later. The animals did not receive any food or water during the measurements. After the transfer of the last animal the room of measurement was darkened.

7. Male and female reproduction data

Male reproduction data

The pairing partners, the number of mating days until vaginal sperm was detected in the female animals, and the gestational status of the females were recorded for F0 breeding pairs.
For the males, mating and fertility indices were calculated for F1 litters according to the following formulas:

$$\text{Male mating index (\%)} = \frac{\text{number of males with confirmed mating} *}{\text{number of males placed with females}} \; x\,100$$

*defined by a female with vaginal sperm or with implants in utero

$$\text{Male fertility index (\%)} = \frac{\text{number of males proving their fertility} *}{\text{number of males placed with females}} \; x\,100$$

*defined by a female with implants in utero

Female reproduction and delivery data

$$\text{Female mating index (\%)} = \frac{\text{number of females mated} *}{\text{number of females placed with males}} \; x\,100$$

*defined by a female with vaginal sperm or with implants in utero

$$\text{Female fertility index (\%)} = \frac{\text{number of females pregnant} *}{\text{number of females mated} **} \; x\,100$$

*defined by a female with implants in utero
**defined as the number of females with vaginal sperm or with implants in utero

$$\text{Gestation index (\%)} = \frac{\text{number of females with live pups on the day of birth}}{\text{number of females pregnant} *} \; x\,100$$

*defined as the number of females with implants in utero

The total number of pups delivered and the number of liveborn and stillborn pups were noted, and the live birth index was calculated for F1 litters according to the following formula:

$$\text{Live birth index (\%)} = \frac{\text{number of liveborn pups at birth}}{\text{total number of pups born}} \; x\,100$$

The implantations were counted and the postimplantation loss (in %) was calculated according the following formula:

$$\text{Post implantation loss (\%)} = \frac{\text{number of implantations} - \text{number of pups delivered}}{\text{number of implantations}} \; x\,100$$

8. Litter/Pup data

Litter data

Pup number and status at delivery

All pups delivered from the F0 parents were examined as soon as possible on the day of birth to determine the total number of pups and the number of liveborn and stillborn pups in each litter. At the same time, the pups were also being examined for macroscopically evident changes. Pups, which died before the first determination of their status on the day of birth, were defined as stillborn pups.

Pup number and status at delivery

In general, a check was made for any dead or moribund pups twice daily on workdays (once in the morning and once in the afternoon) or as a rule, only in the morning on Saturdays, Sundays or public holidays.

The number and percentage of dead pups on the day of birth (PND 0) and of pups dying between PND 1 - 4 (lactation period) were determined. Pups which died accidentally or were sacrificed due to maternal death were not included in these calculations. The number of live pups/litter was calculated on the day after birth, and on lactation day 4. The viability index was calculated according to the following formula:

$$\text{Viability index (\%)} = \frac{\text{number of live pups on day 4 after birth}}{\text{number of live pups on the day of birth}} \ x\ 100$$

Sex ratio

On the day of birth (PND 0) the sex of the pups was determined by observing the distance between the anus and the base of the genital tubercle; normally, the anogenital distance is considerably greater in male than in female pups. The sex of the pups was finally confirmed at necropsy.

The sex ratio was calculated at day 0 and day 4 after birth according to the following formula:

$$\text{Sex ratio} = \frac{\text{number of live male or female pups on day } 0/4}{\text{number of live male and female pups on day } 0/4} \ x\ 100$$

Pup clinical observations

The live pups were examined daily for clinical symptoms (including gross-morphological findings) during the clinical inspection of the dams.

Pup body weight data

The pups were weighed on the day after birth (PND 1) and on PND 4. Pups' body weight change was calculated from these results. The individual weights were always determined at about the same time of the day (in the morning). “Runts” were defined on the basis of the body weights on PND 1. "Runts" are pups that weigh less than 75% of the mean weight of the respective control pups.

Pup necropsy observations
All pups with scheduled sacrifice on PND 4 were sacrificed under isoflurane anesthesia with CO_2. All pups were examined externally and eviscerated; their organs were assessed macroscopically. All stillborn pups and all pups that died before PND 4 were examined externally, eviscerated and their organs were assessed macroscopically. All pups were discarded after their evaluation.

9. Clinical pathology

In the morning blood was taken from the retrobulbar venous plexus from fasted animals. The animals were anaesthetized using isoflurane (Isoba®, Essex GmbH Munich, Germany). The blood sampling procedure and subsequent analysis of blood and serum samples were carried out in a randomized sequence. For urinalysis the individual animals were transferred to metabolism cages (withdrawal of food and water) and urine was collected overnight. Urine samples were evaluated in a randomized sequence. The assays of blood and serum parameters were performed under internal laboratory quality control conditions with reference controls to assure reliable test results. The results of clinical pathology examinations were expressed in International System (SI) units. The following examinations were carried out in the first 5 surviving parental males and the first 5 surviving females with litter (in order of delivery) per group:

Haematology
Blood smears were prepared and stained according to WRIGHT without being evaluated, because of non-ambiguous results of the differential blood cell counts measured by the automated instrument.
Furthermore the following parameters were determined:

Haematology:

	Red blood cells		*White blood cells*		*Clotting Potential*
✓	Erythrocyte count (RBC)	✓	Total leukocyte count (WBC)	✓	Prothrombin time
✓	Hemoglobin (Hb)	✓	Differential blood count		
✓	Hematocrit (Hct)	✓	Platelet count (PLT)		
✓	Mean corp. volume (MCV)				
✓	Mean corp. hemoglobin (MCH)				
✓	Mean corp. Hb. conc. (MCHC)				
✓	Reticulocytes (RET)				

Clinical chemistry

The following parameters were determined using an automatic analyser (Hitachi 917, Roche, Mannheim, Germany):

Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Chloride	✓	Albumin/globulin ratio	✓	Aspartate aminotransferase (AST)
✓	Phosphorus (inorganic)	✓	Bile acids	✓	Alkaline phosphatase (ALP)
✓	Potassium	✓	Bilirubin (total)	✓	γ-glutamyl transpeptidase (γ-GT)
✓	Sodium	✓	Cholesterol		
		✓	Creatinine		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

Urinalysis

The dry chemical reactions on test strips used to determine urine constituents semiquantitatively were evaluated with a reflection photometer. The following parameters were evaluated:

Urinalysis					
	Quantitative parameters:		*Semi quantitative parameters*		
✓	Urine volume	✓	Bilirubin	✓	Protein
		✓	Blood	✓	pH-value
		✓	Color and turbidity	✓	Urobilinogen
		✓	Glucose	✓	Sediment (microscopical exam.)
		✓	Ketones	✓	Specific gravity

10. Sacrifice and pathology:

All parental animals were sacrificed by decapitation under isoflurane anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology; special attention was given to the reproductive organs.

Weight parameters

The following weights were determined in all animals sacrificed on schedule:

1. Anesthetized animals
2. Duodenum
3. Epididymides
4. Testes
5. Thyroid glands

The following weights were determined in 5 animals per sex/test group sacrificed on schedule (females with litters only, same animals as used for clinical pathological examinations):
1. Adrenal glands
2. Brain
3. Heart
4. Kidneys
5. Liver
6. Spleen
7. Thymus

Organ/tissue fixation
The following organs or tissues of all parental animals were fixed in 4% buffered formaldehyde solution or modified Davidson's solution:

1. All gross lesions	17. Ileum	33. Rectum
2. Adrenal glands	18. Jejunum (with Peyer's patches)	34. Salivary glands (mandibular and sublingual)
3. Aorta	19. Kidneys	35. Sciatic nerve
4. Bone marrow (femur)	20. Larynx	36. Seminal vesicles
5. Brain	21. Liver	37. Skeletal muscle
6. Cecum	22. Lungs	38. Spinal cord (cervical, thoracic and lumbar cord)
7. Cervix	23. Lymph nodes (axillary and mesenteric)	39. Spleen
8. Coagulating gland	24. Mammary gland (male and female)	40. Sternum with marrow
9. Colon	25. Nose (nasal cavity)	41. Stomach (forestomach and glandular stomach)
10. Duodenum	26. Ovaries (modified Davidson's solution)	42. Testes (modified Davidson's solution)
11. Eyes with optic nerve	27. Oviducts	43. Thymus
12. Esophagus	28. Pancreas	44. Thyroid glands
13. Extraorbital lacrimal glands	29. Parathyroid glands	45. Trachea
14. Epididymides (modified Davidson's solution)	30. Pharynx	46. Urinary bladder
15. Femur with knee joint	31. Pituitary gland	47. Uterus
16. Heart	32. Prostate gland	48. Vagina

Histopathology
Fixation was followed by histotechnical processing, examination by light microscopy and assessment of findings according to the table below:

Organs	Test group			
	0	1	2	3
Adrenal glands	A4			A4
All gross lesions	A2	A2	A2	A2
Bone marrow (femur)	A4			A4
Brain	A4			A4
Cecum	A4			A4
Cervix	A1			A1
Coagulating glands	A1			A1
Colon	A4			A4
Duodenum (cross and longitudinal section)	A4			A4
Epididymides	A1			A1
Heart	A4			A4
Ileum	A4			A4
Jejunum	A4			A4
Kidneys	A4			A4
Liver	A4			A4
Lungs	A4			A4
Lymph nodes (axillary)	A1	A1	A1	A1
Lymph nodes (mesenteric)	A4			A4
Ovaries	A1			A1
Oviducts	A1			A1
Prostate gland	A1			A1
Peyer's patches	A4			A4
Rectum	A4			A4
Sciatic nerve	A4			A4
Seminal vesicles	A1			A1
Spinal cord (cervical, thoracic, lumbar)	A4			A4
Spleen	A1	A1	A1	A1
Stomach (forestomach and glandular stomach)	A4			A4
Testes	A1			A1
Thymus	A4			A4
Thyroid glands	A1	A1	A1	A1
Trachea	A4			A4
Urinary bladder	A4			A4
Uterus	A1			A1
Vagina	A1			A1

1 = all animals/test group
2 = all animals affected/test group
4 = 5 animals per sex/test group, females with litters only, same animals as used for clinical pathological examinations

Special attention was given on stages of spermatogenesis in the male gonads. A correlation between gross lesions and histopathological findings was attempted.

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

The stability of the test substance in drinking water was demonstrated over a period of 4 days at room temperature, which was the maximum storage time period. All determined concentrations were in the range between 90% and 110% of the nominal concentration.
Concentration control analysis confirmed the correctness of the concentrations of the test item in drinking water. The determined values were found to be in the range from 90% - 110% (see Table 5.8.1-89).

Table 5.8.1-89: Concentration control analysis of the test substance in drinking water

Name of sample	Amount [ppm]	Nominal concentration [ppm]	Nominal concentration [%]
3R	102.003	100	102.0
4R	501.726	500	100.3
5R	1991.874	2000	99.6
7	96.155	100	96.2
8	473.979	500	94.8
9	2003.457	2000	100.2

B. OBSERVATIONS

1. Clinical signs of toxicity
No abnormal clinical signs of toxicity were observed.

2. Mortality
No animal died prematurely during the study.

3. Detailed clinical observations
Summary clinical observations for males and females (except gestation/lactation period)
No abnormal clinical signs were observed.

Summary clinical observations for females during gestation
One sperm-positive F0 female of test group 1 and two sperm-positive F0 females of the test group 3 did not deliver any F1 pups. No other clinical signs were observed.

Summary clinical observations for females during lactation
One female animal of test group 3 showed complete litter loss. One female animal of test group 1 showed insufficient maternal care and did not nurse the pups properly during lactation period.

4. Functional observational battery

Deviations from "zero values" were obtained in several rats. However, as most findings were equally distributed between test-substance treated groups and controls, were without a dose-response relationship or occurred in single rats only, these observations were considered as incidental.
No test substance-related effects were observed in "Home cage observations", "Open field observations", and in "Sensorimotor tests/reflexes". Quantitative parameters revealed significantly increased values of landing foot splay test in males of test group 2 (500 ppm; +27.4%). Due to the lack of dose response relationship this was assessed as being incidental.

5. Motor activity measurement

Male animals of test group 1, 2 and 3 showed a significantly lower value at interval 1 when compared to control males. Female animals of test group 3 (2000 ppm) showed a significantly lower value in interval 7. There were no significant deviations concerning the overall motor activity (summation of all intervals) in male and female animals. All described findings were assessed as being incidental and not related to treatment.

C. BODY WEIGHT AND BODY WEIGHT GAIN

Body weight was significantly decreased in females of test group 3 (2000 ppm) on gestation day 20 (-6.2%) and during lactation days 1 and 4 (up to -9.9%).

Table 5.8.1-90: Mean body weights of male rats administered Ethylene urea

	Males			
Dose level [ppm]	0	100	500	2000
	Body weight [g]			
- Day 0 (pre-mating)	316.2 ± 8.5	316.9 ± 9.5	317.3 ± 9.8	315.9 ± 13.7
- Day 7 (pre-mating)	344.8 ± 11.8	345.8 ± 11.1	341.9 ± 16.2	333.5 ± 25.5
- Day 13 (pre-mating)	362.7 ± 11.1	361.1 ± 18.6	360.0 ± 20.5	356.5 ± 27.0
- Day 8 (mating)	376.3 ± 18.1	379.1 ± 14.3	373.0 ± 27.9	367.0 ± 31.2
- Day 0 (post-mating)	390.5 ± 23.8	387.0 ± 18.2	387.0 ± 28.8	393.9 ± 46.1

Table 5.8.1-91: Mean body weights of female rats administered Ethylene urea

	Females			
Dose level [ppm]	0	100	500	2000
	Body weight [g]			
- Day 0 (pre-mating)	208.5 ± 8.9	208.1 ± 6.5	214.0 ± 6.9	211.8 ± 11.8
- Day 7 (pre-mating)	218.3 ± 8.5	218.5 ± 7.3	223.8 ± 10.8	215.0 ± 11.0
- Day 13 (pre-mating)	220.4 ± 7.7	225.0 ± 8.8	224.9 ± 7.6	221.0 ± 9.8
- Day 0 (gestation)	220.5 ± 8.5	224.0 ± 12.7	226.2 ± 10.0	218.0 ± 8.4
- Day 7 (gestation)	250.3 ± 12.3	261.4 ± 16.2	255.5 ± 13.0	244.4 ± 8.8
- Day 14 (gestation)	277.4 ± 14.8	285.9 ± 14.3	283.3 ± 11.0	272.1 ± 8.8
- Day 20 (gestation)	343.3 ± 17.3	344.1 ± 17.3	342.6 ± 11.9	**322.1 ± 16.6***
- Day 1 (lactation)	257.5 ± 15.5	260.5 ± 21.5	258.8 ± 13.7	**236.2 ± 14.9***
- Day 4 (lactation)	273.8 ± 14.8	281.6 ± 20.1	276.5 ± 12.7	**246.6 ± 12.8****

*: $p \leq 0.05$; **: $p \leq 0.01$

Body weight change value was significantly reduced in males of test group 3 (2000 ppm) from day 0 to 7 (-38.3%). Body weight change values were significantly reduced during gestation days 14-20 (-24.3%) and during the entire gestation period from day 0-20 (-15.3%) in female animals of test group 3 (2000 ppm).

Table 5.8.1-92: Mean body weight gains of male rats administered Ethylene urea

	Males			
Dose level [ppm]	0	100	500	2000
	Body weight gain [g] (% control)			
- Day 0-7 (pre-mating)	28.6	28.9 (1.0)	24.6 (-14.0)	**17.7* (-38.3)**
- Day 7-13 (pre-mating)	17.9	15.3 (-14.4)	18.0 (0.9)	23.0 (28.7)
- Day 0-13 (pre-mating)	46.5	44.2 (-4.9)	42.6 (-8.3)	40.6 (-12.6)

*: $p \leq 0.05$

Table 5.8.1-93: Mean body weight gains of female rats administered Ethylene urea

	Females			
Dose level [ppm]	0	100	500	2000
	Body weight gain [g] (% control)			
- Day 0-7 (pre-mating)	9.8	10.5 (6.8)	9.8 (-0.1)	3.2 (-67.1)
- Day 7-13 (pre-mating)	2.0	6.5 (220.9)	1.1 (-43.3)	6.0 (199.0)
- Day 0-13 (pre-mating)	11.8	16.9 (43.2)	10.9 (-7.4)	9.2 (-21.8)
- Day 0-7 (gestation)	29.8	37.4 (25.6)	29.3 (-1.7)	26.4 (-11.6)
- Day 7-14 (gestation)	27.1	24.5 (-9.6)	27.8 (2.5)	27.8 (2.2)
- Day 14-20 (gestation)	65.9	58.2 (-11.8)	59.3 (-10.0)	**49.9* (-24.3)**
- Day 0-20 (gestation)	122.8	120.1 (-2.2)	116.4 (-5.2)	**104.0* (-15.3)**
- Day 1-4 (lactation)	16.3	21.1 (29.8)	17.8 (9.2)	10.4 (-36.2)

*: $p \leq 0.05$

D. FOOD CONSUMPTION

Food consumption during gestation was significantly decreased in females of test group 3 (2000 ppm) from study days 7-14 and 14-20 (up to -9.5%) and during the entire gestation period from day 0 to 20 (-8.8%) (see Table 5.8.1-95).
During lactation period the food consumption was significantly decreased (-40.2%) in females of test group 3 (2000 ppm).

Table 5.8.1-94: Food consumption of male rats administered Ethylene urea

	Males			
Dose level [ppm]	0	100	500	2000
	Food consumption per day [g] (% control)			
- Day 0-7 (pre-mating)	23.8	23.9 (0.7)	23.4 (-1.6)	22.4 (-5.8)
- Day 7-13 (pre-mating)	23.4	23.2 (-0.6)	23.5 (0.4)	22.3 (-4.7)
- Day 0-13 (pre-mating)	23.6	23.6 (0.1)	23.4 (-0.6)	22.3 (-5.3)

Table 5.8.1-95: Food consumption of female rats administered Ethylene urea

	Females			
Dose level [ppm]	0	100	500	2000
	Food consumption per day [g] (% control)			
- Day 0-7 (pre-mating)	15.9	15.9 (0.3)	16.3 (2.3)	15.4 (-2.9)
- Day 7-13 (pre-mating)	15.4	16.3 (5.7)	16.4 (6.3)	15.1 (-2.3)
- Day 0-13 (pre-mating)	15.7	16.1 (2.8)	16.3 (4.1)	15.3 (-2.6)
- Day 0-7 (gestation)	20.2	20.6 (1.7)	20.4 (0.9)	18.5 (-8.4)
- Day 7-14 (gestation)	22.2	23.5 (5.5)	22.8 (2.5)	**20.3* (-8.9)**
- Day 14-20 (gestation)	24.4	24.6 (0.7)	24.8 (1.3)	**22.1** (-9.5)**
- Day 0-20 (gestation)	22.2	22.8 (2.7)	22.5 (1.6)	**20.2* (-8.8)**
- Day 1-4 (lactation)	35.0	34.0 (-2.7)	33.7 (-3.6)	**20.9** (-40.2)**

*: $p \leq 0.05$; **: $p \leq 0.01$

E. WATER CONSUMPTION AND COMPOUND INTAKE

Water consumption was significantly reduced in females of test group 3 (2000 ppm) on lactation day 4 (-37.9%).
The intake of the test substance (in mg/kg bw/day) was calculated on the most recent individual body weights in each test group and the overall approximate values are shown below (see Table 5.8.1-96).

Table 5.8.1-96: Overview on mean test substance intake of male and female animals

	Test group 1 (100 ppm)	Test group 2 (500 ppm)	Test group 3 (2000 ppm)
F0 males (premating)	7.4	36.9	155.0
F0 females (premating)	10.2	47.8	191.0
F0 females (gestation)	11.0	53.1	222.0
F0 females (lactation)	17.0	86.8	234.2

F. REPRODUCTION PARAMETERS

1. Male reproduction data

Male mating index

For F0 parental animals, which were placed with females to generate F1 pups, mating was confirmed. Thus, the male mating index was 100% in all test groups.

Male fertility index

Fertility was proven for most of the F0 parental males within the scheduled mating interval to produce F1 litter.
One male of test group 1 and two males of test group 3 did not generate F1 pups. The male fertility index was 100%, 90%, 100%, and 80% for test groups 0-3. This finding reflected the normal range of biological variation inherent in the strain of rats used for this study as all respective values were within the range of the historical control data.

2. Female reproduction data

Female mating index

The female mating index calculated after the mating period for F1 litter was 100% for all test groups. The mean duration until sperm was detected (GD 0) was 2.3, 3.4, 3.3, and 2.2 days in test groups 0-3.

Female fertility index

All sperm positive rats delivered pups with the exception of female Nos. 119 (test group 1), 134 and 138 (test group 3), which were mated with male Nos. 19, 34 and 38 did not become pregnant. The female fertility index was 80% in the high dose group, 100% in the mid dose and control group and 90% in the low dose group. Female animals Nos. 119, 134 and 138 which delivered no pups, showed no implantation sites. These data reflect the normal range of biological variation inherent in the strain of rats used for this study as all respective values were within the range of the historical control data.
The mean duration of gestation was similar in all test groups (21.9 – 22.4 days).

Gestation index

The gestation index was 100% in all test groups.

Live birth indices
The rate of liveborn pups was between 99.2%, 99.0%, 99.1%, and 97.4% for test groups 0-3. One stillborn pup was seen in test group 0-2 and two stillborn pups were seen in test group 3. Thereby, the live birth index was in the range of the rat strain used.

Postimplantation loss
The postimplantation loss was 18.12%, 5.61%, 5.65%, and 9.52% in test groups 0-3. The value in test group was unusually high, but all other values were inside the historical control data.

3. F1 generation litter/pups
Litter data
Pup number and status at delivery
The mean number of delivered F1 pups per dam was evenly distributed between the groups. The mean number of delivered F1 pups was 12.2 (test group 0), 11.2 (test group 1), 11.7 (test group 2) and 9.5 (test group 3).
The single stillborn pup each in test group 0, 1 and 2 and two stillborn pups in test group 3 were incidental and in the normal range of biological variation inherent in the strain of rats used for this study.

Pup viability/mortality
The viability index indicating pup mortality during lactation (PND 0 - 4) was between 98.5% (test group 0), 100% (test group 1), 98.4% (test group 2) and 81.6% (test group 3). For test group 0-2, these values were in the normal range of biological variation inherent in the strain of rats used for this study. For test group 3, a decreased pup viability was noted, which was also outside the historical control range. Although 7 of the 11 dead offspring in this group came from only one litter (No. 135), a relationship to the treatment cannot be excluded. At necropsy four pups of this dam (animal No. 135, test group 3; 2000 ppm) had an empty stomach indicating that this pup mortality was likely to be subsequent to insufficient maternal care.

Sex ratio
The sex distribution and sex ratios of live F1 pups on the day of birth and PND 4 did not show substantial differences between the control and the test substance-treated groups; slight differences were regarded to be spontaneous in nature.

Table 5.8.1-97: Summary of relevant reproduction parameters of male and female animals

Reproductive parameters				
Dose level [ppm]	**0**	**100**	**500**	**2000**
Female mating index [%]	100	100	100	100
Female fertility index [%]	100	90	100	80
Male mating index [%]	100	100	100	100
Male fertility index [%]	100	90	100	80
Mating days until Day 0 pc	2.3	3.4	3.3	2.2
Gestation index [%]	100	100	100	100
Gestation days	22.0	22.1	21.9	22.4
Total number of litters				
• With liveborn pups [%]	100	100	100	100
• With stillborn pups [%]	10	11.1	10	25
• With all pups stillborn [%]	0	0	0	0
Implantation sites (total)	149	107	124	84
Implantation sites (mean)	14.9	11.9	12.4	**10.5***
Pups delivered (total)	122	101	117	76
Pups delivered (mean)	12.2	11.2	11.7	9.5
Postimplantation loss [%]	18.12	5.61	5.65	9.52
Pups liveborn [%]	99.2	99.0	99.1	97.4
Pups stillborn [%]	0.8	1.0	0.9	2.6
Perinatal loss [%]	0.82	0.99	0.85	2.63

*: $p \leq 0.05$; **: $p \leq 0.01$

G. CLINICAL PATHOLOGY

1. Hematology

No treatment-related, adverse changes among hematological parameters were observed. In females of test group 3 (2000 ppm) haemoglobin values were lower compared to controls. This was the only altered red blood cell parameter and the decrease was marginal (mean: -6.6%) although below the historical control range (hemoglobin 8.7-9.5 mmol/L). Therefore, this alteration was regarded as treatment-related but not adverse (Müller et al., 2006, ECETOC Technical Report No. 85, 2002).

Table 5.8.1-98: Relevant group mean haematology parameters in male animals

Concentration [ppm]	RBC [10^{12}/L]	HGB [mmol/L]	HCT [L/L]
0	8.69 ± 0.30	8.9 ± 0.2	0.425 ± 0.011
100	8.11 ± 0.46	8.7 ± 0.2	0.417 ± 0.007
500	8.49 ± 0.27	8.7 ± 0.1	0.418 ± 0.004
2000	8.21 ± 0.31	8.5 ± 0.2	0.406 ± 0.011

Table 5.8.1-99: Relevant group mean haematology parameters in female animals

Concentration [ppm]	RBC [10^{12}/L]	HGB [mmol/L]	HCT [L/L]
0	8.32 ± 0.64	9.1 ± 0.4	0.433 ± 0.019
100	8.20 ± 0.23	8.8 ± 0.3	0.423 ± 0.018
500	8.21 ± 0.31	9.1 ± 0.3	0.433 ± 0.013
2000	8.01 ± 0.35	**8.5 ± 0.1***	0.407 ± 0.011

* = $p \leq 0.05$

2. Clinical chemistry findings

No treatment-related changes among clinical chemistry parameters were observed. In females of test group 3 (2000 ppm), creatinine levels were higher compared to controls, but the mean was within the historical control range, whereas the control mean was below the historical range (see Table 5.8.1-100). Creatinine was the only altered clinical chemistry parameter in this study. Therefore, this change was regarded as incidental and not treatment-related (ECETOC, Technical Report No. 85, 2002).

Table 5.8.1-100: Creatinine parameters (Day 52) and historical control data in female animals

Concentration	Creatinine [mmol/L]
0	49.6 ± 2.5
100	50.2 ± 2.4
500	52.9 ± 3.9
2000	**59.6 ± 7.3***
Historical control data	52.2 – 62.7

3. Urinalysis

No treatment-related changes among urinalysis parameters were observed.

H. PATHOLOGY

1. Organ weight

When compared to the control group 0 (set to 100%), the mean absolute and relative weight of the thyroid glands was significantly increased in males of test group 3 (see Table 5.8.1-101). All other mean absolute and relative weight parameters in males and all weight parameters in females did not show significant differences when compared to the control group 0.
The increased thyroid weights in males of test group 3 (2000 ppm) were considered to be treatment-related.

Table 5.8.1-101: Group mean of absolute and relative thyroid weights (% control) of male animals

Concentration [ppm]	Thyroid (absolute weights) [% control group]	Thyroid (relative weights) [% control group]
Males		
0	100	100
100	94	95
500	110	112
2000	**125****	**129****

** = p≤0.01

2. Gross lesions

All findings occurred individually. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

The female animals, which were not pregnant as well as the male mating partners did not show relevant gross lesions.

3. Histopathology

Thyroid gland

The number of animals with follicular hypertrophy/hyperplasia was increased in males and females of test group 3 (2000 ppm). In affected animals the number of small follicles was increased or the follicular epithelium was higher, varying in size from cuboidal cells to columnar cells (see Table 5.8.1-102). The occurrence of hypertrophy/hyperplasia in animals of test group 3 (2000 ppm) was considered to be treatment-related.

Table 5.8.1-102: Incidence and severity of follicular hypertrophy/hyperplasia in thyroid glands of male and female animals after administration of test substance

Thyroid gland	Male animals				Female animals			
Test group (ppm)	0	1 (100)	2 (500)	3 (2000)	0	1 (100)	2 (500)	3 (2000)
Organs examined	10	10	10	10	10	10	10	10
Hypertrophy/hyperplasia follicular	1	-	1	10	-	1	1	5
• Grade 1	1	-	-	1	-	1	-	2
• Grade 2	-	-	1	7	-	-	1	3
• Grade 3	-	-	-	2	-	-	-	-

Axillary lymph nodes and spleen

In the axillary lymph nodes of control males and females, most follicles were primary follicles; these are non-stimulated follicles without germinal centers. In some treated animals, the number of follicles with germinal centers (secondary follicles) was slightly (grade 2) or severely (grade 4) increased.

Table 5.8.1-103: Incidence and severity of germinal center in axillary lymph nodes of male and female animals after administration of test substance

Axillary lymph nodes	Male animals				Female animals			
Test group (ppm)	0	1 (100)	2 (500)	3 (2000)	0	1 (100)	2 (500)	3 (2000)
Organs examined	10	10	8	10	10	9	10	10
No. germinal center increased	-	2	3	7	-	3	8	9
• Grade 2	-	1	-	4	-	3	1	1
• Grade 4	-	1	-	3	-	-	7	8

In the spleen of control male and female animals, most follicles had no germinal centers. In some treated males and females the number of follicles with germinal centers was slightly (grade 2) or severely (grade 4) increased when compared with control animals.

Table 5.8.1-104: Incidence and severity of germinal center in the spleen of male and female animals after administration of test substance

Spleen	Male animals				Female animals			
Test group (ppm)	0	1 (100)	2 (500)	3 (2000)	0	1 (100)	2 (500)	3 (2000)
Organs examined	10	10	10	10	10	10	10	10
No. germinal center increased	-	3	7	6	-	2	8	9
• Grade 2	-	-	1	1	-	2	3	1
• Grade 4	-	3	6	5	-	-	5	8

The number of males and females with a higher number of secondary follicles (follicles with germinal centers) in the axillary lymph nodes or in the spleen was dose-related increased in all treatment groups. The increase of follicles with germinal centers was considered to be treatment-related.
All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

The female animals of the dose groups, which were not pregnant as well as the male mating partners did not show histopathological findings explaining why the animals were not pregnant. From the female animal of the control group, which was recorded as not pregnant as well as from the male mating partner only the axillary lymph node and the spleen were examined histologically, according to the study plan.

I. DISCUSSION

Ethylene Urea was administered orally via drinking water to groups of 10 male and 10 female Wistar rats (F0 animals) at dose levels of 0 ppm (test group 0), 100 ppm (test group 1), 500 ppm (test group 2) and 2000 ppm (test group 3).
Regarding clinical examinations, signs of general systemic toxicity were not observed in male or female parental animals of test groups 1-2 (100 and 500 ppm) during the entire study. The decreased body weight changes in males during the premating period from day 0 to 7 (-38.3%) and the decreased food consumption in females during the entire gestation period and the decreased body weight in females during lactation days 1 and 4 (up to -9.9%) in test group 3 (2000 ppm) were considered to be adverse and toxicologically relevant effects.
Regarding clinical pathology, no treatment-related effects were observed up to a dose of the compound of 2000 ppm.

Regarding pathology, target organs were the thyroid glands, the axillary lymph nodes and the spleen. In the thyroid glands, a diffuse hypertrophy/ hyperplasia of follicular epithelium was observed in all males (minimal to moderate) and in five out of 10 females (minimal or slight) of test group 3 (2000 ppm). Hypertrophy/ hyperplasia of follicular epithelium was regarded to be responsible for the recorded weight changes in males of test group 3. The occurrence of hypertrophy/ hyperplasia in males and females of test group 3 (2000 ppm) was considered to be treatment-related and adverse. In the axillary lymph nodes of control males and females most follicles represented primary follicles, only few secondary follicles (follicles with germinal centers) were noted. After treatment with the test substance, some males and females showed a higher number of secondary follicles with germinal centers. A comparable effect was observed in the spleen. The number of males and females with a higher number of secondary follicles in the axillary lymph nodes or in the spleen was dose-related increased in all treatment groups. There were no weight changes and no further histopathological findings in lymphatic organs. Furthermore, no changed parameters in the clinical pathology were noted. The increase of follicles with germinal centers was considered to be treatment-related. An increase of germinal centers can be caused by immune-stimulation. According to the literature only a few compounds have immune-stimulating or immune-adjuvant properties. An example of an agent with immune-potentiating properties is Hexachlorobenzene (HCB). In rats, prominent changes following dietary exposure to this substance included elevated IgM levels and an increase in the weights of the spleen and lymph nodes (Kuper, F. et al, 2000). Histopathologically, the spleen showed increased extramedullary hemopoiesis and hyperplasia of B lymphocytes in the marginal zone and follicles. Lymph nodes and Peyer's patches showed an increase in proportions of high endothelial venules (HEV), indicative of activation. Apart from the increase in germinal centers, these findings were not observed in the present study with Ethylene Urea. Immuno-stimulation can also be seen in combination with inflammation; however there were no signs of inflammation in any investigated organ in this study. The cause of the increase of germinal centers is unclear. However, the development of germinal centers is a normal reaction after antigen stimulation and the increase of germinal centers was the only finding in the lymphatic organs. Therefore the increase of germinal centers was regarded as non-adverse. All other findings recorded were considered to be incidental in nature and not related to treatment.

Regarding fertility and reproductive performance, no signs of toxicity were observed in male or female parental animals of all test groups (100, 500 and 2000 ppm) during the entire study.

Regarding developmental toxicity, signs of toxicity were observed in the decreased pup viability index in test group 3 (2000 ppm) and in one litter loss, wherefore a relationship to the treatment cannot be excluded. However, at necropsy four pups of the dam with complete litter loss (animal No. 135, test group 3; 2000 ppm) had an empty stomach indicating that this pup mortality was likely to be subsequent to insufficient maternal care.

III. CONCLUSIONS

Ethylene Urea was administered orally via drinking water to groups of 10 male and 10 female Wistar rats (F0 animals) at dose levels of 0 ppm (test group 0), 100 ppm (test group 1), 500 ppm (test group 2) and 2000 ppm (test group 3). Clinical observations included decreased body weight gains in males and female animals and decreased food consumption in females. Clinical pathology revealed no treatment-related effects. Pathology findings included hypertrophy/hyperplasia of thyroid follicular epithelium in animals of the high dose group and were evaluated as treatment-related and adverse. Increased numbers of secondary follicles with germinal centers in axillary lymph nodes and spleen were regarded non-adverse. No effect on fertility and reproductive performance were observed. Regarding developmental toxicity, signs of toxicity were observed in the decreased pup viability index in test group 3 (2000 ppm) and in one litter loss, wherefore a relationship to the treatment cannot be excluded. However, at necropsy four pups of the dam with complete litter loss had an empty stomach indicating that this pup mortality was likely to be subsequent to insufficient maternal care.
In conclusion, the **NOAEL** (no observed adverse effect level) for **general, systemic toxicity** was 500 ppm (37 mg/kg bw/day in parental males, 57 mg/kg bw/day in parental females), based on the decreased body weight/body weight gain and decreased food consumption. The **NOAEL** for **reproductive performance** and **fertility** was 2000 ppm (155 mg/kg bw/day in parental males, 214 mg/kg bw/day in parental females) for the F0 parental rats. The **NOAEL** for developmental toxicity in the F1 progeny was found to be 500 ppm (37 mg/kg bw/day in parental males, 57 mg/kg bw/day in parental females), based on the decreased pup viability which was likely to be subsequent to insufficient maternal care.

Overall conclusion and reference value derivation:

Ethylene urea is a REACH Tier 2 chemical and a dossier has been submitted to ECHA. Ethylene urea is of low acute toxicity and not irritating to rabbit skin or eye and not a skin sensitizer. Ethylene urea is not genotoxic based on a negative Ames, a negative mammalian genotoxicity test (HPRT), and on read-across considerations to negative tests conducted with a structurally very similar compound with and without metabolic activation. No NOAEL could be derived in a 4-week drinking water study in rats. The target organs were were thyroid glands, liver, testes, epididymides, duodenum, urinary bladder, spleen and lymph nodes. The LOAEL of this study was 1000 ppm (76 mg/kg bw/day in males and 92 mg/kg bw/day in females). An OECD TG 422 study was conducted using lower dose levels (0, 100, 500 and 2000 ppm in drinking water). Hypertrophy/hyperplasia was observed at the top dose in the thyroid follicular epithelium. The increased number of secondary follicles with germinal centers in axillary lymph nodes and spleen were considered to be not adverse. No effect of fertility and reproductive performance were seen. Decreased pup viability was observed in the top dose together with one litter loss. The pups from the dam with the complete litter losses had empty stomachs (which could indicate insufficient maternal care). The NOAEL for reproductive performance and fertility was 2000 ppm. The NOAEL for developmental toxicity was 500 ppm, based on decreased pup viability, likely to be subsequent to insufficient maternal care. The NOAEL for parental toxicity was at 500 ppm which corresponds to 37 mg/kg bw in males and 57 mg/kg bw in female rats. This NOAEL is used as a starting point for reference dose setting.

ADI: An ADI of 0.06 mg/kg bw can be derived based on the NOAEL of 37 mg/kg bw (derived in an OECD TG 422 study), an extrapolation factor of 6 (subacute to subchronic: 3, subchronic to chronic: 2) and a standard extrapolation factor of 100.
AOEL: In the absence of further information, the ADI has been used as a surrogate value for the AOEL in the non-dietary risk assessment. Thus the AOEL is considered to be 0.06 mg/kg bw.

Metabolite: EBIS = Reg.No. 243959 (CAS No. 33813-20-6)

QSAR predictions on EBIS:

OASIS TIMES (V.2.27.16.8; Mutagenicity S-9 activated v09.09) [see molecule 2 of report DocID 2015/1094106]
There were **no** Ames mutagenicity alerts for EBIS or in silico generated metabolites and no structural alerts were received. The parent substance was out of the model applicability domain.

VEGA: Mutagenicity model (CAESAR, version 2.1.12) [see molecule 4 of report DocID 2015/1094107]
EBIS was out of the model applicability domain. The prediction is **'mutagen'** with a **low** overall reliability. The experimental value is **'non-mutagen'.**

VEGA: Mutagenicity model (SarPy; version 1.0.6-DEV) [see molecule 4 of report DocID 2015/1094107]
EBIS could be out of the model applicability domain. The prediction is **'non-mutagen'** with a **moderate** overall reliability. This is in concordance with the experimental value, which is also **'non-mutagen'.**

VEGA: Mutagenicity model (Toxtree; version 1.0.0-DEV) [see molecule 4 of report DocID 2015/1094107]
EBIS was out of the model applicability domain. The prediction is **'non-mutagen'** with a **low** overall reliability. Deficiencies result from:

- only moderately similar compounds with known experimental value in the training set have been found
- similar molecules found in the training set have experimental values that disagree with the predicted value
- accuracy of prediction for similar molecules found in the training set is not adequate
- a prominent number of atom centered fragments of the compound have not been found in
- the compounds of the training set or are rare fragments

Toxicological information on EBIS:

Absorption, distribution, excretion, and metabolism
ETU, EU and various unidentified polar products were identified as metabolites of EBIS in mice. In males (ND/4(S) BR mice) given a single oral dose of either 8.8 or 44 mg/kg (0.05 or 0.25 mmol/kg) ^{14}C-EBIS in olive oil, 54% of the administered dose (radioactivity) was recovered in urine or feces within 48 hr, 40-74% in urine. No unchanged EBIS was detected in urines. At the lower dose 99.7% of urinary radioactivity was unidentified polar products; at the higher level, urinary radioactivity consisted of 10.8% ETU, 10.7% EU, and 78% unidentified polar products.
(Jordan and Neal, 1979, BASF DocID 1979/10171).

Acute toxicity
Iverson F, Newsome WH, Hierlihy SL (1977) Tissue distribution of ethylenethiuram monosulfide (ETM) in the rat. BASF DocID 1977/1001204

Materials and methods: Groups of 5 male Sprague-Dawley rats were administered ETM (= ethylenethiuram monosulfide = EBIS = Reg.No. 243959) orally in corn oil at a rate of 1.0 ml/200 g bw for single dose levels of 50, 100, 150, 200, 250, and 350 mg/kg bw. Controls received corn oil only. The LD_{50} vlaue was calculated by the procedure of Ltchfield and Wilcoxon (1949), based on the 24 hour mortality.

Results: The 24 hour LD_{50} value for EBIS was 240 (303 – 174) mg/kg bw. The slope function is 1.63 (2.15 – 1.23).

Conclusion: The LD_{50} of EBIS is 240 mg/kg bw in rats.

Other databases (e.g. ChemID) report an LD_{50} values for EBIS (CAS No. 33813-20-6) of 380 mg/kg bw, which is in the same order of magnitude.

Genotoxicity

Report:	CA 5.8.1/46 Woitkowiak C., 2014a Reg.No. 243959 (metabolite of BAS 222 F, Metiram) - Salmonella typhymurium / Escherichia coli - Reverse mutation assay 2014/1134381
Guidelines:	OECD 471, EPA 870.5100, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.13/B.14 No. L 142
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

S. typhimurium strains TA98, TA100, TA 1535 and TA 1537 and E. coli strain WP2 uvrA were exposed to Reg.No. 243959 (metabolite of metiram); batch: L83-136, purity: 96.7%) using dimethylsulfoxide (DMSO) as a solvent in the presence and absence of metabolic activation in three independent experiments. Vehicle and positive controls were included in each experiment.
In the plate incorporation assay the test substance was tested in concentrations of 33 to 5200 µg/plate. Due to a strong toxicity found in the first experiment a second was done with concentrations between 0.1 and 100 µg/plate. In the preincubation assay the test substance was tested in concentrations of 0.1 to 100 µg/plate. No biologically relevant increase in the number of revertant colonies was noted in any of the strains tested in presence or absence of metabolic activation in any of the experiments. The positive controls induced the appropriate response in the corresponding strains, thus demonstrating the sensitivity of the test system.

A strong bacteriotoxic effect was observed in the standard plate test depending on the strain and test conditions from about 0.1 µg/plate onwards and in the preincubation assay from about 10 µg/plate onwards. No precipitation of the test substance was found up to the highest tested concentration.

The test substance Reg.No. 243959 (Metabolite of metiram) is not mutagenic in the Salmonella typhimurium / Escherichia coli reverse mutation assay under the experimental conditions of the study.

(BASF DocID 2014/1134381)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

test substance Reg.No. 243959 (Metabolite of BAS 222 F, Metiram)

Description: Solid, yellow

Lot/Batch #: L83-136

Purity: 96.7% (tolerance +/- 1.0%)

Stability of test compound: Stable - expiry date 01-Oct-2014. The homogeneity of the test substance was ensured by mixing prior to preparation of test substance solutions. The stability of the test substance in the vehicle DMSO was verified analytically.

Solvent used: Dimethylsulfoxide (DMSO)

2. Control Materials:

Negative control: In parallel to each experiment, negative control experiments were carried out in order to check for possible contaminants (sterility control) and to determine the spontaneous mutation rate (vehicle control).

Vehicle control: The vehicle control with and without S-9 mix only contained the vehicle used for the test substance at the same concentration and volume for all tester strains.

Solvent/final concentration: 100 µL/plate

Positive control compounds tested without addition of metabolic activation system:

Strain	Mutagen	Solvent	Concentration
TA 100	N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)	DMSO	5 µg/plate
TA 1535	N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)	DMSO	5 µg/plate
TA 1537	9-Aminoacridine (AAC)	DMSO	100 µg/plate
TA 98	4-nitro-o-phenylendiamine (NOPD)	DMSO	10 µg/plate
WP2 uvrA	4-nitroquinoline-N-oxide (4-NQO)	DMSO	5 µg/plate

Positive control compounds tested with addition of metabolic activation system:

Strain	Mutagen	Solvent	Concentration
TA 100	2-aminoanthracene	DMSO	2.5 µg/plate
TA 1535	2-aminoanthracene	DMSO	2.5 µg/plate
TA 1537	2-aminoanthracene	DMSO	2.5 µg/plate
TA 98	2-aminoanthracene	DMSO	2.5 µg/plate
WP2 uvrA	2-aminoanthracene	DMSO	60 µg/plate

3. Activation:

S9 was produced from the livers of induced male Wistar rats. The rats received on three consecutive days intraperitoneal injections of 80 mg phenobarbital and 80 mg β-naphthoflavone orally per kg body weight. 24 hours after the last administration the animals were sacrificed. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction is thawed at room temperature and 1 volume of S9-fraction is mixed with 9 volumes of S9-supplement (cofactors). This preparation, the so-called S9 mix, was kept on ice until used.

The S9 mix was prepared immediately before use and had the following composition:

Component	Concentration
Phosphate buffer (pH 7.4)	15 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM
S9	10%

To demonstrate the efficacy of the S9 mix in this assay, the S9 batch was further characterized with benoz(a)pyrene in the strains TA 100 and TA 98 as required in OECD 471.

4. Test organisms:

S. typhimurium strains: TA98, TA100, TA1535, TA1537
E. coli: WP2 uvrA
Salmonella typhimurium:
The Salmonella strains are checked for the following characteristics at regular intervals: deep rough character (rfa); UV sensitivity (uvrB); ampicillin resistance (R factor plasmid). E. coli WP2 uvrA is checked for UV sensitivity.
Histidine and tryptophan auxotrophy is automatically proven in each experiment via the spontaneous rate.

5. Test concentrations:

Plate incorporation assay:

In the first experiment triplicate plates were prepared for each concentration (neg. control; 33, 100, 333, 1000, 2600 and 5200 µg/plate and positive controls at the concentrations indicated above) and conditions (i.e. with and without S9) for all tester strains indicated above.

Due to strong bacteriotoxicity observed in the 1st experiment the standard plate test will be repeated. In the second experiment triplicate plates were prepared for each concentration (neg. control, 0.3, 1.0, 3.3, 10, 33 and 100 µg/plate – for TA strains with S9 mix and E. coli WP2 uvrA with and without S9 mix; neg. control, 0.1, 0.3, 1.0, 3.3, 10 and 33 µg/plate – for TA strains without S9 mix)

Pre-incubation assay:

In the third experiment the test article / vehicle / positive control substance, bacterial and S9 mix or phosphate buffer were incubated at 37°C for the duration of about 20 minutes. Triplicate plates were prepared for each concentration (neg. control; 0.3, 1.0, 3.3, 10, 33 and 100 µg/plate for TA strains with S9 mix, E. coli WP2 uvrA with and without S9 mix and neg. control, 0.1, 0.3, 1.0, 3.3, 10 and 33 µg/plate for TA strains without S9 mix and positive controls at the concentrations indicated above).

B. TEST PERFORMANCE:

1. Dates of experimental work: 04-Mar-2014 to 27-Mar-2014

2. Plate incorporation assay:
To test tubes containing 2-mL portions of warm soft agar (containing 0.5 mM histidine + 0.5 mM biotin), 0.1 mL test solution or vehicle, 0.1 mL fresh bacterial culture, 0.5 mL S9-mix (in tests with metabolic activation) or 0.5 mL phosphate buffer (in tests without metabolic activation) were added in the given order. After mixing, the samples are poured onto Merckoplate® plates (minimal glucose agar plates). In the experiments with E. coli the warm soft agar contains 0.5 mM tryptophan instead of histidine + biotin.

3. Pre-incubation assay:
100 µL of test solution or vehicle, 0.1 mL bacterial suspension and 0.5 mL S9 mix or phosphate buffer were incubated at 37°C for about 20 minutes. Subsequently, 2 mL of soft agar was added and, after mixing, the samples are poured onto the agar plates.

After incubation in the dark for 48 to 72 hours at 37°C each plate was checked for precipitates and status of the background lawn. Revertant colonies were counted. Means and standard deviations were calculated.

4. Toxicity determination:
Toxicity detected by a decrease in the number of reveratants (factor ≤ 0.6) and/or clearing or diminution of the background lawn (= reduced his^- or trp^- background growth) was recorded for all test groups both with and without S9 mix in all experiments.

5. Statistics:
No special statistical tests were performed.

6. Evaluation criteria:
The test chemical is considered positive in this assay if the following criteria are met:

- A dose-related and reproducible increase in the number of revertant colonies, i.e. about doubling (bacteria strains with high spontaneous mutation rate, like TA 98, TA 100 and E. coli WP2 uvrA) or tripling (bacteria strains with low spontaneous mutation rate, like TA 1535 and TA 1537) of the spontaneous mutation rate in at least one tester strain either without S9 mix or after adding a metabolizing system.

A test substance is generally considered non-mutagenic in this test if:

- The number of revertants for all tester strains was within the historical negative control range under all experimental conditions in at least two experiments carried out independently of each other.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

The test substance was stable over the study period under the storage conditions. The stability of the test substance in the vehicle DMSO was verified analytically (BASF study 01Y0362/12Y115).

B. TOXICITY

A preliminary cytotoxicity assay was not performed in this laboratory. Cytotoxicity was determined in the course of the mutation assay.

A strong bacteriotoxic effect (reduced his^- or trp^- background growth, decrease in the number of his^+ or trp^+ revertants) was observed in the standard plate test depending on the strain and test conditions from about 0.1 µg/plate onward.
In the preincubation assay strong bacteriotoxicity (reduced his^- or trp^- background growth, decrease in the number of his+ or trp+ revertants) was observed depending on the strain and test conditions from about 10 µg/plate onward.

C. MUTATION ASSAYS

Neither in the plate incorporation nor in the preincubation experiment with and without metabolic activation was a biologically relevant increase in number of revertants observed in any strain tested [see Table 5.8.1-105]. The positive controls yielded revertant numbers in a range expected for the respective strains and thus demonstrated the sensitivity of the test system. Precipitation was not observed up to the maximum concentration.

III. CONCLUSION

According to the results of the present study, the test substance Reg.No. 243959, metabolite of metiram) is not mutagenic in the Salmonella typhimurium / Escherichia coli reverse mutation assay under the experimental conditions chosen.

Table 5.8.1-105: Bacterial gene mutation assay with Reg.No. 243959 - Mean number of revertants

Experiment 1: Plate incorporation assay										
Strain	TA 98		TA 100		TA 1535		TA 1537		E. coli	
Metabolic activation	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Neg. control (DMSO)	35.3	16.3	57.3	46.7	8.7	11.7	6.7	5.7	60.3	59.7
Reg.No. 243959										
33 µg/plate	3.0[B]	3.0[B]	38.3	4.0[B]	10.7	5.0[B]	0.0[B]	0.0[B]	52.0	41.3[B]
100 µg/plate	0.0[B]	0.0[B]	57.3	0.0[B]	8.7	0.0[B]	0.0[B]	0.0[B]	42.3	20.0[B]
333 µg/plate	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]
1000 µg/plate	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]
2600 µg/plate	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]
5200 µg/plate	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]	0.0[B]
Pos. control[§]	2497.3	374.0	2867.3	3732.3	264.3	5342.7	257.7	1022.0	274.7	864.0
Experiment 2: Plate incorporation assay										
Strain	TA98		TA 100		TA 1535		TA 1537		E. coli	
Metabolic activation	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Neg. control (DMSO)	29.7	16.0	52.3	43.0	10.0	8.0	7.0	9.3	63.0	47.7
Reg.No. 243959										
0.1 µg/plate		22.3		35.7		9.0		5.0		
0.3 µg/plate	24.0	17.3	60.0	40.0	9.7	7.0	7.3	4.0	77.0	64.0
1.0 µg/plate	19.7	17.7	66.7	37.7	7.7	7.3	9.0	6.0	63.7	60.7
3.3 µg/plate	21.0	18.7	57.7	42.7	12.0	5.7	6.0	5.3	64.0	32.0
10 µg/plate	34.0	17.7	57.7	37.7	11.3	6.0[B]	10.0	5.3	72.7	63.7
33 µg/plate	25.0	0.0[B]	54.0	0.0[B]	11.3	2.7	7.0	0.0[B]	66.0	70.0
100 µg/plate	19.7		46.3		13.3[B]		6.7		72.0	3.0[B]
Pos. control[§]	1589.0	427.3	1965.0	4846.0	262.7	5353.3	146.3	2127.0	167.3	738.3
Experiment 3: Preincubation assay										
Strain	TA 98		TA 100		TA 1535		TA 1537		E. coli	
Metabol. activation	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Neg. control (DMSO)	32.0	24.0	61.3	36.0	14.3	13.7	15.3	13.3	83.3	74.3
Reg.No. 243959										
0.1 µg/plate		23.7		45.7		13.7		11.7		
0.3 µg/plate	27.0	22.0	66.0	44.3	12.7	8.3	11.7	9.3	83.3	69.7
1.0 µg/plate	30.3	21.3	67.3	51.7	14.7	12.3	15.3	9.3	76.3	71.3
3.3 µg/plate	23.3	24.3	48.3	44.7	9.0	12.0	13.0	15.7	71.0	64.7
10 µg/plate	35.0	30.7	69.0	50.0	10.0	10.3	9.0	0.0[B]	75.7	76.0
33 µg/plate	34.3	0.0[B]	51.0	0.0[B]	9.0	0.0[B]	11.3	0.0[B]	81.0	54.3
100 µg/plate	37.7		55.0		8.0		10.0		70.3	0.0[B]
Pos. control[§]	2246.7	416.0	2332.3	3681.3	290.3	3855.0	160.3	1429.7	191.3	701.7

§ Compound and concentrations see Material and Methods (I.A.2.) above
B reduced background growth
Due to strong toxicity in the plate incorporation assay the repetition of the experiment with lower concentrations was performed as second experiment. Accordingly, the preincubation assay was the 3rd experiment.

Report:	CA 5.8.1/47 Schulz M.,Landsiedel R., 2014a Reg.No. 243959 (metabolite of BAS 222 F, Metiram) - In vitro micronucleus assay in V79 cells (cytokinesis block method) 2014/1172991
Guidelines:	Commission Regulation EU No. 640/2012 of 06 July 2012 - B.49: In vitro Mammalian Cell Micronucleus Test, OECD 487 (2010)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

Reg.No. 243959 (metabolite of metiram; batch L83-136; purity 96.7%) was tested in vitro for its potential to induce micronuclei in V79 cells of the Chinese hamster in the absence and presence of metabolic activation by S9 mix in two independent experiments with concentrations of 0.31 to 50 µg/mL. The exposure periods were either 4 hours or 24 hours (without S9 only) and the preparation intevalls were 24 or 44 hours (with S9 only) after start of treatment.
The test substance did not induced a statistically significant and biologically relevant increase in the number of micronucleated cells after continuous treatment for 4 or 24 hours with or without S9 mix.
Cytotoxicity indicated by clearly reduced cell count was observed at least at the highest applied test substance concentration in all experimental parts of this study, except in the 1st Experiment in the presence of S9 mix. However, in both experiments in the absence and presence of S9 mix at least the highest applied concentrations were not scorable for cytogenetic damage due to strong cytotoxicity (low proliferation rate). Likewise no test substance precipitation in culture medium observed.
Vehicle and positive controls revealed the expected results of micronucleated cells thereby ensuring the validity of the test.
Based on the results of this study Reg.No. 243959 is considered not to have a chromosome-damaging (clastogenic) effect nor to induce numerical chromosomal aberrations (aneugenic activity) under in vitro conditions in V79 cells in the absence and presence of metabolic activation.

(BASF DocID 2014/1172991)

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** Reg.No. 243959 (metabolite of BAS 222 F, metiram)
   Description: Solid, yellow
   Lot/Batch #: L83-136
   Purity: 96.7% (tolerance ± 1.0%)
   Stability of test compound: Stable - Expiry date 01-Oct-2014. The homogeneity of the test substance was ensured by mixing prior to preparation of test substance solutions.
   The stability of the test substance at room temperature in the vehicle DMSO over a period of 4 hours was verified analytically.
   Solvent used: Dimethylsulfoxide (DMSO)

2. **Control Materials:**
   Negative control: A negative control was not employed in this study.
   Solvent control: DMSO
   Positive control, -S9: Ethylmethanesulfonate (EMS, 400 and 500 µg/mL, dissolved in MEM without FCS)
   Positive control, +S9: Cyclophosphamide (CPA, 0.5 and 1.0 µg/mL, dissolved in MEM without FCS)

3. **Activation:** S9 was produced from male Wistar rats. The rats were induced by intraperitoneal applications of 80 mg/kg bw phenobarbital and by oral administrations of 80 mg/kg bw β-naphthoflavone each, on three consecutive days. The livers were prepared 24 hours after the last treatment. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction was thawed at room temperature and 1 volume of S9-fraction was mixed with 9 volumes of S9-supplement (cofactors). This preparation, the so-called S9 mix, was kept on ice until used.

   The S9-mix was prepared immediately before use and had the following composition:

Component	Concentration
Phosphate buffer (pH 7.4)	15 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM
S9	10%

4. Test organisms: Chinese hamster V79 cells
This is a continuous cell line with a high proliferation doubling time of 12-14 hours, a high plating efficiency (≥90%) and a stable karyotype (modal number of 22 chromosomes).

5. Culture medium/conditions:

Culture media: Minimal essential medium with Earle's salts (MEM) containing a L-glutamine source supplemented with 10% (v/v) fetal calf serum (FCS), 1% (v/v) penicillin/streptomycin (10000 IU / 10000 µg/mL) and 1% (v/v) amphotericine B (250 µg/mL). During exposure to the test substance in the presence of S9 mix MEM medium was used without FCS supplementation.

Cell culture: Deep-frozen cell stocks were thawed at 37°C in a water bath, and volumes of 0.5 mL were transferred into 25 cm² plastic flasks containing about 5 mL MEM supplemented with 10% (v/v) fetal calf serum (FCS). Cells were grown with 5% (v/v) CO_2 at 37°C and ≥ 90% humidity and subcultured twice weekly. Cell monolayers were suspended in culture medium after detachment with 0.25% (w/v) trypsin solution.

Cell cycle and harvest time: The cell cycle of the untreated V79 cells lasts for about 12 to 14 hours under the selected culture conditions. Thus, a harvest time of 24 hours is about 2 times the normal cell cycle length. V79 cells are an asynchronous cell population, i.e. at the time of test substance treatment there are different cell stages. Since the effect on these cell stages may vary for different test substances, more than one harvest time after treatment may be appropriate. Furthermore, substance-induced mitotic delay may considerably delay the first post-treatment mitosis. Therefore, delayed harvest times (e.g. 44 hours) and prolonged exposure periods (e.g. 24 hours treatment) were considered.

6. Test concentrations:

a) Preliminary toxicity assay: 14.84 to 1900 µg/mL with and without metabolic activation

Exposure period: 4 hours with and without metabolic activation; 24 hours without metabolic activation

Harvest time: 24 hours

b) Cytogenicity assay:

1st experiment:	0.78, 1.56, 3.13, 6.25, 12.50, 25.00 and 50.00 µg/mL with and without S9 mix (4 hours exposure, 24 hour preparation interval)
2nd experiment:	0.31, 0.63, 1.25, 2.50, 5.00 and 10.00 µg/mL without metabolic activation (24 hour exposure, 24- hour preparation interval); 0.63, 1.25, 2.50, 5.00, 10.00 and 20.00 µg/mL with metabolic activation (4 hour exposure, 44 hours preparation interval)

B. TEST PERFORMANCE:

1. Dates of experimental work: 17-Mar-2014 to 17-Sep-2014

2. Preliminary cytotoxicity assay:

In the pretest for toxicity based on the purity and the molecular weight of the test substance 1900 µg/mL (approx. 10.8 mM) Reg.No. 243959 (metabolite of metiram) was used as top concentration. The pretest was performed following the method described for the main experiment. As indication of test substance toxicity relative cell count and cell attachment (morphology) were determined for dose selection. pH and solubility were additionally determined.

3. Cytogenicity Assay:

Two independent experiments were performed. In experiment I, the exposure period was 4 hours with and without S9 mix. In experiment II, the exposure periods were 4 hours with S9 mix and 24 hours without S9 mix. The cells were prepared 24 hours after start of treatment with the test item, except for the 4 hour treatment interval in the presence of metabolic activation (experiment II). In this experimental part cells were harvested after 44 hours to consider possibly occurring mitotic delay.

Cell seeding / treatment:	Routinely grown cells that did not exceed a max. of 15 passages and reached a confluency of at least 50%, were detached by trypsination and used to prepare a single cell suspension with the required cell count (3-5x10^5 cells per culture, depending on the schedule) in MEM incl. 10% (v/v) FCS. 5 mL cell suspension were transferred into 25 cm² cell culture flasks using a dispenser. Subsequently, the test cultures were incubated. After an attachment period of about 20 - 24 hours, the medium was removed from the flasks and the treatment medium was added. In case of experiments without metabolic activation the treatment medium consisted of 4 mL MEM medium with FCS plus 1 mL positive control or test substance preparation/ vehicle, respectively.

In case of metabolic activation the treatment medium consisted of 3 mL MEM medium without FCS, 1 mL positive control or test substance preparation/ vehicle and 1 mL S9-mix, respectively. The cultures were incubated for the respective exposure period at 37°C, 5% (v/v) CO_2 and ≥ 90% humidity.

At the end of the exposure period, the medium was removed and the cultures were rinsed twice with 5 mL HBSS (Hanks Balanced Salt Solution). Subsequently, 5 mL MEM incl. 10% FCS supplemented with Cytochalasin B (CytB, final concentration: 3 µg/mL; stock: 0.6 mg/mL in DMSO) was added and incubated for the respective recovery time. In the case of 24-hour continuous exposure, CytB was added to the treatment medium at start of treatment, and cell preparation was started directly at the end of exposure. At 44 hours preparation interval in the presence of S9 mix the supplementation of CytB was 24 hours before preparation of the cultures.

Cell harvest, preparation of slides and staining:

Just before preparation the culture medium was completely removed. Single cell suspensions were prepared from each test group by trypsination. Then, the cell numbers per flask of each single cell suspension were determined using a cell counter. Subsequently, $5x10^4$ cells per slide were centrifuged at 1400 rpm for 7 minutes onto labelled slides using a Cytospin centrifuge. After drying, the slides were fixed in 90% (v/v) methanol for 10 minutes. Before scoring, the slides were stained with a mixture of 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) and propidium iodide (PI) in Fluoroshield™ at a concentration of 0.25 µg/mL each. By the use of the combination of both fluorescence dyes it can be differentiated between DNA (DAPI; excitation: 350 nm, emission: 460 nm) and cytoplasm (PI; excitation: 488 nm, emission: 590 nm).

Analysis of micronuclei and cytotoxicity:

Cytospin slides were scored by fluorescence microscopy. At least 1000 cells per culture, means at least 2000 cells per test group, were evaluated and the number of micronuclei-containing binucleated cells was recorded. Analysis of micronuclei was carried out following the criteria: The diameter of the micronucleus is less than 1/3 of the main nucleus; the micronucleus and main nucleus retain the same color; the micronucleus is not linked to the main nucleus and is located within the cytoplasm of the cell; only cells clearly surrounded by a nuclear membrane were scored. Cultures with few isolated cells were not analyzed for micronuclei.

Cell count:

Before preparing the cytospin slides the cell count was determined from trypsinized cultures. The data are given tabulated in percentage compared with the concurrent negative control. Thus, a relative cell count of 45% indicates 55% cytotoxic/cytostasis.

Proliferation Index (CBPI):

$$CBPI = \frac{((\text{No. mononucleated cells}) + (2 \text{ x No. binucleated cells}) + (3 \text{ x No. mulitnucleated cells}))}{(\text{Total number of cells})}$$

The CBPI was used to calculate the % cytostasis (relative inhibition of cell growth compared to the respective vehicle control group) - a CBPI of 1 (all cells are mononucleate) is equivalent to 100% cytostasis.

$\% \text{ Cytostasis} = 100 - 100 \{(CBPI_T - 1) / (CBPI_C - 1)\}$

(T- test substance treated culture; C = vehicle control)

Cell morphology:

At the end of the treatment period, the cultures of all test groups were examined microscopally with regard to cell morphology, which is a further indication of cytotoxicity.

4. Statistics:

The statistical evaluation of the data was carried out using the MUVIKE program system (BASF SE). The proportion of cells containing micronuclei was calculated for each group. A comparison of each dose group with the concurrent vehicle control group was carried out using Fisher's exact test for the hypothesis of equal proportions. This test is Bonferroni-Holm corrected versus the dose groups separately for each time and was performed one-sided. If the results of this test were statistically significant compared with the respective vehicle control, labels (* $p \leq 0.05$, ** $p \leq 0.01$) have been be printed in the tables.

5. Evaluation criteria:

A test item is considered “positive” in this assay if the following criteria are met:

- A significant, dose-related and reproducible increase in the number of cells containing micronuclei was observed.
- The number of micronucleated cells exceeded both the value of the concurrent vehicle control and the range of our laboratory’s historical negative control data.

A test substance is generally considered “negative” in this test system if:

- The number of micronucleated cells in the test groups is not distinctly increased above the concurrent vehicle control and is within our laboratory’s historical negative control data range.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS:

The stability of the test substance at room temperature in the vehicle DMSO over a period of 4 hours was verified analytically (see BASF study code 01Y0362/12Y115).

B. PRELIMINARY CYTOTOXICITY ASSAY:

A preliminary cytotoxicity assay was performed to determine appropriate test substance concentrations for the main test. Cytotoxicity indicated by clearly reduced cell numbers of about or below 40 – 50% was observed at all applied concentrations either after 4 hours test substance treatment in the absence and presence of S9 mix or after 24 hours continuous treatment in the absence of metabolic activation.

C. CYTOGENICITY ASSAYS:

In all experimental parts after 4 and 24 hours test substance treatment in the absence and presence of S9 mix the values (0.4 - 1.1% micronucleated cells)were close to the concurrent vehicle control values (0.4 - 0.7% micronucleated cells) and within historical negative control data range (0.1 - 1.8% micronucleated cells) [see Table 5.8.1-106 and Table 5.8.1-107].
Besides, in the 2nd Experiment in the absence of S9 mix a single statistically significant value compared to the respective vehicle control values was obtained at an intermediate concentration of 0.63 µg/mL (1.1% versus 0.4% micronucleated cells). However, this observation occurred due to the low rate of micronucleated cells in the concurrent vehicle control group. This value was clearly within our historical negative control data range and, therefore, this finding has to be regarded as biologically irrelevant [see Table 5.8.1-106].

The positive control substances EMS (without S9 mix; 500 µg/mL) and CPP (with S9 mix; 0.5 µg/mL) induced statistically significant increased micronucleus frequencies in two independently performed experiments and the values were within the historical positive control data range.

In addition, in both main experiments in the absence and the presence of S9 mix growth inhibition indicated by reduced cell counts was observed at least at the highest applied test substance concentrations. In this study, cell attachment/morphology was adversely influenced (grade > 2) at about 10 µg/mL and above in the presence of S9 mix. In the absence of S9 mix cell attachment/morphology was adversely influenced at 25 µg/mL and above in the 1st Experiment and from 0.63 to 2.50 µg/mL in the 2nd Experiment. The slides were not scorable for cytogenetic damage due to strong cytotoxicity and/or poor quality at least at the highest applied test substance concentration.
Osmolarity and pH values were not influenced by test substance treatment. No precipitation of the test substance in culture medium was observed

Table 5.8.1-106: Summary of results of micronucleus test with Reg.No. 243959 (metabolite of metiram) - without metabolic activation

					Genotoxicity	Cytotoxicity	
Exp.	Exposure/ Preparation interval	Test groups	S9 mix	Prec.*	Micronucleated cells** [%]	Proliferation index cytostasis (CBPI) [%]	Relative cell count [%]
1	4/24 hrs	Vehicle control[1]	-	n.d.	0.7	0.0	100.0
		0.78 µg/mL	-	-	0.7	-2.6	76.3
		1.56 µg/mL	-	-	0.9	-1.5	71.1
		3.13 µg/mL	-	-	0.6	1.4	41.5
		6.25 µg/mL	-	-	n.s.	n.s.	40.1
		12.50 µg/mL	-	-	n.s.	n.s.	18.1
		25.00 µg/mL	-	-	n.s.	n.s.	24.1
		50.00 µg/mL	-	-	n.s.	n.s.	19.5
		Positive control[2]	-	n.d.	2.1[S]	2.1	61.7
		Positive control[3]	-	n.d.	3.2[S]	0.9	70.3
2	24/24 hrs	Vehicle control[1]	-	n.d.	0.4	0.0	100.0
		0.31 µg/mL	-	-	0.4	-1.5	77.2
		0.63 µg/mL	-	-	1.1[S]	-2.2	70.4
		1.25 µg/mL	-	-	0.9	7.0	67.5
		2.50 µg/mL	-	-	n.s.	n.s.	36.2
		5.00 µg/mL	-	-	n.s.	n.s.	51.5
		10.00 µg/mL		-	n.s.	n.s.	49.6
		Positive control[2]		n.d.	3.2[S]	7.9	87.6

* Precipitation in culture medium at the end of exposure period (macroscopic)
** Relative number of binucleated cells with micronuclei per 2000 cells scored per test group
[S] Frequency statistically significant higher than corresponding control values
n.d. Not determined;
n.s. not scorable due to strong toxicity
[1] DMSO 1% (v/v) [2] EMS 400 µg/mL; [3] EMS 500 µg/mL

Table 5.8.1-107: Summary of results of micronucleus test with Reg.No. 243959 (metabolite of metiram) - with metabolic activation

					Genotoxicity	Cytotoxicity	
Exp.	Exposure/ Preparation interval	Test groups	S9 mix	Prec.*	Micronucleated cells** [%]	Proliferation index cytostasis (CBPI) [%]	Relative cell count [%]
1	4/24 hrs	vehicle control[1]	+	n.d.	0.5	0.0	100.0
		0.78 µg/mL	+	-	n.d.	n.d.	94.9
		1.56 µg/mL	+	-	n.d.	n.d.	99.9
		3.13 µg/mL	+	-	0.4	7.1	86.5
		6.25 µg/mL	+	-	0.4	8.0	84.3
		12.50 µg/mL		-	0.5	9.6	63.2
		25.00 µg/mL		-	n.s.	n.s.	52.4
		50.00 µg/mL		-	n.s.	n.s.	57.5
		positive control[2]	+	n.d.	5.3[S]	29.8	82.9
2	4/44 hrs	vehicle control[1]	+	n.d.	0.4	0.0	100.0
		0.63 µg/mL	+	-	0.5	3.0	100.3
		1.25 µg/mL	+	-	0.6	-0.9	89.0
		2.50 µg/mL	+	-	0.6	0.3	67.0
		5.00 µg/mL	+	-	0.4	-0.1	43.0
		10.00 µg/mL		-	1.0	4.8	37.6
		20.00 µg/mL		-	n.s.	n.s.	26.7
		positive control[2]	+	n.d.	3.0[S]	-16.7	73.3

* Precipitation in culture medium at the end of exposure period (macroscopic)
** Relative number of binucleated cells with micronuclei per 2000 cells scored per test group
S Frequency statistically significant higher than corresponding control values
n.d Not determined;
n.s. not scorable due to strong toxicity
[1] DMSO 1 % (v/v); [2] CPP 0.5 µg/mL

III. CONCLUSION

Based on the results of the study it is concluded that Reg.No. 243959, metabolite of metiram has not the potential to induce micronuclei (clastogenic and/or aneugenic activity) under the in vitro conditions in V79 cells in the absence and presence of metabolic activation.

Short term toxicity

Report:	*CA 5.8.1/48*
	Freudenthal R.I. et al., 1976a
	Subacute toxicity of Ethylenebisisothiocyanate sulfide in the laboratory rat
	1977/1001203
Guidelines:	*none*
GLP:	*no*

Materials and Methods:
Groups of rats (60 male and 60 female Sprague-Dawley rats/group, 30 of each sex were used as controls) were fed EBIS in the diet at dosage levels of 0, 1, 10, 100, or 1000 mg/kg bw for up to 90 days. At 30 day intervals, ten rats of each sex (five of each sex from the control group) were designated for T_3 and T_4 thyroid function tests, for hematology, organ weights, and for gross and microscopic tissue analysis. The remaining 10 rats of each sex and group (5 controls/sex/group) were used for ^{125}I Uptake studies. Positive controls for thyroid function tests were utilized to assure quality of the clinical procedures. These controls were administered just prior to sacrifice and clinical assay and were not necessarily included in the feeding trial. Positive controls included aminotriazole (administered to groups of 6 rats of each sex daily for 4 days by gavage) at a dose of 4000 mg/kg bw and methimazole (administered to groups of 6 rats of each sex twice daily for 4 days by gavage at a dose of 0.6 mg/kg bw).

Results:
EBIS was toxic at 1000 ppm inducing a reversible paralysis of the hind legs followed by death within 8-14 days if once affected the animals were continued on the dosing regimen. Animals removed from the EBIS diet recovered from the paralysis, only to again become ataxic on reexposure to the treated diet. Clinical signs accompanying hind limb paralysis were severely reduced feed consumption, weight loss, shaggy coats, and an unresponsive disposition. No histological lesion could be identified in either H&E stain or Luxol fast blue-stained sections of brain, spinal cord or peripheral nerves taken from paralysed animals. The ability to reverse the paralysis by removing the animals from the test diets coupled with the lack of a histologically observable lesion was considered to suggest a biochemical lesion. Thyroid function was substantially affected at 1000 ppm as indicated by marked decreases in serum T_4, free thyroxine index (FTI), and iodide uptake. However, no observable toxic signs and no significant EBIS-related changes were noted in thyroid function tests or on clinical, gross, and microscopic examination of rats fed 100 ppm or less of EBIS for 30, 60, or 90 days, indicating a no observed effect level (NOAEL) in this study of 100 ppm (equivalent to 4.4 – 9.5 mg/kg bw [in the Monograph, as well as in the abstract of the original paper, erroneously values between 67-31 mg/kg bw were given]), based on mortality, reversible hindlimb paralysis, other toxic signs and thyroid functional changes at 1000 ppm.

Conclusion:
The NOAEL of this study is considered to be 100 ppm (equivalent to 4.4 – 9.5 mg/kg bw)

Report:	*CA 5.8.1/49*
	Chernoff N. et al., 1979a
	Perinatal toxicity of Maneb, Ethylene thiourea and Ethylenebisisothiocyanate sulfide in rodents
	1979/10167
Guidelines:	*none*
GLP:	*no*

Materials and methods:
EBIS was examined alongside maneb and ETU in two rodent species for its potential to induce perinatal toxicity. EBIS was administered by oral gavage in corn oil at doses of 0 (control), 7.5, 15 and 30 mg/kg bw to groups of pregnant rats on days 7-21 of gestation and 0 (control), 50, 100 and 200 mg/kg bw for mice on days 7-16 of gestation daily. Rats were killed on day 21 of gestation and mice on day 18 and uteri were removed, weighed and examined to determine the number of live, dead, and resorbed foetuses. Maternal livers were removed and weighed. Fetuses were weighed as a litter and examined for gross abnormalities. Half of the foetuses were fixed in Bouin´s solution and necropsied. The remaining foetuses were fixed in 65% ethanol, cleared with KOH and stained with Alizarin Red S for skeletal examination. Rats also received EBIS at 0, 15, or 30 mg/kg bw/day from day 7 of gestation and continuing through day 15 postpartum. After birth, litters were culled to four males and four females, weighed weekly and examined for development of eye opening, startle reflex, and air righting. The rats were weaned on day 22 postpartum and the females were discarded. Males continued to be weighed weekly and in postnatal week 6, two males from each litter were tested in a circular open field for 4 minutes on 2 consecutive days on a blind basis for latency to leave the circle, defecations, urinations, rearings, and activity.

Results:
In toxicity studies conducted to determine dose levels for perinatal toxicity studies, EBIS produced death in rats at 75 mg/kg bw/day and hind limb paralysis at 50 mg/kg bw/day. Decreased body weight gain and death was observed in mice given doses above 100 mg/kg bw/day. EBIS given to rats and mice during gestation did not result in adverse fetal effects although average maternal weight gain was decreased in rats at 30 mg/kg bw/day, and the average liver to body weight ratio was increased at the high dose tested for rats (30 mg/kg bw/day) and mice (200 mg/kg bw/day). Postnatal studied with EBIS were uneventful with regard to reproductive parameters. EBIS did not produce significant dose-related alterations in the behavioural development of perinatally exposed rat neonates. Postnatal observations with EBIS indicated decreased fetal body weight at day 1 and day 22 in females only as well as delayed eye opening *at the top dose of 30 mg/kg bw. No effects were seen in this study at 15 mg/kg bw.* The parental NOAEL (mouse) was 100 mg/kg bw/day, based on increased liver-to-body weight ratio at 200 mg/kg bw/day. The fetal NOAEL (mosue) was >200 mg/kg bw/day, highest dose tested. In rats, the parental NOAEL was 15 mg/kg bw/day, based on reduced maternal growth and increased relative liver weight at 30 mg/kg bw/day and hindlimb paralysis at 50 mg/kg bw at 50 mg/kg bw/day. The fetal NOAEL was > 30 mg/kg bw/day, highest dose tested.

Conclusion:
The lowest NOAELs in developmental toxicity studies were 15 mg/kg bw for dams and >30 mg/kg bw (development). The lowest NOAEL from these studies is 15 mg/kg bw.

Overall conclusion and reference value derivation:
The LD50 value of EBIS is 240 mg/kg bw. An Ames test and an in vitro Micronucleus test did not give evidence for a genotoxic activity of EBIS with and without metabolic activation. The NOAEL determined in repeated dose toxicity tests was determined to be 100 ppm (4.4 – 9.5 mg/kg bw) in a 90 day rat study. Developmental toxicity studies conducted in rats and mice did not give evidence for a teratogenic potential of EBIS. The lowest $NOAEL_{maternal}$ was determined to be 15 mg/kg bw.

ARfD: An ARfD of 0.15 mg/kg bw can be derived based on the $NOAEL_{maternal}$ of 15 mg/kg bw and a standard factor of 100.
ADI: An ADI of 0.02 mg/kg bw can be derived based on the NOAEL of appr. 4 mg/kg bw determined in a 90-day study using an extrapolation factor of 2 (extrapolating from subchronic to chronic exposure) and a standard factor of 100.
AOEL: An AOEL of 0.04 mg/kg bw can be derived based on the NOAEL of appr. 4 mg/kg bw determined in the 90-day rat study and a standard factor of 100.

Metabolite: Reg.No. 6002546 = Jaffes Base

QSAR predictions on Jaffes base:

OASIS TIMES (V.2.27.16.8; Mutagenicity S-9 activated v09.09) [see molecule 5 of report DocID 2015/1094106]
There were **no** Ames mutagenicity alerts for Jaffes Base or in silico generated metabolites and no structural alerts were received. The parent substance was out of the model applicability domain.

VEGA: Mutagenicity model (CAESAR, version 2.1.12) [see molecule 8 of report DocID 2015/1094107]
Jaffes Base was out of the model applicability domain. The prediction is **'mutagen'** with a **low** overall reliability. Deficiencies result from:

- only moderately similar compounds with known experimental value in the training set have been found
- similar molecules found in the training set have experimental values that disagree with the predicted value
- accuracy of prediction for similar molecules found in the training set is not optimal

VEGA: Mutagenicity model (SarPy; version 1.0.6-DEV) [see molecule 8 of report DocID 2015/1094107]
Jaffes Base was out of the model applicability domain. The prediction is **'non-mutagen'** with a **low** overall reliability. Deficiencies result from:

- only moderately similar compounds with known experimental value in the training set have been found
- similar molecules found in the training set have experimental values that disagree with the predicted value
- accuracy of prediction for similar molecules found in the training set is not optimal

VEGA: Mutagenicity model (Toxtree; version 1.0.0-DEV) [see molecule 8 of report DocID 2015/1094107]
Jaffes Base was in the model applicability domain. The prediction is **'non-mutagen'** with a **high** overall reliability. Minor deficiencies result from:

- only moderately similar compounds with known experimental value in the training set have been found

Toxicological information on Jaffes Base:

Genotoxicity

Report:	CA 5.8.1/50 Woitkowiak C., 2015c Reg.No. 6002546 (metabolite of BAS 222 F, Metiram) - Salomonella typhimurium / Escherichia coli - Reverse mutation assay 2014/1315335
Guidelines:	OECD 471, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.13/B.14 No. L 142, EPA 870.5100
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

S. typhimurium strains TA98, TA100, TA 1535 and TA 1537 and E. coli strain WP2 uvrA were exposed to Reg.No. 6002546 (Jaffes Base, metabolite of metiram); batch: UPA-771-001-MNZ-102, purity: 96.7%) using water as a solvent in the presence and absence of metabolic activation in two independent experiments. Vehicle and positive controls were included in each experiment. In the preincubation test (PIT) as well as in the standard plate test (SPT) the test substance was tested in concentrations of 33 to 5200 µg/plate. A weak bacteriotoxic effect was observed in the preincubation test only in tester strain TA 1535 after the addition of a metabolizing system at a concentration of 5200 µg/plate. No biologically relevant increase in the number of revertant colonies was noted in any of the strains tested in presence or absence of metabolic activation in any of the experiments. The positive controls induced the appropriate response in the corresponding strains, thus demonstrating the sensitivity of the test system.

No precipitation of the test substance was found up to the highest tested concentration with and without S9 mix.

The test substance Reg.No. 6002546 (Jaffes Base, metabolite of metiram) is not mutagenic in the Salmonella typhimurium / Escherichia coli reverse mutation assay under the experimental conditions of the study.

(BASF DocID 2014/1315335)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

test substance Reg.No. 6002546 (Jaffes Base, metabolite of BAS 222 F, Metiram)

Description:	Solid, beige
Lot/Batch #:	UPA-771-001-MNZ-102
Purity:	96.7%
Stability of test compound:	Stable - expiry date 22-Dec-2016. The homogeneity of the test substance was ensured by mixing prior to preparation of test substance solutions. The stability of the test substance in the vehicle water was verified analytically.
Solvent used:	ultrapure water

2. Control Materials:

Negative control:	In parallel to each experiment, negative control experiments were carried out in order to check for possible contaminants (sterility control) and to determine the spontaneous mutation rate (vehicle control).
Vehicle control:	The vehicle control with and without S-9 mix only contained the vehicle used for the test substance at the same concentration and volume for all tester strains.
Solvent/final concentration:	100 µL/plate

Positive control compounds tested without addition of metabolic activation system:

Strain	Mutagen	Solvent	Concentration
TA 100	N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)	DMSO	5 µg/plate
TA 1535	N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)	DMSO	5 µg/plate
TA 1537	9-Aminoacridine (AAC)	DMSO	100 µg/plate
TA 98	4-nitro-o-phenylendiamine (NOPD)	DMSO	10 µg/plate
WP2 uvrA	4-nitroquinoline-N-oxide (4-NQO)	DMSO	5 µg/plate

Positive control compounds tested with addition of metabolic activation system:

Strain	Mutagen	Solvent	Concentration
TA 100	2-aminoanthracene	DMSO	2.5 µg/plate
TA 1535	2-aminoanthracene	DMSO	2.5 µg/plate
TA 1537	2-aminoanthracene	DMSO	2.5 µg/plate
TA 98	2-aminoanthracene	DMSO	2.5 µg/plate
WP2 uvrA	2-aminoanthracene	DMSO	60 µg/plate

3. Activation:

S9 was produced from the livers of induced male Wistar rats. The rats received on three consecutive days intraperitoneal injections of 80 mg phenobarbital and 80 mg β-naphthoflavone orally per kg body weight. 24 hours after the last administration the animals were sacrificed. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction is thawed at room temperature and 1 volume of S9-fraction is mixed with 9 volumes of S9-supplement (cofactors). This preparation, the so-called S9 mix, was kept on ice until used.

The S9 mix was prepared immediately before use and had the following composition:

Component	Concentration
Phosphate buffer (pH 7.4)	15 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM
S9	10%

To demonstrate the efficacy of the S9 mix in this assay, the S9 batch was further characterized with benoz(a)pyrene in the strains TA 100 and TA 98 as required in OECD 471.

4. Test organisms: S. typhimurium strains: TA98, TA100, TA1535, TA1537
E. coli: WP2 uvrA
Salmonella typhimurium:
The Salmonella strains are checked for the following characteristics at regular intervals: deep rough character (rfa); UV sensitivity (uvrB); ampicillin resistance (R factor plasmid). E. coli WP2 uvrA is checked for UV sensitivity.
Histidine and tryptophan auxotrophy is automatically proven in each experiment via the spontaneous rate.

5. Test concentrations:

Standard plate test (SPT): In this experiment triplicate plates were prepared for each concentration (neg. control; 33, 100, 333, 1000, 2600 and 5200 µg/plate and positive controls at the concentrations indicated above) and conditions (i.e. with and without S9) for all tester strains indicated above.

Preincubation test (PIT): In this experiment the test article / vehicle / positive control substance, bacterial and S9 mix or phosphate buffer were incubated at 37°C for the duration of about 20 minutes. Triplicate plates were prepared for each concentration (neg. control; 33, 100, 333, 1000, 2600 and 5200 µg/plate for all tester strains indicated above).

B. TEST PERFORMANCE:

1. Dates of experimental work: 25-Feb-2015 to 13-Mar-2015

2. Standard plate test:
To test tubes containing 2-mL portions of warm soft agar (containing 0.5 mM histidine + 0.5 mM biotin), 0.1 mL test solution or vehicle, 0.1 mL fresh bacterial culture, 0.5 mL S9-mix (in tests with metabolic activation) or 0.5 mL phosphate buffer (in tests without metabolic activation) were added in the given order. After mixing, the samples are poured onto minimal glucose agar plates. In the experiments with E. coli the warm soft agar contains 0.5 mM tryptophan instead of histidine + biotin.

3. Pre-incubation assay:
100 µL of test solution or vehicle, 0.1 mL bacterial suspension and 0.5 mL S9 mix or phosphate buffer were incubated at 37°C for about 20 minutes. Subsequently, 2 mL of soft agar was added and, after mixing, the samples are poured onto the agar plates.

After incubation in the dark for 48 to 72 hours at 37°C each plate was checked for precipitates and status of the background lawn. Revertant colonies were counted. Means and standard deviations were calculated.

4. Toxicity determination:
Toxicity detected by a decrease in the number of reveratants (factor ≤ 0.6) and/or clearing or diminution of the background lawn (= reduced his^- or trp^- background growth) was recorded for all test groups both with and without S9 mix in all experiments.

5. Statistics:
No special statistical tests were performed.

6. Evaluation criteria:
The test chemical is considered positive in this assay if the following criteria are met:

- A dose-related and reproducible increase in the number of revertant colonies, i.e. about doubling (bacteria strains with high spontaneous mutation rate, like TA 98, TA 100 and E. coli WP2 uvrA) or tripling (bacteria strains with low spontaneous mutation rate, like TA 1535 and TA 1537) of the spontaneous mutation rate in at least one tester strain either without S9 mix or after adding a metabolizing system.

A test substance is generally considered non-mutagenic in this test if:

- The number of revertants for all tester strains was within the historical negative control range under all experimental conditions in at least two experiments carried out independently of each other.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

The test substance was stable over the study period under the storage conditions. The stability of the test substance in the vehicle water was verified analytically (BASF study 01Y0312/14Y079).

B. TOXICITY

A preliminary cytotoxicity assay was not performed in this laboratory. Cytotoxicity was determined in the course of the mutation assay.

No bacteriotoxic effect (reduced his^- or trp^- background growth, decrease in the number of his^+ or trp^+ revertants) was observed in the standard plate test up to the highest required concentration.
In the preincubation assay weak bacteriotoxicity (slight decrease in the number of his^+ revertants) was observed only in tester strain TA 1535 with S9 mix at a concentration of 5200 µg/plate.

C. MUTATION ASSAYS

Neither in the plate incorporation nor in the preincubation experiment with and without metabolic activation was a biologically relevant increase in number of revertants observed in any strain tested [see Table 5.8.1-108]. The positive controls yielded revertant numbers in a range expected for the respective strains and thus demonstrated the sensitivity of the test system. Precipitation was not observed up to the maximum concentration.

III. CONCLUSION

According to the results of the present study, the test substance Reg.No. 6002546 (Jaffes Base), metabolite of metiram) is not mutagenic in the Salmonella typhimurium / Escherichia coli reverse mutation assay under the experimental conditions chosen.

Table 5.8.1-108: Bacterial gene mutation assay with Reg.No. 6002546 - Mean number of revertants

Experiment 1: Plate incorporation assay										
Strain	TA 98		TA 100		TA 1535		TA 1537		E. coli	
Metabolic activation	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Neg. control (water)	29.3±2.1	22.3±3.8	104.7±8.7	91.0±5.6	10.0±5.2	11.0±2.6	9.7±0.6	6.7±2.1	27.0±2.0	22.3±7.0
Reg.No. 6002546										
33 µg/plate	29.7±4.5	20.0±5.3	108.7±10.1	108.7±5.7	10.7±2.3	10.0±2.6	11.7±4.7	10.0±0.0	23.0±8.7	28.7±4.2
100 µg/plate	25.3±7.2	22.3±5.1	109.7±18.1	106.0±9.6	11.3±2.3	12.7±2.3	11.0±1.7	10.0±1.0	28.0±5.3	21.3±5.1
333 µg/plate	27.7±3.5	25.3±11.2	115.3±14.2	112.0±15.7	11.3±0.6	13.3±1.5	10.7±9.0	10.3±2.1	28.7±2.1	26.3±4.0
1000 µg/plate	25.7±2.5	23.3±1.5	110.0±5.6	100.0±10.4	12.0±5.3	16.3±1.5	9.7±2.9	7.7±2.1	33.3±4.0	23.7±2.5
2600 µg/plate	28.7±0.6	22.3±6.4	113.7±8.5	102.7±8.1	9.3±1.5	10.0±2.6	13.0±3.5	10.0±1.7	29.7±2.5	17.7±3.1
5200 µg/plate	27.3±1.5	20.3±6.4	98.0±3.5	91.7±5.1	16.7±2.9	13.3±1.5	13.0±1.7	9.7±4.7	30.0±1.7	31.0±12.5
Pos. control§	1877.0±139.3	470.3±23.2	2748.7±210.4	4539.7±115.0	329.3±41.8	5356.3±63.1	199.0±17.8	1499.3±109.1	86.0±17.3	1598.0±48.0
Experiment 2: Preincubation assay										
Strain	TA 98		TA 100		TA 1535		TA 1537		E. coli	
Metabol. activation	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Neg. control (water)	19.0±3.5	18.3±4.9	102.3±17.6	90.7±3.1	10.3±3.5	90.7±3.1	8.0±3.5	5.3±2.3	25.0±8.7	23.7±5.5
Reg.No. 6002546										
33 µg/plate	20.3±4.9	14.3±5.0	81.0±8.9	91.7±13.3	7.7±0.6	11.0±2.0	6.7±2.5	4.3±1.5	22.0±6.6	21.7±2.1
100 µg/plate	26.0±3.6	18.0±3.0	96.3±12.1	94.3±4.0	10.0±0.0	8.7±2.9	6.0±3.0	6.0±3..6	19.3±6.0	24.0±6.1
333 µg/plate	31.7±0.6	16.7±5.7	98.3±2.3	77.3±11.6	10.0±1.7	9.0±1.0	7.7±2.5	8.0±1.7	20.0±4.6	18.3±5.5
1000 µg/plate	23.7±7.4	16.0±3.6	99.3±9.1	96.3±6.7	8.0±1.7	11.0±6.1	8.3±1.5	4.7±2.1	21.0±5.0	17.3±2.5
2600 µg/plate	24.0±6.0	17.3±5.9	88.7±4.0	83.3±11.6	10.7±3.8	11.0±2.0	8.0±3.5	5.7±2.5	19.3±0.6	23.7±6.7
5200 µg/plate	23.7±6.4	19.3±0.6	91.3±12.6	88.7±2.3	6.7±2.5	12.0±4.4	6.3±2.1	9.0±2.6	24.3±3.2	30.3±4.7
Pos. control§	199.0±73.7	429.7±56.1	2195.0±236.2	1927.3±60.4	169.7±17.4	2959.3±13.3	175.0±16.5	1218±294.1	88.0±20.8	452.0±112.3

§ Compound and concentrations see Material and Methods (I.A.2.) above

Report:	CA 5.8.1/51 Sokolowski A., 2015b Reg.No. 6002546 (metabolite of BAS 222F, Metiram): Micronucleus test in human lymphocytes in vitro 2014/1315332
Guidelines:	OECD 487 (2014), Commission Regulation EU No. 640/2012 of 06 July 2012 - B.49: In vitro Mammalian Cell Micronucleus Test
GLP:	yes (certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Executive Summary

Reg.No. 6002546 (metabolite of metiram; batch UPA-771-001-MNZ-102; purity 96.7%) was tested in vitro for its potential to induce micronuclei in human lymphocytes in the absence and presence of metabolic activation by S9 mix in two independent experiments with concentrations of 574.7 to 1760.0 µg/mL. The exposure periods were either 4 hours or 20 hours (without S9 only) and the total culture period were 88 hours for all experiments.
The test substance did not induced a statistically significant and biologically relevant increase in the number of micronucleated cells after continuous treatment for 40 hours with or without S9 mix.
Cytotoxicity indicated as cytostasis was not observed in both experiments. No test substance precipitation in culture medium was observed up to the highest concentrations in both experiment with and without metabolic activation.
Vehicle and positive controls revealed the expected results of micronucleated cells thereby ensuring the validity of the test.
Based on the results of this study Reg.No. 6002546 is considered to be non-mutagenic in this in vitro micronucleous test, when tested up to cytotoxic or precipitating or the highest evaluable concentration.

(BASF DocID 2014/1315332)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Reg.No. 6002546 (metabolite of BAS 222 F, metiram)
Description:	Solid, beige
Lot/Batch #:	UPA-771-001-MNZ-102
Purity:	96.7%
Stability of test compound:	Stable - Expiry date 22-Dec-2016. Stable in culture medium over 4 h (see BASF Doc ID 2015/1101909)
Solvent used:	culture medium

2. Control Materials:

Negative control:	A negative control was not employed in this study.
Solvent control:	culture medium
Positive control, -S9:	Mitomycin C (MMC, 2 µg/mL, dissolved in deionized water)
	Demecolcin (continuous treatment, 150 ng/mL, dissolved in deionized water)
Positive control, +S9:	Cyclophosphamide (CPA, 12.5 and 17.5 µg/mL, dissolved in saline)

3. Activation:

Phenobarbital/β-naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared and stored according to the currently valid version of the Harlan CCR SOP for rat liver S9 preparation. Each batch of S9 was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test.

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained:

Component	Concentration
Sodium-ortho-phosphate buffer (pH 7.4)	100 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM

4. Test organisms:

Human lymphocytes
Blood samples were drawn from healthy non-smoking donors not receiving medication. For this study, blood was collected from a female donor (32 years old) for Experiment I and from a female donor (36 years old) for Experiment II. The lymphocytes of the respective donors have been shown to respond well to stimulation of proliferation with PHA and to positive control substances. All donors had a previously established low incidence of micronuclei in their peripheral blood lymphocytes.

5. Culture medium/conditions:

Culture media:	Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1.1) containing a L-glutamine source supplemented with 10% (v/v) fetal bovine serum (FCS), 1% (v/v) penicillin/streptomycin (100 IU / 100 µg/mL), 10 mM HEPES, the mitogen PHA (3 µg/mL) and the anticoagulant heparin (125 U.S.P.-U/mL).
Cell culture:	Blood cultures were established by preparing an 11% mixture of whole blood in medium within 30 hrs after blood collection. All incubations were done at 37°C with 5.5% CO_2 in humidified air.
Cell cycle and harvest time:	Human lymphocytes were stimulated for proliferation by the addition of the mitogen PHA to the culture medium for a period of 48 hours. The cell harvest time point was approximately 2 – 2.5 x AGT (average generation time). Any specific cell cycle time delay induced by the test item was not accounted for directly.

6. Test concentrations:

a) Preliminary toxicity assay:	11.4 to 1760.0 µg/mL with and without metabolic activation Exposure period: 4 hours with and without metabolic activation; 20 hours without metabolic activation Harvest time: 40 hours
b) Cytogenicity assay:	
1st experiment (I):	574.7, 1005.7, 1760.0 µg/mL with and without S9 mix (4 hours exposure, 40 hour preparation interval)
2nd experiment (II):	574.7, 1005.7 and 1760.0 µg/mL without metabolic activation (20 hour exposure, 40- hour preparation interval); 574.7, 1005.7 and 1760.0 µg/mL with metabolic activation (4 hour exposure, 40 hours preparation interval)

B. TEST PERFORMANCE:

1. Dates of experimental work: 18-Feb-2015 to 15-Apr-2015

2. Preliminary cytotoxicity assay:

In the pretest for toxicity based on the purity and the molecular weight of the test substance 1760.0 µg/mL Reg.No. 6002546 (metabolite of metiram) was used as top concentration. The pretest was performed following the method described for the main experiment. Cytotoxicity is characterized by the percentages of reduction in the CBPI in comparison with the controls (% cytostasis) by counting 500 cells per culture in duplicate. pH and solubility were additionally determined.

3. Cytogenicity Assay:
Two independent experiments were performed. In experiment I, the exposure period was 4 hours with and without S9 mix. In experiment II, the exposure periods were 4 hours with S9 mix and 20 hours without S9 mix. For the experiments with the 4 hour treatment interval the cells were cultured for additionally 16 hours without test substance (recovery period). Therefore, all cells, regardless which experiment were prepared 40 hours after start of treatment with the test item.

Cell seeding / treatment:	Pulse exposure: About 48 hrs after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium was added. After 4 hrs the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose $\cdot H_2O$, 192 mg/L $Na_2HPO_4 \cdot 2\ H_2O$ and 150 mg/L KH_2PO_4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation. The cultures were incubated for the respective exposure period at 37°C, 5.5% CO_2 in humidified air. Continuous exposure (without S9 mix): About 48 hrs after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once as described. After washing the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation. The cultures were incubated for the respective exposure period at 37°C, 5.5% CO_2 in humidified air.

Cell harvest, preparation of slides and staining:

The cultures were harvested by centrifugation 40 hrs after beginning of treatment. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in approximately 5 mL "saline G" and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

Analysis of micronuclei and cytotoxicity:

Evaluation of the slides was performed using NIKON microscopes with 40 x objectives. The micronuclei were counted in cells showing a clearly visible cytoplasm area. The criteria for the evaluation of micronuclei are described in the publication of Countryman and Heddle (1976). The micronuclei have to be stained in the same way as the main nucleus. The area of the micronucleus should not extend the third part of the area of the main nucleus. 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the CBPI was determined in 500 cells per culture and cytotoxicity is expressed as % cytostasis. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

Proliferation Index (CBPI):

$$CBPI = \frac{((\text{No. mononucleated cells}) + (2 \text{ x No. binucleated cells}) + (3 \text{ x No. mulitnucleated cells}))}{(\text{Total number of cells})}$$

$$\% \text{ Cytostasis} = 100 - 100 \{(CBPI_T - 1) / (CBPI_C - 1)\}$$

(T- test substance treated culture; C = vehicle control)

4. Statistics:

The statistical evaluation of the data was carried out using the Chi square test.

5. Evaluation criteria:

A test item is considered “positive” in this assay if the following criteria are met:

- the number of micronucleated cells is not in the range of the historical laboratory control data and
- either a concentration-related increase in three test groups or a statistically significant increase in the number of micronucleated cells is observed.

A test substance is generally considered “negative” in this test system if:

- The number of micronucleated cells in the test groups is not distinctly increased above the concurrent vehicle control and is within our laboratory’s historical control data.

If the above mentioned criteria for the test item are not clearly met, the test item will be classified as equivocal or a confirmatory experiment may be performed. However, results may remain questionable regardless of the number of times the experiment is repeated.
An increase in the number of micronucleated mononucleate cells may indicate that the test item has aneugenic potential.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS:

The stability of the test substance at room temperature in the vehicle DMSO over a period of 4 hours was verified analytically (see BASF study code 01Y0312/14Y079).

B. PRELIMINARY CYTOTOXICITY ASSAY:

A preliminary cytotoxicity assay was performed to determine appropriate test substance concentrations for the main test. In the pre-test for toxicity, no precipitation of the test item was observed at the end of treatment in the absence and presence of S9 mix. Since the cultures fulfilled the requirements for cytogenetic evaluation, this preliminary test was designated Experiment I.
Using a Cytokinesis-block proliferation index (CBPI) as an indicator for toxicity, no cytotoxic effects were observed in Experiment I after 4 hours treatment in the absence and presence of S9 mix. Therefore, 1760.0 µg/mL were chosen as top treatment concentration for Experiment II.

C. CYTOGENICITY ASSAYS:

In all experimental parts after 4 and 20 hours test substance treatment in the absence and presence of S9 mix the values (0.25 – 0.60% micronucleated cells) were close to the concurrent vehicle control values (0.30 - 1.00% micronucleated cells) and within historical negative control data range (0.05 - 1.65% micronucleated cells) [see Table 5.8.1-109 and Table 5.8.1-110].

Either Demecolcin (150.0 ng/mL), MMC (2.0 µg/mL) or CPA (12.5 or 17.5 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei.

In both experiments, in the absence and presence of S9 mix, no precipitation of the test item in the culture medium was observed at the end of treatment.

No relevant influence on osmolarity was observed. The pH was adjusted to physiological values using small amounts of 2 M HCL to achieve a solution with the test item.

No relevant cytotoxicity, indicated by reduced CBPI and described as cytostasis could be observed up to the highest applied concentration.

Table 5.8.1-109: Summary of results of micronucleus test with Reg.No. 6002546 (metabolite of metiram) - without metabolic activation

					Genotoxicity	Cytotoxicity	
Exp.	Exposure/ Preparation interval	Test groups	S9 mix	Prec.[a]	Micronucleated cells[b] [%]	Proliferation index (CBPI)	Cytostasis [%]
I	4/40 hrs	Vehicle control[1]	-	n.d.	0.30	1.74	-
		574.7 µg/mL	-	-	0.35	1.68	7.9
		1005.7 µg/mL	-	-	0.55	1.78	n.c.
		1760.0 µg/mL	-	-	0.40	1.78	n.c.
		Positive control[2]	-	n.d.	**8.20***	1.14	80.7
II	20/40 hrs	Vehicle vcntrol[1]	-	n.d.	0.55	1.78	-
		574.7 µg/mL	-	-	0.50	1.76	2.7
		1005.7 µg/mL	-	-	0.25	1.64	18.4
		1760.0 µg/mL	-	-	0.45	1.47	39.6
		Positive control[3]		n.d.	**3.60***	1.45	42.8

a Precipitation in culture medium at the end of exposure period (macroscopic)
b Relative number of binucleated cells with micronuclei per 2000 cells scored per test group
* Frequency statistically significant higher than corresponding control values
n.d. Not determined;
n.c. not calculated as the CBPI was equal or higher than solvent control value
[1] culture medium; [2] MMC 2.0 µg/mL; [3] Demecolcin 150 ng/mL

Table 5.8.1-110: Summary of results of micronucleus test with Reg.No. 6002546 (metabolite of metiram) - with metabolic activation

					Genotoxicity	Cytotoxicity	
Exp.	Exposure/ Preparation interval	Test groups	S9 mix	Prec.[a]	Micronucleated cells[b] [%]	Proliferation index (CBPI)	Cytostasis [%]
I	4/40 hrs	Vehicle control[1]	+	n.d.	1.00	1.84	-
		574.7 µg/mL	+	-	0.50	1.94	n.c.
		1005.7 µg/mL	+	-	0.50	1.84	0.6
		1760.0 µg/mL	+	-	0.55	1.84	0.0
		Positive control[2]	+	n.d.	**3.10***	1.63	25.7
II	4/40 hrs	Vehicle control[1]	+	n.d.	0.50	1.86	-
		574.7 µg/mL	+	-	0.60	1.82	5.0
		1005.7 µg/mL	+	-	0.25	1.80	6.6
		1760.0 µg/mL	+	-	0.30	1.78	9.9
		Positive control[3]	+	n.d.	**2.25***	1.69	19.7

a Precipitation in culture medium at the end of exposure period (macroscopic)
b Relative number of binucleated cells with micronuclei per 2000 cells scored per test group
* Frequency statistically significant higher than corresponding control values
n.d Not determined;
n.s. not scorable due to strong toxicity
[1] culture medium; [2] CPA 17.5 µg/mL; [3] CPA 12.5 µg/mL

III. CONCLUSION

Based on the results of the study it is concluded that Reg.No. 6002546, metabolite of metiram has not the potential to induce micronuclei (clastogenic and/or aneugenic activity) under the in vitro conditions in human lymphocytes in the absence or presence of metabolic activation.

Overall conclusion:
The metabolite Reg.No. 6002546 is considered to be not genotoxic based on the available data from QSAR evaluations and in vitro studies. The structure of Reg.No. 6002546 is falling under Cramer Class III class. Thus, human exposure of 1.5 µg/kg bw/day is considered a safe value.

Metabolite: Reg.No. 4670450 = TDIT

QSAR predictions on TDIT:

OASIS TIMES (V.2.27.16.8; Mutagenicity S-9 activated v09.09) [see molecule 7 of report DocID 2015/1094106]
There were **no** Ames mutagenicity alerts for TDIT or in silico generated metabolites and no structural alerts were received. The parent substance was out of the model applicability domain.

VEGA: Mutagenicity model (CAESAR, version 2.1.12) [see molecule 13 of report DocID 2015/1094107]
TDIT was out of the model applicability domain. The prediction is **'mutagen'** with a **low** overall reliability. Deficiencies result from:

- no similar compounds with known experimental value in the training set have been found
- some similar molecules found in the training set have experimental values that disagree with the predicted value
- accuracy of prediction for similar molecules found in the training set is not optimal
- some atom centered fragments of the compound have not been found in the compounds of the training set or are rare fragments

VEGA: Mutagenicity model (SarPy; version 1.0.6-DEV) [see molecule 13 of report DocID 2015/1094107]
TDIT was out of the model applicability domain. The prediction is **'non-mutagen'** with a **low** overall reliability. Deficiencies result from:

- only moderately similar compounds with known experimental value in the training set have been found
- some similar molecules found in the training set have experimental values that disagree with the predicted value
- accuracy of prediction for similar molecules found in the training set is not optimal
- some atom centered fragments of the compound have not been found in the compounds of the training set or are rare fragments

VEGA: Mutagenicity model (Toxtree; version 1.0.0-DEV) [see molecule 13 of report DocID 2015/1094107]
TDIT was out of the model applicability domain. The prediction is **'non-mutagen'** with a **low** overall reliability. Deficiencies result from:

- no similar compounds with known experimental value in the training set have been found
- similar molecules found in the training set have experimental values that disagree with the predicted value
- accuracy of prediction for similar molecules found in the training set is not optimal
- a prominent number of atom centered fragments of the compound have not been found in the compounds of the training set or are rare fragments

Toxicological information on TDIT:

Genotoxicity

Report:	CA 5.8.1/52 Woitkowiak C., 2015e Reg.No. 4670450 (metabolite of BAS 222 F, Metiram) - Salmonella typhimurium / Escherichia coli - Reverse mutation assay 2015/1042058
Guidelines:	OECD 471, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.13/B.14 No. L 142, EPA 870.5100
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

S. typhimurium strains TA98, TA100, TA 1535 and TA 1537 and E. coli strain WP2 uvrA were exposed to Reg.No. 4670450 (TDIT, metabolite of metiram); batch: L83-138, purity: 91.4%) using DMSO as a solvent in the presence and absence of metabolic activation in three independent experiments. Vehicle and positive controls were included in each experiment.
In the plate incorporation assay the test substance was tested in concentrations of 33 to 5500 µg/plate. Due to a strong toxicity found in the first experiment a second was done with concentrations between 3.3 and 1000 µg/plate. In the preincubation assay the test substance was tested in concentrations of 1.0 to 333 µg/plate. A bacteriotoxic effect was observed in both tests depending on the strain and test conditions from about 100 µg/plate onward. No biologically relevant increase in the number of revertant colonies was noted in any of the strains tested in presence or absence of metabolic activation in any of the experiments. The positive controls induced the appropriate response in the corresponding strains, thus demonstrating the sensitivity of the test system.

No precipitation of the test substance was found up to the highest tested concentration with and without S9 mix.

The test substance Reg.No. 4670450 (TDIT, metabolite of metiram) is not mutagenic in the Salmonella typhimurium / Escherichia coli reverse mutation assay under the experimental conditions of the study.

(BASF DocID 2015/1042058)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

test substance Reg.No. 4670450 (TDIT, metabolite of BAS 222 F, Metiram)

Description: Solid, beige

Lot/Batch #: L83-138

Purity: 91.4% (tolerance ± 1.0%)

Stability of test compound: Stable - expiry date 01-Nov-2016. The homogeneity of the test substance was ensured by mixing prior to preparation of test substance solutions. The stability of the test substance in the vehicle DMSO was verified analytically.

Solvent used: DMSO

2. Control Materials:

Negative control: In parallel to each experiment, negative control experiments were carried out in order to check for possible contaminants (sterility control) and to determine the spontaneous mutation rate (vehicle control).

Vehicle control: The vehicle control with and without S-9 mix only contained the vehicle used for the test substance at the same concentration and volume for all tester strains.

Solvent/final concentration: 100 µL/plate

Positive control compounds tested without addition of metabolic activation system:

Strain	Mutagen	Solvent	Concentration
TA 100	N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)	DMSO	5 µg/plate
TA 1535	N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)	DMSO	5 µg/plate
TA 1537	9-Aminoacridine (AAC)	DMSO	100 µg/plate
TA 98	4-nitro-o-phenylendiamine (NOPD)	DMSO	10 µg/plate
WP2 uvrA	4-nitroquinoline-N-oxide (4-NQO)	DMSO	5 µg/plate

Positive control compounds tested with addition of metabolic activation system:

Strain	Mutagen	Solvent	Concentration
TA 100	2-aminoanthracene	DMSO	2.5 µg/plate
TA 1535	2-aminoanthracene	DMSO	2.5 µg/plate
TA 1537	2-aminoanthracene	DMSO	2.5 µg/plate
TA 98	2-aminoanthracene	DMSO	2.5 µg/plate
WP2 uvrA	2-aminoanthracene	DMSO	60 µg/plate

3. Activation:

S9 was produced from the livers of induced male Wistar rats. The rats received on three consecutive days intraperitoneal injections of 80 mg phenobarbital and 80 mg β-naphthoflavone orally per kg body weight. 24 hours after the last administration the animals were sacrificed. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction is thawed at room temperature and 1 volume of S9-fraction is mixed with 9 volumes of S9-supplement (cofactors). This preparation, the so-called S9 mix, was kept on ice until used.

The S9 mix was prepared immediately before use and had the following composition:

Component	Concentration
Phosphate buffer (pH 7.4)	15 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM
S9	10%

To demonstrate the efficacy of the S9 mix in this assay, the S9 batch was further characterized with benoz(a)pyrene in the strains TA 100 and TA 98 as required in OECD 471.

4. Test organisms: S. typhimurium strains: TA98, TA100, TA1535, TA1537
E. coli: WP2 uvrA
Salmonella typhimurium:
The Salmonella strains are checked for the following characteristics at regular intervals: deep rough character (rfa); UV sensitivity (uvrB); ampicillin resistance (R factor plasmid). E. coli WP2 uvrA is checked for UV sensitivity.
Histidine and tryptophan auxotrophy is automatically proven in each experiment via the spontaneous rate.

5. Test concentrations:

Standard plate test (SPT): In the first experiment triplicate plates were prepared for each concentration (neg. control; 33, 100, 333, 1000, 2600 and 5200 µg/plate and positive controls at the concentrations indicated above) and conditions (i.e. with and without S9) for all tester strains indicated above.

Due to strong bacteriotoxicity observed in the 1st experiment the standard plate test will be repeated. In the second experiment triplicate plates were prepared for each concentration (neg. control, 3.3, 10, 33, 100, 333 and 1000 µg/plate and positive controls at the concentrations indicated above) and conditions (i.e. with and without S9) for all tester strains indicated above.

Preincubation test (PIT): In this experiment the test article / vehicle / positive control substance, bacterial and S9 mix or phosphate buffer were incubated at 37°C for the duration of about 20 minutes. Triplicate plates were prepared for each concentration (neg. control; 1.0, 3.3, 10, 33, 100 and 333 µg/plate for all tester strains indicated above).

B. TEST PERFORMANCE:

1. Dates of experimental work: 04-Feb-2015 to 19-Feb-2015

2. Standard plate test:

To test tubes containing 2-mL portions of warm soft agar (containing 0.5 mM histidine + 0.5 mM biotin), 0.1 mL test solution or vehicle, 0.1 mL fresh bacterial culture, 0.5 mL S9-mix (in tests with metabolic activation) or 0.5 mL phosphate buffer (in tests without metabolic activation) were added in the given order. After mixing, the samples are poured onto minimal glucose agar plates. In the experiments with E. coli the warm soft agar contains 0.5 mM tryptophan instead of histidine + biotin.

3. Pre-incubation assay:
100 µL of test solution or vehicle, 0.1 mL bacterial suspension and 0.5 mL S9 mix or phosphate buffer were incubated at 37°C for about 20 minutes. Subsequently, 2 mL of soft agar was added and, after mixing, the samples are poured onto the agar plates.

After incubation in the dark for 48 to 72 hours at 37°C each plate was checked for precipitates and status of the background lawn. Revertant colonies were counted. Means and standard deviations were calculated.

4. Toxicity determination:
Toxicity detected by a decrease in the number of reveratants (factor ≤ 0.6) and/or clearing or diminution of the background lawn (= reduced his^- or trp^- background growth) was recorded for all test groups both with and without S9 mix in all experiments.

5. Statistics:
No special statistical tests were performed.

6. Evaluation criteria:
The test chemical is considered positive in this assay if the following criteria are met:

- A dose-related and reproducible increase in the number of revertant colonies, i.e. about doubling (bacteria strains with high spontaneous mutation rate, like TA 98, TA 100 and E. coli WP2 uvrA) or tripling (bacteria strains with low spontaneous mutation rate, like TA 1535 and TA 1537) of the spontaneous mutation rate in at least one tester strain either without S9 mix or after adding a metabolizing system.

A test substance is generally considered non-mutagenic in this test if:

- The number of revertants for all tester strains was within the historical negative control range under all experimental conditions in at least two experiments carried out independently of each other.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

The test substance was stable over the study period under the storage conditions. The stability of the test substance in the vehicle water was verified analytically (BASF study 01Y0544/14Y068).

B. TOXICITY

A preliminary cytotoxicity assay was not performed in this laboratory. Cytotoxicity was determined in the course of the mutation assay.

A bacteriotoxic effect (reduced his- or trp- background growth, decrease in the number of his^+ or trp^+ revertants) was observed in the standard plate test depending on the strain and test conditions from about 100 µg/plate onward.
In the preincubation assay bacteriotoxicity (decrease in the number of his^+ or trp^+ revertants) was observed depending on the strain and test conditions from about 100 µg/plate onward.

C. MUTATION ASSAYS

Neither in the plate incorporation nor in the preincubation experiment with and without metabolic activation was a biologically relevant increase in number of revertants observed in any strain tested [see
Table **5.8.1-111**]. The positive controls yielded revertant numbers in a range expected for the respective strains and thus demonstrated the sensitivity of the test system. Precipitation was not observed up to the maximum concentration.

III. CONCLUSION

According to the results of the present study, the test substance Reg.No. 4670450 (TDIT), metabolite of metiram) is not mutagenic in the Salmonella typhimurium / Escherichia coli reverse mutation assay under the experimental conditions chosen.

Table 5.8.1-111: Bacterial gene mutation assay with Reg.No. 4670450 - Mean number of revertants

Experiment 1: Plate incorporation assay										
Strain	TA 98		TA 100		TA 1535		TA 1537		E. coli	
Metabolic activation	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Neg. control (DMSO)	37.7±2.9	27.3±7.0	91.3±7.1	96.7±9.3	13.3±3.5	13.0±4.4	9.0±4.4	7.0±2.6	27.7±6.1	25.0±5.6
Reg.No. 4670450										
33 µg/plate	36.3±4.9	26.0±1.0	87.7±11.8	78.0±18.7	11.7±2.3	9.0±1.7	8.3±1.2	6.3±1.2	22.0±1.0	26.3±13.7
100 µg/plate	26.3±8.5	21.0±5.3	97.3±4.0	82.3±9.1	7.3±0.6	13.0±5.6	6.3±4.0	8.0±3.5	36.3±7.6	23.7±6.7
333 µg/plate	32.3±1.2	17.3±4.2	72.7±16.2	66.7±9.9	7.3±1.5	12.0±3.6	5.0±3.6	3.7±1.5	18.3±2.3	13.0±2.0
1000 µg/plate	7.7±4.0	2.7±0.6	13.0±7.5	7.3±2.5	2.7±0.6	2.0±1.0	0.0±0.0	0.0±0.0	16.7±7.4	18.0±6.2
2750 µg/plate	0.0±0.0[B]	0.0±0.0[B]	0.0±0.0[B]	0.0±0.0[B]	0.0±0.0[B]	0.0±0.0[B]	0.0±0.0[B]	0.0±0.0[B]	4.0±2.6[B]	0.0±0.0[B]
5500 µg/plate	0.0±0.0[B]	0.0±0.0[B]	0.0±0.0[B]	0.0±0.0[B]	0.0±0.0[B]	0.0±0.0[B]	0.0±0.0[B]	0.0±0.0[B]	0.0±0.0[B]	0.0±0.0[B]
Pos. control[§]	1786.0±211.7	432.7±6.5	2324.3±329.9	4637.7±296.2	261.0±13.9	5217.0±121.4	226.7±3.1	1101.3±152.8	99.3±7.0	1241.0±62.6
Experiment 2: Plate incorporation assay										
Strain	TA98		TA 100		TA 1535		TA 1537		E. coli	
Metabolic activation	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Neg. control (DMSO)	28.7±0.6	17.7±1.2	101.7±8.1	99.7±0.6	12.3±2.5	11.7±2.9	8.0±0.0	6.7±1.2	23.3±2.3	23.3±4.0
Reg.No. 4670450										
3.3 µg/plate	32.0±6.1	17.0±8.7	101.3±3.8	79.3±4.5	13.0±1.7	16.3±2.5	9.7±2.3	8.3±2.1	27.0±5.6	29.3±2.3
10 µg/plate	28.3±9.6	20.7±2.3	99.7±6.4	93.0±15.7	12.0±3.5	12.7±2.1	8.3±3.1	8.0±3.6	27.0±6.1	23.3±2.3
33 µg/plate	36.3±1.5	19.7±3.8	82.3±7.8	85.7±7.4	11.0±2.6	11.7±4.0	7.0±2.6	9.7±0.6	23.3±3.8	24.7±7.4
100 µg/plate	24.3±6.1	27.0±5.6	80.0±1.7	78.0±2.0	10.3±1.2	10.0±3.0	6.3±3.2	7.7±1.2	24.3±2.5	18.3±2.1
333 µg/plate	12.7±4.6	8.0±0.0	60.7±3.5	44.7±4.2	7.7±0.6	6.3±4.2	3.3±0.6	2.7±0.6	15.0±4.4	15.3±6.4
1000 µg/plate	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	8.0±2.0	2.3±2.1
Pos. control[§]	2038.3±76.9	447.3±17.2	2399.7±314.0	4418.3±112.0	244.3±11.9	5066.0±184.1	211.0±6.1	1772.0±150.4	111.0±7.8	1424.7±50.0
Experiment 3: Preincubation assay										
Strain	TA 98		TA 100		TA 1535		TA 1537		E. coli	
Metabol. activation	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Neg. control (DMSO)	23.3±5.7	17.3±0.6	86.0±9.6	100.3±17.6	8.7±2.1	10.7±1.5	5.3±0.6	7.0±2.6	27.0±6.2	27.7±8.5
Reg.No. 4670450										
1.0 µg/plate	24.0±2.6	16.7±2.3	71.7±3.5	80.7±14.0	12.3±1.5	8.3±2.5	6.7±0.6	6.0±2.0	24.3±9.3	22.0±5.3
3.3 µg/plate	24.0±3.5	14.3±2.1	87.7±9.9	84.7±8.5	10.0±1.0	13.0±1.0	7.0±3.6	4.7±1.5	23.0±4.4	23.7±6.4
10 µg/plate	29.7±1.5	17.0±2.6	95.3±10.0	82.7±6.0	11.0±1.0	8.7±4.7	6.0±2.0	6.0±3.5	23.3±4.9	32.0±7.2
33 µg/plate	25.0±2.6	14.3±6.8	93.3±8.4	71.3±4.5	9.3±2.3	11.3±2.3	7.7±0.6	6.0±2.6	22.7±5.5	23.7±8.6
100 µg/plate	15.3±2.5	11.0±3.5	75.7±6.4	59.7±6.8	12.0±4.0	6.7±3.5	5.0±2.6	5.7±1.2	17.7±6.1	21.7±2.1
333 µg/plate	8.7±1.2	0.3±0.6	50.0±1.7	16.3±6.0	4.7±2.5	1.7±0.6	1.3±1.5	0.3±0.6	14.0±3.5	12.0±3.6
Pos. control[§]	1457.7±38.7	388.0±10.5	1843.0±109.3	2454.3±135.0	242.7±5.0	3047.3±130.4	139.7±21.8	1086.7±335.0	94.3±34.0	435.7±68.1

§ Compound and concentrations see Material and Methods (I.A.2.) above
B reduced background growth.
Due to strong toxicity in the plate incorporation assay the repetition of the experiment with lower concentrations was performed as second experiment. Accordingly, the preincubation assay was the 3rd experiment.

Report:	CA 5.8.1/53 Sokolowski A., 2015a Reg.No. 4670450 (metabolite of BAS 222 F, Metiram): Micronucleus test in human lymphocytes in vitro 2014/1315331
Guidelines:	OECD 487 (2014), Commission Regulation EU No. 640/2012 of 06 July 2012 - B.49: In vitro Mammalian Cell Micronucleus Test
GLP:	yes (certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Executive Summary

Reg.No. 4670450 (metabolite of metiram; batch L83-138; purity 90.3%) was tested in vitro for its potential to induce micronuclei in human lymphocytes in the absence and presence of metabolic activation by S9 mix in three independent experiments with concentrations of 44.1 to 261.2 µg/mL. The exposure periods were either 4 hours or 20 hours (without S9 only) and the total culture period were 88 hours for all experiments.
The test substance did not induced a statistically significant and biologically relevant increase in the number of micronucleated cells after continuous treatment for 40 hours with or without S9 mix.
Cytotoxicity indicated as cytostasis was observed only in experiment IIB in the absence of S9 mix at the highest evaluated concentration. Test substance precipitation in culture medium was observed in the highest concentrations in experiment I with and without S9 mix and in experiment IIA with metabolic activation.
Vehicle and positive controls revealed the expected results of micronucleated cells thereby ensuring the validity of the test.
Based on the results of this study Reg.No. 4670450 is considered not to be non-mutagenic in this in vitro micronucleous test, when tested up to cytotoxic or precipitating or the highest evaluable concentration.

(BASF DocID 2014/1315331)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Reg.No. 4670450 (metabolite of BAS 222 F, metiram)
Description:	Solid, beige
Lot/Batch #:	L83-138
Purity:	90.3% (tolerance ± 1%)
Stability of test compound:	Stable - Expiry date 01-Nov-2016. The stability of the test substance at room temperature in the vehicle DMSO over a period of 4 hours was verified analytically.
Solvent used:	Dimethylsulfoxide (DMSO)

2. Control Materials:

Negative control:	A negative control was not employed in this study.
Solvent control:	DMSO
Positive control, -S9:	Mitomycin C (MMC, 2 µg/mL, dissolved in deionized water) Demecolcin (continuous treatment, 150 and 50 ng/mL, dissolved in deionized water)
Positive control, +S9:	Cyclophosphamide (CPA, 17.5 µg/mL, dissolved in saline)

3. Activation:

Phenobarbital/β-naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared and stored according to the currently valid version of the Harlan CCR SOP for rat liver S9 preparation. Each batch of S9 was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test.

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained:

Component	Concentration
Sodium-ortho-phosphate buffer (pH 7.4)	100 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM

4. Test organisms:

Human lymphocytes
Blood samples were drawn from one healthy non-smoking male donor (24 years old) not receiving medication. The lymphocytes of this donor have been shown to respond well to stimulation of proliferation with PHA and to positive control substances. The donor had a previously established low incidence of micronuclei in his peripheral blood lymphocytes.

5. Culture medium/conditions:

Culture media: Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1.1) containing a L-glutamine source supplemented with 10% (v/v) fetal bovine serum (FCS), 1% (v/v) penicillin/streptomycin (100 IU / 100 µg/mL), 10 mM HEPES, the mitogen PHA (3 µg/mL) and the anticoagulant heparin (125 U.S.P.-U/mL).

Cell culture:	Blood cultures were established by preparing an 11 % mixture of whole blood in medium within 30 hrs after blood collection. All incubations were done at 37°C with 5.5% CO_2 in humidified air.
Cell cycle and harvest time:	Human lymphocytes were stimulated for proliferation by the addition of the mitogen PHA to the culture medium for a period of 48 hours. The cell harvest time point was approximately 2 – 2.5 x AGT (average generation time). Any specific cell cycle time delay induced by the test item was not accounted for directly.

6. Test concentrations:

a) Preliminary toxicity assay:	14.4 to 2215.0 µg/mL with and without metabolic activation Exposure period: 4 hours with and without metabolic activation; 20 hours without metabolic activation Harvest time: 40 hours
b) Cytogenicity assay:	
1st experiment (I):	44.1, 77.1, 135.0 µg/mL with and without S9 mix (4 hours exposure, 40 hour preparation interval)
2nd experiment (IIA):	48.7, 85.3 and 149.3 µg/mL without metabolic activation (20 hour exposure, 40- hour preparation interval); 85.3, 149.3 and 261.2 µg/mL with metabolic activation (4 hour exposure, 40 hours preparation interval)
3rd experiment (IIB):	50.0, 100.0 and 150.0 µg/mL without metabolic activation (20 hour exposure, 40- hour preparation interval)

B. TEST PERFORMANCE:

1. Dates of experimental work: 03-Dec-2014 to 24-Mar-2015

2. Preliminary cytotoxicity assay:

In the pretest for toxicity based on the purity and the molecular weight of the test substance 2215 µg/mL Reg.No. 4670450 (metabolite of metiram) was used as top concentration. The pretest was performed following the method described for the main experiment. Cytotoxicity is characterized by the percentages of reduction in the CBPI in comparison with the controls (% cytostasis) by counting 500 cells per culture in duplicate. pH and solubility were additionally determined.

3. Cytogenicity Assay:

Three independent experiments were performed. In experiment I, the exposure period was 4 hours with and without S9 mix. In experiment IIA, the exposure periods were 4 hours with S9 mix and 20 hours without S9 mix. The experimental part without S9 mix was repeated and corresponds to experiment IIB. For the experiments with the 4 hour treatment interval the cells were cultured for additionally 16 hours without test substance (recovery period). Therefore, all cells, regardless which experiment were prepared 40 hours after start of treatment with the test item.

Cell seeding / treatment:

Pulse exposure: About 48 hrs after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm^2 cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium was added. After 4 hrs the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose $\cdot H_2O$, 192 mg/L $Na_2HPO_4 \cdot 2\ H_2O$ and 150 mg/L KH_2PO_4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation. The cultures were incubated for the respective exposure period at 37°C, 5.5% CO_2 in humidified air.

Continuous exposure (without S9 mix): About 48 hrs after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm^2 cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once as described. After washing the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation. The cultures were incubated for the respective exposure period at 37°C, 5.5% CO_2 in humidified air.

Cell harvest, preparation of slides and staining:

The cultures were harvested by centrifugation 40 hrs after beginning of treatment. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in approximately 5 mL "saline G" and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

Analysis of micronuclei and cytotoxicity:

Evaluation of the slides was performed using NIKON microscopes with 40 x objectives. The micronuclei were counted in cells showing a clearly visible cytoplasm area. The criteria for the evaluation of micronuclei are described in the publication of Countryman and Heddle (1976). The micronuclei have to be stained in the same way as the main nucleus. The area of the micronucleus should not extend the third part of the area of the main nucleus. 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the CBPI was determined in 500 cells per culture and cytotoxicity is expressed as % cytostasis. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

Proliferation Index (CBPI):

$$CBPI = \frac{((\text{No. mononucleated cells}) + (2 \text{ x No. binucleated cells}) + (3 \text{ x No. mulitnucleated cells}))}{(\text{Total number of cells})}$$

% Cytostasis = 100 - 100 {($CBPI_T$ - 1) / ($CBPI_C$ - 1)}
(T- test substance treated culture; C = vehicle control)

4. Statistics:

The statistical evaluation of the data was carried out using the Chi square test.

5. Evaluation criteria:

A test item is considered “positive” in this assay if the following criteria are met:

- the number of micronucleated cells is not in the range of the historical laboratory control data and
- either a concentration-related increase in three test groups or a statistically significant increase in the number of micronucleated cells is observed.

A test substance is generally considered “negative” in this test system if:

- The number of micronucleated cells in the test groups is not distinctly increased above the concurrent vehicle control and is within our laboratory’s historical control data.

If the above mentioned criteria for the test item are not clearly met, the test item will be classified as equivocal or a confirmatory experiment may be performed. However, results may remain questionable regardless of the number of times the experiment is repeated.
An increase in the number of micronucleated mononucleate cells may indicate that the test item has aneugenic potential.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS:

The stability of the test substance at room temperature in the vehicle DMSO over a period of 4 hours was verified analytically (see BASF study code 01Y0544/14Y068).

B. PRELIMINARY CYTOTOXICITY ASSAY:

A preliminary cytotoxicity assay was performed to determine appropriate test substance concentrations for the main test. In the pre-test for toxicity, precipitation of the test item was observed with the unaided eye at the end of treatment at 135.0 µg/mL and above in the absence and presence of S9 mix. Since the cultures fulfilled the requirements for cytogenetic evaluation, this preliminary test was designated Experiment I.
Clear toxic effects were observed after 4 hours treatment with 413.3 µg/mL and above in the absence and presence of S9 mix. Therefore, 800.0 µg/mL (with and without S9 mix) were chosen as top concentration in Experiment IIA. The experimental part without S9 mix was repeated with a top dose of 400.0 µg/mL due to lack of cytotoxicity in evaluable concentrations.

C. CYTOGENICITY ASSAYS:

In all experimental parts after 4 and 20 hours test substance treatment in the absence and presence of S9 mix the values (0.15 – 0.60% micronucleated cells) were close to the concurrent vehicle control values (0.25 - 0.70% micronucleated cells) and within historical negative control data range (0.05 - 1.65% micronucleated cells) [see Table 5.8.1-112 and Table 5.8.1-113].

Either Demecolcin (50.0 or 150.0 ng/mL), MMC (2.0 µg/mL) or CPA (17.5 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei.

In Experiment I, precipitation of the test item in the culture medium was observed at 135.0 µg/mL and above in the absence and presence of S9 mix. In Experiment IIA, precipitation occurred at 457.1 µg/mL and above in the absence of S9 mix and at 261.2 µg/mL and above in the presence of S9 mix. In Experiment IIB precipitation occurred at 200.0 µg/mL and above in the absence of S9 mix at the end of treatment.
No relevant influence on osmolarity or pH was observed. The osmolarity is generally high compared to the physiological level of approximately 300 mOsm. This effect however, is based on a final concentration of 1 % DMSO in medium. As the osmolarity is measured by freezing point reduction, 1 % of DMSO has a substantial impact on the determination of osmolarity.
In Experiment I and IIA in the absence and presence of S9 mix, no cytotoxicity was observed up to the highest evaluable concentration. In Experiment IIB in the absence of S9 mix, clear cytotoxicity was observed at the highest evaluated concentration.

Table 5.8.1-112: Summary of results of micronucleus test with Reg.No. 4670450 (metabolite of metiram) - without metabolic activation

					Genotoxicity	Cytotoxicity	
Exp.	Exposure/ Preparation interval	Test groups	S9 mix	Prec.*	Micronucleated cells** [%]	Proliferation index (CBPI)	Cytostasis [%]
I	4/40 hrs	Vehicle control[1]	-	n.d.	0.50	1.69	-
		44.1 µg/mL	-	-	0.45	1.73	n.c.
		77.1µg/mL	-	-	0.35	1.70	n.c.
		135.0 µg/mL	-	+	0.25	1.72	n.c.
		Positive control[2]	-	n.d.	9.10[S]	1.26	63.1
IIA	20/40 hrs	Vehicle vcntrol[1]	-	n.d.	0.25	1.82	-
		48.7 µg/mL	-	-	0.35	1.79	4.3
		85.3 µg/mL	-	-	0.35	1.69	16.6
		149.3 µg/mL	-	-	0.25	1.52	36.7
		Positive control[3]		n.d.	2.75[S]	1.59	28.0
IIB	20/40	Vehicle vcntrol[1]	-	n.d.	0.70	1.70	-
		50.0 µg/mL	-	-	0.40	1.60	13.7
		100.0 µg/mL	-	-	0.45	1.42	40.7
		150.0 µg/mL	-	-	0.50	1.26	63.1
		Positive control[4]	-	n.d.	3.90[S]	1.77	n.c.

* Precipitation in culture medium at the end of exposure period (macroscopic)
** Relative number of binucleated cells with micronuclei per 2000 cells scored per test group
S Frequency statistically significant higher than corresponding control values
n.d. Not determined;
n.c. not calculated as the CBPI was equal or higher than solvent control value
[1] DMSO 1% (v/v); [2] MMC 2.0 µg/mL; [3] Demecolcin 150 ng/mL; [4] Demecolcin 50 ng/mL

Table 5.8.1-113: Summary of results of micronucleus test with Reg.No. 4670450 (metabolite of metiram) - with metabolic activation

					Genotoxicity	Cytotoxicity	
Exp.	Exposure/ Preparation interval	Test groups	S9 mix	Prec.*	Micronucleated cells** [%]	Proliferation index (CBPI)	Cytostasis [%]
I	4/40 hrs	vehicle control[1]	+	n.d.	0.35	2.08	
		44.1 µg/mL	+	-	0.45	1.99	8.2
		77.1µg/mL	+	-	0.35	1.98	9.4
		135.0 µg/mL	+	+	0.15	1.88	17.8
		positive control[2]	+	n.d.	2.65[S]	1.49	54.7
IIA	4/40 hrs	vehicle control[1]	+	n.d.	0.40	1.84	-
		85.3 µg/mL	+	-	0.60	1.72	13.5
		149.3 µg/mL	+	-	0.50	1.74	10.9
		261.2 µg/mL	+	+	0.40	1.54	35.1
		positive control[2]	+	n.d.	3.40[S]	1.27	68.3

* Precipitation in culture medium at the end of exposure period (macroscopic)
** Relative number of binucleated cells with micronuclei per 2000 cells scored per test group
S Frequency statistically significant higher than corresponding control values
n.d Not determined;
n.s. not scorable due to strong toxicity
[1] DMSO 1 % (v/v); [2] CPA 17.5 µg/mL

III. CONCLUSION

Based on the results of the study it is concluded that Reg.No. 4670450, metabolite of metiram has not the potential to induce micronuclei (clastogenic and/or aneugenic activity) under the in vitro conditions in human lymphocytes in the absence or presence of metabolic activation.
Overall conclusion:
The metabolite Reg.No. 4670450 is considered to be not genotoxic based on the available data from QSAR evaluations and in vitro studies. The structure of Reg.No. 4670450 is falling under Cramer Class III class. Thus, human exposure of 1.5 µg/kg bw/day is considered a safe value.

Metabolite: Ethylene Diamine (EDA):

QSAR predictions on EDA:

OASIS TIMES (V.2.27.16.8; Mutagenicity S-9 activated v09.09) [see molecule 11 of report DocID 2015/1094106]
There were **no** Ames mutagenicity alerts for EDA or in silico generated metabolites and no structural alerts were received. The parent substance was in the model applicability domain. However, the observed mutagenicity in the presence of S9 yielded a positive result.

VEGA: Mutagenicity model (CAESAR, version 2.1.12) [see molecule 9 of report DocID 2015/1094107]
EDA was in the model applicability domain. The prediction is **'non-mutagen'** with a **high** overall reliability.

VEGA: Mutagenicity model (SarPy; version 1.0.6-DEV) [see molecule 9 of report DocID 2015/1094107]
EDA was in the model applicability domain. The prediction is **'non-mutagen'** with a **high** overall reliability.

VEGA: Mutagenicity model (Toxtree; version 1.0.0-DEV) [see molecule 9 of report DocID 2015/1094107]
EDA was out of the model applicability domain. The prediction is **'non-mutagen'** with a **low** overall reliability. Deficiencies result from:

- only moderately similar compounds with known experimental value in the training set have been found
- similar molecules found in the training set

Toxicological information on EDA:

Ethylenediamine (CAS No. 107-15-3) was evaluated under REACh (Tier 1) and an OECD SIDS from 2002 is available (http://www.inchem.org/documents/sids/sids/Ethylenediamine.pdf).
The summary conclusion found in the OECD SIDS concerning human health is as followed:

"Acute toxicity of ethylenediamine (LD50, rat, oral range from 637 mg/kg bw to 1850 mg/kg bw; LC50, rat, inhalation >29 mg/L and LD50, rabbit, dermal 560 mg/kg bw) is considered to be low to moderate. Due to the high alkalinity, ethylenediamine is corrosive to the skin and eyes. It is a dermal and respiratory sensitizer in humans and has been reported to cross-sensitize for chemicals of similar structure. In repeated dose studies, decreased body weight along with decreased water and feed consumption were observed. Every attempt was made to minimize the irritating nature of EDA and reduce the pH by using EDA-2HCL. Hepatocellular pleomorphism was noted in every study following dietary administration of longer than 13 weeks duration. Gavage administration resulted in effects in the eyes and kidneys. Kidney effects consisted of degenerative and regenerative changes in the tubular epithelium. The Lowest-Observable-Adverse-Effect -Level (LOAEL) is 100 mg/kg bw/day with a No-Observable-Effect-Level (NOAEL) of 20 mg/kg bw/day observed in the chronic dietary feeding study in rats. Ethylenediamine was rapidly excreted with most of the material eliminated in the urine within 24 hours. Ethylenediamine has produced weakly positive results, 2-3 times greater than control values, in several Ames tests, which may or may not be related to an impurity. Subsequent studies conducted with purer material were negative. All other tests including several in vitro assays (CHO gene mutation, sister chromatid exchange with CHO cells and UDS with primary rat hepatocytes) and a rat dominant lethal assay were negative. The weight of evidence from both in vitro and in vivo tests indicates that ethylenediamine is unlikely to be genotoxic.
In rat chronic bioassays via two routes of exposure (oral and dermal) there was no carcinogenic effect. In developmental toxicity studies conducted in rats, growth retardation was noted at maternally toxic levels. However, there was no evidence of developmental toxicity at maternally toxic doses when compared with a pair-fed control. The NOAEL for maternal toxicity was 50 mg/kg bw, the developmental NOAEL was 250 mg/kg bw. There was no effect on reproductive parameters at levels, which produced parental toxicity."

DNEL's as extracted from the dissemination dossier from the ECHA homepage:

Systemic long term effects for workers:
DNEL (inhalation) = 25 mg/m^3
DNEL (dermal) = 3.6 mg/kg bw/day

Systemic long term effects for general population:
DNEL (inhalation) = 12.5 mg/m^3
DNEL (dermal) = no hazard identified
DNEL (oral) = 0.275 mg/kg bw/day

An OEL has been derived by the Japan Society for Occupational Health: It is 10 ppm (25 mg/m^3), which is not lower, than the European derived DNEL of 12.5 mg/m^3 (BASF DocID 2014/1325913).

A summary of the toxicological studies is provided in the following table.

Table 5.8.1-114: Overview over toxicological studies conducted with EDA or EDA * 2HCl

Study	Species	Protocol / Endpoint	Results
Acute oral toxicity	Rat	LD50	637 – 1850 mg/kg bw
Acute inhalation toxicity	Rat	LC50	Above 29 mg/L
Acute dermal toxicity	Rabbits	LD50	560 mg/kg bw
Irritation	Rabbits	Skin irritation	70% aqueous solution burns within 6-1w min
Irritation	Rabbits	Eye irritation	Severe irritation with permanent damage
Sensitization	Guinea pig	Modified Maguire maximization	Sensitizing
Repeated dose toxicity	Rat	7-day dietary study (0, 200, 630, 1940 mg/kg bw)	LOAEL: 630 mg/kg bw (body weight gain, liver and kidney weights ↓) NOAEL: 200 mg/kg bw
Repeated dose toxicity	Rat	3 months dietary (target doses: 0, 50, 250, 1000 mg/kg bw)	LOAEL: 250 mg/kg bw (water consumption ↓, clinical chemistry, hepatocellular pleomorphism NOAEL: 50 mg/kg bw
Repeated dose toxicity	Rat	16 day oral gavage (0, 0, 100, 200, 400, 800, 1600 mg/kg bw)	LOAEL: 400 mg/kg bw (kidney histopathology, deaths at higher doses) NOAEL: 100 mg/kg bw
Repeated dose toxicity	Rat	3 months oral gavage (0, 100, 200, 400, 600, 800 mg/kg bw	LOAEL: 100 mg/kg bw (body weight gain, thymus weight ↓, eyes, kidney, uterus (secondary to inanition) histopathology
Repeated dose toxicity	Mouse	7-day dietary study (0, 156, 625, 2500 mg/kg bw)	LOAEL: 2500 mg/kg bw (body weight gain ↓, food consumption ↓, liver, kidney weights ↓) NOAEL: 625 mg/kg bw
Repeated dose toxicity	Mouse	16 day oral gavage (0, 50, 100. 200, 400, 600 mg/kg bw	LOAEL: 100 mg/kg bw (kidney; deaths at higher doses) NOAEL: 50 mg/kg bw
Repeated dose toxicity	Mouse	3 months oral gavage (0, 25, 50, 100, 200, 400 mg/kg bw)	LOAEL: 400 mg/kg bw (kidney histopathology) NOAEL: 200 mg/kg bw
Ames test	Salmonella typhimurium		With S9: weakly positive in TA 100 and TA 1535; without S9: negative
Gene mutation	CHO cells		Negative
Sister chromatid exchange	CHO cells		Negative
Unscheduled DNA	Rat		Negative

Study	Species	Protocol / Endpoint	Results
Synthesis	hepatocytes		
Dominant Lethal test	Rat		Negative
SLRL test	Drosophila		Negative
Carcinogenicity	Fischer F344 Rat	2-year dietary (0, 20, 100, 350/360 mg/kg bw)	No carcinogenicity **NOAEL: 20 mg/kg bw**
Carcinogenicity	Male Mouse	Lifetime (25 µL of 1% solution was applied 3 times/week)	No carcinogenicity
Reproduction toxicity	Rat	2-Generation toxicity (0, 50, 150, 500 mg/kg bw	LOAEL (adults): 150 mg/kg bw (body weight gain ↓, livre weight ↓, kidney weight ↑, hepatocellular pleomorphism) NOAEL (adults): 50 mg/kg bw NOAEL (offspring): 500 mg/kg bw NOAEL (reproduction): 500 mg/kg bw
Developmental toxicity	Rat	GD 6 – 15 (0, 50, 250, 1000 mg/kg bw)	No teratogenicity LOAEL (dams) 250 mg/kg bw **NOAEL (dams): 50 mg/kg bw** LOAEL (pups): 1000 mg/kg bw NOAEL (pups): 250 mg/kg bw

In repated dose studies, decreased body weight water and feed consumption and liver pleomorphism have been noted in nearly every study via the dietary route. However, when EDA was given by oral gavage, effects were noted in the eyes and kidney. Kidney effects consisted of degenerative and regenerative changes in the tubular epithelium. In developmental toxicity studies, growth retardation awas ntoed at maternally toxic leels. However, there was no evidence of developemtnal toxictiy at maternally toxic doses when compared with a pair-fed control. There was no effect of reproductive parameters at levels, which produced parental toxicity. The rat was more sensitive than the mouse. The lowest LOAEL was 100 mg/kg bw with a NOAEL of 20 mg/kg bw in the chronic dietary feeding study in rats. The weight of evidence suggests that ethylene diamine is not genotoxic. It was also negative in chronic bioassays following administration via two routes, oral and dermal. The NOAEL from the chronic rat study is used for ADI derivation. For ARfD derivation the maternal NOAEL of the teratogenicity study has been used as a starting point.

Proposed reference values for EDA:

ADI: 0.2 mg/kg bw, based on the NOAEL of 20 mg/kg bw derived in a rat 2-year study
ARfD: 0.5 mg/kg bw, based on the maternal NOAEL, derived in the rat teratogenicity study

Further crop and livestock metabolites (including metabolites M222F001, M222F008 = hydantoin = CAS 461-72-3, M222F013)

In addition to the relevant metabolites discussed before three further crop and livestock metabolites (M222F001, M222F008, M222F013) were evaluated regarding their genotoxic potential. M222F001 and M222F013 are most likely artefacts seen in only one crop residue study (see Chapter MCA 6.7).

QSAR predictions on crop and livestock metabolites:

Table 5.8.1-115: Overview on QSAR evaluation of additional crop and livestock metabolites of metiram

	QSAR prediction of mutagenicity (Ames)				
	OASIS Times (v2.27.16.8), Ames, with S9		**VEGA**		
			VEGA, Caesar 2.1.12	**VEGA, SarPy 1.0.6-DEV**	**VEGA, TOXTREE 1.0.0-DEV**
	Prediction (compound evaluated)	**Overall prediction***	**Prediction (compound evaluated)**	**Prediction (compound evaluated)**	**Prediction (compound evaluated)**
Metiram	Negative Out of domain	-	Positive Out of domain	Negative Out of domain	Negative Out of domain
M222F001	Negative Out of domain (No.9 in DocID 20151094106)	Negative Out of domain	Negative Out of domain (No.12 in DocID 20151094107)	Negative Could be out of domain	Negative Could be out of domain
M222F008 (Hydantoin)	Negative Out of domain (No.6 in DocID 2015/1094106)	Negative Out of domain (No.6 in DocID 2015/1094106)	Negative In domain (Experimental data: negative) (No.7 in DocID 2015/1094107)	Negative In domain (Experimental data: negative)	Negative Out of domain
M222F013	Negative Out of domain (No.8 in DocID 2015/1094106)	Negative Out of domain	Negative Out of domain (No.6 in DocID 2015/1094107)	Negative Could be out of domain	Negative Out of domain

*: Overall prediction of compound evaluated including presumed in silico metabolites.

For metabolites M222F001 and M222F013 the QSAR predictions were all negative with low to moderate reliabilities. No structural alerts were found.
For metabolite M222F008 (Hydantoin) all QSAR predictions were negative. In the CAESAR and SarPy model the prediction was negative with a high reliability. Furthermore, "negative" experimental data were reported within these two predictions.

For Hydantoin, an Ames test reported by Forster et al. (1992) yielded negative results for Hydantoin in strains TA97, TA98, TA100, and TA102 in the presence and absence of metabolic activation (DocID 1992/1005457). Comparing the structures of hydantoin, with those of dimethylhydantoin and ethylene urea, it can be reasonably assumed, that hydantoin is not genotoxic, based on read-across to the negative genotoxicity data generated for ethylene urea (see above) and dimethylhydantoin (CAS: 77-71-4). Dimethylhydantoin is a REACH compound, whic has been tested extensively for genotoxicity. A summary of the conducted genotoxicity studies can be found on the ECHA homepage. Summaries of negative Ames tests, a DNA damage and repair, an in vitro chromosome aberration test in CHO cells, a mouse lymphoma and read-across to an in vitro chromosome aberration test in human lymphocytes are available. (http://apps.echa.europa.eu/registered/data/dossiers/DISS-a134044b-66ba-3218-e044-00144f67d031/AGGR-ae41142a-76b0-481d-a0aa-2e7039378ee5_DISS-a134044b-66ba-3218-e044-00144f67d031.html#GEN_APPL_SUM_HD)

Hydantoin
(CAS 461-72-3)

Dimethylhydantoin
(CAS 77-71-4)

Ethylene urea
(CAS: 120-93-4)

Figure 5.8.1-16: Structures of hydantoin, dimethylhydantoin and ethylene urea

Thus it can be concluded, that Hydantoin is not likely to have genotoxic properties, based on a negative Ames assay and on read-across considerations to dimethylhydantoin and ethylene urea.

CA 5.8.2 Supplementary studies on the active substance

The conduct of an immunotoxicity study on metiram was required by US EPA, as summarized in US-EPA DCI from 2007.

Report:	CA 5.8.2/1 [redacted] 2011b BAS 222 29 F (Metiram TC) - Immunotoxicity study in female Wistar rats - Administration via the diet for 4 weeks 2011/1038260
Guidelines:	EPA 870.7800
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

Metiram (batch 300015, purity 91.5%) was administered via the diet to female Wistar rats at dietary dose levels of 0, 150, 500, and 1000 ppm, respective 0, 12, 40 and 76 mg/kg bw/day for 4 weeks. Cyclophosphamide monohydrate (4.5 mg/kg bw/day) was used as a positive control.
SRBC IgM antibody titers were similar between control and metiram treated animals. No immunopathological findings were observed after treatment of animals with metiram, and thus a NOAEL of 1000 ppm was set for immunotoxicity. Based on clinical findings in the 500 and 1000 ppm group, including reduced food consumption, lower mean body weights and body weight gains, a NOAEL of 150 ppm was set for general systemic toxicity.
The administration of the positive control cyclophosphamide monohydrate (4.5 mg/kg bw/d) led to effects indicative of immunotoxicity, i.e. reduced absolute and relative spleen and thymus weight. Furthermore, SRBC IgM antibody titers were significantly lower compared to the control group. In addition, an impaired body weight development was observed after treatment of animals with cyclophosphamide.
Based on the results of the immunotoxicity study with metiram the NOAEL was identified at 1000 ppm (76 mg/kg bw/day) for immunotoxicity and 150 ppm (12 mg/kg bw/day) for general systemic toxicity.

(DocID 2011/1038260)

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 29 F (Metiram TC)
   Description: solid, beige
   Lot/Batch #: 300015
   Purity: 91.5%
   Stability of test compound: The test substance was stable over the study period (Expiry date 30.06.2011).

2. Positive control:	Cyclophosphamide Monohydrate (CAS No.: 6055-19-2)
Description:	solid, white
Lot/Batch #:	1362353
Purity:	100%
Stability of test compound:	The test substance was stable over the study period (Expiry date 22.09.2012).

3. Test animals:	
Species:	Rat
Strain:	Crl:WI (Han)
Sex:	Female (more sensitive gender as shown in former studies)
Age:	36 ± 1 days at delivery; 42 ± 1 at the beginning of the administration period
Weight at dosing:	125 – 143 g
Source:	[redacted]
Acclimation period:	6 days
Diet:	Kliba maintenance diet rat/mouse Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water ad libitum
Housing:	Group housing (4 animals per cage) in H-Temp (PSU, TECNIPLAST, Hohenpeißenberg, Germany) cages; floor area about 2065 cm^2.
Environmental conditions:	
Temperature:	20 - 24°C
Humidity:	30 - 70%
Air changes:	Fully air-conditioned rooms; 15 of air-changes per hour
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN AND METHODS

The study was conducted at BASF SE, Experimental Toxicology and Ecology, 67056 Ludwigshafen, Germany.

1. In life dates:	26-Oct-2010 - 30-Nov-2010) (Dates of experimental work: 26-Oct-2010 to 11-Feb-2011)

2. Animal assignment and treatment:
Metiram was administered to groups of 8 female Wistar rats at dietary concentrations of 0, 150, 500 and 1000 ppm for 4 weeks. Cyclophosphamide monohydrate (4.5 mg/kg bw/d) was administered by gavage as a solution in drinking water to a group of 8 female animals.

All animals were immunized 6 days before blood sampling and necropsy using 0.5 ml sheep red blood cells ($4x10^8$ SRBC/ml) administered intraperitoneally. The animals were assigned to the treatment groups by means of a computer generated randomization lists based on body weights.

3. Test substance preparation and analysis:
The test substance metiram was weighed out for each concentration and was mixed with a small amount of food. Then corresponding amounts of food, depending on test group, were added to this premix in order to obtain the desired concentrations. Mixing was carried out for about 10 minutes in a laboratory mixer. Details of the mixers used are retained with the raw data. The test substance preparations were mixed once before the start of the administration period.
Cyclophosphamide monohydrate (positive control substance) was administered as a solution. To prepare the solution, the appropriate amount of Cyclophosphamide monohydrate was weighed out depending on the desired concentration. Then the vehicle (drinking water) was filled up to the desired volume, subsequently mixed using a magnetic stirrer. The positive control substance-preparations were prepared once, split into daily aliquots and kept frozen at -18°C. The mixtures were administered after reaching room temperature.

Metiram pure active ingredient is inaccessible. For dithiocarbomates, it is accepted by the authorities to quantify these compounds by the analyses of carbon disulfide as a common moiety. Therefore, established maximum residue limits (MRL´s) for foods are always based on carbon disulfide determination.
The stability of the test substance metiram in the diet over a period of up to 42 days was proven before the start of the study (BASF Project No. 09L00361). The stability of Cyclophosphamide Monohydrate (positive control substance) in the vehicle (drinking water) over a period of 32 days (in the freezer) was proven before the start of the study

Homogeneity analyses of the test-substance preparations were performed in samples of all concentrations at the start of the administration period. These samples also served for concentration control analyses. Concentration control analyses of Cyclophosphamide monohydrate (positive control substance) were performed before the beginning of the study. From a strict chemical viewpoint, a homogeneity assay was not necessary because the compound is completely soluble in water at the concentration being dosed.

4. Statistics:
Means and standard deviations of each test group were calculated for several parameters. Further statistical analyses were performed according to following tables:

Statistics of clinical examinations

Parameters	Statistical test	Markers in the tables	References
Body weight and body weight change (Groups 0, 1, 2, 3)	A comparison of each test group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means	* for p ≤ 0.05 ** for p ≤ 0.01	DUNNETT, C.W. (1955): A multiple comparison procedure for comparing several treatments with a control. JASA, Vol. 50, 1096-1121 DUNNETT, C.W. (1964). New tables for multiple comparisons with a control. Biometrics, Vol. 20, 482-491
Body weight and body weight change (Groups 0 and 4)	comparison of the dose group with the control group was performed using the t-test (two-sided) for the hypothesis of equal means	* for p ≤ 0.05 ** for p ≤ 0.01	WELCH B.L. (1947): The generalization of Student’s problem when several different population valiances are involved. Biometrika, 34, 28-35

Statistics of clinical pathology

Parameters	Statistical test	Markers in the tables	References
Clinical pathology parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the negative control group was performed using WILCOXON-test (two-sided) for the equal medians.	* for p ≤ 0.05 ** for p ≤ 0.01	SIEGEL, S. (1956): Nonparametric statistics for the behavioural sciences. McGraw-Hill New York

Statistics of pathology

Parameter	Statistical test	Markers in the tables	References
Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each test group with the control group was performed using the WILCOXON test for the hypothesis of equal medians	* for $p \leq 0.05$ ** for $p \leq 0.01$	HETTMANNSPERGER, T.P. (1984): Statistical Inference based on Ranks, John Wiley & Sons New York, 132-140. International Mathematical and Statistical Libraries, Inc., 2500 Park West Tower One, Houston, Texas 77042-3020, USA, nakl-1 - nakl-3 MILLER, R.G. (1981): Simultaneous Statistical Inference Springer-Verlag New York Inc., 165-167 NIJENHUIS, A. and S.W. WILF (1978): Combinatorial Algorithms, Academic Press, New York, 32-33

C. METHODS

1. Observations:

The animals were examined for evident signs of toxicity or mortality twice daily on working days and once daily on weekends and public holidays. If animals were in a moribund state, they were sacrificed and necropsied.

The clinical condition of the test animals were recorded individually. Detailed clinical observations of all animals were performed in a standard arena prior to the administration period and weekly thereafter. The standard arena had a size of 50 x 37.5 cm with walls of 25 cm height. If applicable the findings were ranked according to the degree of severity. The following parameters were examined:

1. abnormal behavior during handling
2. fur
3. skin
4. posture
5. salivation
6. respiration
7. activity/arousal level
8. tremors
9. convulsions
10. abnormal movements
11. impairment of gait
12. lacrimation
13. palpebral closure
14. exophthalmus
15. feces (appearance/consistency)
16. urine
17. pupil size

2. Body weight:
The body weight of the animals was determined before the start of the administration period in order to randomize the animals, at the start of the exposure period and thereafter twice weekly. The difference between the body weight on the respective day of weighing and the body weight on day 0 was calculated as body weight change.

3. Food/water consumption, food efficiency and compound intake:
Group food consumption was determined weekly (as representative value over 1 day) for each cage. The average food consumption was used to estimate the mean food consumption in grams per animal and day.
Water consumption was observed daily by visual inspection of the water bottles for any overt changes in volume.

The mean daily intake of test substance (group means) was calculated based upon individual values for body weight and food consumption.

$$Substance\ intake\ for\ day\ x = \frac{FC_x \times C}{BW_x}$$

BW_x = body weight on study day x [g]; FC_x= mean daily food consumption on study day x [g]; C = concentration in the food on study day x [mg/day]

4. Clinical pathology:
In the morning blood was taken from the retro-orbital venous plexus from fasted animals. The animals were anaesthetized using isoflurane (Isoba®, Essex GmbH Munich, Germany). The blood sampling procedure and subsequent analysis of serum samples were carried out in a randomized sequence. The following examinations were carried out in 8 female animals per test group.

Immunotoxicological examinations
Primary T-cell dependent antibody response (anti-SRBC IgM ELISA)

- The ELISA was performed according to TEMPLE et al. (1995).
- In deviation to the mentioned reference, a standard curve (7 standards in a two-fold dilution) was established using anti-SRBC IgM positive serum pool, for comparison to subsequent test runs (arbitrary lab units/ml; stock standard aliquots stored at -80 °C).
- Each serum sample was applied to the ELISA in two dilutions: negative control and dosed animals: 1:256 and 1:512; positive control animals: 1:64 and 1:128
- Generally, the 1:256 dilution for the negative control and dosed animals and the 1:64 dilution for the positive control animals was reported.
- Result deviations (lab units/µl) in both dilutions of more than 25 % were confirmed
- OD values of the sample dilutions outside of the linear range of the standard curve were repeated with lower or higher dilutions, as appropriate.
- Generally, two in house controls were measured with each test run.
- The ELISA was measured with a Sunrise MTP-reader, Tecan AG, Maennedorf, Switzerland, and evaluated with the Magellan-Software of the instrument producer.

5. Sacrifice and pathology:
All animals were sacrificed by decapitation under isoflurane anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology.

Weight assessment was carried out on all animals sacrificed at scheduled dates. The following weights were determined:
- Anesthetized animals
- Spleen
- Thymus

The following organs or tissues were fixed in 4% buffered formaldehyde solution:
- All gross lesions
- Spleen
- Thymus

At the request of the sponsor histotechnical processing and examination was not performed.

II. RESULTS AND DISCUSSION

A. ANALYSES

Stability analyses
The stability of the test substance metiram in the diet was demonstrated over a period of up to 42 days at room temperature. As the mixtures were stored no longer than this time period, the stability was guaranteed. The stability of Cyclophosphamide monohydrate (positive control substance) in drinking water was demonstrated for 7 days at room temperature and 32 days stored in the freezer.

Homogeneity control analyses
Considering the low standard deviation in the homogeneity analysis, it was concluded that Metiram was distributed homogeneously in feed. Due to the fact that Cyclophosphamide monohydrate (positive control substance) was a solution in drinking water no homogeneity analysis was performed.

Concentration control analyses
The mean values in the range of 98.7 – 111.0 % of the nominal concentration demonstrated the correctness of the concentrations of Metiram in Kliba lab diet mouse/rat "GLP". The mean value of Cyclophosphamide Monohydrate in drinking water was found at 100.2% of the nominal concentration. These results demonstrated the correctness of the concentrations of Cyclophosphamide Monohydrate in drinking water.

B. OBSERVATIONS

1. Clinical signs of toxicity

One animal of test group 3 (1000 ppm), i.e. animal No. 27, had a poor general condition from study day 22 onwards, showed a high-stepping gait from study day 24 onwards and a reduced nutritional condition from study day 26 onwards.

2. Mortality

No mortality was observed throughout the study period.

C. BODY WEIGHT AND BODY WEIGHT GAIN

Mean body weight in test group 3 (1000 ppm) was significantly lower from study day 7 onwards, with a maximum of -18.76% on study day 28 versus test group 0 (control group). For this reason body weight change values were also significantly decreased in test group 3 (1000 ppm) from study day 7 onwards, with a maximum of -65.65% on study day 17 versus test group 0 (control group).
Mean body weight in test group 2 (500 ppm) was significantly lower from study day 3 onwards (except on study day 10) with a maximum of -10.79% on study day 28 compared to test group 0 (control group). Therefore, body weight change values were also significantly lower from study day 14 onwards with a maximum of -30.29% on study day 24 compared to test group 0 (control group).
No test substance-related impairment of body weight data was observed in animals of test group 1 (150 ppm).
During the administration period the mean body weight of test group 4 (positive control group) was slightly lower duration the administration period, but significantly on study days 3 (-3.46%), 7 (-4.27%) and 28 (-6.22%). Therefore, body weight change was significantly lower on study day 7 (-21.35%) and on study day 28 (-16.04%).

Figure 5.8.2-1: Body weight development in rats administered metiram for 4 weeks

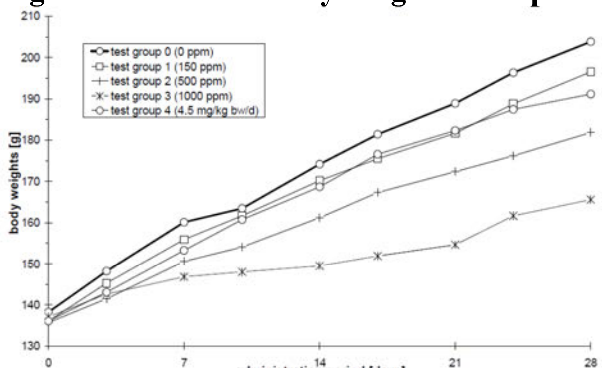


Table 5.8.2-1: Body weight and body weight gain data of rats administered metiram for 4 weeks

	Females				
Dose level [ppm]	0	50	150	450	Positive control
Body weight [g]					
- Day 0	138	136	136	137	136
- Day 28	204	197	182**	166**	191*
% (compared to control)		-3.58	-10.79	-18.76	-6.22
Overall body weight gain (Day 0 – 28) [g]	65.6	60.5	46.2**	28.5**	55.1*
% (compared to control)		-7.81	-29.66	-56.53	-16.0

* $p \leq 0.05$; ** $p \leq 0.01$ (Dunnett's test, two sided); $ calculated from the weekly means

D. FOOD/WATER CONSUMPTION AND COMPOUND INTAKE

Reduced food consumption was observed over the entire study period in test group 3 (1000 ppm) and in the 2nd half of the study in test group 2 (500 ppm). No relevant changes were seen for test group 1 (150 ppm).
Animals which received Cyclophosphamide monohydrate as positive control showed a clearly reduced food consumption at the end of the study.

Table 5.8.2-2: Food consumption data of rats administered metiram for 4 weeks

	Females				
Dose level [ppm]	0	50	150	450	Positive control
Food consumption [g]					
- Day 6-7	12.49	12.86	12.70	9.89	14.18
% (compared to control)		2.96	1.68	-20.82	13.53
- Day 13-14	14.14	13.15	13.75	11.92	12.05
% (compared to control)		-7.00	-2.76	-15.70	-14.78
- Day 20-21	14.72	13.36	13.16	12.18	14.16
% (compared to control)		-9.24	-10.60	-17.22	-3.80
- Day 27-28	16.54	15.13	13.48	12.82	11.81
% (compared to control)		-8.47	-18.45	-22.47	-28.58

* $p \leq 0.05$; ** $p \leq 0.01$ (Dunnett's test, two sided)

No test substance-related findings were observed regarding water consumption in all animals treated with Metiram and in animals which received Cyclophosphamide monohydrate as positive control.
The approximate mean daily test substance intake over the entire study period is shown below (see Table 5.8.2-3):

Table 5.8.2-3: Mean daily test substance intake calculated over the entire study period in test groups 1 – 3.

Test group	Concentration in the diet (ppm)	Mean daily test substance intake (mg/kg bw/day)
1	150	12
2	500	40
3	1000	76

E. CLINICAL PATHOLOGY

Primary T-cell dependent antibody response (anti-SRBC IgM ELISA)
Six days after immunization no difference between control and substance treated animals was observed. SRBC titers were significantly lower in animals treated with Cyclophosphamide monohydrate [see Table 5.8.2-4].

Table 5.8.2-4: SRBC titers of female Wistar rats after administration of metiram for 4 weeks

Sex	Females				
Dose [ppm]	Values (Mean ± SD)				
	0	150	500	1000	Pos. control
SRBC [LU/µL]	3.37 ± 2.43	8.44 ± 12.09	5.66 ± 3.18	5.40 ± 4.05	1.12 ± 0.43**

**: $p \leq 0.01$ (Wilcoxon-test, Two-sided)

F. NECROPSY

1. Organ weight

When compared with control group 0 (set to 100%), the terminal body weights of test group 2 (88%) and test group 3 (80%) were significantly decreased, which was a treatment-related effect (see Table 5.8.2-5). The absolute thymus weights of animals of test group 1 (80%), test group 2 (79%) and test group 3 (77%) were also significantly decreased, which was considered to be a secondary effect to lower body weights. Relative thymus weights were not decreased compared to the controls.
The relative spleen weights of test group 2 (110%) and test group 3 (127%) were significantly increased, which was considered to be incidental.
The positive control group (test group 4: Cyclophosphamide Monohydrate) revealed a significant decrease of terminal body weight, absolute spleen and thymus weights, which was the expected result.

Table 5.8.2-5: Terminal body and organ weights of rats administered metiram for 4 weeks

Sex	Females								
Dose [ppm]	Values					% (compared to control)			
	0	50	150	450	Pos. control	50	150	450	Pos. control
Terminal body weight [g]	191	181	168	152	176**	95	88	80	92
Spleen abs. [g]	0.423	0.429	0.408	0.428	0.321**	101	96	101	76
rel. [%]	0.222	0.237	0.244*	0.281**	0.183**	107	110	127	82
Thymus abs. [mg]	479.6	386.0	377.8	367.4	264**	80	79	77	55
rel. [%]	0.252	0.213	0.226	0.242	0.15**	85	90	96	60

* $p \leq 0.05$; ** $p \leq 0.01$; Wilcoxon test, two sided

2. Gross and histopathology

All gross lesions observed were considered incidental and not related to treatment. In the absence of gross lesions no histopathological investigations were carried out.

III. CONCLUSIONS

Under the conditions of the study metiram did not reveal any signs of immunotoxicity when administered via the diet over a period of 4 weeks to male Wistar rats. The NOAEL for the immunotoxicologically relevant endpoints was set to 1000 ppm (76 mg/kg bw/day), the highest dose tested. The NOAEL for general systemic toxicity was set to 150 ppm (12 mg/kg bw/day) based on clinical parameters. The oral administration of the positive control substance cyclophosphamide monohydrate (4.5 mg/kg bw/day) led to severe findings indicative of immunotoxicity. This was represented by significantly lower SRBC IgM antibody titres as well as reduced spleen and thymus weights. Thus, assay sensitivity was verified in the present immunotoxicity study performed in male Wistar rats.

Report: CA 5.8.2/2
[redacted]., 2014
BAS 222 29 F (Metiram TK) and BAS 222 28 F – Study for comparing the toxicity in male Wistar rats – Administration via the diet for 28-days
2014/1313072

Guidelines: OECD 407, OECD 408, (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.7 No. L 142

GLP: no

Executive Summary

The aim of the study was to compare the toxicological profile of metiram and BAS 222 28 F (Polyram) after 4 weeks of administration via the diet.
Metiram was administered via the diet to groups of 10 male Wistar rats at concentrations of 0 (test group 0), 500 (test group 1) and 1500 ppm (test group 2) over a period of 4 weeks. BAS 222 28 F (Polyram) was administered via the diet to groups of 10 male Wistar at concentrations of 634 ppm (test group 3) and 1901 ppm (test group 4) over a period of 4 weeks. The tested dietary concentrations of metiram and Polyram represent equivalent contents of the active ingredient in the administered diets. The concentrations are 91.5% in METIRAM and 72.2% in BAS 222 28 F.
The administration of metiram and BAS 222 28 F (Polyram) via the diet to male Wistar rats for 4 weeks revealed comparable signs of toxicity at concentrations of 500 and 634 ppm (metiram: 41 mg/kg bw/d, and BAS 222 28 F: 51 mg/kg bw/d, respectively) as well as at concentrations of 1500 and 1901 ppm (metiram: 126 mg/kg bw/d, and BAS 222 28 F: 150 mg/kg bw/d, respectively). In the high dose groups impaired food consumption and body weights, decreased T4 levels, increased relative thyroid gland weights and hypertrophy/hyperplasia of follicular cells (minimal to moderate. In the mid dose the following effects were observed: decreased T4 levels and hypertrophy/hyperplasia of follicular cells (minimal).
(DocID 2014/1313072)

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: BAS 222 29 F (Metiram TK) and BAS 222 28 F (Polyram)

Description: solid, beige (BAS 222 29 F)
solid, bright brown (BAS 222 28 F)

Lot/Batch #: 300015 (BAS 222 29 F)
36035488Q0 (BAS 222 28 F)

Purity: 91.5% (BAS 222 29 F)
72.2% (BAS 222 28 F)

Stability of test compound: The stability of the test substance under storage conditions over the test period was guaranteed by the sponsor, and the sponsor holds this responsibility.

2. Vehicle:	plain diet
3. Test animals:	
Species:	Rat
Strain:	Crl:WI (Han)
Sex:	Male (most sensitive gender with regard to effects on the thyroid glands, the target organ of BAS 222 29 F)
Age:	37 ± 1 days at delivery; 42 ± 1 at the beginning of the administration period
Weight at dosing:	152.2 – 153.9 g
Source:	
Acclimation period:	5 days
Diet:	Kliba maintenance diet rat/mouse Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water ad libitum
Housing:	Group housing (5 animals per cage) in polysulfonate cages (TECNIPLAST, Hohenpeißenberg, Germany) cages; floor area about 2065 cm².
Environmental conditions:	
Temperature:	20 - 24°C
Humidity:	30 - 70%
Air changes:	Fully air-conditioned rooms; 15 of air-changes per hour
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN AND METHODS

The study was conducted at BASF SE, Experimental Toxicology and Ecology, 67056 Ludwigshafen, Germany.

1. In life dates: 17-Dec-2013 - 19-Jan-2014)
(Dates of experimental work: 17-Dec-2013 to xx-May-2014)

2. Animal assignment and treatment:
Metiram and BAS 222 28 F were administered to groups of 10 male Wistar rats at dietary concentrations of 500 and 1500 ppm and 634 and 1901 ppm for 4 weeks, respectively. For comparison, the concentration was calculated based upon the different purity of the test substances. One group with 10 male rats received control diet and served as control group.
The animals were assigned to the treatment groups by means of a computer generated randomization lists based on body weights. All remaining animals were sacrificed after a fasting period (withdrawal of food) for at least 16 hours.

3. Test substance preparation and analysis:
For each concentration the test substance was weighed out and mixed with a small amount of food. Then corresponding amounts of food, depending on test group, were added to this premix in order to obtain the desired concentrations. Mixing was carried out for about 10 minutes in a laboratory mixer. The test substance preparations were mixed once before the start of the administration period.

The analyses of the test-substance preparations were carried out as a separate study at the test facility Competence Center Analytics of BASF SE, 67056 Ludwigshafen, Germany, under the responsibility of a Study Director of this test facility. The study was carried out in compliance with the Principles of Good Laboratory Practice.

The stability of metiram in the diet at room temperature for 42 days was determined before the start of the study.

A stability of BAS 222 28 F in the diet did not exist. Therefore, concentration control analyses directly after mixing and at the end of the feeding period were drawn for determining the stability.

Homogeneity was verified in all concentrations (was used as a concentration control at the same time) at the start of the study (and for BAS 222 28 F additionally at the end of the study).
Reserve samples from all preparations were drawn directly after mixing and at the end of each feeding period.

4. Statistics:
Means and standard deviations of each test group were calculated for several parameters. Further statistical analyses were performed according to following tables:

Statistics of clinical examinations

Parameters	**Statistical test**	**Markers in the tables**	**References**
Body weight, body weight change	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means	* for $p \leq 0.05$ ** for $p \leq 0.01$	DUNNETT, C.W. (1955): A multiple comparison procedure for comparing several treatments with a control. JASA, Vol. 50, 1096-1121 DUNNETT, C.W. (1964). New tables for multiple comparisons with a control. Biometrics, Vol. 20, 482-491
Feces, rearing, grip strength forelimbs, grip strength hindlimbs, foot-splay test, motor activity	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON test (two-sided) for the equal medians	* for $p \leq 0.05$ ** for $p \leq 0.01$	SIEGEL, S. (1956): Non-parametric statistics for the behavioural sciences. McGraw-Hill New York

Statistics of clinical pathology

Parameters	Statistical test	Markers in the tables	References
Blood parameters	For parameters with **bidirectional** changes: Non-parametric one-way analysis using KRUSKAL-WALLIS test. If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the hypothesis of equal medians For parameters with **unidirectional** changes: Pairwise comparison of each dose group with the control group using the WILCOXON-test (one-sided) **with Bonferroni-Holm adjustment** for the hypothesis of equal medians	* for $p \leq 0.05$ ** for $p \leq 0.01$	SIEGEL, S. (1956): Non-parametric statistics for the behavioural sciences. McGraw-Hill New York Holm (1979): A Simple Sequentially Rejective Multiple Test Procedure. Scand. J. Statist. 6, 65-70
Urinalysis parameters (apart from pH, urine volume, specific gravity, color and turbidity	Pairwise comparison of each dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal medians	* for $p \leq 0.05$ ** for $p \leq 0.01$	SIEGEL, S. (1956): Non-parametric statistics for the behavioural sciences. McGraw-Hill New York
Urine pH, volume, specific gravity, color and turbidity	Non-parametric one-way analysis using KRUSKAL-WALLIS test. If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the hypothesis of equal medians. Urine color and turbidity are not evaluated statistically.	* for $p \leq 0.05$ ** for $p \leq 0.01$	SIEGEL, S. (1956): Non-parametric statistics for the behavioural sciences. McGraw-Hill New York

Statistics of pathology

Parameter	Statistical test	Markers in the tables	References
Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each test group with the control group was performed using the WILCOXON test for the hypothesis of equal medians	* for $p \leq 0.05$ ** for $p \leq 0.01$	HETTMANNSPERGER, T.P. (1984): Statistical Inference based on Ranks, John Wiley & Sons New York, 132-140. International Mathematical and Statistical Libraries, Inc., 2500 Park West Tower One, Houston, Texas 77042-3020, USA, nakl-1 - nakl-3 MILLER, R.G. (1981): Simultaneous Statistical Inference Springer-Verlag New York Inc., 165-167 NIJENHUIS, A. and S.W. WILF (1978): Combinatorial Algorithms, Academic Press, New York, 32-33

C. METHODS

1. Observations:

The animals were examined for evident signs of toxicity or mortality twice daily on working days and once daily on weekends and public holidays. If animals were in a moribund state, they were sacrificed and necropsied.
The clinical condition of the test animals were recorded individually. Detailed clinical observations of all animals were performed in a standard arena prior to the administration period and weekly thereafter. The standard arena had a size of 50 x 37.5 cm with walls of 25 cm height. If applicable the findings were ranked according to the degree of severity. The following parameters were examined:

1. abnormal behavior during handling
2. fur
3. skin
4. posture
5. salivation
6. respiration
7. activity/arousal level
8. tremors
9. convulsions
10. abnormal movements
11. gait abnormalities
12. lacrimation
13. palpebral closure
14. exophthalmos
15. feces (appearance/consistency)
16. urine
17. pupil size

2. Body weight:

The body weight of the animals was determined before the start of the administration period in order to randomize the animals, at the start of the exposure period and weekly thereafter. The difference between the body weight on the respective day of weighing and the body weight on day 0 was calculated as body weight change.

3. Food/water consumption, food efficiency and compound intake:
Group food consumption was determined weekly (as representative value over 1 day) for each cage. The average food consumption was used to estimate the mean food consumption in grams per animal and day.
Water consumption was observed daily by visual inspection of the water bottles for any overt changes in volume.

The mean daily intake of test substance (group means) was calculated based upon individual values for body weight and food consumption.

$$\text{Substance intake for day x} = \frac{FC_x \times C}{BW_x}$$

BW_x = body weight on study day x [g]; FC_x= mean daily food consumption on study day x [g]; C = concentration in the food on study day x [mg/day]

4. Functional observational battery:
A functional observational battery (FOB) was performed in all animals at the end of the administration period starting at about 10:00 a.m. At least one hour before the start of the FOB the rats were transferred to single-animal polycarbonate cages (floor area about 800 cm2). Drinking water was provided ad libitum, but no food was offered during the measurements. The FOB started with passive observations without disturbing the rats, followed by removal from the home cage, open field observations in a standard arena and sensory motor tests as well as reflex tests.
The findings were ranked according to the degree of severity, if applicable. The observations were performed at random.

Home cage observations:
The rats were observed in their closed home cages; during this period any disturbing activities (touching the cage or rack, noise) were avoided during these examinations in order not to influence the behavior of the rats. Attention was paid to:

1. Posture
2. Tremors
3. Convulsions
4. Abnormal movements
5. Gait abnormalities

Open field observations:
The rats were transferred to a standard arena (50 × 50 cm with sides of 25 cm height) and observed for at least 2 minutes. The following parameters were examined:

1. behavior when removed from cage	11. tremors
2. fur	12. convulsions
3. skin	13. abnormal movements / stereotypes
4. salivation	14. gait abnormalities
5. nasal discharge	15. acitivity/arousal level
6. lacrimation	16. feces (number of fecal pellets/ appearance/consistency) within two minutes
7. eyes/pupil size	17. urine (amount/color) within two minutes
8. posture	18. rearing within two minutes
9. palpebral closure	
10. respiration	

For sensorimotor tests and reflexes the animals were removed from the open field. The following tests were performed:

1. approach response	9. vocalization
2. touch response	10. pain perception ("tail pinch")
3. vision ("visual placing response")	11. grip strength of forelimbs
4. pupillary reflex	12. grip strength of hind limbs
5. pinna reflex	13. landing foot-splay test
6. audition ("startle response")	14. other findings
7. coordination of movements (righting response)	
8. behavior during "handling"	

5. Motor activity measurement:

Motor activity (MA) was measured from 14:00 h onwards on the same day as the FOB was performed. The examinations were performed using the TSE Labmaster System supplied by TSE Systems GmbH, Bad Homburg, Germany. For this purpose, the rats were placed in new clean polycarbonate cages (floor area about 800 cm²) with a small amount of bedding for the duration of the measurement. Eighteen beams were allocated per cage. The number of beam interrupts was counted over 12 intervals for 5 minutes per interval. The sequence in which the rats were placed in the cages was selected at random. On account of the time needed to place the rats in the cages, the starting time was "staggered" for each animal. The measurement period began when the 1st beam was interrupted and finished exactly 1 hour later. No food or water was offered to the rats during these measurements and the measurement room was darkened after the transfer of the last rat. The program requires a file name for the measured data to be stored. This name consists of the reference number and a serial number.

6. Clinical pathology:

In the morning blood was taken from the retro-orbital venous plexus from fasted animals. The animals were anaesthetized using isoflurane. The blood sampling procedure and subsequent analysis of serum samples were carried out in a randomized sequence. For urinalysis the individual animals were transferred to metabolism cages (withdrawal of food and water) and urine was collected overnight. Urine samples were evaluated in a randomized sequence.

Blood smears were prepared and stained according to WRIGHT without being evaluated, because of non-ambiguous results of the differential blood cell counts measured by the automated instrument.
The following clinical pathology parameters were examined in all animals:

Haematology:					
	Red blood cells		*White blood cells*		*Clotting Potential*
✓	Erythrocyte count (RBC)	✓	Total leukocyte count (WBC)	✓	Prothrombin time (HQT)
✓	Hemoglobin (HGB)	✓	Differential blood count		
✓	Hematocrit (HCT)	✓	Platelet count (PLT)		
✓	Mean corp. volume (MCV)				
✓	Mean corp. hemoglobin (MCH)				
✓	Mean corp. Hb. conc. (MCHC)				
✓	Reticulocytes (RET)				

Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Chloride	✓	Bilirubin (total)	✓	Aspartate aminotransferase (AST)
✓	Phosphorus (inorganic)	✓	Cholesterol	✓	Alkaline phosphatase (ALP)
✓	Potassium	✓	Creatinine	✓	γ-glutamyltransferase (GGT)
✓	Sodium	✓	Globulin (by calculation)		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		
		✓	Bile acids		

Hormones:					
✓	Total triiodothyronine (T3)				
✓	Total thyroxine (T4)				
✓	Thyroid stimulating hormone (TSH)				

Urinalysis:						
	pH	✓	Blood			
✓	Protein	✓	Specific gravity			
✓	Glucose	✓	Sediment			
✓	Ketones	✓	Color turbidity			
✓	Urobilinogen	✓	volume			
✓	bilirubin					

7. Sacrifice and pathology:

All animals were sacrificed by decapitation under isoflurane anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology.

Weight assessment was carried out on all animals sacrificed at scheduled dates. The following weights were determined:

- Anesthetized animals
- Adrenal glands
- Brain
- Epididymides
- Heart
- Kidneys
- Liver
- Spleen
- Testes
- Thymus
- Thyroid glands

The following organs or tissues were fixed in 4% buffered formaldehyde solution or in modified Davidson's solution:

1. All gross lesions	17. Ileum	33. Sciatic nerve
2. Adrenal glands	18. Jejunum (with Peyer's patches)	34. Seminal vesicles
3. Aorta	**19. Kidneys**	35. Skeletal muscle
4. Bone marrow (femur)	20. Larynx	36. Skin
5. Brain	**21. Liver**	37. Spinal cord (cervical, thoracic and lumbar cord)
6. Cecum	22. Lungs	38. Spleen
7. Coagulating gland	23. Lymph nodes (axillary and mesenteric)	39. Sternum with marrow
8. Colon	24. Mammary gland (male)	40. Stomach (forestomach and glandular stomach)
9. Duodenum	25. Nose (nasal cavity)	**41. Testes**

10. Epididymides	26. Pancreas	**42. Thymus**
11. Esophagus	27. Parathyroid glands	**43. Thyroid glands**
12. Extraorbital lacrimal glands	28. Pharynx	44. Trachea
13. Eyes with optic nerve	29. Pituitary gland	45. Urinary bladder
14. Femur with knee joint	30. Prostate	
15. Harderian glands	31. Rectum	
16. Heart	32. Salivary glands (mandibular and sublingual)	

Bold tissues were histologically examined in all animals per test group

II. RESULTS AND DISCUSSION

A. ANALYSES

Stability analyses
The stability of the test substance metiram in the diet was demonstrated over a period of up to 42 days at room temperature. As the mixtures were stored no longer than this time period, the stability was guaranteed.
A stability of BAS 222 28 F in the diet did not exist. Therefore, concentration control analyses directly after mixing and at the end of the feeding period were drawn for determining the stability.

Homogeneity control analyses
Considering the low relative standard deviation in the homogeneity analysis, it was concluded that metiram and BAS 222 28 F was distributed homogeneously in feed.

Concentration control analyses
The values of metiram and BAS 222 28 F in ground Kliba maintenance diet mouse/rat "GLP" meal were found to be in the range between 90-110% of the nominal concentrations. These results demonstrated the correctness of the concentrations of metiram and BAS 222 28 F in feed.

B. OBSERVATIONS

1. Clinical signs of toxicity
No test substance-related, adverse findings were observed in all test groups.

2. Mortality
No animal died prematurely in the present study.

C. BODY WEIGHT AND BODY WEIGHT GAIN

Mean body weights were significantly lower from study day 7 until study day 28 in male animals of test group 2 (metiram; 1500 ppm; -12% on study day 28) and test group 4 (BAS 222 28 F, 1901 ppm; -7.3% on study day 28) when compared to the control group.

Mean body weight change values of male animals were significantly lower during the entire study period, i.e. -25% in test group 2 (metiram; 1500 ppm) and -14% in test group 4 (BAS 222 28 F, 1901 ppm) between study days 0 to 28.

No relevant changes in body weight parameters were observed for male animals of test groups 1 and 3 (metiram; 500 ppm and BAS 222 28 F; 634 ppm).

Figure 5.8.2-2: Body weight development in rats administered metiram and BAS 222 28 F for 4 weeks.

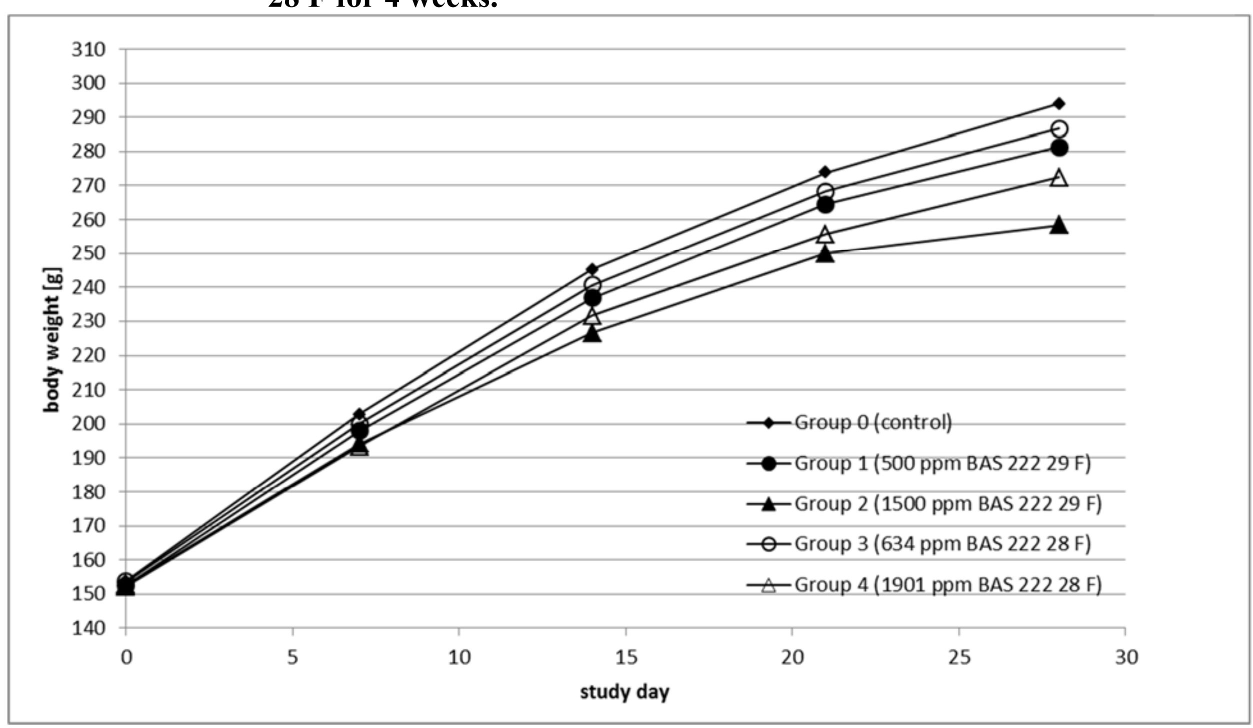


Table 5.8.2-6: Body weight and body weight gain data of rats administered metiram and BAS 222 28 F for 4 weeks

	Males				
Group	0	1	2	3	4
Test substance	none	Metiram	Metiram	BAS 222 28 F	BAS 222 28 F
Dose level [ppm]	0	500	1500	634	1901
Body weight [g]					
- Day 0	153.9	152.5	152.7	153.7	152.2
- Day 28	294.0	281.3	**258.3****	186.7	**272.5****
% (compared to control)		-4.3	-12.1	-2.5	-7.3
Overall body weight gain (Day 0 – 28) [g]	140.1	128.8	**105.7****	133.0	**120.3***
% (compared to control)		-8.1	-24.6	-5.1	-14.1

* p ≤ 0.05; ** p ≤ 0.01 (Dunnett's test, two sided)

D. FOOD CONSUMPTION AND COMPOUND INTAKE

Test substance-related impairments of food consumption were observed for animals of test groups 2 (metiram; 1500 ppm) and 4 (BAS 222 28 F, 1901 ppm). No treatment-related effects were observed in animals of 1 (metiram; 500 ppm) and 3 (BAS 222 28 F, 634 ppm).

Table 5.8.2-7: Food consumption data of rats administered metiram and BAS 222 28 for 4 weeks

	Males				
Group	0	1	2	3	4
Test substance	none	Metiram	Metiram	BAS 222 28 F	BAS 222 28 F
Dose level [ppm]	0	500	1500	634	1901
Food consumption [g]					
- Day 6-7	18.9	18.3	18.4	17.9	14.8
% (compared to control)		-3.2	-2.6	-5.3	-21.7
- Day 13-14	22.1	20.1	19.4	20.9	20.0
% (compared to control)		-8.8	-12.2	-5.2	-9.5
- Day 20-21	21.9	20.8	19.0	21.8	23.1
% (compared to control)		-5.5	-13.4	-0.9	5.2
- Day 27-28	22.6	20.8	20.3	21.2	18.9
% (compared to control)		-8.2	-10.2	-6.2	-16.4

* $p \leq 0.05$; ** $p \leq 0.01$ (Dunnett's test, two sided)

The mean daily test substance intake in mg/kg body weight/day (mg/kg bw/d) over the entire study period was calculated and is shown in the following Table 5.8.2-8:

Table 5.8.2-8: Mean daily test substance intake calculated over the entire study period in test groups 1 – 4.

Test group	Concentration in the diet (ppm)	Mean daily test substance intake (mg/kg bw/day)
1 (Metiram)	500	41
2 (Metiram)	1500	126
3 (BAS 222 28 F)	634	51
4 (BAS 222 28 F)	1901	150

E. FUNCTIONAL OBSERVATIONAL BATTERY

In the home cage observations, open field observations, sensorimotor test/reflexes and quantitative parameter no test substance-related effects were observed. The mean grip strength of hindlimbs in 5 male animals of test group 1 (500 ppm; metiram) examined on study day 25 was decreased compared to the control group. This finding was assessed as being incidental and not related to treatment as no dose-response relationship occurred.

F. MOTOR ACTIVITY MEASUREMENT

No significant deviations occurred concerning the overall motor activity (summation of all intervals) as well as the single intervals.

G. CLINICAL PATHOLOGY

Hematology:
No treatment-related changes among hematological parameters were observed.

At the end of the study in males of test group 4 (BAS 222 28 F, 1901 ppm), absolute and relative basophil cell counts were higher compared to controls, but the means were within historical control ranges (absolute basophil counts 0.00-0.06 Giga/L, relative basophil counts 0.0-0.9%). In males of test groups 1 and 2 (metiram, 500 and 1500 ppm), relative monocyte cell counts were decreased, but the values were also within the historical control range (relative monocyte counts 0.9-2.5%). Therefore, the mentioned alterations were regarded as incidental and not treatment-related.

Clinical chemistry:
No treatment-related, adverse changes among clinical chemistry parameters were observed.

At the end of the study in males of test group 1 (metiram, 500 ppm) glucose levels were higher compared to controls, but the values were not dose-dependently changed. Therefore, this change was regarded as incidental and not treatment-related.
In males of test group 2 (metiram, 1500 ppm) cholesterol levels were increased and in males of test group 4 (BAS 222 28 F, 1901 ppm) creatinine values were decreased. Both alterations were isolated without any other accompanied changes of clinical pathology parameters and therefore they were regarded as treatment-related but not adverse.

Hormones:
At the end of the administration period, in males of test groups 1 and 2 (metiram, 500 and 1500 ppm) as well as in those of test groups 3 and 4 (BAS 222 28 F, 634 and 1901 ppm) T4 levels were decreased by 27 and 25% in the mid dose and by 35 and 37% in the top dose (please see table below).
Additionally, in males of test groups 2 (metiram, 1500 ppm) T3 levels were higher compared to controls, but the increase was marginal (mean values <15% increase). This alteration was regarded as incidental and not treatment-related.

Table 5.8.2-9: Thyroid hormone levels determined in blood of 10 animals at terminal sacrifice (day 29)

Test substance and dose group [ppm]	Control	Metiram		Polyram (BAS 222 28 F)	
		500 ppm	1500 ppm	634 ppm	1901 ppm
T3 [nmol/l]	1.25 (0.17)	1.18 (0.15)	1.43** (0.16)	1.21 (0.14)	1.16 (0.18)
T4 [nmol/l]	88.73 (15.05)	64.75** (10.69)	57.31** (5.75)	66.98** (10.05)	56.05** (9.76)
TSH [µg/l]	5.76 (0.75)	6.10 (1.35)	10.72 (9.38)	6.33 (1.31)	8.65 (4.40)

**p<0.01

Urinalyses:
No treatment related changes among urinalysis parameters were observed.

H. NECROPSY

1. Organ weight

Comparing the absolute organ weights to control group 0 (set to 100%), the following mean absolute weights were significantly decreased in one or more test groups (Table 5.8.2-10). All other mean absolute weight parameters did not show significant differences when compared to the control group 0.

Table 5.8.2-10: Absolute organ weights

	Male animals			
Test group (ppm)	1 Metiram (500)	2 Metiram (1500)	3 BAS 222 28 F, Polyram (634)	4 BAS 222 28 F, Polyram (1901)
Terminal body weight	95%*	89%**	97%	91%**
Thymus	80%*	71%**	80%*	70%**

*p ≤ 0.05; **p ≤ 0.01

When compared to control group 0 (set to 100%), the mean relative weights of the following organs were significantly increased or decreased in one or more test groups (Table 5.8.2-11).
The following weights were outside the historical control range: terminal body weight of test group 2 (Metiram, 1500 ppm), relative brain weights, relative kidney weights, relative testes weights and relative thyroid gland weights in test groups 2 (Metiram, 1500 ppm) and 4 (BAS 222 28 F, Polyram, 1901 ppm). There was only a histopathological correlate in thyroid glands to explain the weight change. As there was no histological correlate for increased weights of brain, testis and kidney, they were considered to be of questionable significance and likely secondary to the body weight decrease.

All other mean relative weight parameters did not show significant differences when compared to the control group 0.

Table 5.8.2-11: Relative organ weights

	Male animals			
Test group (ppm)	1 Metiram (500)	2 Metiram (1500)	3 BAS 222 28 F, Polyram (634)	4 BAS 222 28 F, Polyram (1901)
Adrenal glands	107%	114%*	104%	106%
Brain	103%	111%**	100%	108%
Epididymides	111%*	114%**	102%	116%**
Kidneys	103%	114%**	107%**	112%**
Liver	104%*	115%**	104%	113%**
Testes	103%	113%**	102%	113%*
Thymus	83%*	79%*	83%*	77%**
Thyroid glands	111%	138%**	107%	134%**

*p ≤ 0.05; **p ≤ 0.01

2. Gross and histopathology

Foci were detected on the epididymis in 4 of 10 males of test group 1 (Metiram, 500 ppm) and 3 of 10 males of test group 2 (Metiram, 1500 ppm), as well as in each one male of test groups 3 (BAS 222 28 F, Polyram, 634 ppm) and 4 (BAS 222 28 F, Polyram, 1901 ppm).

All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

Treatment-related findings were observed in the thyroid glands of males of all treated test groups with comparable severity and incidence with the two test substances. Incidences and grading are shown in the Table 5.8.2-12 below:

Table 5.8.2-12: Histopathological observations

Thyroid glands	**Male animals**				
Test group (ppm)	0 Control (0)	1 Metiram (500)	2 Metiram (1500)	3 BAS 222 28 F, Polyram (634)	4 BAS 222 28 F, Polyram (1901)
No. of animals	10	10	10	10	10
Hypertrophy/hyperplasia, follicular cell		1	9	5	10
• Grade 1		1	2	1	2
• Grade 2			3	4	4
• Grade 3			4		4

Sperm granulomata in the epididymis correlating to foci detected macroscopically were noted in animals treated with both test substances. This finding can occur in up to 30% of control animals in studies at BASF SE. The incidence of this finding was not dose-dependent and it was not observed in previous studies with this test substance. Therefore, this finding was regarded as incidental.

All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

III. CONCLUSIONS

The administration of metiram and BAS 222 28 F (Polyram) via the diet to male Wistar rats for 4 weeks revealed comparable signs of toxicity at concentrations of 500 and 634 ppm (metiram: 41 mg/kg bw/d, and BAS 222 28 F: 51 mg/kg bw/d, respectively) as well as at concentrations of 1500 and 1901 ppm (metiram: 126 mg/kg bw/d, and BAS 222 28 F: 150 mg/kg bw/d, respectively) considering the effects on body weight, throid weights, thyroid histopathology and thyroid hormone changes.

Report:	*CA 5.8.2/3*
	█ 1979a
	Metiram (containing 2.2% ethylenethiourea) oral toxicity study in rhesus monkeys (repeated dosage for 26 weeks with recovery period)
	1979/0082
Guidelines:	*none*
GLP:	*no*

Executive summary:
In a study over 26 weeks metiram technical (purity: 96.82% metiram) with an ETU content of 2.2% was administered by gavage to four Rhesus monkeys (Macaca mulatta) per sex at three dose levels (0; 5; 15 and 75 mg/kg bw). Suspensions of metiram were prepared using 0.5% mucilage of tragacanth in destilled water as a vehicle. After administration had been completed, one monkey per sex and dose group was observed for an additional period of 15 weeks. One satellite group with two animals per sex and dose group (5 and 75 mg/kg bw) was used additionally to investigate the thyroid function (^{131}I-uptake). In the following study summary, the thyroid-related effects are described in more detail compared to the original entry in the Monograpth. The 6-months monkey has been considered as additional information, as the outcomes of regulatory studies in standard laboratory animal species rat, mouse and dog are assessable with more confidence, also in comparison to other AIs with similar toxicological data packages. Further – as with dog studies, the group sizes are small: including each 4 males and 4 female animals.

The following observations were made:
Salivation and occasional vomiting were observed as intolerance reactions to the test substance. Body weight gain, drinking water and food consumption were unaffected.
The hematological and clinicochemical examinations merely showed a dose-dependent adverse effect on the parameters of the thyroid function. From 15 mg/kg bw of the test substance onward, the values for T3 and T4 were below the control. After 24 weeks, T4 was reduced even at the lowest dose level (5 mg/kg bw), but that was due to an unusually high control value (see below). During the observation period, a normalization of T4 was observed in the animals used. The T3 values, however, continued to be reduced. The thyroid function tests of the satellite group using ^{131}I did not show any quantitative changes of the protein-bound ^{131}I in the plasma during the first 16 weeks of test substance administration. After 26 weeks, this parameter was increased in the high dose compared with the control. Both at 16 and 27 week 5 mg/kg dose was without effect. The uptake of ^{131}I by the thyroid was reduced in the two high dose groups at the beginning of the study, but it had returned to normal values after 16-week test substance administration. After 26 weeks, these values were even above the control at the two dose levels. In the highest dose group, the total uptake of iodine in the thyroid was increased, but the protein-bound fraction showed no change.

Statistical analysis revealed that the mean of absolute and relative thyroid weight for the group receiving 75 mg/kg/day was significantly increased when compared with the control group at the end of the study. The remaining organ weights were considered to be within normal limits, however statistical analysis also revealed that the relative liver weights for the group 75 mg/kg were significantly increased when compared to the control values. In the Monograph it is said, that there was an increase of testicular weights and a decrease of ovary weights at all the doses considered. The notifier would consider the increased prostatic and testicular weights, seen in a few animals to be a reflection of the stage of sexual maturity of these animals (e.g. animals #303 (5 mg/kg bw) and #311 (15 mg/kg bw) with considerably higher prostate and testicular weights, in which some spermatogenesis was evident, while the other male animals were sexually immature). Thus these findings are not considered to be treatment-related.

At the completion of a 1-week recovery period, the relative thyroid weights of animals receiving 75 mg/kg were increased. Histopathology findings: minimal thyroid follicular epithelial hyperplasia was seen in 6/6 monkeys receiving 75 mg/kg/day and in 2/6 monkeys receiving 15 mg/kg/day of metiram, but was not seen in control monkeys or monkeys receiving 5 mg/kg/day. No other histopathological abnormalities or variations from normal were encountered in the tissues examined that were attributable to treatment.

Thyroid weights

At terminal sacrifice 3 males and 3 females were killed and the thyroids were weighed. The individual values are given in the table below; a mean value over both sexes was calculated in the study. However as the sex-specific number of investigated animals was too low to allow for a statistical analysis, no statistics had been performed on the parameter thyroid weights.

Table 5.8.2-12: Individual absolute and relative thyroid weights determined at terminal sacrifice

Dose	Animal number	Sex	Abs. thyroid (left) R [g]	Abs. thyroid (right) [g]	Rel. thyroid weight [%]
Control	291	M	0.15	0.15	0.0100
	293	M	0.42	0.46	0.0314
	295	M	0.27	0.29	0.0151
	292	F	0.14	0.15	0.0105
	294	F	0.15	0.16	0.0113
	296	F	0.40	0.42	0.0293
Mean					**0.0179**
5 mg/kg bw	299	M	0.30	0.33	0.0225
	301	M	0.40	0.38	0.0190
	303	M	0.56	0.72	0.0267
	300	F	0.24	0.19	0.0156
	302	F	0.25	0.26	0.0170
	304	F	0.17	0.18	0.0106
Mean					**0.0186**
15 mg/kg bw	307	M	0.23	0.24	0.0174
	309	M	0.21	0.21	0.0145
	311	M	0.59	0.72	0.0323
	308	F	0.56	0.59	0.0418
	310	F	1.06	1.18	0.0815
	312	F	0.20	0.20	0.0136
Mean					**0.0335**
75 mg/kg bw	315	M	0.29	0.25	0.0220
	317	M	1.12	1.00	0.0707
	319	M	0.79	0.97	0.0457
	316	F	0.66	0.66	0.0463
	318	F	0.70	0.70	0.0410
	320	F	0.62	0.62	0.0397
Mean					**0.0442**

Although the group sizes are too small to perform a proper sex-specific evaluation, there seems to be an indication for higher relative thyroid weights in the treated groups dosed ≥ 15 mg/kg bw.

Necropsy results of the thyroid

Thyroid follicular hyperplasia was seen in several monkeys receiving 75 or 15 mg/kg bw/day as shown in the table below. Also here the sexes are combined.:

Table 6.8.2-14: Summary table of histopathological changes in the thyroid after terminal sacrifice

Dose levels [mg/kg bw]	Within normal limits	A few follicles with cuboidal cells	Majority of follicles with cuboidal cells	Follicles with cuboidal cells and papillary folding
Control	6/6	-	-	-
5	6/6	-	-	-
15	4/6	-	2/6	-
75	-	-	5/6	1/6

Thyroid hormones

The total thyroid hormones T3 and T4 were determined before treatment, 6, 12 and 24 weeks after treatment in each animal. All hematology and biochemistry parameter had been analysed before treatment, in order to learn about the natural variations depending on normal biological variations and the robustness of the used assays. For analysis of T4 the mean value out of 32 samples was 3.95 µgI% with a standard deviation of 1.68. The 95% ranges were 0.5 – 7.4 µgI%. The variation was lower for the T3 values (also taken out of 32 pre-treatment samples) with mean values of 1.52 +/- 0.25 ng/ml and a 95% range of 1.0 – 2.0. TSH, which is known to be a sensitive and robust parameter of thyroid hormone status was not measured.
The following table shows the individual values. A statistical analysis had been conducted after the study report has been published using a one-sided Wilcoxon test:

Table 6.8.2-15: Mean total T3 and T4 hormone values in male and female animals at different timepoints

Dose groups	Sex	Before treatment	6 weeks	12 weeks	24 weeks
T3 [ng/ml] (SD)					
Cont.	M	1.6 (0.3)	2.5 (0.4)	2.9 (0.7)	2.5 (0.8)
5	M	1.5 (0.2)	2.8 (0.3)	2.6 (0.1)	2.3 (0.7)
15	M	1.6 (0.3)	2.3 (0.4)	2.8 (0.7)	1.9 (0.5)
75	M	1.4 (0.2)	1.9 (0.6)	2.0 (0.4)*	1.9 (0.5)
Cont.	F	1.5 (0.2)	2.8 (0.1)	2.6 (0.1)	2.3 (0.7)
5	F	1.5 (0.4)	2.7 (0.6)	2.8 (0.5)	2.5 (0.9)
15	F	1.4 (0.2)	2.2 (1.1)	2.4 (0.2)	1.9 (0.5)
75	F	1.8 (0.2)	2.1 (0.9)	2.2 (0.2)*	1.9 (0.5)
T4 [µgI%] (SD)					
Cont	M	3.2 (1.2)	3.8 (0.7)	4.3 (0.5)	6.7 (1.0)
5	M	3.5 (0.9)	4.0 (0.9)	3.3 (1.0)	4.4 (0.5)*
15	M	5.1 (1.0)	3.9 (1.2)	2.9 (0.4)*	4.3 (0.6)*
75	M	3.9 (1.5)	4.0 (0.9)	2.5 (0.5)*	4.7 (0.9)*
Cont	F	4.3 (0.9)	4.0 (0.9)	3.6 (0.8)	5.1 (0.9)
5	F	4.0 (2.6)	3.5 (0.6)	3.3 (0.4)	4.5 (0.4)
15	F	3.3 (1.3)	3.3 (0.5)	3.4 (0.7)	4.4 (0.8)
75	F	4.3 (2.7)	4.2 (1.2)	2.7 (1.1)	4.3 (0.5)

*$p<0.05$

Statistically significant changes have been seen for the T3 values in the 75 mg/kg bw group at the 12 weeks time point only. At the same time point T4 values were statistically significantly decreased at 15 and at 75 mg/kg bw. At the 24-week time point the T4 values of the controls are relatively high, as well as the standard deviation, in this group the individual values were 5.50, 6.50, 6.80 and 8.00.

Enlarged thyroid glands were seen in male and female animals at ≥ 15 mg/kg bw. At ≥ 15 mg/kg bw also histopathological changes were seen in the thyroids of the investigate danimals (follicles with cuboidal cells). In order to properly interprete the thyroid-related findings of this study and to derive the correct NOAEL, all data needs to be taken into account, especially the correlation between histopathological findings and the thyroid hormone measurements.

Based on the facts, that

- the robustness of the thyroid hormone measurements is low (only 4 animals per sex/dose and high standard deviations),
- the mean control group value at 24 week time point is unusually high,
- the T4 changes at 5, 15, 75 mg/kg bw do not show a dose-response relationship at the 24 week time point
- more robustly assessed T3 values were changed at the 12 week time point at 75 mg/kg bw dose group in males and females only
- and the lacking correlation between thyroid weights, thyroid enlargements and histopathological thyroid changes

the NOAEL of this study using metiram spiked with 2.2% ETU is 5 mg/kg bw.

Conclusion:
After treatment of male and female monkeys with metiram spiked with 2.2% ETU, the thyroid was a target organ, as indicated by increased relative thyroid organ weights, increased incidences of histopathological findings in the thyroid at doses of ≥ 15 mg/kg bw and decreased T3 values at the 12 week time point of the study at 75 mg/kg bw dose group in males and females. Decreased T4 values were seen at the 12 week time point in males only at doses of ≥ 15 mg/kg bw. Based on the described findings, the NOAEL under this test conditions is established at 5 mg/kg bw/day.

Report:	*CA 5.8.2/4* *Tu A.S. et al., 1985a* *Evaluation of Metiram in the C3H-10T 1/2 cell system for transformation and promotion activities* *1985/209*
Guidelines:	*none*
GLP:	*no*

Executive summary:
Metiram was investigated in the *in vitro* cell transformation test in permanent C3H-10T 1/2 cells (mouse fibroblasts). The aim of the study was 2fold: First to detect the ability of metiram to induce cell transformation, which would suggest potential carcinogenic properties of the test substance. Secondly, to investigate whether metiram can enhance the rate of transformation in cells, which had been previously initiated with a suboptimal concentration of a known carcinogen MNNG. Transformation of cells induces visible changes which are denominated as type-II or type-III foci. Despite its poor solubility, metiram proved to be relatively cytotoxic in pretests. At a concentration of 1 µg/ml, growth of colonies was not observed.
In the first part of the assay (direct transformation) metiram did not lead to any significant increase of transformed cells (the so called type-II and type-III foci) under the test conditions chosen in a dose range of 0.1 - 1 µg/ml. Merely in the second assay in which promotion of transformation was evaluated an increase of type-II foci but not of the type-III foci was observed at a concentration of 0.3 µg/ml.

Conclusion:
Under the test conditions given, metiram seemed to enhance the rate of the cell transformation of cells previously exposed to a known carcinogen, but it showed no direct transformation potential. In this study, direct genotoxicity is not the cause of the positive test result in the promotion assay either.

Report: CA 5.8.2/5
Sakr S.A.,Shalaby S.Y., 2012a
Metiram-induced histological and histochemical alternations in liver and kidney of pregnant mice
2012/1368182
Guidelines: none
GLP: no

Executive Summary of Literature

Pregnant mice were administered orally with 28.4 mg/kg bw of metiram (purity 80%) for day 2 to day 19 of gestation. The treated animals and their controls were sacrificed by decapitation on the day 19th of gestation. Their livers and kidneys were removed and fixed in 10% formalin. Fixed materials were embedded in paraffin wax and sections of 5 micrometer thickness were cut. Slides were stained with haematoxylin and eosin for histological examination. For histochemical study specimens were fixed in Carnoy's fluid. Periodic acid Schiff's reaction was used for demonstration of polysaccharides. Total proteins were detected using the mercury bromophenol blue method. Results obtained in this work showed that treating pregnant mice with metiram induced many histopathological changes in the liver and kidney cortex. Examination of liver of metiram-treated mice displayed apparent signs of degenerative changes. The normal structural organization of the hepatic lobules was impaired and the characteristic cord-like arrangement of the normal liver cells was lost. In addition, severe inflammatory leucocytic infiltrations were abundant. Such inflammatory infiltration is spread over several liver areas and around the blood vessels. Enlargement and congestion of blood vessels, especially veins were observed. The hepatocytes were clearly manifested by marked cytoplasmic vacuolization and most cells showed nuclei with signs of karyolysis and pyknosis. Moreover an obvious fatty degeneration indicated by large number of fatty droplets with different size was observed. The histochemical observation revealed reduction of total carbohydrates and total proteins in the hepatocytes. Looking at the kidneys of animals treated with metiram showed degeneration and deterioration of the cortical constituents. The epithelial linning of the renal tubules appeared with cloudy swelling and vacuolated cytoplasm with pyknotic nuclei. Intertubular leucocytic infiltrations were observed. A number of glomerular capillaries were suffering from severe signs of glomerular congestion, while others were completely damaged. Histochemical observations also revealed reduction of total carbohydrates and total proteins.

Conclusion of the author:

Metiram orally applied to pregnant mice during gestation day 2 and 19 induces histological and histochemical alterations in liver and kidney of these mice. It is suggested that the histological and histochemical alterations observed in liver and kidney of pregnant mice by metiram may be mediated by depletion of antioxidants and elevation of lipid peroxidation.

Conclusion of the applicant:

That metiram causes liver and kidney damages in mice is described by the authors in other literature. Why they used in this study pregnant mice is not described since a different outcome is not expected. That the alteration observed in liver and kidney are mediated by depletion of antioxidants is speculative since no measurements concerning oxidative stress were done. Since no new substantiated information about the mode of action of metiram is given in this study, this study may at most be used as supplemental information but is not considered relevant for risk assessment.

Classification of study: Supplemental information

CA 5.8.3 Endocrine disrupting properties

Metiram is not classifiable for carcinogenicity or reproduction toxicity and it is thus not falling under the interim criteria for endocrine disruption, as depicted from the 1107/2009. Up to now, there is no other scientific criteria available on the regulatory definition of an endocrine disrupting compound.

Nevertheless the target organ in rats and dogs after dietary administration of metiram is the thyroid. Furthermore, there is evidence, that ethylene thiourea (ETU) – a known thyroid-active compound – is formed during *in vivo* metabolism of metiram in rats. The thyroid-related toxicity observed in studies conducted with metiram is considered to be related – at least in major parts – to internal (systemic) ETU formation. This is based on observations in in vivo rat metabolism studies and in the detections of ETU (and to a minor extent ethylene urea) in urine of rats dosed metiram for 90-days (see Chapter MCA 5.7). Based on the already evaluated studies a mean coversion of metiram into ETU of 3% was determined gavage dosing of radioactive metiram to rats. This value is taking into account the differences in molecular weights and the conversion from one molecule of metiram into 4 molecules of ETU. According to the more recently conducted in vivo rat metabolism study the analyzed percentages of ETU in urine (when dose-normalized to molecular weight) were 6.67% after a single dose of 5 mg/kg bw, 3.62% after a single dose of 50 mg/kg bw and 4.55% after repeated doses of 50 mg/kg bw, also indicating no accumulating properties of ETU in the body (see Chapter MCA 5.1, table 5.1.1-12). A similar metabolic/ (degradation pattern has been seen in an in vitro comparative metabolism study using hepatocytes of humans, rats, mice, dogs and rabbits (see Chapter MCA 5.1).

There are no in vitro mechanistic studies available, investigating the thyroidal mode of action of metiram. The compound is a complex, which is insoluble in aqueous solution. In DMSO metiram is rapidly degrading forming degradates, which are in quality and quantity not identical to what happens in pure aqueous solution or *in vivo*. For the major metabolite ETU in vitro mechanistic studies on thyroidal mode of action are available. These studies indicate a reversible inhibition of the thyroid peroxidase.

Any assessment on the endocrine activity of metiram should be done by evaluating the studies conducted with the active ingredient metiram. Therefore, in the following a short overview over the relevant metiram studies (subchronic, chronic and reproduction toxicity) and NOAELs/LOAELs identified for thyroid toxicity is given and an assessment on different susceptibilities towards expected exposure to humans is provided. The subchronic studies conducted with ETU-spiked metiram are not further discussed in this chapter, as the addition of 2.2% ETU to the technical batch is not representing a realistic exposure situation. For some endpoints (long term and carcinogenicity, teratogenicity) only studies with metiram spiked with 2.2% ETU have been conducted, representing a worst-case approach in terms of thyroid-related hazard assessment of the parent.

Subacute and subchronic toxicity studies

A 28-day side-by-side comparing the toxicity of metiram and polyram (BAS 222 28 F) and two 90-day rat studies were conducted with metiram in rats: 90-day neurotoxicity study including neurofunctional parameter and the 90-day subchronic neurotoxicity study. Additionally the 2-generation toxicity study is included in this overview, as additional thyroid parameters (weights, histopathology and hormones) were included in this study. In dogs a 19-week and a 1-year toxicity study has been conducted. The 19-week study is not further evaluated in this chapter, as in this study the dietary administrations to dogs has been changed several times during the administration period and no conclusive picture on the toxicity and the NOAEL of the study could be reached. The 1-year dog study is included in the following targeted evaluation. The thyroid effects are summarized in the table below.

Table 5.8.3-1: Evaluation of the thyroid-related parameter in the subacute and subchronic studies in rats and dogs

Study Species	Test substance	NOAEL/LOAEL	Effects
28-day dietary study Wistar rats	Metiram* Batch 300015	< 500 ppm (41 mg/kg bw) / 500 ppm (41 mg/kg bw)	Thyroid weights ↑, follicular hypertrophy ↑, T4 levels ↓
90-day dietary study incl. neurofunctional parameter Wistar rats	Metiram	320 ppm (25.4 mg/kg bw) / 960 ppm (81 mg/kg bw)	Thyroid weights ↑ without histopathological correlate, thyroid hormone changes
90-day neurotoxicity study Wistar rats	Metiram Batch 300015	200 ppm (13/17 mg/kg bw in males & females) / 900 ppm (59/71 mg/kg bw in males & females	Males: T4 levels ↓ Females: thyroid weights ↑, thyroid follicular hypertrophy (one female), T4 levels ↓
2-Generation toxicity study	Metiram Batch 300015	Males:** < 100 mg/kg bw (9 mg/kg bw)/100 ppm (9 mg/kg bw) Females: 350 ppm (31 mg/kg bw)	Males:** ≥ 100 ppm: follicular hypertrophy/hyperplasia ≥ 350 ppm: T4 levels ↓, TSH levels ↑ 1000 ppm: thyroid weight ↑; unilateral follicular cell adenoma in thyroid gland of each one male in F0 and F1 Females: ≥ 350 ppm: follicular hypertrophy/hyperplasia 1000 ppm: thyroid weight ↑, T4 levels ↓
1-year dog study	Metiram	80 ppm (2.59 mg/kg bw)/1000 ppm (29.9 mg/kg bw)	≥ 1000 ppm: thyroid weight ↑ (not statistically significant at 1000 ppm, but only at 3000 ppm) thyroid follicular hyperplasia

*as there were no differences seen between the toxicity of metiram and polyram, only the effects of metiram are shown in this table.
**in this table only the effects in the parental generation are shown

After dietary administration of metiram to rats, increased weights of the thyroid, thyroid follicular hyperplasia/hypertrophy and decreased T4 levels are seen. In most cases hormone level changes occur at the same doses, than pathological or organ weight effects; it seems, that thyroid histopathology is the most sensitive parameter. The lowest NOAEL in rat studies is < 9 mg/kg bw (a BMD of 5.5 mg/kg bw has been calculated), with a LOAEL of 9 mg/kg bw in male rats. The NOAEL for the dog was found to be 2.59 mg/kg bw (with a calculated BMDL of 3.25 mg/kg bw) in a 1-year dog study, the respective LOAEL was 29.9 mg/kg bw.

Long term and carcinogenicity studies in rats and mice

The chronic and carcinogenicity study conducted with metiram (spiked with 2.2% ETU) did not give evidence for a carcinogenic response. The slightly increased incidence of benign thyroid adenoma was within historical control data of studies conducted during the same time span. The lowest NOAEL for rats was 3.1 and 3.8 mg/kg bw for male and female rats, thereby also covering the thyroid effects seen in the parental animals of the reproduction toxicity study.

Reproduction toxicity

With regard to reproduction toxicity of metiram the enhanced reproduction toxicity study (enhanced means the additional of a detailed thyroid investigation of parentals and offspring including thyroid hormone determination) did not give evidence for reproduction or developmental toxicity of metiram or for effects on fertility. There was also no evidence for any treatment-related thyroid-related reproduction toxicity seen in the offspring animals, like e.g. body weight development, behaviour, sexual maturation, brain weights, or any effects on the thyroid in offspring. Table 5.8.3.-2 gives an overview over the treatment-related effects in maternal adults and offspring animals. Statistically significantly decreased T4 levels (-23%) were only seen at 1000 ppm (92 mg/kg bw) females of the F0 generation. In F1 females at 350 ppm a statistically significant decrease in T4 levels was seen, but not at the next higher dose of 1000 ppm. The thyroid follicular hypertrophies/hyperplasia was non significantly increased above historical controls at ≥ 350 ppm in the F1 females only. It has to be noticed, that the actual substance intake is higher in the F1 parental compared to the F0 parental generation.

Table 5.8.3-2: Thyroid (related) effects seen in parental females and offspring during the enhanced 2-generation toxicity study in rats

Test material	Doses Study type	Effects on parental females	Effects on reproduction/pups
Metiram, 91.5%	100 (9), 350 (31), 1000 (92) ppm OECD 416, 2011	F0 generation: ≥ 350 ppm: body weight/body weight gain ↓, terminal body weight ↓, food consumption ↓, 1000 ppm: thyroid weight ↑, T4 levels ↓ (-23%) F1 generation: ≥ 350 ppm: body weight/body weight gain ↓, food consumption ↓, follicular hypertrophy/hyperplasia 1000 ppm: thyroid weight ↑	F1 pups 1000 ppm: body weight on some days during lactation (-5 - -15%)↓*, T4 (-20%)↓ f (PND4), TSH (120%)↑ f (PND4), F2 pups: body weight at some days during lactation (-5 - -8%)↓* No effects on reproduction, fertility or development

*related to maternal body weight decrease

While the decreae in T4 levels in parental F0 animal was marginal, no thyroid hormone changes were evident in females at 31 mg/kg bw. The offspring generally was less susceptible to develop signs of toxicity in this enhanced 2-generation toxicity study. Only the F1 pups, not the F2 pups showed lower T4 levels at PND 4, but not at PND 21. At none of the sacrifices (conducted at PND 4 and PND 21) thyroid pathological effects were observed in the offspring animals. For more details please refer to Chapter MCA5.6.

It can be concluded, that metiram is not affecting the fertility, offspring and development of rats. The marginal effects on T4 levels (-23%) was only seen in the top dose of the F0 generation, no consistent effects on T4 levels were seen in the F1 females. At this dose level the only parameter affected in the offspring, where slightly lower pup body weights at some days during lactation were observed.

Although the metiram 2-generation toxicity study included additional thyroid parameters (thyroid weight and histopathological examination and thyroid hormone level determination in adults and offspring), developmental neurotoxicity and the learning behaviour was not assessed in this study. However in this study, the effects on the thyroid were determining the NOAEL setting of the study. Also in the ETU extended one generation toxicity study, the thyroid – together with the pituitary – is the most susceptible organ. This extended one-generation toxicity study includes two developmental neurotoxicity cohorts (PND 22 and PND 78) and all elements of a comparative thyroid toxicity study are covered by the study design. The study is summarized in detail in Chapter MCA5.8.

Also in this extended one-generation toxicity study conducted with ETU, no effects on the reproduction or fertility of the animals were seen. The only effect seen in offspring animals were lower brain sizes – a finding of low relevance - at the 10 mg/kg bw group. No changes in brain histopathology were seen, nor was there any evidence for (learning) behavioural changes in the offspring animals at all doses. The NOAEL of 0.2 mg/kg bw (2.8 ppm) was based on thyroid follicular hyperplasia together with increased thyroid weights and thyroid hormone changes seen at the next higher dose level of 2 mg/kg bw. At 2 mg/kg bw the T4 levels in the female F0 generation were decreased by -35% (1.03 µg/dl vs 1.60 µg/dl in controls), while at the top dose of that study (9.7 mg/kg bw), T4 was decreased by – 75%. At the top dose of 9.7 mg ETU/kg bw, the TSH levels were more than 3-fold increased in F0 females, indicating clearly higher thyroid activity of ETU compared to metiram (for details please see Chapter MCA 5.8).

Conclusion

Metiram is adequately tested in toxicological studies. Metiram has effects on the thyroid – as specified by increased thyroid weights, increased incidences of follicular hyperplasia/hypertrophy and slightly decreased T4 levels (sometimes accompanied by increased TSH levels in male rats only) in rats and dogs.

All relevant thyroid-related parameter during all sensitive windows of exposure can be adequately assessed with the available enhanced 2-generation toxicity study of metiram in comparison with the comprehensive extended one-generation toxicity study conducted with ETU. No effects on reproduction toxicity, fertility, development, neurodevelopment, learning behaviour, or carcinogenicity are observed in toxicological data of metiram, especially no effects which might be (secondary) related to thyroid toxicity. As the rat is considered to be a (over-) sensitive species with regard to thyroid(-related) toxicity (see Chapter MCA5.8), the relevant NOAELs – derived in the available enhanced reproduction toxicity and long-term toxicity studies with metiram are considered to fully protect humans in terms of hazard and risk assessment, including all relevant endocrine endpoints. The overall long-term NOAELs for AOEL- and ADI-setting are based on the 1-year dog study (BMD: 3.25 mg/kg bw) and the 2-year rat study (3.1/3.8 mg/kg bw) supported by a calculated BMD of the enhanced reproduction toxicity study (5.5 mg/kg bw).

Thus it can be concluded, that metiram can be handled safely. From a weight of evidence point of view – taking into account the lacking severity of effects and the uncertainties in the mode of action – metiram itself shall not be considered an endocrine disruptor.

CA 5.9 Medical Data

There were no medical surveillance or monitoring studies available on metiram. Six studies referring to EBDC pesticides use are summarized as well as one reference, comparing urinary ETU levels of different populations.

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

Report: CA 5.9.1/1
Amelsvoort L.G.P.M. van et al., 2008a
Immune effects and exposure to Ethylenebisdithiocarbamate pesticides in re-entry workers in the Netherlands
2008/1102537
Guidelines: none
GLP: no

Limitations of the publication include the inability to demonstrate objectively that the re-entry workers were exposed to higher levels of exposure during the application period than the non-exposed group.

Executive Summary:
The field study was conducted in the Netherlands to investigate the short-term and long-term immune effects of occupational exposure to ethylene-bis-dithiocarbamates (EDBCs), in particular mancozeb. The investigation formed part of the EUROPIT study and included 41 re-entry workers and 40 non-exposed controls. The subjects were investigated before the seasonal application of pesticides (T0) and 30 days after the beginning of the application period (T30). At T0 and T30 immune parameters were determined in blood and all participants filled in a questionnaire level regarding exposure and outcome parameters, and all subjects underwent a comprehensive medical examination at T0. The level of ethylenethiourea (ETU) in urine was determined as indicator of exposure. No relevant adverse immune effects were found in the pesticide exposed workers compared with the non-exposed controls. Also no exposure response relationship between immune effects and ETU was round. However, there were no significant differences between ETU levels among exposed subjects and controls at T30, and no significant change in levels from T0 in either group.

Objective:
To investigate the possible effect of occupational exposure to dithiocarbamate fungicides, in particular mancozeb, on immune function.

Materials and Methods:
This Dutch investigation formed part of the EUROPIT study designed to evaluate potential immunological effects of pesticide exposure with a focus on ethylene-bis-dithiocarbamates (EDBC). The Dutch field study was one of 5 field studies conducted in 4 countries, and included 41 male flower bulb growers (predominantly re-entry workers) with occupational exposure to dithiocarbamate fungicides including mancozeb. The control group consisted of security guard and office personnel (37 men, 3 women).

The study design was largely cross-sectional, but with a longitudinal component. A baseline questionnaire was completed by subjects at the start of the field study after a 30·day exposure-free period (T0). The baseline questionnaire collected information on demographic factors, job history and pesticide use, presence of chronic diseases, allergies, recent episodes of fever and/or infections, use of drugs, alcohol intake and smoking habits. A medical doctor reviewed drugs being taken by subjects for their ability to cause immune changes. In addition, all subjects underwent a comprehensive medical examination at T0, samples of urine and blood were collected, and subjects received a hepatitis B vaccination. Approximately 30 days after the start of the application season of pesticides (time T30), subjects completed a second questionnaire which collected information on smoking and drinking habits, the presence of fever and infections, and use of drugs and pesticides in the past 30 days. The length of follow-up period was chosen because crop treatment with mancozeb usually lasts around one month (and consists of 5-8 applications). At T30 further blood and urine samples were collected, and subjects were given the second course of the Hepatitis B vaccination. The final course was given at 6 months after T0. Immune system investigation was performed at T0 and T30 using a range of blood cellular and serum parameters: complete and differential blood cell counts; lymphocyte clusters of differentiation including CD3 (Total-T cells), CD4 (helper/inducer), CD8 (suppressor/cytotoxic), CD19 (B-cells), and CD56/CD16 (NK cells); and serum immunoglobulins (IgG1, IgG4, IgM). Functional assays were assessed in order to evaluate the capacity of pesticide exposure to affect the immune response to antigens and allergens including specific IgE (allergens tested at T0 and T30 were cat, dog, house dust mite, grass, tree and herb pollen, and fungi) and the anti-HB antibodies after hepatitis B vaccination (at T0, T30, T30 + 2 weeks, and 6 weeks after the final vaccination). EDBC exposure was assessed at T0 and T30 by measuring urinary ethylenethiourea (ETU), a major metabolite of EDBC chemicals in humans. However, the investigators noted that it was not possible to collect urine samples immediately after exposure for technical reasons, and the half-life of EDBCs in human is relatively short, about 10 hours.
The main statistical analyses consisted of comparisons between the exposed and control groups, either baseline values or changes after exposure (differences between T30 and T0). At baseline, least square means were adjusted for age, gender, and current smoking using regression analysis. Subjects with anti-HBs titre above 10 at baseline, or who had already been vaccinated, were excluded from the analysis of anti-HB levels, and mean results were adjusted for baseline levels, smoking status and age. IgG4, IgE, ETU and anti-HBs titre levels were log transformed to account for the skewed, log-normal distribution, and geometric mean levels were calculated. Statistical analyses were performed using the SAS statistical package

Results:
Exposed and control subjects were well matched for age, body mass index, alcohol consumption and smoking habits, but the exposed workers were less educated ($p<0.001$).
Geometric mean levels of urinary ETU in exposed workers at T30 were not significantly different from those at T0 (1.27 and 1.04 mg/mmol creatinine, respectively) and similar to those in controls at T30 and T0 (1.16 and 1.17 mg/mmol creatinine, respectively). However, 29% (12) of the exposed workers reported poisoning symptoms during the exposure period (mainly headaches), compared to none of the controls, but these may not have been due to EDBCs exposure.

At baseline, there was no significant difference between exposed and control subjects in the prevalence of most of the health outcomes (assessed by questionnaire and medical examination). The prevalence of a skin rash for more than 6 months ever was significantly higher among the exposed subjects (29.3% v 7.7%), but for itchy or watering eyes, and medically diagnosed hay fever, prevalences were significantly lower among the exposed subjects. In addition, the differences in most immune parameters at baseline between control and exposed subjects were small and insignificant, but IgM levels which were significantly higher (p=0.003) among controls (1.80; 95% CI 1.48-2.11) than exposed subjects (1.41; 95% CI 1.11-1.72). Total IgE levels were also higher among controls (0.93; 95% CI 0.52-1.67) than exposed subjects (0.53; 95% CI 0.30-0.93), but did not reach statistical significance.
The longitudinal analysis of changes in immune parameters after a 30-day exposure period (T30-T0) showed no significant differences between changes in exposed subjects compared with controls except for the T-lymphocyte and T-helper cell counts, which significantly increased in the controls, but not in the exposed (see Table). No difference in immune response after the hepatitis B vaccination was observed between the exposed and control subjects.

Table 5.9.1-1: Mean differences (T30-T0) with 95% CI of selected immune parameters among exposed and control subjects. Parameters shown are those with significant differences (p<0.1) between exposed and control groups in either the difference parameter or baseline result.

Parameter	Controls	Exposed
	Mean (95% CI)	Mean (95% CI) p value, exposed v control
T·lymphocytes (abs)	205 (92,317)	-15(-123,92) **
T·helper cells (abs)	117.8 (48.2, 187.4)	-13.6 (-80.2,52.5) **
B·lymphocytes (blmfa) (abs)	41.2 (13.4,69.2)	7.6 (-19.0,34.1)
IgG1	-0.29 (-0.86,0.28)	0.45 (-0.12,1.02)
IgM	-0.61 (-0.92, -0.29)	-0.65 (-0.96,-0.34)
IgE	1.48 (-0.11,3.07)	-0.20 (-1.76,1.35)

***p<0.05, ** p<0.01, *** p<0.001**

Conclusions:

The investigators were not able to demonstrate objectively that the exposed group had experienced more exposure to EDBCs than the control group. The investigators concluded that no relevant adverse immune effects were found in the pesticide exposed workers compared with the non-exposed controls.

Report: CA 5.9.1/2
Steerenberg P. et al., 2008b
Toxicological evaluation of the immune function of pesticide workers, a European wide assessment
2008/1102541
Guidelines: none
GLP: no

Limitations of the study include multiple comparisons and the cross-sectional design.

Executive Summary:

This cross-sectional investigation was carried out in five field studies in: the Netherlands (flower bulb growers, mainly re-entry workers), Italy (vineyard workers), Finland (potato farmers), and Bulgaria (workers from a zineb factory and greenhouse workers) to evaluate a possible association between occupational exposure to pesticides, verified by urinary levels of ethylenethiourea (ETU) the main urinary metabolite ethylene-bis-dithiocarbamates (EBDC), and haematological parameters and components of the immune defence. Immunotoxicity was studied by measuring haematological parameters, complement, immunoglobulins, lymphocyte subpopulations, natural killer cells, autoimmunity, and antibody responses to hepatitis B vaccination. A total of 248 workers exposed to EBDC and 231 non-occupationally exposed subjects entered the study. A significantly higher level of ETU in occupationally exposed subjects compared with controls (2.7 ± 8.1 µg/g vs 0.5 ± 3.7 µg/g creatinine) was found 30 days after start of pesticide application. The total study population consisted of 248 pesticide-exposed and 231 non-occupationally exposed workers. At the same time point, statistically significant differences between exposed and controls were found for complement C3 and C4 and the immunoglobulin classes IgG4 and IgA. For complement and IgG4, the levels were slightly increased and the level of IgA was decreased. Erythrocyte and leucocyte counts were also increased among the exposed group, and the CD8 subpopulation was also increased. No effects were found on autoimmune antibodies and antibody response to hepatitis vaccination. In conclusion, pesticide exposure, specifically exposure to EBDC, under various work place conditions in Europe was associated only with some subtle effects on the immune system, which may suggest that occupational exposure to EBDC does not influence the immunologic system in a clinically significant fashion, and does not pose a significant health risk to the exposed subjects.

Objective:

To investigate the possible effect of occupational exposure to EBDC on haematological parameters and components of the immune defence.

Materials and Methods:
The cross-sectional investigation formed part of the EUROPIT study designed to evaluate potential toxicological effects of pesticide exposure on the human immune system. Five field studies were conducted in four countries. The field studies included 40 flower bulb growers and 39 controls in the Netherlands, 52 potato farmers and 48 non-exposed biological farmers in Finland, 47 vineyard workers and 45 controls in Italy, 61 greenhouse workers and 56 controls from Bulgaria and a further group of Bulgarian subjects including 49 workers from a zineb producing facility and 53 unexposed controls mainly from the same factory. Overall there were 248 EBDC exposed and 231[2] non-occupationally exposed workers.
Blood and urine samples were collected twice in pesticide-exposed workers, and once in controls. Sampling in pesticide-exposed workers was performed before the pesticide application period at baseline (T0) and approximately 30 days after the beginning of the application period (T30). The serum and cellular immune parameters investigated included complete and differential blood cell counts, lymphocyte subpopulations (CD3, CD4, CD8, CD19, and CD56/CD16 natural killer cells), α-1-glycoproteins, erythrocyte sedimentation rate (ESR), complement 3 and 4 fractions (C3, C4), anti-nuclei (ANA), anti-smooth muscle (SMA) and anti-mitochondria (AMA) autoantibodies, and serum immunoglobulins (IgG1, IgG4, IgM, IgA, total and specific IgE to most common allergens). Hepatitis B (HB) vaccination of non-immunized subjects was used as a challenge test to assess immune function. Measurement of anti-HB antibodies was carried at the baseline sampling collection time. Subjects that had titres to hepatitis before vaccination were excluded from the analysis. All the other subjects underwent anti-HB vaccination at baseline, and a second anti-HB vaccination was performed 35 days later. Anti-HB antibodies were measured at day 49 after the first vaccination. EBDC exposure was assessed by measuring urinary ethylenethiourea (ETU), a major metabolite of EBBC chemicals in humans, before the EBDBC application season started and again after one month of exposure.

Least square mean averages were calculated comparing the levels between exposed and control individuals at T30 and were adjusted for work package, age, gender, and current smoking using regression analysis. Levels of erythrocyte count and IgG5, IgM, and IgA were log-transformed to account for the skewed, log-normal distribution of these parameters, and geometric mean levels are presented for these parameters. Mixed effect models were used to account for the multilevel data structure and analyses were performed using the SAS and GLIM statistical packages.

Results:
Exposed and control subjects were well matched for age, sex, body mass index, alcohol consumption and smoking habits, but the agricultural nature of the jobs of most exposed workers meant that they were more likely to live in rural areas (63.8% v 37.4%, $p<0.001$) or have lived on a farm (67.6% v 36.3%, $p<0.001$), have low education (33.3% v 9.7%, $p<0.001$), and more likely to have regular sunlight exposure (78.8% v 66.5%, $p<0.01$).

[2] The numbers of exposed subjects and controls given for the 5 individual studies add to 249 and 241, respectively.

Significantly higher levels of ETU were observed in urine of the exposed group at T30 when compared to controls, with geometric means (geometric sd) of 2.7 (8.1) µg/g creatinine and 0.5 (3.7) µg/g creatinine, respectively ($p<0.05$). Erythrocyte count and leucocyte count were significantly higher in exposed subjects (p=0.04 and p=0.00005, respectively) but haemoglobin, haematocrit, thrombocyte count and the percentages of lymphocytes, monocytes, eosinophils, and neutrophils showed no differences between the exposed group and the control group. Among lymphocyte subpopulations, the number of CD8 cells was significantly increased (p=0.01) among exposed subjects, and as a consequence, the ratio CD4/CD8 was significantly decreased (p=0.01). Statistically significant differences were found for complement C3 and C4, IgG4 and IgA, but there were no significant differences for glycoproteins, ESR, IgG4 and IgM. For complement and IgG4, the levels were slightly higher in exposed workers, but IgA levels were lower in exposed workers. .No differences were observed in ANA, SMA, and SMA autoantibodies between the exposed and the control group, and no difference in antibody response to hepatitis B vaccination was observed at day 49 after the first vaccination, with mean (sd) levels of IgG to HB of 2.1(1.9) IU/L and 2.3(2.0) IU/L among exposed and controls, respectively.

Table 5.9.1-2: Blood cellular and serum immunoglobulin parameter with significant differences between the exposed group and control group.

Parameter	Exposed	Control
	Mean (min-max)	**Median (min-max)**
Peripheral blood cell counts		
Erythrocyte count[a] $10^6/mm^3$	4.79 (1.73–4.84)	4.69 (4.60–4.77) *
Leucocyte count[a] $10^3/mm^3$	6.88 (6.65–7.11)	6.17 (5.83–6.51) ***
CD8 T-cytotoxic/suppressor cells	564 (529–600)	485 (433–538) *
CD4/CD8 T-helper/T-suppressor ratio	1.94 (1.82–2.05)	1.94 (1.82–2.05) *
Immunological parameters		
Complement C3 g/L	1.22 (1.19–1.25)	1.16 (1.12–1.20) *
Complement C4 g/L	0.26 (0.25–0.27)	0.24 (0.23–0.25) *
~IgG4 mg/mL	0.0095 (0.0088–0.0102)	0.0071 (0.0064–0.0079) ***
~IgA mg/mL	1.44 (1.31–1.58)	2.34 (2.04–2.68) ***

*$p<0.05$, ** $p<0.01$, *** $p<0.001$
[a] geometric mean

Conclusions:
The investigators concluded that pesticide exposure, specifically exposure to EBDC, under various work place conditions in Europe was associated only with some subtle effects on the immune system, and this may suggest that occupational exposure to EBDC does not influence the immunologic system in a clinically significant fashion, and does not pose a significant health risk to the exposed subjects.

Report: CA 5.9.1/3
Swann G.M.H. et al., 2008b
Occupational exposure to Ethylenebisdithiocarbamates in agriculture and allergy: Results from the Europit field study
2008/1102538
Guidelines: none
GLP: no

Limitations of the study include the low exposure of exposed subjects in one field study, self-reported endpoints, the cross-sectional design and inadequate reporting.

Executive Summary:
The study was conducted in four countries (the Netherlands, Finland, Italy and Bulgaria) to evaluate the possible association between occupational exposure to ethylene-bis-dithiocarbamates (EBDC) and allergy. A total of 248 workers exposed to EBDC and 231 non-occupationally exposed subjects entered the study. Exposure to EBDC was measured as urinary ethylenethiourea (ETU) in samples collected at baseline (T0) and after 30 days of exposure (T30). The effect parameters, evaluated at T0 and T30, included self-reported questionnaire data on allergy, the Phadiatop allergy screening test and specific IgE parameters. Cross-sectional as well as longitudinal comparisons were made, adjusted for potential confounding factors. No association was found between exposure status, EBDC levels and allergic contact dermatitis, allergic rhinitis, food allergy, and atopy as measured by the Phadiatop. The prevalence of skin irritation was elevated in the Dutch field study only, but is more likely a result of plant contact rather than EBDC exposure. It is concluded that the EBDC exposure levels experienced in the field study are not associated with increased prevalence of allergic symptoms or allergy.

Objective:
To investigate the possible effect of occupational exposure to EBDC on allergic symptoms including skin irritation, contact dermatitis, allergic rhinitis and food allergy.

Materials and Methods:
The investigation formed part of the EUROPIT study designed to evaluate potential immunological effects of pesticide exposure with a focus on ethylene-bis-dithiocarbamates (EBDC). Five field studies were conducted in four countries. The field studies included 42 flower bulb growers in the Netherlands and 40 controls, 51 potato farmers and 49 controls in Finland, 48 vineyard workers and 45 controls in Italy, 55 greenhouse workers and 45 controls from Bulgaria, and a further group of Bulgarian study group of 52 workers from a zineb producing facility and 52 unexposed controls (obtained by calculation). Overall there were 248 EBDC exposed and 231 non-occupationally exposed subjects who were selected to achieve optimal comparability in age, gender and educational level.

The study design was predominantly cross-sectional, but with a longitudinal component. Each field study was set up so that baseline data were collected from both groups before the EBDC application season started (time T0) and again for both groups after one month of exposure (time T30). These investigations included blood samples used to determine IgE and a Phadiatop test to detect reactions to a range of common allergens including cat, dog, house dust mite, grass, tree and herb pollen, and a mixture of fungi. At the same time points, EBDC exposure was assessed based on job description and by measuring urinary ethylenethiourea (ETU), a major metabolite of EBDC chemicals in humans. A self-administered questionnaire was used to collect information on other effect parameters including skin irritation, contact dermatitis, allergic rhinitis and food allergy. A questionnaire was also used to collect information on job description, potential confounders including age, country, residence, education, gender, smoking, alcohol consumption, diet, medications and sunlight exposure.

The main statistical analysis consisted of comparisons between the exposed and control groups at baseline or after exposure. The study design allowed for a longitudinal analysis (T30 versus T0), but short term changes in most of the effect parameters were not considered to be relevant by the investigators. Analyses were also made in which ETU concentration at T30 was used as exposure metric because there were substantial differences in ETU levels between the five field studies. ETU concentrations followed a log-normal distribution and were log-transformed. Also mean values for the job held by a subject were used in the analysis because of the high variability in individual values.

Multivariate logistic regression analyses were performed using the self-reported allergies as dependent variables. Models were adjusted for either demographic variables (age, gender, rural residence, and education), or all potential confounders (demographic variables plus smoking, field study, and sunlight exposure). Short-term effects of exposure were evaluated by linear regression analysis of changes in log (IgE) from T0 to T30 adjusted for exposure status, residence, gender, smoking, education, and sunlight exposure. Statistical significance testing for the crude comparison was done by using chi-square or the Student's t-test as indicated. Analyses were performed using the SAS statistical package.

Results:

Exposed and control subjects were well matched for age, sex, body mass index, alcohol consumption and smoking habits, but the agricultural nature of the jobs of most exposed workers meant that they were more likely to live in rural areas (63.8% v 37.4%, $p<0.001$) or have lived on a farm (67.6% v 36.3%, $p<0.001$), were less educated ($p<0.001$), and more likely to have regular sunlight exposure ($p<0.01$).

Geometric mean levels of ETU at T30 were significantly higher in exposed subjects than controls (2 µg/g creatinine and 0.4 µg/g creatinine, $p<0.001$). However, there were wide variations between the five field studies. In the Dutch field study there was no difference between exposed subjects and controls (0.81 µg/g creatinine in both groups). Geometric mean levels of exposed workers in the Bulgarian field study of zineb manufacturing workers were stated to be have been 22.1 µg/g creatinine, and 17.0 µg/g creatinine in controls. The latter figure is very high and inconsistent with the overall geometric mean for control subjects and not consistent with other reports of the EUROPIT study e.g. Steerenberg et al (2008) (see above, BASF DocID 2008/1102541) notes that no major differences in ETU concentrations were found between the four countries. No information was given about the types of EBDC chemicals used, other than one group of Bulgarian subjects worked in a facility that produced zineb.

Prevalences of specific allergens included in the Phadiatop and atopy were not significantly different between exposed and non-exposed subjects at T0 and T30, indeed the prevalence of atopy was higher among the non-exposed than in the exposed (26.2% v 22.3% at T0, 29.2% v 22.3% at T30). Prevalences of self-reported skin irritation, contact dermatitis (ever or current), sensitivity to grass pollen, and food allergy also did not show a significant association with exposure status at T0, but allergic rhinitis was more prevalent among non-exposed than exposed workers (15.5% v 8.5%, $p<0.05$). The prevalence of skin irritation was elevated among exposed workers in the Dutch field study (28.6% v 7.7%), but there was no evidence of increased exposure to EBDC chemicals among exposed workers than non-exposed workers in the Dutch study. Logistic regression analyses also showed no associations with exposure group for skin irritation, contact dermatitis, allergic rhinitis and food allergy (T0 or T30) whether unadjusted or adjusted for confounding factors. There were also no significant associations when ETU level grouped by job was included in the logistic regression analyses instead of exposed versus unexposed. Regression analysis of the difference in log (IgE) from T0 to T30 showed no association with exposure to EBDC chemicals, but a statistically significant negative association with sunlight exposure.

Conclusions:
There was no evidence of an effect of EBDC exposure on the prevalence of allergic contact dermatitis, allergic rhinitis, food allergy, and atopy as measured by the Phadiatop. The prevalence of skin irritation was elevated among exposed workers in the Dutch field study only, but there was no evidence of increased exposure to EBDC chemicals among exposed workers than non-exposed workers in the Dutch study, and it was considered that skin irritation was more likely a result of plant contact rather than EBDC exposure. There was no evidence that exposure to EBDC had affected the immune balance of workers in this study.

Report: CA 5.9.1/4
Boers D. et al., 2008b
Asthmatic symptoms after exposure to Ethylenebisdithiocarbamates and other pesticides in the Europit field studies
2008/1102539
Guidelines: none
GLP: no

Limitations of the publication include the low exposure of exposed workers in one field study, self-reported endpoints and the cross-sectional design.

Executive Summary:

The study was conducted in four countries (the Netherlands, Finland, Italy and Bulgaria) to evaluate a possible association between occupational exposure to ethylene-bis-dithiocarbamates (EBDC) and self-reported asthma and asthmatic symptoms. A total of 248 workers exposed to EBDC and 231 non-occupationally exposed subjects entered the study. Exposure to EBDC was measured as urinary ethylenethiourea (ETU) in samples collected at baseline (before the start of exposure) and after 30 days of exposure. Subjects completed a self-administered questionnaire at baseline. Prevalences of all asthma symptoms were lower (but not statistically significant) among exposed subjects than non-exposed subjects, including a diagnosis of asthma (3.3% v 8.4%). In multiple logistic regression analyses adjusted for all confounders, risks for asthma and all asthma-related symptoms were lower among exposed subjects, and the risk for wheeze was significantly reduced (OR 0.56; 95%CI 0.32-0.98). Furthermore, there were null associations for multivariate analyses that used ETU as determinant for exposure. The results do not suggest an association between exposure to EBDC and/or other pesticides used in the study on asthma and asthmatic symptoms.

Objective:

To investigate the possible effect of occupational exposure to EBDC on asthma and asthmatic symptoms.

Materials and Methods:

The investigation formed part of the EUROPIT study designed to evaluate potential immunological effects of pesticide exposure with a focus on ethylene-bis-dithiocarbamates (EBDC). Five field studies were conducted in four countries. The field studies included 42 flower bulb growers and 40 controls in the Netherlands, 51 potato farmers and 49 controls in Finland, 48 vineyard workers and 45 controls in Italy, 55 greenhouse workers and 45 controls from Bulgaria and a further group of Bulgarian subjects including 52 workers from a zineb producing facility and 50 unexposed controls mainly from the same factory. Overall there were 248 EBDC exposed and 231[3] non-occupationally exposed workers. The Italian controls were matched on main characteristics such as age and sex.

[3] The numbers of controls in the 5 individual studies add to 229.

The study design was prospective but the asthma component was cross-sectional as there was no follow-up data on asthma and asthmatic symptoms. A self-administered questionnaire was used to collect information on asthma and asthmatic symptoms using a respiratory questionnaire partly constructed from the IUATLD (International Union Against Tuberculosis and Lung Disease) questionnaire. The questionnaire included questions on demographic factors, employment history, presence of disease, vaccinations, medications, alcohol consumption, and smoking. Information on pesticides used was also collected for exposed subjects. At the same time points, EBDC exposure was assessed by measuring urinary ethylenethiourea (ETU), a major metabolite of EBDC chemicals in humans, before the EBDC application season started (time T0) and again after one month of exposure (time T30).
A cross-sectional comparison was performed of asthma and asthmatic symptoms among the exposed and non-exposed groups at T0. In addition, analyses were performed using urinary ETU as a continuous variable. A value corresponding to half of the detection limit was assigned to ETU measurements below analytical detection, and values were log-transformed as urinary ETU was not normally distributed. Analyses were also performed using the mean ETU result for the job held by a subject. Multivariate logistic regression analyses were performed using asthma (self-reported), wheezing, chest tightness, asthma attack, and asthma medication as dependent variables. Models were adjusted for either field study or all potential confounders (age, gender, rural residence, education, smoking and field study). Results are presented as Odds Ratios (OR). Analyses were performed using the SPSS statistical package.

Results:
Exposed and control subjects were well matched for age, sex, body mass index, alcohol consumption and smoking habits, but the agricultural nature of the jobs of most exposed workers meant that they were more likely to live in rural areas (63.8% v 37.4%, $p<0.01$) or have lived on a farm (67.6% v 36.3%, $p<0.01$), were less educated ($p<0.01$), and more likely to have regular sunlight exposure ($p<0.01$).
Significantly higher levels of ETU were observed in urine of the exposed group at T30 when compared to controls, with geometric means (geometric sd) of 2.7 (8.1) µg/g creatinine and 0.5 (3.7) µg/g creatinine, respectively ($p<0.01$).
An extremely low level of ETU was noted among exposed subjects in the Dutch field study. No information was given about the types of EBDC chemicals used, other than one group of Bulgarian subjects worked in a facility that produced zineb.
Prevalences of all asthma symptoms were lower (but not statistically significant) among exposed subjects than non-exposed subjects, including a diagnosis of asthma (3.3% v 8.4%). In multiple logistic regression analyses adjusted for all confounders, risks for asthma and all asthma-related symptoms were lower among exposed subjects and the risk for wheeze was significantly reduced (OR 0.56; 95%CI 0.32-0.98). In logistic regression analyses that included urinary ETU as a continuous variable, there were no significant associations with urinary ETU whether individual or grouped values of ETU were used.

Conclusions:
The prevalences of all self-reported asthma and asthmatic symptoms were lower (but not statistically significant) among exposed subjects than non-exposed subjects. In multiple logistic regression analyses adjusted for all confounders, risks for asthma and all asthma-related symptoms were lower among exposed subjects, and the risk for wheeze was significantly reduced (OR 0.56; 95%CI 0.32-0.98). There was no evidence that exposure to EBDC chemicals increased the risk of asthma or asthmatic symptoms.

Report:	CA 5.9.1/5 Dupupet J.-L. et al., 2010b Etude d ergoexpologie aux fongicides Dithiocarbamates aupres de professionnels de trois secteurs agricoles 2010/1232774
Guidelines:	none
GLP:	no

Executive Summary:

The study assessed the exposure of farm workers to dithiocarbamate fungicides by measuring the urinary output of the main metabolite, ethylenethiourea (ETU). There was an increase in urinary excretion of ETU from the first day of seasonal exposure. Wearing gloves did not have a significant protective effect although a protective effect due to wearing a mask during spray preparation was evident as the active substances were non-inhalable fine powders. The impact of the mask during preparation made it difficult to appreciate those effects linked to other behaviours or exposure circumstances. In a multivariate analysis, applying the product whilst in a closed cab provided protection.
The absence of a significant correlation between the biological results and the number of observed contaminations did not allow the conclusion that there is an absence of other independent variables that play a part (specifically the preparation mask).

Objective:

To quantify exposure to wine-growing, horticulture and potato-farming workers exposed to mancozeb by measuring urinary excretion of the main metabolite, ethylenethiourea (ETU), and to correlate the results with observed work

Materials and Methods:

Test material	Not stated
Description	No data
Source	No data
Batch	No data
Purity	No data
Stability	No data

The 56 individuals involved in the study were all male, 32 were farmers and 24 employees. The worker's spraying technique was observed by an occupational health physician or a prevention consultant. Urinary ETU concentrations were determined before (in the morning of the day of observation) and after exposure (48 h to 96 h after the end of exposure on the day of observation). Consideration was taken of food and drink products consumed that may contain dithiocarbamates and thus influence the urinary excretion of ETU.

The median value of urinary concentrations of ETU before and after exposure was compared through a Wilcoxon rank test. Then, to appreciate the potential influence of independent variables from the sample, (habits and daily hygiene, treatment material, personal protection equipment), the median values for the variations of concentrations of ETU prior to and after exposure were compared by the Mann-Whitney rank test if the sample was split in two. When the sample was split into three or more sub-groups then the Kruskal and Wallis rank test was used. Finally a Kendall correlation rank test was used to check the impact of individual characteristics (age, seniority), food, duration of exposure and the number of zone changes, stains or contaminations. After having tested the potential link to each variable with the ETU doses, multivariate analyses were carried out. The variables taken into account were those where a link with ETU doses showed the p value lower than 0.20 in the univariate analysis. Modelling was carried out after transformation of the dependent variable (x ->log(x + 1)) by multiple linear regression. For all statistical tests, the tolerance level of 5% was maintained.

Results:
Only 15 of the group declared that they had been trained in using the fungicides over the course of the last five years. The annual median duration of exposure to dithiocarbamates was 12 days (range: 2 and 30 days) and the median observed for daily treatment was 3 hours 46 minutes (range: lower than 1 and higher than 9 hours).
29 applicators wore a mask appropriate to the slurry preparation and the median elevation of their ETU rate was 0.47µg/g of creatinine against a median elevation of 2.92µg/g of creatinine for the 27 who did not wear it (p=0.012). 15 of the subjects showed onychophagia and 38 did not (3 did not respond regarding this matter) and they had median elevation of their ETU rates of 2.85 and 1.07 respectively (p=0.043).
Wearing of "appropriate clothing" appeared to be protective with a test at the borderline of significance, but of the 25 subjects wearing" appropriate clothing", 19 also wore an "appropriate mask". The "appropriate clothing" variable shows a strong correlation with "appropriate mask" (r=0.444; p< 0.0001).
The longitudinal follow-up showed the persistence of an increased urinary excretion for several days following the end of the exposure period, with daily increases that pertain to re-contaminations. The operator was not re-interviewed about possible returns to the treated plots following the end of direct exposure. However, the rapid decrease in urinary concentrations seven days following the end of the exposure period favours this hypothesis. Short periods of treatment for several successive days does not appear to have caused an accumulation of ETU in urine, in contrast to long consecutive days of treatment (the treatments carried out between d42 and d44 showed a ETU level of 27.4µg/g of creatinine).
Food consumption prior to exposure and in the following days did not significantly influence ETU output.

Conclusions:
This study demonstrated the complexity of advice given for occupational risk prevention linked to exposure to pesticides. With certain formulations in high grain aerosol form the use of respiratory protection is a preventative measure that is, at best, mediocre. Primary prevention consisting of limiting the use of fine powder formulations and, when spraying vegetation, of the implementation of collective protection by an appropriate cab is encouraged. These two provisions, which have demonstrated their effectiveness, should be preferred to the prescription of individual protection equipment.

Report: CA 5.9.1/6
Panganiban L. et al., 2003b
Correlation between blood Ethylenethiourea and thyroid gland disorders among banana plantation workers in the Philippines
2004/1040815
Guidelines: none
GLP: no

Limitations of the study include flawed statistical analyses[4]/interpretation and the cross-sectional design.

Executive Summary:
The study was a cross-sectional investigation of the incidence of thyroid gland disorders among banana plantation workers exposed to ethylene-bis-dithiocarbamates (EBDCs), and the relation between the incidence of thyroid gland disorders and blood and urinary ethylenethiourea (ETU), a metabolite of EBDCs. The study group included 57 directly exposed workers, 31 indirectly exposed workers randomly selected from four banana plantations and 43 comparison workers from an organic farm; all subjects underwent complete medical examinations and laboratory tests. Results showed higher, but not statistically significantly different, mean thyroid-stimulating hormone levels among exposed workers compared with the control group, although the levels were well within normal range. Nine of the exposed farmers had abnormal thyroid ultrasound findings, consisting mostly of solitary nodules, compared with three among the control group, but this was not statistically significantly different, and the lowest prevalence was among the directly exposed group. Analysis of variance showed significantly different blood ETU levels among the directly exposed, indirectly exposed, and control groups ($p < 0.001$). ETU levels in urine were much higher in exposed workers than controls, but stated to be not significantly different ($p = 0.10$). Environmental ETU levels were below the U.S. Environmental Protection Agency remediation levels. A significant correlation between the size of the nodule and blood ETU level was reported among the 7 farmers with solitary thyroid nodules, but there was no evidence that farmers developing solitary nodules or other thyroid gland disorders had higher mean levels of blood and urinary ETU than exposed subjects who did not develop such disorders. The investigators concluded that blood ETU is a more reliable biomarker for EBDC exposure than urinary ETU, and should be part of medical surveillance efforts among workers exposed to EBDCs to detect occurrences of thyroid gland disorders.

Objective:
To investigate the possible correlation between the levels of blood and urinary ETU and the incidence of thyroid gland disorders among banana plantation workers in the Philippines.

[4] *Many of the statistical analyses are clearly incorrect and inadequately described. The correlation between nodule size and blood ETU is based on 7 points and heavily influenced by the worker with the largest nodule and highest blood ETU level. There is little correlation if this point is excluded, but this worker has no urinary ETU value (the urinary ETU correlation with nodule size is greater than the blood ETU correlation with nodule size if this point is excluded). The r^2 values in Table 3 are actually correlations (based on the p values), and the correlation between urinary iodine and nodule size is clearly negative and not positive as stated. The investigators did not compare blood and urinary ETU levels of exposed subjects with nodules with those without nodules, but the levels are clearly not elevated among those with solitary nodules. In addition, there are clearly highly significant differences in urinary ETU between the 3 groups (as for blood ETU), although it is stated that the differences are not significant.*

Materials and Methods:
The study was a cross-sectional investigation of the incidence of thyroid gland disorders among banana plantation workers exposed to ethylene-bis-dithiocarbamates (EBDCs), and the relation between the incidence of thyroid gland disorders and blood and urinary ethylenethiourea (ETU), a metabolite of EBDCs. The study group included 88 workers with a 3-year history of direct or indirect exposure to EBDCs randomly selected from four plantations that had used large quantities of dithiocarbamates during the past 20 years. The 57 directly exposed workers included mixers, sprayers, flagmen, and clean-up labourers. The 31 indirectly exposed workers consisted of supervisors, maintenance crew, and research aides. The most recent exposure of these workers occurred between 1 and 9 days before the study was conducted. The comparison group included 43 workers randomly selected from an organic farm. The comparison subjects were stated to have no exposure to EBDCs, The comparison subjects were stated to reside at least 50 km away from the banana plantations, but the investigators also stated that nearby plantations aerially sprayed EBDCs. The EBDC-exposed workers were examined approximately 14 months before the comparison workers. Face-to-face interviews were used to collect a demographic profile, nutritional history, work background, and exposure data. A physical examination included thyroid gland ultrasound performed by a trained radiologist and a fine needle aspiration biopsy was performed on subjects who had palpable nodules. Urine and blood samples were taken and thyroid-stimulating hormone (TSH) and free thyronine (T4) were determined using immunoradiometric assay and radioimmunoassay methods. Free T_4 was determined only among subjects with elevated TSH levels. The samples were also analysed for iodine in urine, urinary creatinine, and blood and urinary ETU. Soil and air samples were also collected for workers at the 4 banana plantations and the organic farm and tested for ETU. Soil and air results were assigned to individual workers as the investigators performed correlations between biological and environmental sample results, but the procedure for doing this is not described and the numbers of environmental samples taken are not stated. The detection limit for ETU is stated to be 0.2 ppb but Table 3 lists results of < 0.1 ppb. The investigators state that descriptive statistics, Student t-test, analysis of variance, Fisher's exact test, Pearson's correlation analysis, and regression analysis were performed using the SPSS statistical package. It can be deduced from the Results section that some analyses were adjusted for factors such as age, sex, height, weight and dietary preferences. In addition, urinary and blood ETU analyses were log transformed in some analyses. The investigators did not state how they dealt with ETU results below the limit of detection in these analyses.

Results:
The groups were not well matched in terms of gender. All directly exposed workers were male, but 11 (35.5%) of indirectly exposed workers were female and 9 (20.9%) comparison workers. The mean ages of the 3 groups were just significantly different ($p<0.05$): the mean age of control subjects was 33.5 years, directly exposed subjects were 2 years older on average and indirectly exposed subjects were 4.3 years older on average. There were no significant differences regarding history of known thyroid gland disorders and diabetes mellitus in first degree relatives. Directly exposed workers reported handling different types of EBDCs, mostly maneb and mancozeb. Environmental monitoring results showed significantly higher levels of airborne ETU for control workers than workers directly exposed to EBDCs which the investigators attributed to exposure from nearby plantations that use aerial spraying. Levels of ETU in soil were significantly higher for the directly exposed group than comparison subjects. Environmental ETU levels were below the U.S. Environmental Protection Agency remediation levels. Blood ETU levels (untransformed and log transformed) were significantly different between groups ($p<0.001$). Mean and geometric mean urine ETU levels were much higher in exposed workers than control workers, and confidence intervals (not presented) of the means and geometric means of the exposed groups were greatly separated from those of the control groups. However, urine ETU levels (untransformed and log transformed) were stated to be not significantly different between groups ($p<0.1$). Analyses of blood and urine ETU levels adjusted for age and diet were stated to give similar conclusions.

Table 5.9.1-3: Biologic and environmental monitoring results for ETU

Parameter	Directly exposed (57)	Indirectly exposed (31)	Control (43)	p-value
Blood ETU (ppb)				
Mean (SE)	4.45 (0.55)	2.55 (0.60)	0.30 (0.04)	p<0.001
Geometric Mean (SE)	2.55 (0.32)	0.98 (0.42)	0.00 (0.36)	p<0.001
Urine ETU (ppb)				
Mean (SE)	378.34 (50.11)	267.16 (69.90)	26.31 (6.39)	p=0.1
Geometric Mean (SE)	94.73 (0.32)	134.21 (0.49)	14.09 (0.62)	p=0.1
Air ETU (ng/m^3)[1]	8.8	-	16.17	p<0.001
Soil ETU (ng/g)[1]	51.36	-	10.62	P=0.003

[1] It is assumed that results are arithmetic means. Standard error not given.

Enlarged thyroid glands were found in 17 workers from the EBDC-using plantations and 2 of the organic-farm workers, but there was no significant difference in the neck diameter of the two groups (p = 0.07) after controlling for variables such as age, sex, height, and weight. There was no significant difference in the physical diagnosis of goiter in the two groups for both female (p = 0.16) and male (p = 0.79) workers. Mean TSH levels were higher among exposed workers than control workers, but mean levels among the 3 groups were not statistically significantly different after adjusting for age (p=0.24), and neither were urinary iodine levels after correcting for dietary differences (p=0.45). Three exposed workers were stated to have elevated TSH levels compared with controls, but their free T_4 levels were within normal ranges. Nine of the exposed workers and three organic-farm workers had abnormal thyroid ultrasound results but the prevalences were not significantly different (p = 1.0), and there were only 3 cases (5.3%) of abnormal thyroid ultrasound results among directly exposed compared to 3 cases (7.0%) among control subjects. Among the 7 farmers with solitary thyroid nodules, a significant correlation was reported between the size of the nodule and blood ETU level, but the significant association is due to one subject (exposed subject 6 in Table 2) who had a much larger solitary nodule and blood ETU result than the other 6 subjects with solitary nodules. This subject had no urinary ETU result and the correlation between nodule size and urinary ETU is based on 6 subjects. Spearman correlation coefficients were calculated, but not presented. The investigators did not compare mean blood and urinary ETU levels among the exposed subjects with solitary nodules with exposed subjects who did not develop nodules, but it is clear from Table 2 that levels were not elevated.

Conclusions:

There were no statistically significant differences in thyroid function tests of exposed and control groups after controlling for age and dietary preferences, and no statistically significant differences in the prevalence of abnormal thyroid ultrasound results, or solitary nodules in exposed workers compared to controls. There were only 3 abnormal thyroid ultrasound results, including one case with a solitary nodule, in the group of directly exposed workers and the same in the smaller control group. A significant correlation between the size of the nodule and blood ETU level was reported among the 7 farmers with solitary thyroid nodules, but there was no evidence that farmers developing solitary nodules, or other thyroid gland disorders, had higher mean levels of blood and urinary ETU than exposed subjects who did not develop such disorders. The investigators concluded that blood ETU is a more reliable biomarker for EBDC exposure than urinary ETU, and should be part of medical surveillance efforts among workers exposed to EBDC to detect occurrences of thyroid gland disorders.

Report: CA 5.9.1/7
Castorina R. et al., 2010a
Comparison of current-use pesticide and other toxicant urinary metabolite levels among pregnant women in the Chamacos cohort and Nhanes
2010/1232773
Guidelines: none
GLP: no

Prenatal exposure to pesticides has been shown to increase the risk of adverse developmental outcomes in children. Biomonitoring results from the National Health and Nutrition Examination Survey (NHANES) (1999–2002) [Centers for Disease Control and Prevention (CDC) 2005] and other studies suggest that many pregnant women are exposed to a mixture of insecticidal and fungicidal compounds. There are multiple pesticide exposure pathways, but recent research suggests that diet is the dominant route of exposure for the general population. Para-occupational or take-home exposure from farmworkers and ambient exposure from living in proximity to agricultural activity can add to total pesticide exposure in agricultural communities. In this study, ETU exposure data from repeat samples collected from pregnant women late in the first and second trimesters are presented. ETU was measured as analyte in urine which indicates the possible precursor Mancozeb and Maneb with 6259 and 146592 kg active ingredient, respectively, applied in 2000. Between September 1999 and November 2000, 601 pregnant women were enrolled in the CHAMCOS birth cohort study from six local prenatal clinics. Women were eligible to participate in this study if they were ≤ 20 weeks gestation at the time of enrolment, were ≥ 18 years of age, qualified to receive poverty-based government health insurance, and planned to continue receiving prenatal care at a participating health center. Two spot urine samples were collected from CHAMACOS participants at each prenatal 13 ± 5.2 weeks of gestation and at 26 ± 2.6 weeks gestation and analyzed by the Division of Laboratory Sciences, CDC. ETU is water soluble and after lyophilisation it was extracted with dichloromethane; they were then analysed using isotope dilution high-performance liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS/MS).

In the CHAMACOS cohort, the detection frequency of ETU was higher at the first (23.5%) compared with the second (7.7%) sampling time. In addition, the 95th percentile value of ETU was significantly higher in the first compared with the second prenatal sample (1.5 µg/L vs. 0.4 µg/L, respectively; $p < 0.01$). No data are available for ETU from NHANES. Therefore a comparison of CHAMCOS and NHANES metabolite levels is not possible. ETU is the principal metabolite of the EBDC fungicides (maneb, mancozeb and metiram). ETU is more toxic than its precursor compounds. More than 150,000 kg of maneb and mancozeb are used annually in the Salinas Valley region, primarily on lettuce and wine. CHAMACOS is the first study to report repeated urinary ETU levels in a U.S. population and in pregnant women. The authors found that the ETU levels decreased with pregnancy, with higher detection frequencies and levels in samples collected during the first trimester compared with samples collected during the second trimester. The authors do not know if this change is due to differences in exposure or in metabolism over the course of pregnancy. More information on the metabolism and excretion of these compounds during pregnancy is needed.

Conclusion
Urinary ETU levels in the CHAMACOS cohort were similar to those of non-occupational populations studied in Italy (Aprea et al. 1996; Colosio et al. 2006; Saieva et al. 2004), but lower than those reported for pesticide applicators in other countries (Colosio et al. 2002; Panganiban et al. 2004; Steenland et al. 1997).

CA 5.9.2 Data collected on humans

No information was available.

CA 5.9.3 Direct observations

No information was available.

CA 5.9.4 Epidemiological studies

No information was available.

CA 5.9.5 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

No information was available.

CA 5.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

No information was available.

CA 5.9.7 Expected effects of poisoning

No information was available.

Metiram

Document M-CA, Section 6

RESIDUES IN OR ON TREATED PRODUCTS, FOOD AND FEED AND PLANT METABOLISM

Compiled by:

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number
29/Feb/2016	CA 6.1/1: Interim report 2014/1000228 replaced by final report 2015/1260660.	

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 6 RESIDUES IN OR ON TREATED PRODUCTS, FOOD AND FEED AND PLANT METABOLISM

A concordance list of structures and designations of reference compounds used during Consumer Safety studies is given below.

An overview of metabolites identified during consumer safety studies is given below.

Code Numbers			Description	Compound found in	Structure
Substance/ Metabolite Code	Reg No.	Synonyms/ Old nomenclature	Chemical Name CAS-No.		
BAS 222 F	250284	M222F000 Metiram Metiram TK = Metiram TK 85 = Metiram Premix 95 = Metiram–Complex 95% = BAS 222 29 F	Zinc ammoniate ethylenebis (dithiocarbamate) – poly(ethylenethiouram disulfide) 9006-42-2		
M222F001	Not assigned	None	2-[(2-oxoimidazolidine-1-carbonyl)carbamoylamino]acetic acid No CAS available	Rotational Crop	
M222F002	146099	BF222-ETU ETU	2-IMIDAZOLIDINETHIONE; 4,5-Dihydro-1H-imidazol-2-thione 96-45-7	Plant, Animal	
M222F003	27270	BF222-EU EU	Imidazolidine-2-one ETHYLENEUREA 120-93-4	Plant, Animal	

Code Numbers			Description	Compound found in	Structure
Substance/ Metabolite Code	Reg No.	Synonyms/ Old nomenclature	Chemical Name CAS-No.		
M222F004	243959	BF 222-EBIS EBIS ETM	5,6-Dihydro-3H-imidazo-[2,1-c]-1,2,4-dithiazole-3-thione, DIDT Ethylene-bis-(isothiocyanate) sulfide Ethylenethiuram monosulfide, 33813-20-6	Plant, Animal	
M222F005	251072	BF222-Carbimid Carbimid ETT	2-thioxo-imidazolidinyl-thiocarboxamide 695-76-1		
M222F006	247214	BF222-EBIT EBIC	Ethylenediisothiocyanate 3688-02-08		
M222F007	4670450	TDIT M212	2,3,7,8-tetrahydrodiimidazo[2,1-b:1',2'-e][1,3,5]thiadizine-5-thione 75676-85-6	Plant	
M222F008	132345	Hydantoin	2,4-Imidazolidinedione 461-72-3	Plant, Animal,	
M222F009	Not assigned	None	2-thioxoimidazolidine-1-carboxamide No CAS available		
M222F010	Not assigned	None	O-methyl 2-thioxoimidazolidine-1-carbothioate No CAS available		
M222F011	70964	None	4,5-dihydro-1H-imidazole 504-75-6		

Code Numbers			Description	Compound found in	Structure
Substance/ Metabolite Code	Reg No.	Synonyms/ Old nomenclature	Chemical Name CAS-No.		
M222F012	Not assigned	None	2-Imidazolidinol 1628741-67-2		
M222F013	6014473	None	2-oxoimidazolidine-1-carbaldehyde 41731-11-7	Plant	
M222F014	Not assigned	None	No CAS available		
M222F015	6012392	None	1-1'-methanediyldiimidazolidin-2-one 13311-64-3		
M222F016	6014472	None	2-thioxoimidazolidine-1-carbaldehyde 954379-26-1		
M222F017	283749	None	1H-imidazole 288-32-4		
M222F018	Not assigned	None	No CAS available		
M222F019	Not assigned	None	2-hydroxyimidazolidine-1-carbaldehyde No CAS available		
M222F020	Not assigned	None	1-(4,5-dihydro-1H-imidazol-2-yl)imidazolidin-2-ol No CAS available		

Code Numbers			Description	Compound found in	Structure
Substance/ Metabolite Code	Reg No.	Synonyms/ Old nomenclature	Chemical Name CAS-No.		
M222F021	Not assigned	N-ac-EDA	N-acetyl-ethylenediamin 1001-53-2	Plant, Animal	
M222F022	6002546	Jaffe's base	1-(4,5-dihydro-1H-imidazol-2-yl)imidazolidine-2-thione 484-92-4	Plant, Animal	
M222F023	4183259	EDA Ethylenediamine	Ethane-1,2-diamine 107-15-3	Plant, Animal	
M222F024	Not assigned	None	1-methyl-5,6-dihydroimidazo[2,1-c][1,2,4]dithiazole-3-thione No CAS available	Rat	
M222F025	Not assigned	None	3,5-dihydro-2H-imidazo[1,5-b][1,2,5]thiadiazole-6-thione No CAS available	Rat	
M222F026	Not assigned	None	S-methyl 2-thioxoimidazolidine-1-carbothioate No CAS available	Rat	

CA 6.1 Storage stability of residues

The following information is copied from the Monograph prepared by RMS Italy in context of the Annex I inclusion of metiram.

Stability of residues (OECD data point numbers IIA 6.1 and IIIA 8.1)

Metiram is stable in plant matrix for at least 12 months and up to 18 months in grapes The stability of ETU depends on the matrix (stable up to 12 months in tomatoes and processed products, apple juice and sauce, wine; stable up to 3 months in grapes). Limited stability in potatoes –7 days-. Uncertain stability in apples, with recoveries of 0, 12 and 74% at 1 month in 3 different studies. Unknown stability in other crops. The stability of Metiram and ETU in animal matrix is 26 weeks.

Stability investigations for the determination of Metiram and ETU residues in food of plant and animal origin were evaluated in the context of the inclusion in Annex I under Directive 91/414/EEC. These studies evaluated previously are summarized in Table 6.1-1 for the reviewer´s convenience.

Table 6.1-1: Summary of peer-reviewed storage stability studies

Author.	Study	Crop/ Matrix	Analyte	Storage interval	Stable
Larese J. 1989	Storage stability of metiram and ethylenethiourea in frozen potatoes – Addendum: Twelve months ethylenethiourea and metiram stability Enviro-Bio-Tech Ltd., Bernville – USA BASF Aktiengesellschaft, Limburgerhof, Germany BASF DocID 1989/5086 GLP, Unpublished	Potato	Metiram ETU	12 months	yes no
Mamouni, A. 1994	Storage stability of metiram in white grapes RCC Umweltchemie AG, Itingen, Switzerland BASF Aktiengesellschaft, Limburgerhof, Germany BASF DocID 1994/11068 GLP, Unpublished	White grapes	Metiram	18 months	yes
Benz, A. et al 2002	Storage stability study of incurred ETU residues in potatoes. BASF AG, Agrarzentrum Limburgerhof, Limburgerhof, Germany BASF Aktiengesellschaft, Ludwigshafen, Germany BASF DocID 2002/1004096	potatos	ETU (incurred)	No residues found	-
Benz, A. et al 2002	Storage stability study of incurred ETU residues in grapes. BASF AG, Agrarzentrum Limburgerhof, Limburgerhof, Germany BASF Aktiengesellschaft, Ludwigshafen, Germany BASF DocID 2002/1012093	grapes	ETU (incurred)	3 months	yes
Christman, P.J. 1988	Determination of the Stability of Metiram and ETU residues in/on Animal Products Hazleton Laboratories America Inc., Madison USA BASF Aktiengesellschaft, Limburgerhof, Germany BASF DocID 1988/5127 Addendum: to BASF DocID 1988/5100 GLP, Unpublished.	food of animal origin	Metiram ETU	26 weeks	yes yes

Storage stability of food of animal origin

As no new feeding or any residue study of animal origin has been conducted, no new stability study in animal matrices has been executed.

Storage stability of food of plant origin

Multiple freezer storage stability investigations have been performed. The studies were performed applying two different techniques. Both of them are considered as equally suitable in the most recent OECD guideline.

- In the first type of studies, Metiram and its metabolites EU and EBIS were spiked to homogenised and coarsely ground plant material.

- In the second type of studies, the crops (mainly grapes) were treated with Polyram DF, the solo formulation of Metiram. The storage stability of incurred ETU residues was investigated over a period of time, but also depending on different homogenisation techniques. In parallel the Metiram residues were measured.

The entire information available summarised below. The results obtained confirmed the stability of Metiram under frozen conditions. The data indicate limited storage stability over a period of approximately 12 months when samples are stored as entire material. Homogenisation should occur immediately prior to residue analysis. The metabolites show a significant lower stability, depending on matrix type and processing methodology.

~~**Report:** CA 6.1/1~~
~~Meyer M., 2015a~~
~~Interim report - Storage stability study of Metiram and its two Metiram metabolites (EU and EBIS) in plant matrices~~
~~2014/1000228~~
~~**Guidelines:** SANCO/825/00 rev. 8.1 (16 November 2010), EEC 7032/VI/95 rev. 5, OECD 506 (Oct. 2007), OECD-DOC ENV/MC/CHEM(98)17 Paris 1998~~
~~**GLP:** yes~~
~~(certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)~~

~~**As this study is still ongoing, data of the interim report is presented. The final version can be submitted after the last sampling point has been analyzed.**~~

Report: CA 6.1/1
Meyer M., 2016 a
Storage stability study of Metiram and its two Metiram metabolites (EU and EBIS) in plant matrices
2015/1260660
Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), OECD-ENV/JM/MONO/(2007)17, EEC 7032/VI/95 rev. 5, OECD 506 (Oct. 2007)
GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** Metiram (as EBDC and CS_2), EU (M222F003), EBIS (M222F004)

Description: Metiram (as EBDC, CS_2), EU and EBIS

Lot/Batch #: 93.6% / 92.7% (Metiram), 99.6% (EU), 95.5% (EBIS)

Purity: CP052744 / 300014 (Metiram), L83-48 (EU), L83-136 (EBIS)

CAS#: 9006-42-2 (Metiram), 75-15-0 (CS_2), 120-93-4 (EU), 33813-20-6 (EBIS)

Development code: Metiram: BAS 222 F
EU: M222F003
EBIS: M222F004

Spiking levels: 0.05 - 0.5 mg/kg (EBDC)
1.0 mg/kg (CS_2)
0.01 - 0.1 mg/kg (EU)
0.1 mg/kg (EBIS)

2. Test Commodity: Plant matrices
Crop: cucumber, onion, lettuce (CS_2 and EBDC)
rape seed, white bean, potato and grapes, lettuce (EU)
grapes, lettuces, potatoes (EBIS)
Type: not reported
Variety: not reported
Botanical name: *Brassica napus L., Phaseolus vulgaris L., Solanum tuberosum L., Vitis vinifera L., Lactuca sativa L., Allium cepa L., Cucumis sativus L.*
Crop parts(s) or processed commodity: not reported
Sample size: not reported

B. STUDY DESIGN AND METHODS

1. Test procedure

Metiram by EBDC

The storage stability test was carried out in 4 analytical assays, respectively in the time intervals of 0, 12, 18 and 24 months (+ 4 spare sets). The matrices to be tested for EBDC are cucumber, onion and lettuce. The specimens were sprayed with the test item Metiram at a mean concentration level of 3.2 mg/kg for onion, 0.9 mg/kg for lettuce and 0.3 mg/kg for cucumber. The specimens were stored at ≤ -18 °C and analyzed after different time intervals. In preparation of the storage stability the matrices were homogenized in the presence of dry ice.

Metiram by CS_2

The storage stability test was carried out in 4 analytical assays, respectively in the time intervals of 0, 12, 18 and 24 months (+ 4 spare sets). The matrices to be tested for Metiram via CS_2 are cucumber, onion and lettuce. The specimens were sprayed with the test item Metiram at a mean concentration level of 1.3 mg/kg for onion, 0.5 mg/kg for lettuce and 0.3 mg/kg for cucumber. The specimens were stored at ≤ -18 °C and analyzed after different time intervals. In preparation of the storage stability the matrices were homogenized in the presence of dry ice.

EU

The storage stability test was carried out in 6 analytical assays, respectively in the time intervals of 0, 1, 3, 6, 12 and 18 months (+ 5 spare sets). The matrices to be tested for EU were lettuce (aqueous matrix), rape seed (oily matrix), white bean (protein containing matrix), potato (starch containing matrix) and grapes (acid matrix). The specimens were spiked with the test item EU at a concentration level of 0.1 mg/kg. The specimens were stored at ≤ -18 °C and analyzed after different time intervals. In preparation of the storage stability the matrices were homogenized in the presence of dry ice (with exception of the test matrix grapes).

EBIS
Please note the following: At the start of the study no analytical method for EBIS was available, it was developed in study 389443 (IF-13/02308815) and the development turned out to be very complicated and time consuming. In order to meet the scheduled timelines of this study the storage specimens destined for the storage times 12, 18 and 24 months as well as the spare set 3 were prepared and stored deep frozen without having a valid method for EBIS.
During the development work and pretests hints were observed that EBIS might be unstable in the tested matrices. In order to test this, the spare set 3 was analyzed after a storage time of 72 days for the matrices for which a valid method was available at that point (grapes, lettuce and potato). The specimens were spiked with the test item EBIS at a concentration level of 0.1 mg/kg. The specimens were stored at ≤ -18 °C and analyzed after 72 days. In preparation of the storage stability the matrices were homogenized in the presence of dry ice (with exception of the test matrix grapes).

2. Description of analytical procedures

The specimens were analysed for residues of Metiram according to BASF method No. L0089/01 and as carbondisulfide according to BASF method L0234/01. The residues of EU and EBIS were determined with method L0233/01.

Principle of BASF Method L0089/01:
The ethylene-bisdithiocarbamate (EBDC) moiety is formed out of BAS 222 F and extracted from the specimen with a buffer solution consisting of EDTA, cysteine, methanol and sodium hydroxide adjusted to pH 11.0. The formed ethylene-bisdithiocarbamate (EBDC) analyte is methylated with iodmethane prior to chromatography. Specimens are quantified by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). The limit of quantitation (LOQ) of the method is 0.05 mg/kg, apart from rape seed, where the LOQ is 0.1 mg/kg.

Principle of method L0234/01:
Metiram is transformed to CS_2 by means of orthophosphoric acid. Subsequently, the CS_2 is transferred to isooctane with a flow of nitrogen.
The quantification was carried out using gas chromatography with mass spectrometric detection (GC-MS). The limit of quantitation (LOQ) of the method is 0.10 mg/kg (0.056 mg/kg expressed as CS_2). Two mass fragments were determined. One was used for evaluation, the other one for confirmation.

Principle of the Method L0233/01:
EU (Reg.No. 27270) is extracted from plant material with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol/water. After centrifugation, an aliquot is taken and the methanol is evaporated from the extract. The pH is adjusted to 8 before evaporating. The remaining water phase is cleaned by liquid/liquid partition (water/ethylacetate) on an Extrelut column. After concentration of the eluate, the residue is determined by LC-MS/MS. The limit of quantitation (LOQ) of the method is 0.01 mg/kg.

Principle of method L0233/01:
EBIS (Reg.No. 243959) is extracted from plant material with a mixture of acetonitril/formic acid (1000/1, v/v) in the presence of thiourea. After centrifugation, an aliquot is taken and cleaned by dispersive SPE against C18. The supernatant is used for the determination of EBIS by LC-MS/MS. The limit of quantitation (LOQ) of the method is 0.01 mg/kg.

II. RESULTS AND DISCUSSION

Metiram (by EBDC)

Table 6.1-2 shows the month zero results of Metiram (by EBDC), which were used as reference to calculate the residue recoveries [%] in Table 6.1-3.

Table 6.1-2: Month zero specimens for Metiram (by EBDC)

Matrix	Storage intervals [months]	EBDC residue level found Individual results (mg kg-1)				Month zero mean amount (mg/kg)
Onion	0	3.08	3.71	3.33	2.83	3.24
Lettuce	0	0.997	0.508	1.12	1.15	0.944
Cucumber	0	0.365	0.337	0.345	0.299	0.336

The data used to assess the storage stability of Metiram (by EBDC) in onion, lettuce and cucumber are provided in Table 6.1-3. The results are given in mg/kg and in % of month zero mean amount, and not corrected with procedural recoveries.

Table 6.1-3: Storage stability of Metiram (by EBDC) in plant matrices

Matrix	Month	Residue Level found in Stored Sample: Individual results (mg/kg)		Individual results (% of month zero mean amount)		Mean (% of month zero mean amount)	Recovery of freshly spiked specimens, (mean, %)
Onion	12	2.8	3.3	88.1	102.4	**95.2**	87.9
	18	3.4	3.3	104.6	102.8	**103.7**	88.7
	24	3.2	3.3	98.0	102.9	**100.5**	84.5
Lettuce	12	0.111	0.092	11.7	9.7	**10.7**	86.7
	18	0.115	0.095	12.2	10.1	**11.1**	97.9
	24	0.065	0.069	6.9	7.3	**7.1**	87.5
Cucumber	12	0.132	0.150	39.1	44.5	**41.8**	85.3
	18	0.050	0.049	14.8	14.4	**14.6**	99.7
	24	0.032	0.037	9.5	11.1	**10.3**	97.4

The means of the procedural recoveries for Metiram (by EBDC) at 0.05 - 0.5 mg/kg are shown in Table 6.1-4.

Table 6.1-4: Means of procedural recoveries for Metiram (by EBDC)

Matrix	Storage intervals [months]	Recoveries EBDC [%]	
		Mean [%]	RSD [%]
Onion	12, 18, 24	87	2.5
Lettuce	12, 18, 24	91	6.6
Cucumber	12, 18, 24	94	8.0

RSD = Relative Standard Deviation

Metiram (by CS_2)

Table 6.1-5 shows the month zero results of Metiram (by CS_2), which were used as reference to calculate the residue recoveries [%] in Table 6.1-6.

Table 6.1-5: Month zero specimens for Metiram (by CS_2)

Matrix	Storage intervals [months]	CS_2 residue level found				Month zero mean amount (mg/kg)
		Individual results (mg kg-1)				
Onion	0	1.21	1.29	1.24	1.33	1.26
Lettuce	0	0.659	0.525	0.448	0.424	0.514
Cucumber	0	0.376	0.386	0.309	0.303	0.343

The data used to assess the storage stability of Metiram (by CS_2) in onion, lettuce and cucumber are provided in Table 6.1-6. The results are given in mg/kg and in % of month zero mean amount, and not corrected with procedural recoveries.

Table 6.1-6: Storage stability of Metiram (by CS_2) in plant matrices

Matrix	Month	Residue Level found in Stored Sample					Recovery of freshly spiked specimens, (mean, %)
		Individual results (mg/kg)		Individual results (% of month zero mean amount)		Mean (% of month zero mean amount)	
Onion	12	1.0	1.0	79.9	82.1	**81.0**	87.2
	18	0.887	0.921	70.2	72.9	**71.5**	76.2
	24	0.840	0.801	66.5	63.4	**64.9**	79.5
Lettuce	12	0.570	0.587	110.9	114.3	**112.6**	97.9
	18	0.480	0.508	93.4	98.8	**96.1**	82.5
	24	0.425	0.491	82.8	95.6	**89.2**	86.5
Cucumber	12	0.108	0.094	31.5	27.5	**29.5**	78.0
	18	0.059	0.067	17.3	19.5	**18.4**	79.8
	24	0.048	0.049	13.9	14.3	**14.1**	82.3

The means of the procedural recoveries for Metiram (by CS_2) at 1.0 mg/kg are shown in Table 6.1-7.

Table 6.1-7: Means of procedural recoveries for Metiram (by CS_2)

Matrix	Storage intervals [months]	Recoveries CS_2 [%]	
		Mean [%]	RSD [%]
Onion	12, 18, 24	81	8.3
Lettuce	12, 18, 24	89	9.8
Cucumber	12, 18, 24	80	7.9

RSD = Relative Standard Deviation

EU

The data used to assess the storage stability of EU in rape seed, white bean, potato, grape and lettuce are provided in Table 6.1-8.

Table 6.1-8: Storage stability of EU in plant matrices

Matrix	Month	Residue Level found in Stored Sample					Recovery of freshly spiked specimens, (mean, %)
		Individual results (mg/kg)		Individual results (% of nominal)		Mean (% of nominal)	
Rape seed	0	0.078	0.081	77.6	80.8	**79.2**	83.3
	1	0.062	0.067	62.3	66.6	**64.5**	76.7
	3	0.040	0.053	40.3	52.8	**46.5**	84.3
	6	0.043	0.040	42.5	40.3	**41.4**	77.6
	12	0.053	0.051	52.9	51.2	**52.1**	109.3
	18	0.027	0.029	27.4	29.1	**28.3**	79.5
	24	0.039	0.040	39.0	40.3	**39.6**	75.2
White bean	0	0.069	0.064	69.3	63.6	**66.5**	74.5
	1	0.070	0.074	70.2	74.0	**72.1**	72.8
	3	0.094	0.064	93.7	63.6	**78.7**	70.6
	6	0.068	0.050	67.7	49.4	**58.6**	74.1
	12	0.078	0.077	78.2	77.0	**77.6**	98.2
	18	0.068	0.072	67.9	71.9	**69.9**	74.1
	24	0.080	0.067	80.1	66.9	**73.5**	70.9
Potato	0	0.082	0.077	82.2	77.4	**79.8**	85.2
	1	0.068	0.077	67.5	77.2	**72.3**	72.8
	3	0.081	0.082	80.4	81.5	**80.9**	86.3
	6	0.074	0.077	73.6	76.5	**75.1**	85.4
	12	0.076	0.081	75.9	80.7	**78.3**	91.1
	18	0.057	0.078	56.8	78.0	**67.4**	78.1
	24	0.083	0.084	83.0	84.0	**83.5**	95.5
Grapes	0	0.076	0.070	76.1	71.0	**73.5**	71.2
	1	0.049	0.061	48.8	60.7	**54.8**	68.7
	3	0.069	0.071	67.5	69.4	**68.5**	83.7
	6	0.064	0.053	63.4	52.7	**58.0**	85.7
	12	0.049	0.056	50.3	56.8	**53.6**	97.0
	18	0.048	0.034	47.8	33.2	**40.5**	96.5
	24	0.064	0.069	65.6	68.6	**67.1**	92.9

Matrix	Month	Residue Level found in Stored Sample					Recovery of freshly spiked specimens, (mean, %)
		Individual results (mg/kg)		Individual results (% of nominal)		Mean (% of nominal)	
Lettuce	0	0.069	0.072	68.5	72.1	**70.3**	71.0
	1	0.075	0.078	74.9	77.5	**76.2**	81.3
	3	0.060	0.059	60.2	59.3	**59.8**	81.9
	6	0.055	0.049	54.8	49.1	**51.9**	94.7
	12	0.036	0.034	35.5	33.9	**34.7**	76.3
	18	0.035	0.032	34.4	31.8	**33.1**	101.5
	24	0.035	0.035	34.8	34.8	**34.8**	93.4

The means of the procedural recoveries for EU at 0.01 - 0.1 mg/kg are shown in Table 6.1-9.

Table 6.1-9: Means of procedural recoveries for EU

Matrix	Storage intervals [months]	Recoveries EU [%]	
		Mean [%]	RSD [%]
Rape seed	0, 1, 3, 6, 12, 18, 24	~~85~~ 84	14
White bean	0, 1, 3, 6, 12, 18, 24	~~77~~ 76	~~16~~ 15
Potato	0, 1, 3, 6, 12, 18, 24	~~83~~ 85	~~7.8~~ 8.8
Grapes	0, 1, 3, 6, 12, 18, 24	~~84~~ 85	~~15~~ 13
Lettuce	0, 1, 3, 6, 12, 18, 24	~~85~~ 86	14

RSD = Relative Standard Deviation

EBIS

The data used to assess the storage stability of EBIS in potato, grapes and lettuce are provided in Table 6.1-10.

Table 6.1-10: Storage stability of EBIS in plant matrices

Matrix	Days	Residue Level found in Stored Sample					Recovery of freshly spiked specimens, (mean, %)
		Individual results (mg/kg)		Individual results (% of nominal)		Mean (% of nominal)	
Potato	72	0.0060	0.0050	6.0	5.0	5.5	85.6
Grapes	72	0.0079	0.0099	8.3	9.7	9.0	104.4
Lettuce	72	0.0250	0.0240	24.9	24.3	24.6	91.8

The means of the procedural recoveries for EBIS at 0.1 mg/kg are shown in Table 6.1-11.

Table 6.1-11: Means of procedural recoveries for EBIS

Matrix	Storage intervals [days]	Recoveries EBIS [%]			
		Mean [%]	RSD [%]	Overall Mean [%]	Overall RSD [%]
Potato	72	86	-		
Grapes	72	104	-	94	9.2
Lettuce	72	92	-		

RSD = Relative Standard Deviation

III. CONCLUSION

**The results obtained from this storage stability study indicate the following:
(based on non-corrected recoveries of the nominal content)**

**Metiram (measured via EBDC) is stable under deep frozen conditions (≤ -18 °C) in the tested matrix onion for the investigated time period of about 18 months, while Metiram (by EBDC) is not stable in the tested matrices lettuce and cucumber.
Metiram (by CS_2) is stable under deep frozen conditions (≤ -18 °C) in the tested matrices onion and lettuce for the investigated time period of about 18 months (lettuce 24 months), while Metiram (by CS_2) is not stable in the tested matrix cucumber.
The discrepancy for the stability of Metiram in lettuce is actually not explainable, because the principle of measurement should not have any influence on it. Taking into account the stable residue values for metriam within the stability study on incurred ETU residues (BASF DocID 2010/1169547), it can be assumed that Metiram is stable in lettuce at least for 12 months. Nevertheless the results of the EBDC-measurements have to be further clarified.**

EU is stable under deep frozen conditions (≤ -18 °C) in the tested matrices white bean and potato for an investigated time period of about ~~12~~ 24 months, 1 month in lettuce, but not stable in the tested matrices rape seed and grapes, not taking into account the procedural recoveries of freshly spiked specimens. ~~If this is taken into account storage stability of 18 months for white bean and potato, 3 months for Grapes and lettuce as well as 2 months for Rape seed may be provided.~~ If this is taken into account, storage stability of 3 months for grapes and lettuce as well as 1 month for rape seed may be provided.

EBIS is not stable under deep frozen conditions (≤ -18 °C) in the tested matrices potato, grapes and lettuce for the investigated time period of 72 days. As a results of these findings the investigation of the storage stability of EBIS in this study was cancelled and an investigation of the short term storage stability under deep frozen conditions (≤ -18 °C) was carried out in separate study (DocID 2014/1028661).

Report: CA 6.1/2
Schatz N., 2015a
Storage stability of the Metiram metabolite EBIS in plant matrices
2014/1028661

Guidelines: SANCO/825/00 rev. 8.1 (16 November 2010), OECD-DOC ENV/MC/CHEM(98)17 Paris 1998, EEC 7032/VI/95 rev. 5, OECD 506 (Oct. 2007)

GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** EBIS (M222F004)
   Description: EBIS
   Lot/Batch #: L83-136
   Purity: 96.7% / 95.5%
   CAS#: 33813-20-6
   Development code: EBIS: M222F004
   Spiking levels: 0.1 mg/kg

2. **Test Commodity:** Plant matrices
   Crop: rape seed, white bean, potato, grapes, lettuce, onion and cucumber
   Type: not reported
   Variety: not reported
   Botanical name: *Brassica napus L., Phaseolus vulgaris L., Solanum tuberosum L., Vitis vinifera L., Lactuca sativa L., Allium cepa L., Cucumis sativus L.*
   Crop parts(s) or processed commodity: not reported
   Sample size: not reported

B. STUDY DESIGN AND METHODS

1. Test procedure

The stability of EBIS in plant matrices (rape seed, white bean, potato, grapes, lettuce, onion and cucumber) under deep frozen conditions was investigated. The samples were spiked with the test item EBIS at a concentration level of 0.1 mg/kg. The samples were stored at ≤ -18 °C and analysed after different time intervals (0 to at least 60 days). In preparation of the storage stability, the matrices were homogenized in the presence of dry ice, with the exception of grapes, white beans and rape seeds. These matrices were not homogenized prior to the start of the storage stability as the BASF Method L0233/01 is performed with unhomogenized samples.

2. Description of analytical procedures

Samples were analysed with the BASF method L0233/01 (IF-13/02308815) which enables the quantification of residues of EBIS in different matrices.

Principle of the Method L0233/01:
EBIS is extracted with a mixture of acetonitrile/formic acid in combination with a thiourea solution. This step is followed by a clean-up step utilizing C18-EC. Samples are diluted with a solution of acetonitrile/H_2O/formic acid before being determined by LC-MS/MS.
The limit of quantitation (LOQ) of the method is 0.01 mg/kg.

II. RESULTS AND DISCUSSION

The data used to assess the storage stability of EBIS in rape seed, white bean, potato, grape, lettuce, onion, cucumber are provided in Table 6.1-12.

Table 6.1-12: Storage stability of EBIS in plant matrices

Matrix	Day	Residue Level found in Stored Sample					Recovery of freshly spiked samples, (mean, %)
		Individual results (mg/kg)		Individual results (% of nominal)		Mean (% of nominal)	
Rape seed	0	0.090	0.091	88.2	89.6	**88.9**	95.7
	3	0.023	0.024	22.9	23.2	**23.0**	91.8
	5	0.017	0.019	16.4	18.8	**17.6**	93.1
	7	0.015	0.015	14.9	15.3	**15.1**	98.4
	14	0.029	0.028	28.2	28.2	**28.2**	82.7
	31	0.020	0.017	20.3	16.8	**18.6**	88.2
	45	0.025	0.027	25.0	26.2	**25.6**	89.8
	60	0.017	0.016	16.5	15.4	**16.0**	110
White bean	0	0.089	0.092	88.0	90.3	**89.2**	95.5
	3	0.055	0.057	55.6	57.4	**56.5**	84.4
	5	0.058	0.075	58.6	74.0	**66.3**	97.6
	7	0.044	0.046	44.1	45.7	**44.9**	95.2
	14	0.047	0.043	47.4	42.9	**45.2**	84.7
	31	0.047	0.042	46.8	42.3	**44.5**	83.5
	45	0.057	0.057	57.1	57.0	**57.0**	98.6
	63	0.052	0.055	50.9	55.2	**53.0**	109
Potato	0	0.090	0.094	89.3	93.3	**91.3**	98.4
	3	0.032	0.029	31.4	29.3	**30.4**	98.3
	5	0.020	0.028	20.1	28.4	**24.3**	107
	7	0.020	0.020	20.1	19.7	**19.9**	83.9
	14	0.022	0.023	21.8	22.7	**22.3**	93.3
	31	0.016	0.023	16.4	23.1	**19.8**	105
	45	0.015	0.015	14.3	15.3	**14.8**	110
	67	0.0068	0.0077	6.7	7.7	**7.2**	96.5

Matrix	Day	Residue Level found in Stored Sample					Recovery of freshly spiked samples, (mean, %)
		Individual results (mg/kg)		Individual results (% of nominal)		Mean (% of nominal)	
Grapes	0	0.087	0.082	85.8	90.0	**87.9**	83.1
	3	0.063	0.068	62.2	70.5	**66.4**	93.8
	5	0.071	0.068	71.1	67.2	**69.1**	101
	7	0.055	0.055	54.5	56.5	**55.5**	78.7
	14	0.054	0.050	53.2	52.9	**53.0**	84.0
	31	0.036	0.032	36.4	31.8	**34.1**	87.3
	45	0.025	0.024	24.9	23.3	**24.1**	94.3
	67	0.016	0.016	17.0	16.8	**16.9**	104
Lettuce	0	0.099	0.093	97.6	91.9	**94.7**	83.1
	3	0.045	0.050	44.3	49.7	**47.0**	101
	5	0.045	0.039	44.4	38.4	**41.4**	98.0
	7	0.033	0.036	32.3	35.7	**34.0**	78.1
	14	0.038	0.050	37.3	48.9	**43.1**	95.1
	31	0.021	0.026	20.8	25.8	**23.3**	94.2
	45	0.041	0.043	40.6	42.1	**41.4**	110
	60	0.063	0.060	61.9	59.0	**60.5**	109
Onion	0	0.104	0.095	103	93.8	**98.2**	99.6
	3	0.053	0.059	52.2	59.1	**55.6**	101
	5	0.058	0.057	58.2	57.3	**57.8**	106
	7	0.040	0.043	39.8	42.2	**41.0**	87.4
	14	0.044	0.048	43.6	47.8	**45.7**	110
	31	0.034	0.037	33.9	37.1	**35.5**	103
	45	0.034	0.037	33.9	37.5	**35.7**	108
	60	0.049	0.044	48.9	43.6	**46.2**	102
Cucumber	0	0.099	0.101	97.6	101	**99.3**	97.1
	3	0.064	0.068	63.8	66.5	**65.1**	102
	5	0.057	0.048	56.8	47.7	**52.3**	103
	7	0.045	0.046	44.6	45.8	**45.2**	83.6
	14	0.050	0.055	49.6	55.0	**52.3**	93.1
	31	0.041	0.049	40.8	48.9	**44.8**	101
	45	0.041	0.041	40.8	40.0	**40.4**	102
	60	0.043	0.039	42.4	38.7	**40.6**	109

The results obtained from this storage stability study indicate that EBIS is not stable under deep frozen conditions (≤ -18 °C) in any of the tested matrices (rape seed, white bean, potato, grape, lettuce, onion, cucumber) for the investigated time period of about 60 days.
The decline in concentration of EBIS showed a similar progress in all matrices. A fast decrease in concentration of EBIS could be observed during the first 7 days of storage. After this time, the further decrease in concentration took place at a slower rate.

The means of the procedural recoveries at 0.1 mg/kg are shown in Table 6.1-13.

Table 6.1-13: Means of procedural recoveries

Matrix	Storage intervals [days]	Recoveries EBIS [%]	
		Mean [%]	RSD [%]
Rape seed	0, 3, 5, 7, 14, 31, 45, 60	94	8.7
White bean	0, 3, 5, 7, 14, 31, 45, 63	94	11.0
Potato	0, 3, 5, 7, 14, 31, 45, 67	99	8.5
Grapes	0, 3, 5, 7, 14, 30, 45, 67	91	10.0
Lettuce	0, 3, 5, 7, 14, 30, 45, 60	96	12.0
Onion	0, 3, 5, 7, 14, 31, 45, 60	102	7.7
Cucumber	0, 3, 5, 7, 14, 31, 45, 60	99	7.9

RSD = Relative Standard Deviation

III. CONCLUSION

The results obtained from this storage stability study indicate that EBIS is not stable under deep frozen conditions (≤ -18 °C) in any of the tested matrices (rape seed, white bean, potato, grape, lettuce, onion, cucumber) for the investigated time period of about 60 days. The decline in concentration of EBIS showed a similar progress in all matrices. A fast decrease in concentration of EBIS could be observed during the first 7 days of storage. After this time, the further decrease in concentration took place at a slower rate.

Report: CA 6.1/3
Benz-Birck A. et al., 2011a
Storage stability study of incurred ETU residues in lettuce
2010/1169547
Guidelines: none
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** Metiram, ETU, BAS 222 28 F
Description: Metiram (BAS 222 F), ETU, BAS 222 28 F (Metiram: 70% nominal, WG formulation)
Lot/Batch #: 2000-2
Purity: not reported
CAS#: 9006-42-2, BAS 222 F
Development code: Metiram: BAS 222 F
ETU: M222F002
Spiking levels: 0.25 mg/kg (with exception of 0.5 mg/kg in sampling occasion 2, day 0 and 2.5 mg/kg in sampling occasion 4, day 1).

2. **Test Commodity:** Lettuce (Leaf vegetables, herbs and edible flowers)
Crop: Lettuce
Type: not reported
Variety: not reported
Botanical name: *Lactuca sativa L.*
Crop parts(s) or processed commodity: head
Sample size: 12 heads (min. 4 kg)

B. STUDY DESIGN AND METHODS

1. Test procedure

During the 2001 growing season, one field trial was conducted in a representative lettuce growing area in Germany, in order to provide lettuce specimens with incurred residues of ethylenethiourea (ETU), a common metabolite of all ethylenebisdithiocarbamates, for assessing the storage stability in lettuce. In addition, this study also investigated whether or not the method of specimen preparation and the degree of homogenisation (size of surface) had any influence on the rate of degradation.

The aim was to generate sufficiently high (quantifiable) ETU residues in the lettuce specimens. Therefore application rates were significantly exaggerated compared to the usual agricultural practice (GAP) used and the sampling regime corresponded with a worst case scenario. The test item BAS 222 28 F (70% Metiram, WG) was applied four times to head lettuce at a rate of 2.24 kg as/ha (3.2 kg of formulated product/ha). The first application was carried out on 21.05.2001 followed by three further applications 7, 18 and 28 days later. The spray volume used was 400 L/ha.
Head lettuce specimens from the control plot were collected immediately before the 2nd application, at sampling occasion 1. Head lettuce specimens from the treated plot were collected at 3 and 8 days after the 2nd application at sampling occasions 2 and 3. Due to the low residue level in specimens collected at sampling occasion 3, a 3rd application and, after analysis of residue levels at sampling occasion 4, a 4th application was performed. Sampling 6 was performed 3 days after the fourth application. Sampling occasions 2 (3DAA2), 4 (3DAA3) and 6 (3DAA4) were used for investigations on specimen preparation and storage stability.

In order to compare three different methods of specimen preparation, the specimens were either stored unprocessed as entire field specimens and processed under frozen conditions with a wooden hammer on the day of analysis ("F") or were prepared on the day of sampling. Therefore, the lettuce heads of three specimens were segmented into three pieces with a ceramic knife. The thirds were allocated to three mixed samples, which were processed by the following three methods:
1. The sample material was cut into small pieces with a ceramic knife ("MA").
2. The sample material was homogenized with a commercial mill ("MB").
3. The sample material was homogenized with a Stephan mill after addition of dry ice ("MC").

The processed samples were aliquoted and stored frozen until analysis. In addition, control samples, which were prepared from untreated specimens with a ceramic knife on the day of sampling, were aliquoted, frozen and analyzed with each measurement series.
For the storage stability investigation, analysis of ETU was planned on 0, 1, 3 (±2), 7 (±2), 14 (±2), 28 (±2), 42 (±2), 56 (±2), 84 (±7), 112 (±7), 140 (±7), 168 (±7), 230 (±7), 365 (±14) days of storage. This report is the final report describing the result up to 358 days after sampling occasion 2 and up to 396 days after sampling occasion 6, respectively.

2. Description of analytical procedures

The samples were analysed for ETU using BASF method No. 373/1 with some adjustments. These adjustments are incorporated into the revised method 373/2. The validated LOQ of the revised method No. 373/2 was 0.01 mg/kg. In the current study, individual results were extrapolated to the LOQ of 0.01 mg/kg.
Principle of BASF Method no. 373/1: ETU was extracted from the plant material with a mixture of sodium ascorbate, ethyleneurea, ammonium chloride and methanol-water. After centrifugation an aliquot was taken and the methanol was evaporated from the extract. The pH was adjusted to 8 before evaporating. The remaining water phase was cleaned by liquid/liquid (water/dichloromethane) partition on an Extrelut/Al_2O_3 column. After concentration of the eluate, the residue was determined by HPLC using pulsed amperometric detection.

The mean procedural recovery was 76% at a fortification level of 0.25 mg/kg (with exception of 0.5 mg/kg in sampling occasion 2, day 0 and 2.5 mg/kg in sampling occasion 4, day 1).

Before storage, the processed samples were also analysed for Metiram in order to evaluate the initial residue level. The analysis was conducted using BASF method No. 135/4 which determines the active ingredient as CS_2. The limit of quantitation of the method is 0.05 mg/kg. The mean procedural recovery was approximately 81% at a fortification level of 0.5 or 5.0 mg/kg.
Principle of BASF Method no. 135/4: Carbon disulphide was released from Metiram and comparable substances by heating with hydrochloric acid and tin chloride. Reaction with the anion of methanol formed by KOH lead to the formation of a xanthogenate which was determined photometrically at 302 nm. The limit of quantitation is 0.05 mg/kg Metiram for all matrices.

II. RESULTS AND DISCUSSION

Sampling occasion 2 (3 days after the 2nd application)
The corrected initial mean ETU values of lettuce samples after processing with a ceramic knife ("MA") were 0.274 mg/kg, with a commercial mill 0.160 mg/kg and with dry ice in a Stephan mill ("MC") 0.120 mg/kg. The field specimen ("F") stored unprepared for one day and crushed in frozen state with a wooden hammer showed a corrected initial mean value of 0.115 mg/kg.

"MA": The individual results of the ETU concentration in samples stored after processing with a ceramic knife showed a considerable variation. The residue level decreased to values lower than 70% of the corrected initial mean values, but increased again to 75% on storage day 42 and to 69% on storage day 172.
"MB": The residue levels in samples stored after processing with a commercial mill showed a significant decrease and ranged between 21% and 59% of the corrected initial mean values.
"MC": The individual results in samples stored after processing with dry ice in a Stephan mill apparently showed increased residue levels at several storage intervals. The stability was demonstrated over the investigated time period of more than one year.
"F": The individual results in specimens stored unprepared and crushed in frozen state with a wooden hammer showed an apparent increase of the residue level at the majority of storage intervals. The stability was demonstrated for the investigated time interval of more than one year.

Procedural recoveries were recorded for every storage interval and ranged between 48.8% (storage day 5) and 117.95% (storage day 1) for sampling occasion 2. A degradation curve was simulated from the results of measurement series with procedural recoveries over 70% with corrected values. The most evident results are:

- The unprepared field samples ("F") and the samples processed with dry ice in a Stephan mill ("MC") showed the best storage stability over a year and a flat curve at a high residue level (calculated stabilities of "F": 2221 days and "MC": 1553 days, 70% of initial value).
- After homogenisation with a ceramic knife ("MA"), the residue levels started with the highest values, after storage a degradation to a residue level of approx. 0.13 mg/kg was observed
- For samples prepared with a commercial mill ("MB") a rapid decline to a low residue level of approx. 0.05 mg/kg was observed

The data used to assess the storage stability of ETU in lettuce treated with BAS 222 28 F at sampling occasion 2 are provided in Table 6.1-14.

Table 6.1-14: Residues of ETU in stored lettuce at sampling occasion 2

Processing Method	Day	ETU Residue level					Procedural recovery (mean, %)
		Individual results (mg/kg)		% of mean value day 0		Mean value of % of day 0	
Process MA (ceramic knife)	0[1]	0.272	0.276	99	101	**100**	101.6
	Mean value day 0: 0.274						
	1	0.165	0.228	60	83	**72**	114.9
	5	0.128	0.120	47	44	**45**	51.7
	7	0.174	0.179	64	66	**65**	75.5
	13	0.090	0.109	33	40	**36**	94.7
	28	0.118	0.145	43	53	**48**	81.1
	42	0.169	0.244	62	89	**75**	(95.5)[2]
	56	0.097	0.090	36	33	**34**	71.5
	85	0.132	0.123	48	45	**47**	81.0
	112[3]						
	138	0.078	0.105	28	38	**33**	64.3
	172	0.158	0.220	58	81	**69**	65.9
	230	0.131	0.123	48	45	**47**	66.6
	358	0.069	0.061	25	22	**24**	73.0
Process MB (commercial mill)	0[1]	0.181	0.139	113	87	**100**	101.6
	Mean value day 0: 0.160						
	1	0.034	0.032	21	20	**21**	114.9
	5	0.035	0.039	22	25	**23**	51.7
	7	0.063	0.072	40	45	**42**	75.5
	13	0.030	0.047	19	29	**24**	94.7
	28	0.030	0.051	19	32	**25**	81.1
	42	0.060	0.073	37	45	**41**	(95.5)[2]
	56	0.059	0.058	37	36	**37**	71.5
	85	0.058	0.055	36	34	**35**	81.0
	112[3]						
	138	0.048	0.040	30	25	**28**	64.3
	172	0.085	0.104	53	65	**59**	65.9
	230	0.074	0.059	46	37	**42**	66.6
	358	0.036	0.049	23	31	**27**	73.0
Process MC (Stephan mill/dry ice)	0[1]	0.109	0.131	91	109	**100**	101.6
	Mean value day 0: 0.120						
	1	0.203	0.167	169	139	**154**	114.9
	5	0.207	0.182	173	151	**162**	51.7
	7	0.218	0.192	181	160	**171**	75.5
	13	0.045	0.027	37	22	**30**	94.7
	20[4]	0.086	0.121	71	101	**86**	68.8
	28	0.209	0.141	174	117	**146**	81.1
	42	0.331	0.494	275	411	**343**	(95.5)[2]
	56	0.125	0.114	104	95	**99**	71.5
	85	0.157	0.144	131	120	**125**	79.9
	112[3]						
	138	0.068	0.061	57	51	**54**	77.7
	172	0.159	0.223	132	185	**159**	65.9
	230	0.115	0.222	96	185	**140**	80.0
	358	0.112	0.136	93	113	**103**	96.2

Processing Method	Day	ETU Residue level					Procedural recovery (mean, %)
		Individual results (mg/kg)		% of mean value day 0		Mean value of % of day 0	
Field (F) Specimen (unprepared)	0						
	1[1]	0.106	0.123	92	108	**100**	114.9
	Mean value day 1: 0.115						
	5	0.205	0.151	178	132	**155**	51.7
	7	0.145	0.185	127	161	**144**	75.5
	13	0.205	0.259	178	225	**202**	94.7
	28	0.165	0.128	144	112	**128**	81.1
	42	0.109	0.128	95	112	**103**	(95.5)[2]
	56	0.093	0.082	81	71	**76**	71.5
	85	0.116	0.274	101	239	**170**	81.0
	112[3]						
	138	0.046	0.101	40	88	**64**	64.3
	172	0.175	0.146	153	128	**140**	78.3
	230	0.117	0.115	102	100	**101**	66.6
	358	0.074	0.097	65	85	**75**	73.0

1 values of individual results corrected with procedural recovery
2 one procedural recovery determined only
3 analysis of sampling occasion 2, day 112 ± 7 was not reported due incorrect fortification
4 sampling occasion 2, storage day 20: samples prepared according to method "MC" measured only

Additionally, processed lettuce samples and the corresponding unprepared field specimens were analysed for Metiram after six storage intervals. Lettuce samples prepared according to method "MB" showed the lowest residue level of Metiram.

The initial BAS 222 F residues of lettuce from sampling occasion 2 are presented in Table 6.1-15.

Table 6.1-15: Residues of Metiram and CS_2 in lettuce at sampling occasion 2

Day	Process MA		Process MB		Process MC		Field specimens F	
	Residues (mg/kg)		Residues (mg/kg)		Residues (mg/kg)		Residues (mg/kg)	
	Metiram	CS_2[1]	Metiram	CS_2[1]	Metiram	CS_2[1]	Metiram	CS_2[1]
0	15.14 16.55	8.47 9.26	13.86 12.89	7.75 7.21	14.82 15.07	8.29 8.43	15.14 16.55	8.47 9.26
13	14.68 14.53	8.22 8.13	7.61 9.84	4.26 5.50	13.79 13.22	7.71 7.40	16.64 18.11	9.31 10.13
42	10.24 12.94	5.73 7.24	11.03 10.38	6.17 5.81	15.07 13.34	8.43 7.46	16.15 12.83	9.03 7.18
172	10.86 9.79	6.08 5.48	7.99 7.51	4.47 4.20	7.55 9.97	4.23 5.58	11.79 12.38	6.59 6.93
230	14.11 10.59	7.86 5.87	12.13 11.49	6.74 6.37	13.01 12.95	7.26 7.24	13.38 17.58	7.48 9.77
362	11.19 11.98	6.26 6.71	7.85 7.76	4.39 4.34	10.89 10.74	6.09 6.01	11.27 12.48	6.31 6.98

1 calc. factor Metiram to CS_2: 0.5595
n.m. not measured

Sampling occasion 3 (8 days after the 2nd application)
The determination of the ETU concentration in samples processed according to method "MA" showed a considerably decreased residue level (< 0.05 mg/kg). These results indicate that a fast degradation or metabolism of ETU occurred on the field. Metiram was therefore applied for a third time.

Sampling occasion 4 (3 days after the 3rd application)
Five storage intervals were analysed after the third application. The residue levels were still shown to be low and a fourth application was carried out. The further investigation of the stored samples was cancelled.

The data used to assess the storage stability of ETU in lettuce treated with BAS 222 28 F at sampling occasion 4 are provided in Table 6.1-16.

Table 6.1-16: Residues of ETU in stored lettuce at sampling occasion 4

Processing Method	Day	ETU Residue level					Procedural recovery (mean, %)
		Individual results (mg/kg)		% of mean value day 0		Mean value of % of day 0	
Process MA (ceramic knife)	0[1]	0.105	0.097	104	96	**100**	94.7
	Mean value day 0: 0.101						
	1	0.071	0.060	70	60	**65**	110.6
	2	0.028	0.013	28	13	**20**	70.0
	7	0.052	0.071	52	70	**61**	(54.0)[2]
	14	0.028	< 0.01	28	n.a.	**28**	40.8
Process MB (commercial mill)	0[1]	0.105	0.101	102	98	**100**	94.7
	Mean value day 0: 0.103						
	1	0.057	0.052	55	50	**53**	110.6
	2	0.015	< 0.01	15	n.a.	**15**	70.0
	7	0.047	0.060	46	59	**52**	(54.0)[2]
	14	0.010	0.015	10	15	**12**	40.8
Process MC (Stephan mill/dry ice)	0[1]	0.040		100		**100**	94.7
	1	0.116	0.081	287	200	**243**	110.6
	2	0.024	< 0.01	60	n.a.	**60**	70.0
	7	0.203	0.030	503	73	**288**	(54.0)[2]
	14	0.047	< 0.01	116	n.a.	**116**	40.8
Field (F) Specimen (unprepared)	0						
	1[1]	0.115	0.106	104	96	**100**	110.6
	Mean value day 1: 0.111						
	2	0.089	0.078	81	71	**76**	70.0
	7	0.051	0.060	46	54	**50**	(54.0)[2]
	14	0.067	0.015	61	13	**37**	40.8

1 values of individual results corrected with procedural recovery
2 one procedural recovery determined only
n.a. not applicable

The initial BAS 222 F residues of lettuce from sampling occasion 4 are presented in Table 6.1-17.

Table 6.1-17: Residues of Metiram and CS_2 in lettuce at sampling occasion 4

Day	Process MA		Process MB		Process MC		Field specimens F	
	Residues (mg/kg)		Residues (mg/kg)		Residues (mg/kg)		Residues (mg/kg)	
	Metiram	CS_2[1]	Metiram	CS_2[1]	Metiram	CS_2[1]	Metiram	CS_2[1]
0	16.26	9.10	8.57	4.80	12.96	7.25	n.m.	n.m.
	17.88	10.00	13.02	7.28	13.50	7.55	n.m.	n.m.

1 calc. factor Metiram to CS_2: 0.5595
n.m. not measured

Sampling occasion 6 (3 days after the 4th application)
Procedural recoveries for sampling occasion 6 ranged between 45.60% and 110.88%. Recoveries lower than 70% were obtained for several storage intervals and for the initial analysis on day 0. In addition, for a number of measurement series, only one procedural recovery was valid. For this reason, samples from sampling occasion 2 yielded the most valuable results and degradation curves were simulated only on the basis of these data. However, confirming the results obtained from sampling occasion 2, the lettuce samples after homogenisation with a commercial mill (method "MB") contained the lowest ETU levels.

The data used to assess the storage stability of ETU in lettuce treated with BAS 222 28 F at sampling occasion 6 are provided in Table 6.1-18.

Table 6.1-18: Residues of ETU in stored lettuce at sampling occasion 6

Processing Method	Day	ETU Residue level					Procedural recovery (mean, %)
		Individual results (mg/kg)		% of mean value day 0		Mean value of % of day 0	
Process MA (ceramic knife)	0[1]	0.371	0.309	109	91	**100**	(61.9)[2]
	Mean value day 0: 0.340						
	1	0.140	0.122	41	36	**39**	55.0
	4	0.182	0.090	54	26	**40**	55.0
	7	0.106	0.050	31	15	**23**	(103.5)[2]
	14	0.415	0.381	122	112	**117**	69.8
	33	0.204	0.091	60	27	**43**	72.0
	43	0.040	0.036	12	11	**11**	74.4
	56	0.042	0.069	12	20	**16**	69.8
	83	0.130	0.095	38	28	**33**	(81.9)[2]
	111	0.066	0.058	19	17	**18**	71.9
	144	0.185	0.163	55	48	**51**	(77.3)[2]
	173	0.143	0.141	42	42	**42**	70.5
	230	0.034	0.059	10	17	**14**	65.6
	396	0.184	0.140	54	41	**48**	(86.2)[2]

Processing Method	Day	ETU Residue level					Procedural recovery (mean, %)
		Individual results (mg/kg)		% of mean value day 0		Mean value of % of day 0	
Process MB (commercial mill)	0[1]	0.322	0.239	115	85	**100**	(61.9)[2]
	Mean value day 0: 0.281						
	1	0.036	n.m.[3]	13	n.a.	**13**	55.0
	4	0.024	0.044	9	16	**12**	55.0
	7	0.064	0.041	23	15	**19**	(103.5)[2]
	14	0.161	0.120	57	43	**50**	69.8
	33	0.039	0.055	14	20	**17**	72.0
	43	0.025	0.022	9	8	**8**	74.4
	56	< 0.01	0.023	n.a.	8	**8**	69.8
	83	0.055	0.063	20	22	**21**	(81.9)[2]
	111	0.057	0.039	20	14	**17**	71.9
	144	0.121	0.079	43	28	**36**	(77.3)[2]
	173	0.077	0.079	27	28	**28**	70.5
	230	0.043	0.044	15	16	**15**	65.6
	396	0.059	0.073	21	26	**24**	(86.2)[2]
Process MC (Stephan mill/dry ice)	0[1]	0.177	0.116	121	79	**100**	(61.9)[2]
	Mean value day 0: 0.147						
	1	0.239	0.175	163	119	**141**	55.0
	4	0.182	0.185	124	126	**125**	55.0
	7	0.347	0.109	237	74	**155**	(103.5)[2]
	14	0.509	0.595	347	406	**377**	69.8
	33	[3]	0.120	n.a.	82	**82**	48.8
	43	0.119	0.172	81	117	**99**	75.8
	56	0.163	0.148	111	101	**106**	88.5
	83	0.333	0.330	227	225	**226**	(81.9)[2]
	111	0.200	0.148	136	101	**118**	87.6
	144	0.210	0.262	143	179	**161**	75.9
	173	0.332	0.302	226	206	**216**	84.4
	230	0.235	0.204	160	139	**150**	105.2
	396	0.346	0.305	236	208	**222**	98.7
Field (F) Specimen (unprepared)	0			91	109	**100**	55.0
	1[1]	0.462	0.556				
	Mean value day 1: 0.509						
	4	[3]	0.190	n.a.	37	**37**	55.0
	7	0.134	0.176	26	34	**30**	(103.5)[2]
	14	0.394	0.466	77	92	**84**	69.8
	33	0.224	0.112	44	22	**33**	72.0
	43	0.144	0.089	28	18	**23**	74.4
	56	0.093	0.135	18	27	**22**	69.8
	83	0.136	0.188	27	37	**32**	(81.9)[2]
	111	0.107	0.087	21	17	**19**	71.9
	144	0.188	0.171	37	34	**35**	(77.3)[2]
	173	0.084	0.114	17	22	**19**	70.5
	230	0.076	0.143	15	28	**21**	65.6
	396	0.129	0.065	25	13	**19**	(86.2)[2]

n.m. not measured
n.a. not applicable
1 values of individual results corrected with procedural recovery
2 one procedural recovery determined only
3 measurement not evaluated

The initial BAS 222 F residues of lettuce from sampling occasion 6 are presented in Table 6.1-19.

Table 6.1-19: Residues of Metiram and CS_2 in lettuce at sampling occasion 6

Day	Process MA		Process MB		Process MC		Field specimens F	
	Residues (mg/kg)		Residues (mg/kg)		Residues (mg/kg)		Residues (mg/kg)	
	Metiram	CS_2[1]	Metiram	CS_2[1]	Metiram	CS_2[1]	Metiram	CS_2[1]
0	17.04 15.15	9.54 8.48	13.80 11.59	7.72 6.48	17.66 16.84	9.88 9.42	n.m. n.m.	n.m. n.m.

1 calc. factor Metiram to CS_2: 0.5595
n.m. not measured

III. CONCLUSION

The results of the ETU analysis showed an influence of the homogenization method on the rate of degradation.

The residue level in samples after processing with a ceramic knife (method "MA") showed an apparent decrease. In samples processed with a commercial mill (method "MB"), a strong decrease of the residue level right after processing was observed. After processing with dry ice in a Stephan mill (method "MC"), a high residue level and storage stability were observed for the investigated period of more than one year.

After unprocessed storage of the entire lettuce head and crushing in frozen state with a wooden hammer ("F"), the residue levels were high and no degradation was observed for the investigated period of more than one year.

The results obtained after all processing methods showed a certain variability. This effect could be at least partly explained by the variability between the field specimens and by the inhomogeneity of the analyzed plant material.

The inaccuracy of the method of analysis was averaged by analyses in duplicate. Still, a certain variability of the individual results was observed.

CA 6.2 Metabolism, distribution and expression of residues

Annex II Dossier :
Regarding metabolism in plants, studies in apples and potatoes were previously evaluated during the Annex I inclusion process and considered as suitable.
Regarding metabolism in livestock, studies in chicken and goat were previously evaluated during the Annex I process and considered as suitable.

AIR3 Dossier :
A new metabolism study was conducted in lettuce.

Regarding metabolism in plants, the representative uses of the present dossier (grape, potato) are supported by the previously evaluated metabolism studies (covering crop categories of fruit crops and root/tuber vegetables). In order to provide a general view on the metabolism of metiram in foliar applied crops, an additional metabolism study in lettuce, representing a third crop category, was conducted. Taken together the results obtained for three different crop categories (fruits/fruiting vegetables, root/tubers, leafy vegetables) show that metabolism pathways of metiram are comparable and consequently can be extrapolated to foliar applied crops in general. Noteworthy, the metiram metabolism is qualitatively not different from the metabolism elucidated for other dithiocarbamate fungicides.

Regarding metabolism in livestock, the previously evaluated studies in chicken and goat show metabolism pathways comparable to in rat (see section metiram AIR3 dossier MCA 5.1). Noteworthy, the metiram metabolism is qualitatively not different from the metabolism found for other dithiocarbamate fungicides.

In brief, in plants (apple, potato, lettuce) and livestock (goat, hen), metiram desintegrates rapidly into dynamic intermediates which after entering the metabolic carbon pool are incorporated into carbohydrates, protein and other natural products as the terminal residue.

In 2008 and in context of the re-evaluation of MRLs according to Reg. 396/2005, Art. 12, BASF SE has submitted an EU MRL compilation dossier to Italy acting as Rapporteur Member State (see also "*Italy, 2010: Evaluation report on the setting of MRLs for metiram in plant commodities prepared by the evaluating Member State Italy under Article 8 of Regulation (EC) No 396/2005, 15 November 2010, 174 p*"). In this EU MRL compilation dossier (DocID 2008/1042839), the following tables are included.

Metabolism in plants (OECD data point numbers IIA 6.2.1, IIA 6.7, IIIA 8.2 and IIIA 8.7)

Plant groups covered	Apples (fruits), potatoes (root and tuber vegetables), Lettuce (leafy vegetables)
Rotational crops	Wheat, beet, kale
Plant residue definition for monitoring	Metiram (expressed as CS_2)
Plant residue definition for risk assessment	Metiram (expressed as CS_2) ETU (in processed commodities)
Conversion factor (monitoring to risk assessment)	Not applicable

Metabolism in livestock (OECD data point numbers IIA 6.2.2 to IIA 6.2.5, IIA 6.7, IIIA 8.4 and IIIA 8.7)

Animals covered	Goat and hens
Animal residue definition for monitoring	Metiram expressed as CS_2
Animal residue definition for risk assessment	Metiram expressed as CS_2
Conversion factor (monitoring to risk assessment)	Not applicable
Metabolism in rat and ruminant similar (yes/no)	Yes
Fat soluble residue: (yes/no)	No (not applicable)

CA 6.2.1 Metabolism, distribution and expression of residues in plants

Annex II Dossier :
Regarding metabolism in plants, studies in apples and potatoes were previously evaluated during the Annex I inclusion process and considered as suitable.

AIR3 Dossier :
A new metabolism study was conducted in lettuce.

Overall, plant metabolism studies were performed in three crops representing three different crop categories, namely apple (crop category: fruit/fruiting vegetable), potato (crop category: root/tuber), and lettuce (crop category: leafy vegetable). The metabolism pathways in all three crops were comparable and consequently can be extrapolated to foliar applied crops in general. In addition, the pathways were comparable to the pathways known for the other dithiocarabamate fungicides.
In brief, metiram - as are the other dithiocarbamate fungicides - is a non-systemic fungicide. Once deposited on the plant surface, the non-soluble metal ion complex is not translocated further into the plant. Rather, upon contact with a solvent system, the metal complex undergoes chemical desintegration (solvolysis) releasing dynamic intermediates which enter the general metabolic carbon pool and by this are incorporated into biological products (e.g. carbohydrates, amino acids, lipds).

Table 6.2-1: Summary of available metabolism studies in plants

Crop group	Crops	Application	Sampling	Dossier reference
Fruits	apples	1st method of treatment: streaking pure, radiolabelled test material onto surface of apples and leaves as suspension using micropipette 2nd method of treatment: foliar spray application of test material as admixture of radiolabelled and non-radiolabelled BAS 222 F, 4x 3.36 - 4.85 kg a.s./ha (treatment 1: 4.85 kg a.s./ha, treatment 2-4: 3.36 kg a.s./ha)	0, 15, 27 DAT	CA 6.2.1/1
		foliar application, 5x 1.5 kg a.s./ha	83 DAT	CA 6.2.1/2
		foliar spraying to apple plants, 2x 0.240 g a.i/m^2	4 DAT	CA 6.2.1/3
Root / tuber vegetables	potatoes	foliar spraying to potato plants, 4x 1.794 - 3.587 kg a.s./ha (treatment 1-2: 1.794 kg a.s./ha, treatment 3-4: 3.587 kg a.s./ha)	21 DAT	CA 6.2.1/4
		foliar spraying to potato plants, 4x 2.0 kg a.s./ha	1 DAT	CA 6.2.1/5
		foliar spraying to potato plants, 2x 2.0 kg a.s./ha	21 DAT	CA 6.2.1/6
Leafy vegetables	lettuce	post emergence treatment, 3 x 0.2 kg as/ha	7 DAT	CA 6.2.1/7

DAT: days after last treatment

Metabolism in apple (crop category: fruits/fruiting vegetables)

Three metabolism studies in apple are available. They have been evaluated previously and considered valid (Italy, 2010: Evaluation report on the setting of MRLs for metiram in plant commodities prepared by the evaluating Member State Italy under Article 8 of Regulation (EC) No 396/2005, 15 November 2010, 174 p)
In DocID 1990/10669 (report CA 6.2.1/1) foliar application of metiram was shown to result in much higher residues on the surface compared with the inside (pulp), predominantly parent metiram. Regarding transformation products, the predominant endproducts of metiram metabolism in apple were natural product fractions (i.e. sugar, lignin, cellulose, lipids, amino acids etc.). EBIS, EU and ETU were present at 2-4 % TRR. Similar to parent, metabolites were predominantly located at the surface. Notably, EBIS found in whole apple (3.8% TRR) is attributed to predominantly surface (3.2%TRR) and peel (0.41% TRR) and only traces in pulp (0.17% TRR). These results are confirmed by investigations after foliar application of metiram reported in two documents, DocID1986/0524 (report CA 6.2.1/2) and DocID 1986/0526 (report CA 6.2.1/3), regarding the surface (peel) as the predominant location of the residue as well as the parent complex as the predominant component of the residue.

Report:	*CA 6.2.1/1 Hubert T.D., 1990a Metiram: Nature of the residue in apples 1990/10669*
Guidelines:	*EPA 171-4(a)*
GLP:	*yes (certified by United States Environmental Protection Agency)*

Report:	*CA 6.2.1/2 Bieber W.-D.,Kroehn R., 1986a Study on the metabolism of the Metiram complex (ethylene-14C-labelled) in apples 1986/0524*
Guidelines:	*<none>*
GLP:	*yes (certified by <none>)*

Report:	*CA 6.2.1/3 Bieber W.-D.,Kroehn R., 1986b Study on the metabolism of the Metiram complex (thiocarbonyl-14C-labelled) in apples 1986/0526*
Guidelines:	*<none>*
GLP:	*yes (certified by <none>)*

Metabolism in <u>potato</u> (crop category: root/tuber)

Three metabolism studies in potato are available. They have been evaluated previously and considered valid (Italy, 2010: Evaluation report on the setting of MRLs for metiram in plant commodities prepared by the evaluating Member State Italy under Article 8 of Regulation (EC) No 396/2005, 15 November 2010, 174 p)
In DocID 1990/10668 (report CA 6.2.1/4) foliar application of potato plants with metiram was shown to result in similar low levels in peel and pulp, both plant parts which were not directly exposed to the spray application. While immediate decomposition products, notably EBIS, EU, and ETU were present only in minor amounts, major metabolites were biomolecules such as glycine, creatinine, and allantoin. Their subsequent incorporation into natural plant product fractions is indicated by the presence of radioactivity in natural product fractions such as proteins and carbohydrates. These results are confirmed by investigations after foliar application of metiram reported in two documents, DocID1986/0523 (report CA 6.2.1/5) and DocID 1986/0523 (report CA 6.2.1/6), regarding the <u>surface (tops of potato plant)</u> as the predominant location of the residue.

Report:	*CA 6.2.1/4* *Wu J., 1990a* *Metabolism of 14C-Metiram complex in potatoes - Nature of the residue in potatoes: Analysis and quantitation of metabolites* *1990/10668*
Guidelines:	*EPA 171-4, EPA Subdivision O of the Pesticide Assessment Guidelines, EPA 40 CFR 158.125*
GLP:	*yes* *(certified by United States Environmental Protection Agency)*

Report:	*CA 6.2.1/5* *Bieber W.-D.,Kroehn R., 1986c* *Study on the metabolism of the Metiram complex (ethylene-14C-labelled) in potatoes* *1986/0523*
Guidelines:	*<none>*
GLP:	*yes* *(certified by <none>)*

Report:	*CA 6.2.1/6* *Bieber W.-D.,Kroehn R., 1986d* *Study on the metabolism of the Metiram complex (thiocarbonyl-14C-labelled) in potatoes* *1986/0525*
Guidelines:	*<none>*
GLP:	*yes* *(certified by <none>)*

Metabolism in lettuce (crop category: leafy vegetable)

One metabolism study in lettuce is available (DocID 2009/1049027, report* CA 6.2.1/7*). This study was conducted, in addition to the available studies in apple and potato, with the objective to provide metabolism data representative for a third crop category. Based on these new results in lettuce, showing metabolism of metiram comparable to the other two crop categories, a general definition of the residue can be proposed. A summary of the lettuce metabolism study is provided below.
In brief, foliar application is shown to result in high residues on the plant surface (predominantly the unchanged parent compound). The immediate decomposition products characteristic for dithiocarbamates were present in major proportions (sum of EU and ETU at 15% TRR) and minor proportion (EBIS at 2.3% TRR). In addition, M222F007 (TDIT) a compound previously reported as occasionally occuring soil metabolite was identified in this study, however only at very low proportion (1.4% TRR). The subsequent incorporation of the decomposition products into natural plant constituents is indicated by the presence of radioactivity in natural product fractions such as proteins and carbohydrates.

The metabolism study in lettuce is summarized below:

Report:	CA 6.2.1/7 Bross M.,Glaessgen W.E., 2010a Metabolism of 14C-Metiram (14C-BAS 222 F) in lettuce 2009/1049027
Guidelines:	EPA 860.1300: Nature of the Residue in Plants Livestock, EPA 860.1000, EEC 7028/VI/95 rev. 3 Appendix A (EU): Metabolism and distribution in plants (draft of 22 July 1997)
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

I. MATERIAL AND METHODS (2009/1049027)

A. MATERIALS

1.	**Test Material:**	^{14}C-BAS 222 F
	Description:	^{14}C-BAS 222 F (Metiram), labelled in the ethylene bridge (Specific radioactivity: 4.68 MBq/mg)
	Lot/Batch #:	153-3003 (^{14}C-BAS 222 F)
	Purity:	> 98% (by Iodometry)
	CAS#:	9006-42-2
	Stability of test Compound:	The residues in lettuce, treated with BAS 222 F were stable under the chosen experimental conditions (-18°C, 15 months)

2. **Test Commodity:** Leaf vegetables & fresh herbs
Crop: Lettuce
Type: not reported
Variety: Nadine
Botanical name: *Lactuca sativa* L.
Crop parts(s) or processed commodity: head
Sample size: lettuce head: 4412 g

3. **Soil:** Loamy sand soil (USDA scheme loamy sand; German scheme DIN 4220: soil type loamy sand)
The soil physicochemical properties are described below (see Table 6.2-2).

Table 6.2-2: Soil physicochemical properties

Soil Type	pH	Organic Carbon [%]	Sand [%]	Silt [%]	Clay [%]	Maximal water holding capacity [g water/100 g dry soil]	CEC[1] [meq Ba/100 g dry soil]
Loamy sand soil* Loamy sand**	6.5*** 7.0$^{\Delta}$	0.9	82*	12*	6*	29	8

* USDA scheme ** German scheme DIN 4220 *** ($CaCl_2$) Δ (H_2O) 1 cation exchange capacity

B. STUDY DESIGN AND METHODS

1. Test procedure

Metiram (BAS 222 F) belongs to the class of ethylenebis (dithiocarbamate) (EBDC) fungicides. These non-systemic foliar applied compounds with mainly protective activity are widely used to control fungal diseases in many fruit and vegetable crops. The metabolism of ^{14}C-BAS 222 F (Metiram) was investigated in leaf lettuce following post emergence treatment at a nominal application rate of 3 x 0.2 kg as/ha (approximately 1.786 lb/A). The spray applications were performed using radioactive material labelled in the ethylene bridge after mixing with the blank WG formulation BAS 222 28 F. Three applications were carried out in intervals of seven days, and the lettuce heads were harvested seven days after the final treatment to investigate the nature and the level of radioactive residues. All samples were stored in a freezer at ≤ -18°C.

2. Description of analytical procedures

TRR combusted:

For the determination of the TRR combusted, aliquots of homogenized solid plant samples were weighed and combusted by means of an automatic sample. The $^{14}CO_2$ evolved during combustion was trapped by an absorption liquid, and the collected radioactivity was measured by liquid scintillation counting (LSC).

Extraction:
Aliquots of homogenized plant material were extracted three times with methanol. The methanol extracts of the three steps were combined and measured by LSC. The residue was further extracted with water (twice). The aqueous extracts were also combined and analyzed by means of LSC.

The results of the methanol extraction and the water extraction were summarized and referred to as extractable radioactive residues (ERR).

The residue after solvent extraction of each sample was dried and homogenized. Aliquots were combusted for the determination of the residual radioactive residue (RRR).

Partition of extractable radioactive residues on Extrelut columns:
The methanol and water extracts were characterized by partition experiments on Extrelut columns. The partition step was also used for removing the most likely suspended metal complexes from the extracts, since they accumulate during HPLC.

The column was eluted with dichloromethane, followed in some cases by a second elution step with water. The eluates were mixed prior to determination of the radioactivity by LSC measurement of aliquots. The same partition procedure was also carried out for 20 ml aliquots of the NH_40H solubilizate of the residual radioactive residue (RRR, second work up).

Characterization of the RRR:
In order to characterize the residual radioactive residue, an aliquot of the dried residue after solvent extraction (second work up) was homogenized with 1 % NH_40H. A subsample of the NH_40H solubilizate was applied on an Extrelut column and the solubilized metabolites were eluted with dichloromethane. An aliquot of the eluate was concentrated, diluted with water and investigated with HPLC.

The rest of the eluate was adjusted to pH 3.8 and the proteins were precipitated by the addition of acetone, stirring with a glass rod and standing over night at 4 °C. Since no visible sediment had been observed after the first step and no precipitate was to be seen after the second step, the sample was filtered and analyzed by HPLC. Aliquots of the individual liquid samples were analyzed by LSC measurement.

Two further subsamples of the NH_40H solubilizate were partitioned on two parallel Extrelut columns as described above, and the radioactive residues in the dichloromethane eluates were quantitated by LSC.

Determination of Metiram in solid samples as (unlabeled) CS_2 by application of BASF method No. 135/4:
In order to determine the amounts of the parent compound metiram (and / or other metabolites or degradates with the ability to liberate CS_2) in the solid phases of Extrelut columns and in the residues after solvent extraction or solubilization with aqueous ammonia, respectively, the residue analytical method No. 135/4 was applied. This method is based on the decomposition of bis-dithiocarbamates by reductive cleavage with stanneous hydrochloric acid. The carbon disulfide formed is distilled with a stream of nitrogen, washed by passage through adsorption tubes with zinc acetate and sulfuric acid, and adsorbed in a methanolic solution of potassium hydroxide. Quantitation of CS_2 is achieved by photometric measurement of the absorbance at 302 nm of the resulting xanthogenate.

The solid phases of the Extrelut columns or subsamples of the dried residues (RRR or NH_4OH residue) were completely transferred to Erlenmeyer flasks and subjected to the decomposition procedure.

As a deviation to BASF method No. 135/4, an automated HPLC system without a stationary phase (2 ml loop instead of a column) equipped with a flow-through UV detector was used instead of an UV photometer for the quantitation of the xanthogenate.

3. Identification of metabolites

The identification of the metabolites is based on HPLC-MS and HPLC-NMR analyses. All the quantitation of the metabolites is based on the HPLC analysis of the concentrated dichloromethane eluates obtained after Extrelut partition of the methanol extract and the aqueous extract or, in one case, also of the eluate after partition of the NH_4OH solubilizate from the residual radioactive residue, using HPLC system 1 with a Prevail C18 column. The second HPLC system was used for confirmatory purposes.

II. RESULTS AND DISCUSSION

A. TOTAL RADIOACTIVE RESIDUES (TRRs)

Due to the inhomogeneity of the samples, the total radioactive residue (TRR) was calculated by adding the extractable and the residual radioactive residues (ERR + RRR). The total radioactive residue in lettuce head collected seven days after the final treatment accounted for 43.491 to 108.445 mg/kg in three different work ups. The calculated TRR in the second work up which was used for all further quantitative evaluations amounted to 108.445 mg/kg.

The TRRs are summarised in Table 6.2-3.

Table 6.2-3: Total Radioactive Residue (TRR) in lettuce samples after treatment with ^{14}C-BAS 222 F

Matrix	DAT[1)]	TRR determined [2)] [mg/kg]	TRR calculated [3)] [mg/kg]
Lettuce head	7	78.502	43.491
Lettuce head	7	n.d.	108.445
Lettuce head	7	124.164	105.376

1) DAT = Days After Last Treatment; 2) determined by direct combustion; 3) calculated as the sum of ERR + RRR
n.d. not determined

B. EXTRACTION, CHARACTERIZATION AND IDENTIFICATION OF RESIDUES

1. Extraction of residues

Although it is known that the parent molecule is not soluble without any degradation, the samples were extracted with methanol followed by water. Clear purpose of the work up procedures selected was to identify as many metabolites / hydrolytic degradation products as possible for deriving a metabolic degradation pathway. Due to the chemical nature of the parent compound, any differentiation between hydrolytic degradation products formed during the analytical procedures and "real" plant metabolites is not possible.

The extractability of the radioactive residues in lettuce head with methanol and water was relatively low with portions of 22.5 to 33.8% TRR extracted in the three different work ups.

The major part of the radioactive residues detected was not extractable with methanol and water but remained in the residual radioactive residue (RRR, 66.2 to 77.5% TRR in three different work ups).

The results of the extraction of ^{14}C-BAS 222 F derived radioactivity from lettuce samples are shown in Table 6.2-4.

Table 6.2-4: Extractability of radioactive residues in lettuce head after post emergence treatment with ^{14}C-BAS 222 F

Matrix	DAT [1)]	TRR calculated [2)] [mg/kg]	Methanol		Water		ERR [3)]		RRR [4)]	
			mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR
Lettuce head Work up 1[5]	7	43.491	8.757	20.1	5.943	13.7	14.700	33.8	28.791	66.2
Lettuce head Work up 2[6]	7	108.445	16.821	15.5	7.596	7.0	24.417	22.5	84.028	77.5
Lettuce head Work up 3[7]	7	105.376	16.558	15.7	7.895	7.5	24.453	23.2	80.923	76.8

1) DAT = Days After Treatment; 2) TRR was calculated as the sum of ERR + RRR; 3) ERR = Extractable Radioactive Residues; RRR = Residual Radioactive Residue (after solvent extraction); 5) storage time = 5 days; 6) storage time = 278 days; 7) storage time = 460 days

2. Identification, characterization and quantitation of extractable residues

The metabolites in both the methanol extracts and the aqueous extracts were classified by their behaviour during partition on Extrelut columns: about one third to half of the radioactive residues detected in the crude extracts were eluted with dichloromethane while more than half of the radioactive residues remained on the stationary phases, most likely due to their poor solubility. When the residual radioactive residue after solvent extraction was subsequently treated with an aqueous ammonia solution, the major part of the radioactive residues (73.6 % TRR) was solubilized.

The investigations performed indicate that the parent compound metiram is only partially metabolized / degraded. Nearly half of the total radioactive residue was determined to be present in the residual radioactive residue after solvent extraction as unchanged active substance (49.840 mg/kg or 46.0% TRR) by application of the residue analytical method No. 135/4 (decomposition and detection of unlabelled CS_2). Due to its chemical nature as metal ion complex, the parent compound could not be detected in any extract and in the NH_4OH solubilizate from the residual radioactive residue after partition on Extrelut.

As known for most of the metabolites of EBDCs, the identified metabolites may at least in part have been formed also by abiotic processes like solvolysis or hydrolysis during / after extraction. Thus, it remains uncertain whether or to which extent the observed metabolites have been formed by true plant metabolism.

Four metabolites of BAS 222 F were identified by HPLC-MS and HPLC-NMR analysis of the methanol extract after partition on Extrelut. The metabolites detected in higher amounts in leaf lettuce were ETU and EU which occurred mainly in the NH_4OH solubilizate and are already known as degradates or intermediates from other plant metabolism studies. Two further degradation products were identified as EBIS and the metabolite TDIT which has recently been reported to occur also in soil and in water / sediment systems. The hydrolytic degradation product ETU can be generated directly from metiram and / or from EBIS and / or from TDIT, and is further metabolized to EU.

The total concentration of the scarcely resolved metabolites ETU and EU in the extracts and the NH_4OH solubilizate accounted for 15.950 mg/kg corresponding to 14.7% TRR. EBIS was detected in a total concentration of 2.441 mg/kg or 2.3% TRR, and TDIT was detected only in the extractable radioactive residues in an amount of 1.545 mg/kg or 1.4% TRR.

The major part of the radioactive residues detected was not extractable with methanol and water but remained in the residual radioactive residue (RRR). When the RRR after solvent extraction in the second work up (84.028 mg/kg or 77.5% TRR) was treated with an aqueous ammonia solution, most of the residual radioactive residue (79.787 mg/kg or 73.6% TRR) was solubilized, and 12.146 mg/kg or 11.2% TRR were recovered in the NH_4OH residue.

The solubilization step with aqueous ammonia quantitatively released the parent compound metiram from the residues after solvent extraction indicating that the chemical nature as metal ion complex was the reason for the poor solvent extractability of the parent compound.

In addition; the NH_4OH solubilizate contained a portion of soluble radioactive residues (31.910 mg/kg or 29.4% TRR) which was recovered in the dichloromethane eluate after Extrelut partition and subsequently analyzed by HPLC. The major component in this eluted portion was identified as ETU (and / or EU) accompanied by minor amounts of the metabolite EBIS. No evidence was found indicating that the ammonia solubilizate contained considerable amounts of radioactive residues incorporated into the protein pool of lettuce head.

The final non-extractable residues after ammonia solubilization contained only very low amounts of metiram and / or other metabolites or degradate containing a CS_2 substructure (two sulfur atoms bound to the same carbon atom). The major portion of radioactive residues consisted of further degradation products of BAS 222 F without any CS_2 moiety. Most likely, these degradation products were physically associated with or incorporated in insoluble natural products like starch or cell wall polymers.

The results are shown in Table 6.2-5.

Table 6.2-5: Summary of identified and characterized radioactive residues extracted from lettuce head after post emergence treatment with ^{14}C-BAS 222 F (harvested 7 DAT; second work up

Designation	Extracts				Solubilizate with aqueous ammonia Lab0034		Sum	
	Methanol Extract Lab0025		Aqueous Extract Lab0026					
	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]
Total Radioactive Residues (TRR)							**108.445**	**100.0**
Extractable radioactive residues (ERR)	16.821	15.5	7.596	7.0	79.787	73.6	24.417	22.5
Identified in ERR								
EU and / or ETU	1.366	1.2	0.444	0.4	see below		1.810	1.7
EBIS	0.525	0.5	1.152	1.1	see below		1.677	1.6
TDIT	1.307	1.2	0.238	0.2	see below		1.545	1.4
Total Identified in ERR							**5.032**	**4.6**
Characterized in ERR								
Formamide derivative	0.392	0.4	n.d.	n.d.	see below		0.392	0.4
Further HPLC peaks (each below 0.4 mg/kg or 0.4% TRR)	1.449	1.3	0.417	0.4	see below		1.866	1.7
Extrelut solid phase[1]	10.177	9.4	4.041	3.7	see below		14.218	13.1
Total Characterized in ERR							**16.476**	**15.2**
Total Identified and / or Characterized in ERR							**21.508**	**19.8**
Residual radioactive residue (RRR, Lab0027)							84.028	77.5
Identified in RRR (residual radioactive residue)[2]								
ETU and / or EU in supernatant after treatment with aqueous ammonia (Lab0034)					14.141	13.0	14.141	13.0
EBIS in supernatant after treatment with aqueous ammonia (Lab0034)					0.764	0.7	0.764	0.7
TDIT in supernatant after treatment with aqueous ammonia (Lab0034)					n.d.	n.d.	0.000	0.0
Total Identified in RRR[2]							**14.905**	**13.7**

Designation	Extracts				Solubilizate with aqueous ammonia Lab0034		Sum	
	Methanol Extract Lab0025		Aqueous Extract Lab0026					
	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]
Total Radioactive Residues (TRR)							**108.445**	**100.0**
Extractable radioactive residues (ERR)	16.821	15.5	7.596	7.0	79.787	73.6	24.417	22.5
Characterized in RRR[2,3]								
Formamide derivative in supernatant after treatment with aqueous ammonia (Lab0034)					n.d.	n.d.	0.000	0.0
Further HPLC peaks in supernatant after treatment with aqueous ammonia (Lab0034; each below 8.0 mg/kg or 7% TRR)					17.005	15.7	17.005	15.7
Extrelut solid phase of the NH4OH solubilizate (Lab0034)[1,4]					57.698	53.2	57.698	53.2
Total Characterized in RRR[2]							**74.703**	**68.9**
Total Identified and / or Characterized in RRR[2]							**89.608**	**82.6**
Total Identified and Characterized (ERR and RRR)							**111.116**	**102.4**
Final Residue (Lab0035)[3]							**12.146**	**11.2**
Total Identified and Characterized (ERR and RRR) + Final Residue							**123.262**	**113.6**

n. d. = not detected / identified

1) Determination of the non-eluted radioactive residues adsorbed on Extrelut columns by LSC of the decomposition solutions (method No. 135/4) of partition experiments carried out in parallel
2) Treatment with aqueous ammonia was applied for solubilization, or metiram (and / or other metabolites or degradates with the ability to liberate CS_2) was determined as CS_2 applying method No. 135/4 to a parallel subsample
3) Metiram (and / or other metabolites or degradates containing a substructure of two sulfur atoms bound to the same carbon atom, see discussion in the text) was determined as CS2 applying method No. 135/4 (see Table 6.2-6)
4) Since the elution with dichloromethane was less effective in the parallel partition experiments in the case of the NH_4OH solubilizate, the value for the Extrelut solid phases may be over-estimated for Lab0034

Table 6.2-6: Summary of the results of the determination of Metiram as CS_2 in solid remainders (after extraction) of lettuce head after post emergence treatment with ^{14}C-BAS 222 F (harvest 7 DAT; second work up) using BASF method No. 135/4

Sample description	[mg/kg][1]	[% TRR][1]
RRR	49.840	46.0
NH_4OH residue	0.416	0.4

1) Mean values of two parallel determinations

A summary of all identified components in lettuce head after post emergence treatment with ^{14}C-BAS 222 F is given in Table 6.2-7. The total radioactive residues (TRR) were calculated as the sum of ERR and RRR. In contrast, the amounts given in mg/kg are calculated from the TRR values not taking the change of specific radioactivity into account. Therefore, in order to obtain more precise data, metabolite specific correction factors have to be applied (see Conclusion, Table 6.2-8).

Table 6.2-7: Summary of identified and characterized components in lettuce head samples post emergence treatment with ^{14}C-BAS 222 F (harvested 7 DAT)

Designation Metabolite Code (Reg. No.)	Structure	Lettuce head (7 DAT)[1] (second work up)	
		mg/kg	% TRR
Metiram BAS 222 F (250284)[2]		49.840	46.0
Imidazolidine-2-one (ethyleneurea) BF 222-EU / EU[3] (27270)		15.950	14.7
Imidazolidine-2-thione (ethylenethiourea) ETU[3] (146099)			
"Ethylene-bis-(isothiocyanate)sulfide" BF 222-EBIS / DIDT (243959)		2.441	2.3
TDIT (M = 212)		1.545	1.4
Formamide derivative[4] (M = 114)		0.392	0.4

Designation Metabolite Code (Reg. No.)	Structure	Lettuce head (7 DAT)[1] (second work up)	
		mg/kg	% TRR
Further HPLC peaks (unknown)		18.871	17.4
Extrelut solid phases[5]		14.218	13.1
Final residue		12.146	11.2
Sum		**115.404**	**106.4**

1) Including radioactive residues released from RRR
2) Metiram (and / or other metabolites or degradates with the ability to liberate CS_2) was quantitated in the residual radioactive residues (RRR) as CS_2 applying the residue analytical method No. 135/4
3) EU and ETU were not separated by HPLC in system 1; values have to be read as mg/kg,(or % TRR, respectively) of EU and / or ETU
4) Probably an artifact formed of EU
5) Due to its chemical properties, it is likely that the radioactive residues retained on the Extrelut column mainly consist of the parent compound metiram

3. Metabolic pathway

In leaf lettuce, metiram (BAS 222 F) is only partially metabolized within seven days after the last application. At least half of the total radioactive residues were still present as unchanged active substance which was only poorly extractable with methanol and water, but detected in association with the residues after solvent extraction (RRR) and solubilized with aqueous ammonia. The metabolic pathway for the degradation is shown in Figure 6.2-1.

Hydrolysis (or generally solvolysis) is known to play an important role in the decomposition of metiram, and dissolution or extraction can only be achieved under decomposition. Since the solvolytic degradation leads to the formation of essentially the same decomposition products, it can hardly be determined whether the identified metabolites arise from true plant metabolism, from transformation reactions on the plant surface after application, or from artificial degradation upon solvent extraction.

The metabolites detected in higher amounts in lettuce are the known derivatives ETU and EU which occurred particularly in the NH_4OH solubilizate but also constituted major components in the methanol extracts (total concentration of both barely resolved metabolites in extracts and NH_4OH solubilizate: 15.950 mg/kg or 14.7% TRR, compare Table 6.2-7). Two further metabolites were observed in considerable amounts predominantly in the extractable radioactive residues and identified by HPLC-MS as EBIS and TDIT. Several minor unknown degradation products were characterized by their chromatographic properties as medium-polar derivatives eluting between EU / ETU and EBIS in reversed-phase HPLC.

The portion of the residual radioactive residue which was not solubilized by treatment with aqueous ammonia (NH_4OH residue) is composed of metabolites of metiram not containing any CS_2 substructure. These non-extractable residues may have been formed either by physical incorporation of degradation products into insoluble starch / cell wall polymers or via complete metabolization of primarily embedded active substance. Incorporation into proteins (which were precipitated with cold acetone from the NH_4OH solubilizate after Extrelut partition) was not observed in considerable amounts.

Figure 6.2-1: Metabolic pathway of ^{14}C-BAS 222 F in lettuce

Metiram

M 212 (TDIT)

EBIS / DIDT

ETU

EU

Incorporation into non-extractable residues / association with natural products

4. Storage stability

All samples were stored at approximately -18°C during the course of the study. A comparison of the metabolite patterns obtained by HPLC analysis and of the extractability of the stored sample material at the beginning and at the end of the investigation period showed that there was no major change in the nature of the radioactive residues during sample storage over a period of about 15 months.

III. CONCLUSION

The nature of the residues of metiram was investigated in lettuce growing in plastic containers under laboratory conditions. Lettuce heads were applied three foliar spray applications of 2 kg as/ha.The TRR level in lettuce head harvested 7 days after the last application amouted to 108.4 mg/kg. The extractability was relatively low (below 35%). The high RRR is indicative of effective incorporation of the radiocarbon into natural plant constituents (e.g. starch, cell wall polymers). Identification and characterization of the total radioactive residue did show the predominant proportion of the residue is the unchanged parent compound metiram (46% TRR). Importantly, metiram is a metal ion complex which desintegrates upon contact with water. Due to its chemical properties its extraction is only achievable upon desintegration of the complex (solvolysis) which results in formation of dynamic intermediates, e.g. EBIS, ETU, and EU. Within the context of plant metabolism studies it is not possible to determine whether such dynamic intermediates are abiotically formed desintegration products or in fact, metabolites generated by plant metabolism.
Using radio-HPLC, the transformation products ETU (M222F002) and EU (M222F003) were not quantifiable separately. Together, they were present at major amounts (14.7% TRR). Two further degradation products were identified at low amounts as EBIS (M222F004, 2.3% TRR) and as TDIT (M222F007, 1.4% TRR). In addition, a minor portion of radioactive residues was observed after solvent extraction and subsequent treatment with aqueous ammonia: These non-extractable residues are most likely formed by physical association or incorporation of metiram transformation into insoluble natural products such as starch or cell wall polymers. Note, the levels of transformation products reported are 16.0 mg/kg (EU and/or ETU), 2.4 mg/kg (EBIS), 1.5 mg/kg (TDIT) when expressed as metiram parent equivalents. To obtain absolute residue levels a correction factor accounting for the molecular mass difference has to be applied. The following table provides the calculated molecular weight correction factors. It was copied from document BASF DocID2015/1087922 (see section MCA 6.10).

Table 6.2-8: Metiram metabolites: molecular weight correction factors

Metabolite	Units[1)]	Total molecular weight[2)]	Molecular weight correction factor[3)]
M222F004 (EBIS)	4	705.2	0.65
M222F003 (EU)	4	344.4	0.32
M222F002 (ETU)	n.a.[4)]	n.a.	n.a.
M222F023 (EDA)	4	240.4	0.22
M222F021 (N-AcEDA)	4	408.4	0.38
M222F022 (Jaffe`s Base)	2	340.4	0.31
M222F007 (TDIT)	2	426.6	0.39
M222F001	2	460.4	0.42
Glycine	n.a.	n.a.	n.a.
M222F008 (Hydantoin)	4	400.4	0.37
M222F013 (Formamide derivative)	4	456.4	0.42

1) number of molecules that may potentially be formed from one molecule metiram (= n)
2) n x molecular weight [g/mol]
3) calculated as (n x molecular weight)/1088.7 g/mol)
4) n.a. not applicable

Conclusion on section 6.2.1: metabolism in crops

Plant metabolism studies were performed in three crops, namely apple (crop category: fruits/fruiting vegetables), potato (crop category: root/tuber), and lettuce (crop category: leafy vegetable). The metabolism pathways in all three crops were comparable. Noteworthy, the metabolism of metiram in plants is comparable to the well known pathways for dithiocarbamates fungicides.
In brief, metiram, as are the other dithiocarbamate fungicides, is a non-systemic fungicide. Once deposited on the plant surface the non-soluble metal ion complex is not translocated further into the plant. Rather, upon contact with a solvent system, the metal complex undergoes chemical desintegration (solvolysis) releasing dynamic intermediates which enter the general metabolic carbon pool and thereby are incorporated into biological products (e.g. carbohydrates, amino acids, lipds).
In conclusion, the metabolism of metiram can be considered sufficiently elucidated in fruits, roots/tuber and leafy vegetables. Considering this conclusion is based on crops representing three different crop categories (including also rotational crops, see section MCA 6.6) this metabolism data can be extrapolated to plant crops in general thereby allowing a general definition of the relevant residue in commodities of plant origin (foliar spray application, see section MCA 6.7).
Following application of metiram to the target plant, the compound remains on the plant surface. In raw agricultural commodities (RAC) metiram is generally the predominant component of the residue, and as such an appropriate marker compound for enforcement (see section MCA 6.7).
Dynamic intermediates formed upon desintegration of the metiram complex as well as naturally occurring plant constituents are further components of the residue. While naturally occuring plant constituents are not relevant for the definition of the residue, a potential relevance of these dynamic intermediates has been evaluated (see section MCA 6.7 and 6.10 for a detailed relevance assessment).

CA 6.2.2 Poultry

Annex II Dossier :
Regarding metabolism of metiram in poultry, studies in hen were previously evaluated during the Annex I inclusion process and were considered as suitable.

AIR3 Dossier :
No new metabolism study was conducted.

Regarding metiram, the previously evaluated studies support the representative uses of the present dossier: DocID1990/5080 (see MCA 6.2.2/1), DocID 1990/5131 (see MCA 6.2.2/2), DocID 1989/5049 (see MCA 6.2.2/3), DocID1988/5016 (see MCA 6.2.2/4). In brief, chicken were dosed with seven consecutive daily doses of metiram (14C-BAS 222F in capsules, 4 mg/kg bw/day). Analysis of egg and tissues did reveal incorporation of radiocarbon into naturally occuring cell constituents in significant amounts (e.g. lipids and proteins as predominant terminal residue) indicating extensive metabolism of metiram. In addition, several small molecular weight transformation products of metiram were identified (EBIS, ETU, EU, Jaffes Base, EDA/NAcetyl-EDA).
In the framework of a metabolism study, cell constituents such as lipids and proteins are extractable only to a limited extent with the consequence that in certain cases, a high proportion of the radioactivity remains non-identified. This is also the case for the metiram poultry metabolism study: the proportion of non-identified residue reported does reflect the rapid conversion of the dynamic intermediates into natural cell constituents rather than being a deficiency of the study.

In conclusion, the metabolic pathway of metiram in poultry is well elucidated. Noteworthy, it is comparable to the metabolic pathways found in other animals, both ruminants (see section MCA 6.2.3) and rat (see section MCA 5.1.1). In brief, upon contact with water the metal ion complex desintegrates (solvolysis) and releases dynamic intermediates such as EBIS, Jaffes Base, ETU, EU and EDA. These degradation products are stepwise further metabolized to glycine and thereby the radiocarbon is channelled into the metabolic carbon pool. In consequence, the radiocarbon is incorporated into naturally occurring cell constituents such as lipids and proteins as the predominant terminal residue.

Regarding the degradation product ETU potentially occurring in feed items (processed plant commodities), data on metabolism in poultry is not required (the rationale is described in detail in BASF DocID 2002/1006209, *Metiram: Metabolism of ETU in livestock, Statement of the Notifer to ECCO 120, May 22, 2002*) [see KCA 6.2/1 2002/1006209]. In brief, the rationale is : concerning the metiram residue intake of poultry, the contribution of ETU from processed feed items is very low (worst case assumptions result in an estimation of 0.003 mg/animal/d). Furthermore, poultry metabolism studies with metiram show that the ETU is formed in the animal itself and is rapidly eliminated (either by excretion or by further transformation into natural cell constituents). Both facts considered together, ETU levels in commodities of animal origin are insignificant (and thus do not need to be included in a definition of the residue, see section MCA 6.7).

In conclusion, additional poultry studies with ETU as test item are not required nor justified, considering both limited knowledge gain as well as animal welfare perspective.

Report:	*CA 6.2.2/1*
	[redacted] 1990b
	Metabolism of 14C-Metiram complex in laying hens. Analysis and quantitation of metabolites and/or the corresponding natural products in eggs and tissues
	1990/5080
Guidelines:	*EPA 171-4, EPA Subdivision O of the Pesticide Assessment Guidelines, EPA 40 CFR 158.125*
GLP:	*yes*
	(certified by United States Environmental Protection Agency)

Report:	*CA 6.2.2/2*
	[redacted] 1990a
	Executive summary of Metiram hen metabolism studies conducted by the Metiram Task Force
	1990/5131
Guidelines:	*EPA 171-4, EPA Subdivision O of the Pesticide Assessment Guidelines, EPA 40 CFR 158.125*
GLP:	*no*
	(certified by <none>)

Report:	*CA 6.2.2/3*
	[redacted] 1989a
	Metiram and Ethylenethiourea residue analysis: Analysis of tissues and eggs from laying hens dosed with 14C-Metiram
	1989/5049
Guidelines:	*EPA 171-4*
GLP:	*yes*
	(certified by United States Environmental Protection Agency)

Report:	*CA 6.2.2/4*
	[redacted] 989a
	Metabolism feeding study in laying hens using 14C-Metiram
	1988/5016
Guidelines:	*EPA 171-4*
GLP:	*yes*
	(certified by United States Environmental Protection Agency)

CA 6.2.3 Lactating ruminants

Annex II Dossier :
Regarding metabolism of metiram in ruminants, studies in goat were previously evaluated during the Annex I inclusion process and were considered as suitable.

AIR3 Dossier :
No new metabolism study was conducted.

Regarding metiram, the previously evaluated studies support the representative uses of thepresent dossier: DocID1988/7002665 (see CA 6.2.3/1), and DocID1989/10487 (see CA 6.2.3/2).
In brief, goats were dosed with five consecutive daily doses of metiram (14C-BAS 222 F in capsules, 77 mg/day). Noteably, all relevant matrices were analysed, with identification/characterization of metabolite as well as bound residues meeting guideline requirements. Analysis of milk and tissues did reveal significant incorporation of radiocarbon into naturally occurring cell constituents (e.g. with carbohydrates, lipids, proteins as predominant terminal residue) indicating extensive metabolism of metiram : subsequent to decomposition of the metal ion complex, the short lived dynamic intermediates such as EBIS, ETU and EU as well as EDA are further metabolized to glycine thereby channelling radiocarbon into the metabolic cycleIn addition, several small molecular weight transformation of metiram were identified (EBIS, ETU, EU, Jaffes Base, and EDA/NAcetyl-EDA).

In conclusion, the metabolic pathway of metiram in ruminant is well elucidated. Noteworthy, it is comparable to the metabolic pathways found in other animals, both poultry (see section MCA 6.2.2) and rat (see section MCA 5.1.1). In brief, upon contact with water the metal ion complex desintegrates (solvolysis) and releases dynamic intermediates such as EBIS, Jaffes Base, ETU, EU and EDA. These degradation products are stepwise further metabolized to glycine and thereby the radiocarbon is channelled into the metabolic carbon pool. In consequence, the radiocarbon is incorporated into naturally occurring cell constituents such as lipids and proteins as the predominant terminal residue.

Regarding the degradation product ETU potentially occurring in feed items (processed plant commodities), data on metabolism in ruminant is not required (the rationale is described in detail in BASF DocID 2002/1006209, *Metiram: Metabolism of ETU in livestock, Statement of the Notifer to ECCO 120, May 22, 2002*) [see KCA 6.2/1 2002/1006209]. In brief, the rationale is : concerning the metiram residue intake of ruminant, the contribution of ETU from processed feed items is very low (worst case assumptions result in an estimation of 0.17 mg/animal/d for beef cattle and 0.07 mg/animal/d for dairy cow). Furthermore, goat metabolism studies with metiram show that the ETU is formed in the animal itself and is rapidly eliminated (either by excretion or by further transformation into natural cell constituents). Both facts considered together, ETU levels in commodities of animal origin are insignificant (and thus do not need to be included in a definition of the residue, see section MCA 6.7).
In conclusion, additional poultry studies with ETU as test item are not required nor justified, considering both limited knowledge gain as well as animal welfare perspective.

Report: *CA 6.2.3/1*
[redacted] 1989a
Metabolism of 14C-Metiram complex in lactating goats - Analysis and quantification of metabolites and/or the corresponding natural products in milk and tissues
1989/10487
Guidelines: *EPA 171-4*
GLP: *yes*
(certified by United States Environmental Protection Agency)

Report: *CA 6.2.3/2*
[redacted] 1986a
The biokinetics and metabolism of Metiram complex in lactating goats
1986/10190
Guidelines: *<none>*
GLP: *yes*
(certified by <none>)

CA 6.2.4 Pigs

A metiram metabolism study in pigs is not required. Metabolism in ruminants (goat, see MCA 6.2.2) and rat (see MCA 5.1.1) are comparable and can be extrapolated to pigs as well.

CA 6.2.5 Fish

A metiram metabolism study in fish is not required. The representative uses supported in the present dossier, grape and potato, are not considered to serve as feed item for fish.

More specifically, according to Commission regulation 283/2013, metabolism studies in fish may be required where the plant protection product is used in crops whose parts or products, also after processing, are fed to fish and where residues in feed may occur from the intended applications. The conditions under which such a study should be performed are further described in the Working document of the EU Commission SANCO/11187/2013, rev. 3 on the nature of pesticide residues in fish. The document specifies that the accumulation of compounds with low lipophilicity via the diet is known to be negligible and that fish metabolism studies are therefore required for active substances with a log P_{ow} equal or greater than 3 and an expected feed burden above 0.1 mg/kg DM. In the case of metiram representative uses, both criteria do not apply (logP_{ow}<3, expected feed burden <0.1 mg/kg DM) .

Conclusion on section 6.2: metabolism in livestock animals

Livestock metabolism studies were performed in hen (laying poultry) and goat (lactating ruminant). The metabolism pathways in poultry and goat were comparable to rat. Therefore, the findings can be extrapolated to pig and a metabolism study in pig is not required. A metabolism study in fish is not required ($\log P_{ow}<3$, expected feed burden <0.1 mg/kg DM). Noteworthy, the metabolism of metiram in livestock is comparable to the well known pathways for dithiocarbamate fungicides.

The metabolic pathway of metiram in livestock animals, including poultry, ruminants, and pig can be considered sufficiently elucidated. In brief, the parent compound metiram is not resorbed in the gastrointestinal tract. Rather, upon contact with water, the metal ion complex desintegrates (solvolysis) and releases dynamic intermediates such as EBIS, Jaffes Base, ETU, EU and EDA. These degradation products are stepwise further metabolized to glycine and thereby the radiocarbon is channeled into the metabolic carbon pool. In consequence, the radiocarbon is incorporated into naturally occurring cell constituents such as lipids and proteins as the predominant terminal residue.

In conclusion, the terminal residue in livestock commodities is predominantly naturally occurring cell constituents, such as lipids in proteins. Such compound fractions are not relevant for the definition of the residue. In addition, several dynamic intermediates were identified, some of which are CS_2-releasing molecules and therefore are detected by the multi-residue method analysing residues of dithiocarbamates measured as CS_2. A potential relevance of these metabolic intermediates for the residue definition is evaluated in detail in section MCA 6.7.

CA 6.3 Magnitude of residues trials in plants

The present dossier supports the representative formulation WG BAS 222 28 F used in two representative uses, grapes and potatoes. In this section, the residue data for grapes (page 1) and potatoes (page 42) are summarized.

CA 6.3.1 Grapes

In support of the representative use in grapes, a total of 23 cGAP compliant new field trials on wine grapes were conducted. The cGAP is provided in Table 6.3.1-1. The residue trials were performed in various European Member States in N-EU and S-EU during the growing seasons 2012, 2013 and 2014 and thereby fulfill the requirements of seasonal and geographical distribution (see Table 6.3.1-2).

Table 6.3.1-1: Summary of the critical GAPs for the proposed uses in grapes

Crop	Outdoor/ Protected	Growth stage (BBCH)	Maximum number of applications	Minimum application interval (days)	Maximum		Minimum PHI (days)
					Rate (kg as/ha)	Water (L/ha)	
Grapes (wine, table)	Outdoor	05 - 19* 53 - 79**	2* - 3**	7	0.84* - 1.40**	150 - 300* 150 - 1300**	56

* Pests or Group of pests controlled: Phomopsis viticola, Pseudopezicula tracheiphila
** Pests or Group of pests controlled: Plasmopara viticola, Guignardia bidwellii, Pseudopezicula tracheiphila

Table 6.3.1-2: Number of residue trials conducted per geographical region and vegetation period

Crop	Vegetation Period	Number of Trials					Reference
		EU North	Country	EU South	Country	Total	
Grape	2014	4	DE, HU, UK	4	ES, IT, FR, GR	8	6.3.1/1
Grape	2013	5	FR, DE	5	ES, IT, FR, GR	10	6.3.1/2 6.3.1/3
Grape	2012	3	DE, UK	3	ES, GR	6	6.3.1/4
Total number of trials per Region		**12**		**12**	**Total number of trials**	**24**	

Table 6.3.1-3: Overall summary of residue data for metiram from grape residue trials

Crop	Region	RAC	n	Residue level (mg/kg)								
				CS_2			Metiram, determined as CS_2			Metiram, determined as EBDC		
				Min.	HR	STMR	Min.	HR	STMR	Min.	HR	STMR
Grapes	N-EU	fruit	11	<0.056	0.88	0.41	<0.1	1.57	0.74	<0.05	3.08	1.30
	S-EU	fruit	12	0.062	0.65	0.305	0.11	1.20	0.55	0.23	2.7	1.305

Table 6.3.1-4: Overall summary of residue data for ETU, EU and EBIS from grape residue trials

Crop	Region	RAC	n	Residue level (mg/kg)								
				ETU			EU			EBIS		
				Min.	HR	STMR	Min.	HR	STMR	Min.	HR	STMR
Grapes	N-EU	fruit	11	<0.01	0.035	0.01	<0.01	0.061	0.01	<0.01	0.04	0.01
	S-EU	fruit	12	<0.01	0.046	0.01	<0.01	0.029	0.019	<0.01	0.05	0.015

The study reports are summarized below.

Table 6.3.1-5: Grape residue data: summary of maximum storage interval and storage stability

Crop	DocID	Study No.	Maximum storage interval (days)*					Storage stability (days)				
			Metiram by CS_2	Metiram by EBDC	ETU	EU	EBIS	Metiram by CS_2	Metiram by EBDC	ETU	EU	EBIS
Grapes	2015/1000322	731173	95	138	74	90	90 Δ	547	547	90	90	0
	2014/1161880	389449_1	264	242	203 Δ	203 Δ	203 Δ					
	2014/1000221	389460	371	368	237 Δ	237 Δ	237 Δ					
	2012/1272626	389449	318	444	311 Δ	431 Δ	444 Δ					

* of deep frozen samples from harvest until extraction
Δ freezer storage stability not confirmed (see section MCA 6.1)

Table 6.3.1-6: Grape residue data: summary of storage intervals of PHI samples

DocID	Study No.	PHI Specimen No.*	Storage of PHI samples* (days)				
			Metiram by CS_2	Metiram by EBDC	ETU	EU	EBIS
2015/1000322	731173	L1403550007			15		15 Δ
		L1403550008	13	15	9	9	
		L1403560007	35	48	15	15	15 Δ
		L1403580007			15	15	15 Δ
		L1403580008	9	49			
		L1403590007	19	59	12	12	12 Δ
		L1403600007	18	20	14	14	14 Δ
		L1403610008	33	53	27	48	27 Δ
		L1403620007	39	102	35	35	35 Δ
2014/1161880	389449_1	L1300150007	168	146	108 Δ	108 Δ	107 Δ
		L1300160007			148 Δ	148 Δ	147 Δ
		L1300160008	201	179			
2014/1000221	389460	L1300860007				142 Δ	143 Δ
		L1300860008	209	176	142 Δ		
		L1300870007		189			
		L1300870008	215		147 Δ	147 Δ	146 Δ
		L1300880007	209		135 Δ	135 Δ	136 Δ
		L1300880008		168			
		L1300890007	206	176	136 Δ	136 Δ	137 Δ
		L1300900007			122 Δ		
		L1300900008	207	155		121 Δ	120 Δ
		L1300910007	240		148 Δ	148 Δ	149 Δ
		L1300910008		180			
		L1300920007	228	195	155 Δ	155 Δ	156 Δ
		L1300930007		217		177 Δ	178 Δ
		L1300930008	243		176 Δ		

DocID	Study No.	PHI Specimen No.*	Storage of PHI samples* (days)				
			Metiram by CS_2	Metiram by EBDC	ETU	EU	EBIS
2012/1272626	389449	L1205320007	241	344	231 Δ	350 Δ	365 Δ
		L1205330007					371 Δ
		L1205330008	239	365	230 Δ	349 Δ	
		L1205350007	222	325	212 Δ	331 Δ	346 Δ
		L1205360007			255 Δ		389 Δ
		L1205360008	255	383		367 Δ	
		L1205370007	248		238 Δ	357 Δ	372 Δ
		L1205370008		344			
		L1205390007	264	367	254 Δ	373 Δ	388 Δ

* PHI samples (when higher residues were detected in specimens sampled at a later sampling event then these residue values were taken)
Δ freezer storage stability not confirmed (see section MCA 6.1*)*

Report: CA 6.3.1/1
Meyer M.,Gabriel J.E., 2015a
Study on the residue behaviour of Metiram (BAS 222 F) in wine grapes after treatment with BAS 222 28 F under field conditions in Germany, United Kingdom, Hungary, Southern France, Italy, Spain and Greece, 2014
2015/1000322

Guidelines: EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, EU Regulation Regulation 544/2011 (10 June 2011) implementing Regulation No 1107/2009, EEC 7029/VI/95 rev. 5 (July 22 1997), EEC 7525/VI/95 rev. 9 (March 2011), SANCO/3029/99 rev. 4 (11 July 2000)

GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
Description: BAS 222 28 F: 700 g/kg of BAS 222 F (metiram), WG formulation
Lot/Batch #: FRE-000977
Purity: Not relevant
CAS#: Metiram (BAS 222 F): 9006-42-2
Development code: Metiram: BAS 222 F
ETU: M222F002
EU: M222F003
EBIS: M222F004
Spiking levels:
BAS 222 F (by EBDC): 0.05, 0.5, 49 mg/kg
BAS 222 F (by CS_2): 0.1, 1.0, 10 mg/kg
ETU and EU: 0.01, 0.1, 0.2 mg/kg
EBIS: 0.01, 0.1, 0.5 mg/kg

2. **Test Commodity:** berries and small fruits
Crop: wine grapes
Type: *Vitis vinifera L.,*
Variety: Regent, Weißburgunder, Sauvignon Blanc, Cserszegi Füszeres, Cabernet, Muscat, Rossese, Moscatel
Crop parts(s) or processed Commodity: grapes (fruits)
Sample size: 1.0 kg (12 brunches)

B. STUDY DESIGN AND METHODS

1. Test procedure

During the growing season of 2014, eight field trials with wine grapes were conducted in Germany, the United Kingdom, Hungary, Southern France, Greece, Italy and Spain, in order to determine the magnitude of the residues of metiram (BAS 222 F) after application of BAS 222 28 F.
The WG formulation BAS 222 28 F was applied three times (69 - 72, 62 - 65 and 55 - 57 days before harvest) at single rates of 1.4 kg as/ha for BAS 222 F (2.0 kg/ha of BAS 222 28 F) in a spray volume of 800 L/ha in order to determine the magnitude of the residues of the active ingredient on Raw Agricultural Commodities (RAC).

Treated grape fruit specimens of all trials were sampled at 0 DALA, at 48 - 49 DALA, at 55 - 57 DALA and at 61 - 63 DALA.

Table 6.3.1-7 Application and sampling details for trials conducted in 2014

Region	No. of trials	No. of Appl.	F, G, I[2]	Method	Test Item	Active Substance	Application		Target Timing	
							Rate (kg a.s./ha)	Water vol. (L/ha)	Appl. (DBH)[3]	Sampl. (DALA)[1]
EU North & South	8	3	F	-	BAS 222 28 F (WG)	Metiram BAS 222 F	1.4	800	1st appl.: 69 - 72 2nd appl.: 62 - 65 3rd appl.: 55 - 57	0 48 - 49 55 - 57 61 - 63

1) Days after last application,
2) Field, Glasshouse or Indoor,
3) Days before harvest

2. Description of analytical procedures

The specimens were analysed for residues of metiram according to BASF method No. L0089/01 and as carbondisulfide according to BASF method L0234/01. The residues of ETU were determined according to the BASF method No. L0176/01. The residues of EU and EBIS were determined with method L0233/01.
The limit of quantitation (LOQ) for metiram was 0.05 mg/kg. The limit of quantitation (LOQ) for metiram BAS 222 F (by CS_2) was 0.10 mg/kg. The limit of quantitation (LOQ) for ETU, EU and EBIS was 0.01 mg/kg.
The results of procedural recovery experiments averaged at about 100% for BAS 222 F (by EBDC) at fortification levels between 0.05 and 49.0 mg/kg, at 88.8% for BAS 222 F (by CS_2) at fortification levels between 0.1 and 10.0 mg/kg and at 98.8% for ETU and at 83.9% for EU at fortification levels between 0.01 and 0.2 mg/kg and at 92.2% for EBIS at fortification levels between 0.01 and 0.5 mg/kg.

<u>BASF method L0089/01 was used to determine Metiram as EBDC:</u>
The ethylene-bisdithiocarbamate (EBDC) moiety was formed out of BAS 222 F and extracted from the specimen with a buffer solution consisting of EDTA, cysteine, methanol and sodium hydroxide adjusted to pH 11.0. The formed ethylene-bisdithiocarbamate (EBDC) analyte was methylated with iodmethane prior to C18 SPE clean up. Specimens were quantified by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). The limit of quantitation (LOQ) was 0.05 mg/kg.

<u>BASF method L0234/01 was used to determine Metiram as CS_2</u>
Metiram was transformed to CS_2 by means of orthophosphoric acid. Subsequently, the CS_2 was transferred to isooctane with a flow of nitrogen. The quantification was carried out using GC-MS. The limit of quantitation (LOQ) was 0.10 mg/kg.

<u>BASF method L0176/01 was used to determine ETU:</u>
ETU was extracted from the specimen material with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol-water. After centrifugation an aliquot was taken and the methanol was evaporated from the extract. The pH was adjusted to 8 before evaporating (in some cases after evaporation). The remaining water phase was cleaned by liquid/liquid (water/dichloromethane) partition on an Extrelut column. After concentration of the eluate, the residue was determined by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

<u>EU was determined according to BASF method L0233/01:</u>
The extraction from EU was performed with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol-water and was followed by a clean-up by liquid-liquid partition on an Extrelut column where ethyl acetate was used for the elution.
Water was added prior the evaporation of the specimen extract. For the final reconstitution of the specimen extract a solution of H_2O/MeOH was used. The determination was performed by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

<u>EBIS was determined according to BASF method L0233/01:</u>
The extraction from EBIS was performed with a mixture of acetonitrile/formic acid in combination with a thiourea solution and was followed by a clean-up step utilizing C18-EC. Specimens were diluted with a solution of acetonitrile/H_2O/formic acid before being determined by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

The results of procedural recovery experiments are summarized in Table 6.3.1-8.

Table 6.3.1-8 Summary of recoveries

Matrix	Fortification Level (mg/kg)	Summary Recoveries			
		n	Mean (%)	SD (+/-)	RSD (%)
Method No. L0089/01		**Metiram (by EBDC)**			
Grapes / Fruit	0.05, 0.5, 49	6	100	5.5	5.4
Method No. L0234/01		**Metiram (by CS_2)**			
Grapes / Fruit	0.1, 1.0, 10	11	88.8	12	13
Method No. L0176/01		**ETU**			
Grapes / Fruit	0.01, 0.1, 0.2	14	98.8	11	11
Method No. L0233/01		**EU**			
Grapes / Fruit	0.01, 0.1, 0.2	14	83.9	11	13
Method No. L0233/01		**EBIS**			
Grapes / Fruit	0.01, 0.1, 0.5	14	92.2	13	15

II. RESULTS AND DISCUSSION

Residue data of the field trials is summarized in Table 6.3.1-9 to Table 6.3.1-12. Detailed information is shown in Table 6.3.1-13 to Table 6.3.1-16.

Samples from untreated control plots did not contain apparent residues of CS_2, EBDC, ETU, EU and EBIS, i.e. residues were below the corresponding LOQ. The maximum storage interval of deep frozen samples from harvest until extraction was 138 days for metiram (by EBDC), 95 days for metiram (by CS_2), 74 days for ETU and EBIS and 90 days for EU. All specimens taken at the PHI (55 - 57 DALA) were analysed for ETU, EU and EBIS within 30 days after sampling, apart from those taken in trial L140362 which were analyzed 35 days after sampling.
The mean of the concurrent recoveries was within the acceptable range of 70 – 110% for CS_2, EBDC, ETU, EU and EBIS.

Table 6.3.1-9: Summary of metiram level in BAS 222 28 F treated wine grapes (Trials L140355 – L140356 and L140358 – L140362)

Region	Matrix	DALA [1]	BBCH	Range of CS_2 Residues [2] [mg/kg]	Range of Metiram Residues (by CS_2) [mg/kg]	Range of Metiram Residues (by EBDC) [mg/kg]
EU North & South	Grapes (fruit)	0	73 - 79	0.53 - 3.1	0.96 - 5.6	1.5 - 11
		48 - 49	79 - 85	0.14 - 0.88	0.26 - 1.6	0.41 - 4.3
		55 - 57	87 - 89	0.10 - 0.65	0.18 - 1.2	0.32 - 2.7
		61 - 63	89	< 0.056 - 0.54	< 0.10 - 0.97	0.24 - 2.7

[1] DALA = Days after Last Application
[2] the conversion factor from CS_2 to metiram is 1.79

Table 6.3.1-10: Summary of ETU, EU and EBIS level in BAS 222 28 F treated wine grapes (Trials L140355 – L140356 and L140358 – L140362)

Region	Matrix	DALA [1]	BBCH	Range of ETU Residues [mg/kg]	Range of EU Residues [mg/kg]	Range of EBIS Residues [mg/kg]
EU North & South	Grapes (fruit)	0	73 - 79	0.018 – 0.16	< 0.010 – 0.050	0.032 – 0.31
		48 - 49	79 - 85	0.014 – 0.059	0.016 – 0.063	0.011 – 0.037
		55 - 57	87 - 89	<0.010 - 0.046	0.012 – 0.061	< 0.010 – 0.030
		61 - 63	89	<0.010 - 0.038	0.013 – 0.054	< 0.010 – 0.035

[1] DALA = Days after Last Application

Table 6.3.1-11: Summary of metiram level in BAS 222 28 F treated wine grapes (Trials L140357)

Region	Matrix	DALA [1]	BBCH	Range of CS_2 Residues [2] [mg/kg]	Range of Metiram Residues (by CS_2) [mg/kg]	Range of Metiram Residues (by EBDC) [mg/kg]
EU North	Grapes (fruit)	0	73	0.31	0.56	0.89
		48	79	< 0.056	< 0.10	0.11
		57	89	< 0.056	< 0.10	0.050
		62	89	< 0.056	< 0.10	0.053

[1] DALA = Days after Last Application

[2] the conversion factor from CS_2 to metiram is 1.79

Table 6.3.1-12: Summary of ETU, EU and EBIS level in BAS 222 28 F treated wine grapes (Trials L140357)

Region	Matrix	DALA [1]	BBCH	Range of ETU Residues [mg/kg]	Range of EU Residues [mg/kg]	Range of EBIS Residues [mg/kg]
EU North	Grapes (fruit)	0	73	< 0.010	< 0.010	0.019
		48	79	< 0.010	< 0.010	< 0.010
		57	89	< 0.010	< 0.010	< 0.010
		62	89	< 0.010	< 0.010	< 0.010

[1] DALA = Days after Last Application

Table 6.3.1-13: Level of metiram in BAS 222 28 F treated wine grapes (N-EU)

Study details		Crop	Country	Formulation Application rate	GS[3] at last appl. (BBCH)	DALA[2]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	CS_2[1]	Metiram (by CS_2)	Metiram (by EBDC)
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731173 2015/1000322 L140355 yes 2014	Grapes	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	79	0 49 56 62	fruit fruit fruit fruit	3.1 0.36 0.31 0.41	5.5 0.65 0.55 0.74	11 2.6 1.9 2.4
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731173 2015/1000322 L140356 yes 2014	Grapes	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	78	0 49 56 63	fruit fruit fruit fruit	1.7 0.52 0.52 0.20	3.1 0.93 0.94 0.35	6.3 1.9 1.8 0.63
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731173 2015/1000322 L140357 yes 2014	Grapes	United Kingdom	**Note: trial underdosed** BAS 222 28 F: 3x 0.4 kg metiram/ha Spray volume: 227-233 L/ha	73	0 48 57 62	fruit fruit fruit fruit	0.31 <0.056 <0.056 <0.056	0.56 <0.1 <0.1 <0.1	0.89 0.11 0.050 0.053
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731173 2015/1000322 L140358 yes 2014	Grapes	Hungary	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	77	0 49 56 63	fruit fruit fruit fruit	1.7 0.63 0.28 0.45	3.1 1.1 0.51 0.81	6.4 1.9 1.2 1.3

1) the conversion factor from metiram to CS_2 is 1.79
2) Days after last application
3) Growth stage
* mean value of two individual extractions
_ underlined values were used for MRL calculation

Table 6.3.1-14: Level of metiram in BAS 222 28 F treated wine grapes (S-EU)

Study details		Crop	Country	Formulation Application rate	GS[3] at last appl. (BBCH)	DALA[2]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	CS_2[1]	Metiram (by CS_2)	Metiram (by EBDC)
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731173 2015/1000322 L140359 yes 2014	Grapes	France (S)	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	79	0 49 57 63	fruit fruit fruit fruit	0.53 0.14 0.10 <0.056	1.0 0.26 0.18 <0.1	1.5 0.41 0.32 0.24
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731173 2015/1000322 L140360 yes 2014	Grapes	Greece	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	75	0 48 57 61	fruit fruit fruit fruit	1.1 0.73 0.29 0.27	2.0 1.3 0.52 0.49	3.1 3.0 1.2 0.96
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731173 2015/1000322 L140361 yes 2014	Grapes	Italy	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	79	0 48 55 62	fruit fruit fruit fruit	0.59 0.24 0.23 0.54	1.1 0.43 0.42 0.97	2.2 1.0 0.84 2.7
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731173 2015/1000322 L140362 yes 2014	Grapes	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	77	0 48 56 63	fruit fruit fruit fruit	3.1 0.88 0.65 <0.056	5.6 1.6 1.2 <0.1	9.0 4.3 2.7 1.0

1) the conversion factor from metiram to CS_2 is 1.79
2) Days after last application
3) Growth stage
For calculation purposes for example < 0.056 was set to 0.056
_ underlined values were used for MRL calculation

Table 6.3.1-15: Level of ETU, EU and EBIS in BAS 222 28 F treated wine grapes (N-EU)

Study details		Crop	Country	Formulation Application rate	GS[2] at last appl. (BBCH)	DA LA[1]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	ETU*	EU*	EBIS*
Study code: Doc ID: Trial No. GLP: Year:	731173 2015/1000322 L140355 yes 2014	Grapes	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	79	0 49 56 62	fruit fruit fruit fruit	0.065 0.027 0.027 0.025	0.023 0.019 0.018 0.021	0.31 0.019 0.017 0.017
Study code: Doc ID: Trial No. GLP: Year:	731173 2015/1000322 L140356 yes 2014	Grapes	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	78	0 49 56 63	fruit fruit fruit fruit	0.065 0.031 0.035 0.019	0.025 0.026 0.034 0.023	0.17 0.029 0.019 <0.01
Study code: Doc ID: Trial No. GLP: Year:	731173 2015/1000322 L140357 yes 2014	Grapes	United Kingdom	**Note: trial underdosed** BAS 222 28 F: 3x 0.4 kg metiram/ha Spray volume: 227-233 L/ha	73	0 48 57 62	fruit fruit fruit fruit	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	0.019 < 0.01 < 0.01 < 0.01
Study code: Doc ID: Trial No. GLP: Year:	731173 2015/1000322 L140358 yes 2014	Grapes	Hungary	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	77	0 49 56 63	fruit fruit fruit fruit	0.055 0.027 0.016 0.014	0.050 0.063 0.061 0.054	0.080 0.013 <0.01 <0.01

1) Days after last application
2) Growth stage
For calculation purposes for example < 0.01 was set to 0.01
* The limit of quantitation (LOO) for ETU, EU and EBIS was 0.01 mg/kg

Table 6.3.1-16: Level of ETU, EU and EBIS in BAS 222 28 F treated wine grapes (S-EU)

Study details		Crop	Country	Formulation Application rate	GS[2] at last appl. (BBCH)	DALA[1]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	ETU*	EU*	EBIS*
Study code:	731173	Grapes	France (S)	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	79	0	fruit	0.018	0.011	0.032
Doc ID:	2015/1000322					49	fruit	0.014	0.021	0.011
Trial No.	L140359					57	fruit	< 0.01	0.017	< 0.01
GLP:	yes					63	fruit	< 0.01	0.013	< 0.01
Year:	2014									
Study code:	731173	Grapes	Greece	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	75	0	fruit	0.027	0.012	0.088
Doc ID:	2015/1000322					48	fruit	0.018	0.019	0.028
Trial No.	L140360					57	fruit	0.011	0.015	<0.01
GLP:	yes					61	fruit	< 0.01	0.014	<0.01
Year:	2014									
Study code:	731173	Grapes	Italy	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	79	0	fruit	0.046	<0.01	0.097
Doc ID:	2015/1000322					48	fruit	0.039	0.016	0.024
Trial No.	L140361					55	fruit	0.017	0.012	0.030
GLP:	yes					62	fruit	0.038	0.029	0.035
Year:	2014									
Study code:	731173	Grapes	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	77	0	fruit	0.16	0.014	0.26
Doc ID:	2015/1000322					48	fruit	0.059	0.040	0.037
Trial No.	L140362					56	fruit	0.046	0.027	0.026
GLP:	yes					63	fruit	0.022	0.024	<0.01
Year:	2014									

1) Days after last application
2) Growth stage
For calculation purposes for example < 0.01 was set to 0.01
* The limit of quantitation (LOO) for ETU, EU and EBIS was 0.01 mg/kg

Trials L140355 – L140356 and L140358 – L140362

In the specimens taken 0 DALA the residues of metiram (by EBDC) ranged from 1.5 – 11 mg/kg, they decreased to 0.41 – 4.3 mg/kg in the specimens taken 48-49 DALA and further to 0.32 – 2.7 mg/kg in those taken 55 – 57 DALA (PHI). In the specimens from the last sampling (61 – 63 DALA) they remained at this level.

No residues of metiram were found above the limit of quantitation in any of the analysed untreated specimens, apart from the specimens taken in trail L140360 were the following residue concentrations were found: 0.81 mg/kg (0 DALA), 0.99 mg/kg (48 DALA), 0.21 mg/kg (57 DALA) and 0.91 mg/kg (61 DALA).

The specimens taken 0 DALA showed residues of CS_2 in a range of 0.53 – 3.1 mg/kg (0.56 – 5.6 mg/kg expressed as metiram). In the 48 – 49 DALA specimens they decreased to 0.14 - 0.88 mg/kg (0.26 – 1.6 mg/kg expressed as metiram), a further decrease to 0.10 - 0.65 mg/kg (0.18 – 1.2 mg/kg expressed as metiram) was observed in the specimen taken 55 – 57 DALA (PHI). < 0.056 - 0.54 mg/kg (< 0.10 – 0.97 mg/kg expressed as metiram) were found the specimens of the final sampling (61 - 63 DALA).

No residues of CS_2 were found above the limit of quantitation in any of the analysed untreated specimens, apart from the specimens taken in trail L140360 were the following residue concentrations were found: 0.19 mg/kg (0.34 mg/kg expressed as metiram) (0 DALA), 0.53 mg/kg (0.94 mg/kg expressed as metiram) (48 DALA), 0.38 mg/kg (0.68 mg/kg expressed as metiram) (57 DALA) and 0.20 mg/kg (0.36 mg/kg expressed as metiram) (61 DALA).

In the specimens taken 0 DALA residues of ETU ranged from 0.018 – 0.16 mg/kg. They decreased to 0.014 – 0.059 mg/kg in the specimens taken 48 - 49 DALA and further to < 0.01 – 0.046 mg/kg in the PHI specimens (55 – 57 DALA). In the specimens taken at the last sampling (61 – 63 DALA) < 0.01 – 0.038 mg/kg were found.

No residues of ETU were found above the limit of quantitation in any of the analysed untreated specimens.

The 0 DALA specimens showed EU residues in a range of < 0.01 – 0.050 mg/kg, they showed a slight increase in the specimens taken 48 – 49 DALA (0.016 – 0.063 mg/kg). In the specimens from the following sampling events the residues remained on this level (0.012 – 0.061 mg/kg (55 – 57 DALA (PHI)) and 0.013 – 0.054 mg/kg (61 – 63 DALA).

No residues of EU were found above the limit of quantitation in any of the analysed untreated specimens.

The EBIS residues in the 0 DALA specimens ranged from 0.032 – 0.31 mg/kg, they showed a decrease to 0.011 – 0.037 mg/kg in the 48 – 49 DALA specimens. In the specimens from the following sampling events the residues remained on this level (< 0.010 – 0.030 mg/kg (55 – 57 DALA (PHI)) and < 0.010 – 0.035 mg/kg (61 – 63 DALA).

No residues of EBIS were found above the limit of quantitation in any of the analysed untreated specimens.

Trial L140357

Trial L140357 will be reported separately as the calculation of the spray volume and the test item rate was based on the canopy dimensions rather than the plot dimensions which let to an underdosing of about 70%.

In the metiram (by EBDC) specimen taken at 0 DALA the residue concentration was determined at 0.89 mg/kg. It decreased to 0.11 mg/kg in the specimen taken 48 DALA and further to 0.050 mg/kg in the 57 DALA specimen (PHI). In the 62 DALA specimen it remained at this level (0.053 mg/kg). The untreated specimens of trial L140357 showed no residues of metiram above the limit of quantitation.

The CS_2 specimen taken 0 DALA showed residues of CS2 at 0.31 mg/kg (0.56 mg/kg expressed as metiram). No residues above the LOQ were found in the specimsn taken 48, 57 and 62 DALA. The untreated specimens of trial L140357 showed no residues of CS2 above the limit of quantitation.

No residues of ETU above the LOQ were found in any of the treated specimens of trial L140357. No residues of ETU above the LOQ were found in any of the untreated specimens of trial L140357.

No residues of EU above the LOQ were found in any of the treated specimens of trial L140357. No residues of EU above the LOQ were found in any of the untreated specimens of trial L140357.

No residues of EBIS were found in any of the treated specimens of trial L140357, apart from the 0 DALA specimen, where 0.019 mg/kg were found. No residues of EBIS above the LOQ were found in any of the untreated specimens of trial L140357.

III. CONCLUSION

Eight field trials with wine grapes were conducted in Europe (North and South) in season 2014. BAS 222 28 F was applied three times at single rates of 1.4 kg/ha BAS 222 F (2.0 kg/ha of WG formulation BAS 222 28 F) in a spray volume of 800 L/ha. (Note, seven field trials are considered further, omitting one trial L40357 since underdosed by 70%.)
Residue analysis of samples taken at different time intervals after the last application (0, 48-49, 55-57, and 61-63 DALA) showed a decline of residue levels.
At the recommended PHI of 56 days (when higher residues were detected in specimens sampled at a later sampling event then these residue values were taken), residues of metiram ranged between 0.18-1.2 mg/kg (determination as CS_2) and 0.32 – 27 mg/kg (determination as EBDC). Residues of CS_2 ranged between 0.10 - 0.65 mg/kg. Residues of ETU ranged between <0.010 – 0.046 mg/kg. Residues of EU ranged between 0.012 – 0.061 mg/kg and residues of EBIS ranged between 0.010 – 0.035 mg/kg.
Samples from untreated control plots did not contain apparent residues of CS_2, EBDC, ETU, EU and EBIS, i.e. residues were below the corresponding LOQ (except for trial L140360 for CS_2 and EBDC residues of 0.91 and 0.38 mg/kg (0.68 mg/kg metiram, determination as CS_2) were found at PHI 57-61). The maximum storage interval of deep frozen samples from harvest until extraction was 138 days for metiram (by EBDC), 95 days for metiram (by CS_2), 74 days for ETU and EBIS and 90 days for EU. All specimens taken at the PHI (55 - 57 DA~A) were analyzed for ETU, EU and EBIS within 30 days after sampling, apart from those taken in trial L140362 which were analyzed 35 days after sampling. The mean of the concurrent recoveries was within the acceptable range of 70 – 110% for CS_2, EBDC, ETU, EU and EBIS.

Report: CA 6.3.1/2
Meyer M., 2014b
Study on the residue behaviour of Metiram (BAS 222 F) in wine grapes after treatment with BAS 222 28 F under field conditions in the United Kingdom and Italy, 2013
2014/1161880

Guidelines: EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, EEC 79/117, EEC 91/414, EU Regulation Regulation 544/2011 (10 June 2011) implementing Regulation No 1107/2009, EEC 7029/VI/95 rev. 5 Appendix B (July 22 1997), EEC 7525/VI/95 rev. 9 (March 2011), SANCO/825/00 rev. 8.1 (16 November 2010)

GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
Description: BAS 222 28 F: 700 g/kg of BAS 222 F (metiram), WG formulation
Lot/Batch #: FRE-000825
Purity: Not relevant
CAS#: Metiram (BAS 222 F): 9006-42-2
Development code: Metiram: BAS 222 F
ETU: M222F002
EU: M222F003
EBIS: M222F004
Spiking levels: BAS 222 F (by EBDC): 0.05, 0.5, 10 mg/kg
BAS 222 F (by CS_2): 0.1, 1.0, 10 mg/kg
ETU, EU, EBIS: 0.01, 0.1, 0.25 mg/kg

2. **Test Commodity:** berries and small fruits
Crop: wine grapes
Type: *Vitis vinifera L.,*
Variety: Sauvignon Blanc, Rossese
Crop parts(s) or processed Commodity: grapes (fruits)
Sample size: 1.0 kg (12 bunches)

B. STUDY DESIGN AND METHODS

1. Test procedure

During the growing season of 2013, two field trials with wine grapes (field conditions) were conducted in the United Kingdom and Italy in order to determine the magnitude of the residues of metiram (BAS 222 F) after application of BAS 222 28 F.
The WG formulation BAS 222 28 F was applied three times (70, 62 - 63 and 56 days before harvest) at single rates of 1.4 kg as/ha for BAS 222 F in a spray volume of 800 L/ha in order to determine the magnitude of the residues of the active ingredient on Raw Agricultural Commodities (RAC).
Specimens were collected at 0, 49, 56 and 62 - 63 days after the last application (DALA).

Table 6.3.1-17 Application and sampling details for trials conducted in 2013

Region	No. of trials	No. of Appl.	F, G, I[2]	Method	Test Item	Active Substance	Application		Target Timing	
							Rate (kg a.s./ha)	Water vol. (L/ha)	Appl. (DBH)[3]	Sampl. (DALA)[1]
EU North & South	2	3	F	-	BAS 222 28 F (WG)	Metiram BAS 222 F	1.4	800	1st appl.: 70 2nd appl.: 62 - 63 3rd appl.: 56	0 49 56 62 - 63

1) Days after last application,
2) Field, Glasshouse or Indoor,
3) Days before harvest

2. Description of analytical procedures

The specimens were analysed for residues of metiram according to BASF method No. L0089/01 and as carbondisulfide according to BASF method L0234/01. The residues of ETU were determined according to the BASF method No. L0176/01. The residues of EU and EBIS were determined with BASF method L0233/01 developed at SGS INSTITUT FRESENIUS GmbH.
The limit of quantitation (LOQ) of the method L0089/01 was 0.05 mg/kg and for metiram as carbondisulfide it was 0.10 mg/kg. The LOQ of the method L0176/01 was 0.01 mg/kg. The LOQ for EU and EBIS were 0.01 mg/kg with the applied method.
The results of procedural recovery experiments averaged at about 102% for BAS 222 F (by EBDC) at fortification levels between 0.05 and 10 mg/kg, at 77% for BAS 222 F (by CS_2) at fortification levels between 0.1 and 10.0 mg/kg, at 85% for ETU, at 82% for EU and at 97% for EBIS at fortification levels between 0.01 and 0.25 mg/kg.

<u>BASF method L0089/01 was used to determine Metiram as EBDC:</u>
The ethylene-bisdithiocarbamate (EBDC) moiety was formed out of BAS 222 F and extracted from the specimen with a buffer solution consisting of EDTA, cysteine, methanol and sodium hydroxide adjusted to pH 11.0. The formed ethylene-bisdithiocarbamate (EBDC) analyte was methylated with iodmethane prior to C18 SPE clean up. Specimens were quantified by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).
The limit of quantitation (LOQ) was 0.05 mg/kg.

<u>BASF method L0234/01 was used to determine Metiram as CS_2</u>
Metiram was transformed to CS_2 by means of orthophosphoric acid. Subsequently, the CS_2 was transferred to isooctane with a flow of nitrogen. The quantification was carried out using GC-MS. The limit of quantitation (LOQ) was 0.10 mg/kg.

<u>BASF method L0176/01 was used to determine ETU:</u>
ETU was extracted from the specimen material with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol-water. After centrifugation an aliquot was taken and the methanol was evaporated from the extract. The pH was adjusted to 8 before evaporating (in some cases after evaporation). The remaining water phase was cleaned by liquid/liquid (water/dichloromethane) partition on an Extrelut column. After concentration of the eluate, the residue was determined by LC-MS/MS.
The limit of quantitation (LOQ) was 0.01 mg/kg.

<u>EU was determined according to BASF method L0233/01:</u>
The extraction from EU was performed with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol-water and was followed by a clean-up by liquid-liquid partition on an Extrelut column where ethyl acetate was used for the elution.
Water was added prior the evaporation of the specimen extract. For the final reconstitution of the specimen extract a solution of H_2O/MeOH was used. The determination was performed by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

<u>EBIS was determined according to BASF method L0233/01:</u>
The extraction from EBIS was performed with a mixture of acetonitrile/formic acid in combination with a thiourea solution and was followed by a clean-up step utilizing C18-EC. Specimens were diluted with a solution of acetonitrile/H_2O/formic acid before being determined by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

The results of procedural recovery experiments are summarized in Table 6.3.1-18.

Table 6.3.1-18 Summary of recoveries

Matrix	Fortification Level (mg/kg)	Summary Recoveries			
		n	Mean (%)	SD (+/-)	RSD (%)
Method No. L0089/01		**Metiram (by EBDC)**			
Grapes / Fruit	0.05, 0.5, 10	3	102	4.4	4.3
Method No. L0234/01		**Metiram (by CS_2)**			
Grapes / Fruit	0.1, 1.0, 10	4	77	13	16
Method No. L0176/01		**ETU**			
Grapes / Fruit	0.01, 0.1, 0.25	3	85	11	13
Method No. L0233/01		**EU**			
Grapes / Fruit	0.01, 0.1, 0.25	3	82	7.5	9.2
Method No. L0233/01		**EBIS**			
Grapes / Fruit	0.01, 0.1, 0.25	3	97	4.7	4.9

II. RESULTS AND DISCUSSION

Residue data of the field trials is summarized in Table 6.3.1-19 and Table 6.3.1-20. Detailed information is shown in Table 6.3.1-21 to Table 6.3.1-24.
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Samples from untreated control plots did not contain apparent residues of CS_2, EBDC, ETU, EU and EBIS, i.e. residues were below the corresponding LOQ. The maximum storage interval of deep frozen samples from harvest until extraction was 242 days for metiram (by EBDC), 264 days for metiram (by CS_2) and 203 days each for ETU, EU and EBIS. The mean of the concurrent recoveries was within the acceptable range of 70 – 110% for CS_2, EBDC, ETU, EU and EBIS.

Table 6.3.1-19 Summary of metiram level in BAS 222 28 F treated wine grapes

Region	Matrix	DALA [1]	BBCH	Range of CS_2 Residues [2] [mg/kg]	Range of Metiram Residues (by CS_2) [mg/kg]	Range of Metiram Residues (by EBDC) [mg/kg]
EU North & South	Grapes (fruit)	0	73 - 81	0.96 - 2.32	1.71 - 4.16	3.12 - 8.91
		49	83 - 85	< 0.056 - 0.38	< 0.10 - 0.69	0.10 - 1.89
		56	85 - 89	<0.056 - 0.24	< 0.10 - 0.42	0.16 - 1.26
		62 - 63	89	< 0.056 - 0.29	< 0.10 - 0.52	0.084 - 1.41

[1] DALA = Days after Last Applicati-on
[2] the conversion factor from CS_2 to metiram is 1.79

Table 6.3.1-20 Summary of ETU, EU, and EBIS level in BAS 222 28 F treated wine grapes

Portion analysed	No. of specimens	DALA [1)]	BBCH	Range of ETU Residues [mg/kg]	Range of EU Residues [mg/kg]	Range of EBIS Residues [mg/kg]
EU North & South	Grapes (fruit)	0	73 - 81	0.015 - 0.039	< 0.01 - 0.013	0.039 - 0.20
		49	83 - 85	< 0.01 - 0.015	< 0.01 - 0.013	< 0.01 - 0.034
		56	85 - 89	< 0.01 - 0.013	< 0.01 - 0.016	< 0.01 - 0.017
		62 - 63	89	< 0.01	< 0.01 - 0.018	< 0.01 - 0.020

[1)] DALA = Days after Last Application

Table 6.3.1-21 Level of metiram in BAS 222 28 F treated wine grapes (N-EU)

Study details		Crop	Country	Formulation Application rate	GS[3] at last appl. (BBCH)	DALA[2]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	CS_2[1]	Metiram (by CS_2)	Metiram (by EBDC)
Study code:	389449_1	Grapes	United Kingdom	BAS 222 28 F: 3x 1.4 kg metiram/ha	73	0	fruit	0.96	1.71	3.12
Doc ID:	2014/1161880					49	fruit	<0.056	<0.1	0.10
Trial No.	L130015					56	fruit	<0.056	<0.1	0.16
GLP:	yes			Spray volume: 800 L/ha		62	fruit	<0.056	<0.1	0.084
Year:	2013									

1) the conversion factor from metiram to CS_2 is 1.79
2) Days after last application
3) Growth stage
For calculation purposes for example < 0.056 and <0.1 was set to 0.056 and 0.1
_ underlined values were used for MRL calculation

Table 6.3.1-22 Level of metiram in BAS 222 28 F treated wine grapes (S-EU)

Study details		Crop	Country	Formulation Application rate	GS[3] at last appl. (BBCH)	DALA[2]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	CS_2[1]	Metiram (by CS_2)	Metiram (by EBDC)
Study code:	389449_1	Grapes	Italy	BAS 222 28 F: 3x 1.4 kg metiram/ha	81	0	fruit	2.32	4.16	8.91
Doc ID:	2014/1161880					49	fruit	0.38	0.69	1.89
Trial No.	L130016					56	fruit	0.24	0.42	1.26
GLP:	yes			Spray volume: 800 L/ha		63	fruit	0.29	0.52	1.41
Year:	2013									

1) the conversion factor from metiram to CS_2 is 1.79
2) Days after last application
3) Growth stage
_ underlined values were used for MRL calculation

Table 6.3.1-23 Level of ETU, EU, EBIS in BAS 222 28 F treated wine grapes (N-EU)

Study details		Crop	Country	Formulation Application rate	GS[2] at last appl. (BBCH)	DALA[1]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	ETU	EU	EBIS
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389449_1 2014/1161880 L130015 yes 2013	Grapes	United Kingdom	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	73	0 49 56 62	fruit fruit fruit fruit	0.015 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	0.039 <0.01 <0.01 <0.01

1) Days after last application
2) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

Table 6.3.1-24 Level of ETU, EU, EBIS in BAS 222 28 F treated wine grapes (S-EU)

Study details		Crop	Country	Formulation Application rate	GS[2] at last appl. (BBCH)	DALA[1]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	ETU	EU	EBIS
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389449_1 2014/1161880 L130016 yes 2013	Grapes	Italy	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	81	0 49 56 63	fruit fruit fruit fruit	0.039 0.015 0.013 <0.01	0.013 0.013 0.016 0.018	0.20 0.034 0.017 0.020

1) Days after last application
2) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

In specimens taken at 0 DALA residues of metiram (by EBDC) ranged from 3.12 - 8.91 mg/kg. They decreased to 0.10 – 1.89 mg/kg in the specimens taken 49 DALA and further to 0.16 - 1.26 mg/kg (56 DALA). In the specimens of the final sampling (62 – 63 DALA) 0.084 - 1.41 mg/kg were found.
No residues above the limit of quantitation were found in any of the analyzed untreated specimens.

The specimens taken 0 DALA showed residues of CS_2 (Metiram by CS_2) in a range of 0.96 – 2.32 mg/kg (1.71 – 4.16 mg/kg expressed as BAS 222 F). In the 49 DALA specimens they decreased to < 0.056 – 0.38 mg/kg (< 0.10 – 0.69 mg/kg expressed as BAS 222 F), a further decrease to < 0.056 – 0.24 mg/kg (< 0.10 – 0.42 mg/kg expressed as BAS 222 F) was observed in the specimen taken 56 DALA. Residues in a range of < 0.056 – 0.29 mg/kg CS2 (< 0.10 – 0.52 mg/kg expressed as BAS 222 F) were found the specimens of the final sampling (62 - 63 DALA).
No residues of BAS 222 F were found above the limit of quantitation in any of the analyzed untreated specimens.

In specimens taken at 0 DALA residues of ETU ranged from 0.015 - 0.039 mg/kg and showed a decline over time in the following samplings < 0.01 - 0.015 mg/kg (49 DALA), < 0.01 - 0.013 mg/kg (56 DALA) and < 0.01 mg/kg (62 - 63 DALA).
No residues of ETU above the limit of quantitation were found in any of the analyzed untreated specimens.

In specimens taken at 0 DALA residues of EU ranged < 0.01 – 0.018 mg/kg for all four sampling events (< 0.01 – 0.013 (0 DALA), < 0.01 – 0.013 (49 DALA), < 0.01 – 0.016 mg/kg (56 DALA) and < 0.01 – 0.018 mg/kg (62 - 63 DALA)).
No residues of ETU above the limit of quantitation were found in any of the analyzed untreated specimens.

In specimens taken at 0 DALA residues of EBIS ranged from 0.039 - 0.20 mg/kg and showed a decline over time < 0.01 - 0.034 mg/kg (49 DALA), < 0.01 - 0.017 mg/kg (56 DALA) and < 0.01 - 0.020 mg/kg (62 - 63 DALA).
No residues of EU above the limit of quantitation were found in any of the analyzed untreated specimens.

III. CONCLUSION

Two field trials with wine grapes were conducted in Europe (North and South) in season 2013. BAS 222 28 F was applied three times at single rates of 1.4 kg/ha BAS 222 F (2.0 kg/ha of WG formulation BAS 222 28 F) in a spray volume of 800 L/ha. Residue analysis of samples taken at different time intervals after the last application (0, 49, 56 and 62 - 63 DALA) showed a decline of residue levels.
At the recommended PHI of 56 days (when higher residues were detected in specimens sampled at a later sampling event then these residue values were taken), residues of metiram ranged between <0.10 – 0.52 mg/kg (determined as CS_2) and 0.16 - 1.41 mg/kg (determination as EBDC). Residues of CS_2 ranged between <0.056 – 0.29 mg/kg. Residues of ETU ranged between <0.01 - 0.013 mg/kg. Residues of EU ranged between <0.01 – 0.018 mg/kg mg/kg and residues of EBIS ranged between <0.01 - 0.020 mg/kg.
Samples from untreated control plots did not contain apparent residues of CS_2, EBDC, ETU, EU and EBIS, i.e. residues were below the corresponding LOQ. The maximum storage interval of deep frozen samples from harvest until extraction was 242 days for metiram (by EBDC), 264 days for metiram (by CS_2) and 203 days each for ETU, EU and EBIS. The mean of the concurrent recoveries was within the acceptable range of 70 – 110% for CS_2, EBDC, ETU, EU and EBIS.

Report: CA 6.3.1/3
Klimmek S.,Gizler A., 2014a
Study on the residue behaviour of Metiram (BAS 222 F) in grapes (vine) after three applications with BAS 222 28 F under field conditions in Germany, Northern France, Southern France, Greece, Italy and Spain, 2013
2014/1000221

Guidelines: none

GLP: yes
(certified by Freie und Hansestadt Hamburg, Behoerde fuer Soziales, Familie, Gesundheit und Verbraucherschutz, Hamburg, Germany)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
   Description: BAS 222 28 F: 700 g/kg of BAS 222 F (metiram), WG formulation
   Lot/Batch #: FRE-000825
   Purity: Not relevant
   CAS#: Metiram (BAS 222 F): 9006-42-2
   Development code: Metiram: BAS 222 F
   ETU: M222F002
   EU: M222F003
   EBIS: M222F004
   Spiking levels:

BAS 222 F (by EBDC):	0.05, 0.5, 2.0, 20 mg/kg
BAS 222 F (by CS_2):	0.1, 0.5, 1.0, 10 mg/kg
ETU and EU:	0.01, 0.1 mg/kg
EBIS:	0.01, 0.1, 0.5 mg/kg

2. **Test Commodity:** berries and small fruits
   Crop: wine grapes
   Type: *Vitis vinifera L.,*
   Variety: Johanniter, Bacchus, Pinot Noir, Sauvignon, Cabernet Sauvignon, Muscat, Trebbiano, Palomino Fino
   Crop parts(s) or processed Commodity: grapes (fruits)
   Sample size: ≥ 1.0 kg (≥ 12 brunches)

B. STUDY DESIGN AND METHODS

1. Test procedure

During the growing season of 2013, eight field trials with wine grapes (field conditions) were conducted in Germany, Northern and Southern France, Greece, Italy and Spain in order to determine the magnitude of the residues of metiram (BAS 222 F) after application of BAS 222 28 F.

The WG formulation BAS 222 28 F was applied three times (70 ± 1, 63 ± 1 and 56 ± 1 days before harvest) at single rates of 1.4 kg as/ha for BAS 222 F (2.0 kg/ha of BAS 222 28 F) in a spray volume of 800 L/ha in order to determine the magnitude of the residues of the active ingredient on Raw Agricultural Commodities (RAC).

Treated grapes (vine) (bunches) specimens of all trials were sampled at 0 DALA, at 48 - 50 DALA, at 55 - 57 DALA and at 61 - 65 DALA.

Table 6.3.1-25 Application and sampling details for trials conducted in 2013

Region	No. of trials	No. of Appl.	F, G, I[2]	Method	Test Item	Active Substance	Application		Target Timing	
							Rate (kg a.s./ha)	Water vol. (L/ha)	Appl. (DBH)[3]	Sampl. (DALA)[1]
EU North & South	8	3	F	-	BAS 222 28 F (WG)	Metiram BAS 222 F	1.4	800	1st appl.: 70 ± 1 2nd appl.: 63 ± 1 3rd appl.: 56 ± 1	0 48 - 50 55 - 57 61 - 65

1) Days after last application,
2) Field, Glasshouse or Indoor,
3) Days before harvest

2. Description of analytical procedures

The specimens were analysed for residues of metiram according to BASF method No. L0089/01 and as carbondisulfide according to BASF method L0234/01. The residues of ETU were determined according to the BASF method No. L0176/01. The residues of EU and EBIS were determined with method L0233/01.

The limit of quantitation (LOQ) for metiram was 0.05 mg/kg. The limit of quantitation (LOQ) for metiram BAS 222 F (by CS_2) was 0.10 mg/kg. The limit of quantitation (LOQ) for ETU, EU and EBIS was 0.01 mg/kg.

The results of procedural recovery experiments averaged at about 86.3% for BAS 222 F (by EBDC) at fortification levels between 0.05 and 20 mg/kg, at 77.8% for BAS 222 F (by CS_2) at fortification levels between 0.1 and 10.0 mg/kg and at 83.8% for ETU and at 79.3% for EU at fortification levels between 0.01 and 0.1 mg/kg and at 100% for EBIS at fortification levels between 0.01 and 0.5 mg/kg.

<u>BASF method L0089/01 was used to determine Metiram as EBDC:</u>
The ethylene-bisdithiocarbamate (EBDC) moiety was formed out of BAS 222 F and extracted from the specimen with a buffer solution consisting of EDTA, cysteine, methanol and sodium hydroxide adjusted to pH 11.0. The formed ethylene-bisdithiocarbamate (EBDC) analyte was methylated with iodmethane prior to C18 SPE clean up. Specimens were quantified by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). The limit of quantitation (LOQ) was 0.05 mg/kg.

<u>BASF method L0234/01 was used to determine Metiram as CS_2</u>
Metiram was transformed to CS_2 by means of orthophosphoric acid. Subsequently, the CS_2 was transferred to isooctane with a flow of nitrogen. The quantification was carried out using GC-MS. The limit of quantitation (LOQ) was 0.10 mg/kg.

<u>BASF method L0176/01 was used to determine ETU:</u>
ETU was extracted from the specimen material with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol-water. After centrifugation an aliquot was taken and the methanol was evaporated from the extract. The pH was adjusted to 8 before evaporating (in some cases after evaporation). The remaining water phase was cleaned by liquid/liquid (water/dichloromethane) partition on an Extrelut column. After concentration of the eluate, the residue was determined by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

<u>EU was determined according to BASF method L0233/01:</u>
The extraction from EU was performed with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol-water and was followed by a clean-up by liquid-liquid partition on an Extrelut column where ethyl acetate was used for the elution.
Water was added prior the evaporation of the specimen extract. For the final reconstitution of the specimen extract a solution of H_2O/MeOH was used. The determination was performed by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

<u>EBIS was determined according to BASF method L0233/01:</u>
The extraction from EBIS was performed with a mixture of acetonitrile/formic acid in combination with a thiourea solution and was followed by a clean-up step utilizing C18-EC. Specimens were diluted with a solution of acetonitrile/H_2O/formic acid before being determined by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

The results of procedural recovery experiments are summarized in Table 6.3.1-26.

Table 6.3.1-26 Summary of recoveries

Matrix	Fortification Level (mg/kg)	Summary Recoveries			
		n	Mean (%)	SD (+/-)	RSD (%)
Method No. L0089/01		**Metiram (by EBDC)**			
Grapes / Fruit	0.05, 0.5, 2, 20	10	86.3	15	18
Method No. L0234/01		**Metiram (by CS_2)**			
Grapes / Fruit	0.1, 0.5, 1.0, 10	20	77.8	10	13
Method No. L0176/01		**ETU**			
Grapes / Fruit	0.01, 0.1	8	83.8	7.8	9.3
Method No. L0233/01		**EU**			
Grapes / Fruit	0.01, 0.1	10	79.3	4.7	5.9
Method No. L0233/01		**EBIS**			
Grapes / Fruit	0.01, 0.1, 0.5	10	100	11	11

II. RESULTS AND DISCUSSION

Residue data of the field trials is summarized in Table 6.3.1-27 and
Table **6.3.1-28**. Detailed information is shown in Table 6.3.1-29 to Table 6.3.1-32.

Samples from untreated control plots did not contain apparent residues of CS_2, EBDC, ETU, EU and EBIS, i.e. residues were below the corresponding LOQ. The maximum storage interval of deep frozen samples from harvest until extraction was 368 days for metiram (BAS 222 F) (by EBDC), 371 days for metiram (by CS_2) and 237 days for ETU, EU and EBIS. The mean of the concurrent recoveries was within the acceptable range of 70 – 110% for CS_2, EBDC, ETU, EU and EBIS.

Table 6.3.1-27: Summary of metiram level in BAS 222 28 F treated wine grapes

Region	Matrix	DALA [1]	BBCH	Range of CS_2 Residues [2] [mg/kg]	Range of Metiram Residues (by CS_2) [mg/kg]	Range of Metiram Residues (by EBDC) [mg/kg]
EU North & South	Grapes (fruit)	0	75 - 81	0.30 - 3.56	0.54 - 6.38	0.46 - 9.86
		48 - 50	83 - 89	0.11 - 0.37	0.20 - 0.67	0.18 - 2.12
		55 - 57	81 - 89	<0.056 - 0.48	< 0.10 - 0.86	0.07 - 2.03
		61 - 65	89	< 0.056 - 0.58	< 0.10 - 1.03	0.09 - 2.05

[1] DALA = Days after Last Applicati-on
[2] the conversion factor from CS_2 to metiram is 1.79

Table 6.3.1-28: Summary of ETU, EU, and EBIS level in BAS 222 28 F treated wine grapes

Region	Matrix	DALA [1)]	BBCH	Range of ETU Residues [mg/kg]	Range of EU Residues [mg/kg]	Range of EBIS Residues [mg/kg]
EU North & South	Grapes (fruit)	0	75 - 81	< 0.01 - 0.06	< 0.01 - 0.01	0.01 - 0.21
		48 - 50	83 - 89	< 0.01 - 0.03	< 0.01 - 0.02	< 0.01 - 0.04
		55 - 57	81 - 89	< 0.01	< 0.01 - 0.02	< 0.01 - 0.05
		61 - 65	89	< 0.01 - 0.03	< 0.01 - 0.02	< 0.01 - 0.05

[1)] DALA = Days after Last Application

Table 6.3.1-29: Level of metiram in BAS 222 28 F treated wine grapes (N-EU)

Study details		Crop	Country	Formulation Application rate	GS[3] at last appl. (BBCH)	DALA[2]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	CS_2[1]	Metiram (by CS_2)	Metiram (by EBDC)
Study code: Doc ID: Trial No. GLP: Year:	389460 2014/1000221 L130086 yes 2013	Grapes	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	79	0 50 56 62	fruit fruit fruit fruit	2.21 0.36 0.40 0.44	3.95 0.64 0.72 0.78	1.97 2.12 1.70 2.05
Study code: Doc ID: Trial No. GLP: Year:	389460 2014/1000221 L130087 yes 2013	Grapes	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	75	0 49 57 65	fruit fruit fruit fruit	2.79 0.37 0.48 0.49	5.00 0.67 0.86 0.88	7.50 1.24 1.93 1.49
Study code: Doc ID: Trial No. GLP: Year:	389460 2014/1000221 L130088 yes 2013	Grapes	France (N)	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	77	0 49 56 63	fruit fruit fruit fruit	0.64* 0.16* 0.11* 0.09*	1.14* 0.28* 0.21* 0.18*	6.59 0.63 0.31 0.34
Study code: Doc ID: Trial No. GLP: Year:	389460 2014/1000221 L130089 yes 2013	Grapes	France (N)	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	77	0 49 56 63	fruit fruit fruit fruit	0.30* 0.11* < 0.056* < 0.056*	0.54* 0.20* < 0.1* < 0.1*	0.46* 0.18* 0.067* 0.088*

1) the conversion factor from metiram to CS_2 is 1.79
2) Days after last application
3) Growth stage
* mean value of two individual extractions
_ underlined values were used for MRL calculation

Table 6.3.1-30: Level of metiram in BAS 222 28 F treated wine grapes (S-EU)

Study details		Crop	Country	Formulation Application rate	GS[3] at last appl. (BBCH)	DALA[2]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	CS_2[1]	Metiram (by CS_2)	Metiram (by EBDC)
Study code:	389460	Grapes	France (S)	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	79 - 81	0	fruit	1.39	2.49	5.06
Doc ID:	2014/1000221					49	fruit	0.37	0.67	1.32
Trial No.	L130090					56	fruit	0.462	0.82	2.03
GLP:	yes					63	fruit	0.58	1.03	1.52
Year:	2013									
Study code:	389460	Grapes	Greece	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	75 / 77	0	fruit	3.56	6.38	9.86
Doc ID:	2014/1000221					48	fruit	0.22	0.40	0.46
Trial No.	L130091					55	fruit	0.32	0.58	0.61
GLP:	yes					63	fruit	0.29	0.53	0.81
Year:	2013									
Study code:	389460	Grapes	Italy	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	80	0	fruit	1.67	2.99	5.61
Doc ID:	2014/1000221					48	fruit	0.11	0.20	0.34
Trial No.	L130092					55	fruit	0.13	0.24	0.52
GLP:	yes					61	fruit	0.095	0.17	0.31
Year:	2013									
Study code:	389460	Grapes	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	77	0	fruit	1.60	2.87	6.15
Doc ID:	2014/1000221					49	fruit	0.33	0.59	1.30
Trial No.	L130093					56	fruit	0.28	0.51	1.41
GLP:	yes					63	fruit	0.35	0.62	1.40
Year:	2013									

1) the conversion factor from metiram to CS_2 is 1.79
2) Days after last application
3) Growth stage
For calculation purposes for example < 0.056 was set to 0.056
_ underlined values were used for MRL calculation

Table 6.3.1-31: Level of ETU, EU, EBIS in BAS 222 28 F treated wine grapes (N-EU)

Study details		Crop	Country	Formulation Application rate	GS[2] at last appl. (BBCH)	DALA[1]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	ETU*	EU*	EBIS*
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389460 2014/1000221 L130086 yes 2013	Grapes	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	79	0 50 56 62	fruit fruit fruit fruit	0.02 0.01 < 0.01 0.01	< 0.01 0.01 0.01 0.01	0.17 0.02 0.03 0.02
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389460 2014/1000221 L130087 yes 2013	Grapes	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	75	0 49 57 65	fruit fruit fruit fruit	0.05 < 0.01 < 0.01 0.01	< 0.01 0.01 < 0.01 0.01	0.17 0.02 0.02 0.04
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389460 2014/1000221 L130088 yes 2013	Grapes	France (N)	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	77	0 49 56 63	fruit fruit fruit fruit	0.02 < 0.01 < 0.01 < 0.01	0.01 < 0.01 < 0.01 < 0.01	0.06 < 0.01 < 0.01 < 0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389460 2014/1000221 L130089 yes 2013	Grapes	France (N)	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	77	0 49 56 63	fruit fruit fruit fruit	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0.01 < 0.01 < 0.01 < 0.01

1) Days after last application
2) Growth stage
For calculation purposes for example < 0.01 was set to 0.01
* The limit of quantitation (LOO) for ETU, EU and EBIS was 0.01 mg/kg

Table 6.3.1-32: Level of ETU, EU, EBIS in BAS 222 28 F treated wine grapes (S-EU)

Study details		Crop	Country	Formulation Application rate	GS[2] at last appl. (BBCH)	DALA[1]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	ETU*	EU*	EBIS*
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389460 2014/1000221 L130090 yes 2013	Grapes	France (S)	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	79 - 81	0 49 56 63	fruit fruit fruit fruit	0.02 < 0.01 < 0.01 < 0.01	0.01 0.02 0.02 0.02	0.08 < 0.01 0.01 0.03
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389460 2014/1000221 L130091 yes 2013	Grapes	Greece	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	75 / 77	0 48 55 63	fruit fruit fruit fruit	0.05 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0.21 < 0.01 0.01 0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389460 2014/1000221 L130092 yes 2013	Grapes	Italy	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	80	0 48 55 61	fruit fruit fruit fruit	0.03 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0.12 < 0.01 0.01 0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389460 2014/1000221 L130093 yes 2013	Grapes	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	77	0 49 56 63	fruit fruit fruit fruit	0.06 0.03 < 0.01 0.03	< 0.01 0.02 0.02 0.02	0.14 0.04 0.05 0.05

1) Days after last application
2) Growth stage
For calculation purposes for example < 0.01 was set to 0.01
* The limit of quantitation (LOO) for ETU, EU and EBIS was 0.01 mg/kg

In the treated grape specimens taken 0 DALA the residue concentrations of metiram (by EBDC) ranged from 0.46 – 9.86 mg/kg. The residue concentration of metiram decreased to 0.18 – 2.12 mg/kg in specimens taken at 48 - 50 DALA and to 0.07 – 2.03 mg/kg (55 - 57 DALA). In the specimens of the final sampling (61 -65 DALA) residue concentrations of metiram ranged between 0.09 – 2.05 mg/kg.
No residues above the limit of quantitation (0.05 mg/kg) were found in any of the analysed untreated specimens with exception of five specimens. In the untreated specimens derived from Trial L130088 (France-North) at the sampling event 0 DBLA metiram residues were determined as 0.09 mg/kg and 0.06 mg/kg at 49 DALA. In the untreated specimens derived from trial L130090 (France-South) at the sampling event 0 DBLA metiram residues were determined as 0.09 mg/kg, 0.06 mg/kg at 49 DALA and 0.011 mg/kg at 63 DALA

The metiram (by CS_2) residues in the treated grape specimens taken at 0 DALA ranged from 0.54 – 6.38 mg/kg. The residue concentration of metiram decreased to 0.20 – 0.67 mg/kg in specimens taken at 48 - 50 DALA and to < 0.1 – 0.86 mg/kg (55 - 57 DALA). In the specimens of the final sampling (61 - 65 DALA) residue concentrations of metiram ranged between < 0.1 – 1.03 mg/kg.
No residues above the limit of quantitation (0.1 mg/kg) were found in any of the analysed untreated specimens with exception of two specimens derived from Greek Trial L130091. Here, residues were detected in the specimen taken at 0 DBLA (0.23 mg/kg and in specimens taken at 63±2 DALA (0.15 mg/kg).

The ETU residues in the treated grape specimens taken at 0 DALA ranged from < 0.01 – 0.06 mg/kg. The residue concentration of ETU decreased to < 0.01 – 0.03 mg/kg in specimens taken at 48 - 50 DALA and to < 0.01 mg/kg at 55 - 57 DALA. In the specimens of the final sampling (61 – 65 DALA) residue concentrations of ETU ranged between < 0.01 – 0.03 mg/kg.
No residues of ETU above the limit of quantitation were found in any of the analysed untreated specimens.

The EU residues in the treated grape specimens taken at 0 DALA ranged from < 0.01 mg/kg - 0.01 mg/kg. The residue concentrations of EU remained relatively stable at the following sampling events. At 48 - 50 DALA the residues ranged between < 0.01 mg/kg - 0.02 mg/kg EU, at 55 - 57 DALA they ranged between < 0.01 - 0.02 mg/kg EU. In the specimens of the final sampling (61 - 65 DALA) residue concentrations of EU ranged between < 0.01 mg/kg - 0.02 mg/kg.
No residues of EU above the limit of quantitation were found in any of the analyzed untreated specimens.

The EBIS residues in the treated grape specimens taken at 0 DALA ranged from 0.01 mg/kg - 0.21 mg/kg. The residue concentration of EBIS decreased to < 0.01 mg/kg - 0.04 mg/kg in specimens taken at 48 - 50 DALA and to < 0.01 - 0.05 mg/kg at 55 - 57 DALA. In the specimens of the final sampling (61 - 65 DALA) residue concentrations of EBIS ranged between < 0.01 mg/kg - 0.05 mg/kg.

No residues of EBIS above the limit of quantitation (0.01 mg/kg) were found in any of the analyzed untreated specimens.

III. CONCLUSION

Eight field trials with wine grapes were conducted in Europe (North and South) in season 2013. BAS 222 28 F was applied three times at single rates of 1.4 kg/ha BAS 222 F (2.0 kg/ha of WG formulation BAS 222 28 F) in a spray volume of 800 L/ha. Residue analysis of samples taken at different time intervals after the last application (0, 48-50, 55-57, and 61-65 DALA) showed a decline of residue levels.
At the recommended PHI of 56 days (when higher residues were detected in specimens sampled at a later sampling event then these residue values were taken), residues of metiram ranged between <0.1 - 1.03 mg/kg (determination as CS_2) and 0.07 – 2.05 mg/kg (determination as EBDC). Residues of CS_2 ranged between <0.056-0.58 mg/kg. Residues of ETU ranged between <0.010 – 0.03 mg/kg. Residues of EU ranged between <0.01 – 0.02 mg/kg and residues of EBIS ranged between <0.01 – 0.05 mg/kg.
Samples from untreated control plots did not contain apparent residues of CS_2, EBDC, ETU, EU and EBIS, i.e. residues were below the corresponding LOQ (except for trial L130090 for EBDC, residues of 0.011 mg/kg at 63 DALA and in trial L130091 residues of metiram by CS_2 of 0.15 mg/kg at 63 DALA were found). The maximum storage interval of deep frozen samples from harvest until extraction was 368 days for metiram (BAS 222 F) (by EBDC), 371 days for metiram (by CS_2) and 237 days for ETU, EU and EBIS. The mean of the concurrent recoveries was within the acceptable range of 70 – 110% for CS_2, EBDC, ETU, EU and EBIS.

Report: CA 6.3.1/4
Meyer M., 2014a
Study on the residue behaviour of Metiram (BAS 222 F) in wine grapes after treatment with BAS 222 28 F under field conditions in Germany, the United Kingdom, Spain and Greece, 2012
2012/1272626

Guidelines: EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, EEC 79/117, EEC 91/414, EEC 1607/VI/97 rev. 2 10.06.1999, EEC 7029/VI/95 rev. 5 Appendix B (July 22 1997), EEC 7525/VI/95 rev. 7, SANCO/3029/99 rev. 4 (11 July 2000)

GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
   Description: BAS 222 28 F: 700 g/kg of BAS 222 F (metiram), WG formulation
   Lot/Batch #: 80449975L0
   Purity: Not relevant
   CAS#: Metiram (BAS 222 F): 9006-42-2
   Development code: Metiram: BAS 222 F
   ETU: M222F002
   EU: M222F003
   EBIS: M222F004
   Spiking levels:
   BAS 222 F (by EBDC): 0.05, 0.5, 1.0 mg/kg
   BAS 222 F (by CS_2): 0.1, 1.0, 10 mg/kg
   ETU and EU: 0.01, 0.1 mg/kg
   EBIS: 0.01, 0.05, 0.1 mg/kg

2. **Test Commodity:** berries and small fruits
   Crop: wine grapes
   Type: *Vitis vinifera L.,*
   Variety: Riesling, Johanniter, Sauvignon Blanc, Moscatel, Muscat, Moscatel de Alejandria
   Crop parts(s) or processed Commodity: grapes (fruits)
   Sample size: 1.0 kg (12 bunches)

B. STUDY DESIGN AND METHODS

1. Test procedure

During the growing season of 2012, six field trials with wine grapes (field conditions) were conducted in Germany, the United Kingdom, Spain and Greece in order to determine the magnitude of the residues of metiram (BAS 222 F) after application of BAS 222 28 F.
The WG formulation BAS 222 28 F was applied three times (69 - 74, 61 - 67 and 55 - 57 days before harvest) at single rates of 1.4 kg as/ha for BAS 222 F in a spray volume of 800 L/ha in order to determine the magnitude of the residues of the active ingredient on Raw Agricultural Commodities (RAC).
Specimens were collected at 0, 49 - 50, 55 - 57 and 62 - 63 days after the last application (DALA).

Table 6.3.1-33: Application and sampling details for trials conducted in 2012

Region	No. of trials	No. of Appl.	F, G, I[2]	Method	Test Item	Active Substance	Application		Target Timing	
							Rate (kg a.s./ha)	Water vol. (L/ha)	Appl. (DBH)[3]	Sampl. (DALA)[1]
EU North & South	6	3	F	-	BAS 222 28 F (WG)	Metiram BAS 222 F	1.4	800	1st appl.: 69 - 74 2nd appl.: 61 - 67 3rd appl.: 55 - 57	0 49 - 50 55 - 57 62 - 63

1) Days after last application,
2) Field, Glasshouse or Indoor,
3) Days before harvest

2. Description of analytical procedures

The specimens were analysed for residues of metiram according to BASF method No. L0089/01 and as carbondisulfide according to BASF method L0234/01. The residues of ETU were determined according to the BASF method No. L0176/01. The residues of EU and EBIS were determined with BASF method L0233/01 developed at SGS INSTITUT FRESENIUS GmbH.
The limit of quantitation (LOQ) of the method L0089/01 was 0.05 mg/kg and for metiram as carbondisulfide it was 0.10 mg/kg. The LOQ of the method L0176/01 was 0.01 mg/kg. The LOQ for EU and EBIS were 0.01 mg/kg with the applied method.
The results of procedural recovery experiments averaged at about 89% for BAS 222 F (by EBDC) at fortification levels between 0.05 and 1.0 mg/kg, at 84% for BAS 222 F (by CS_2) at fortification levels between 0.1 and 10.0 mg/kg, at 90% for ETU, at 72% for EU and at 93% for EBIS at fortification levels between 0.01 and 0.1 mg/kg.

BASF method L0089/01 was used to determine Metiram as EBDC:
The ethylene-bisdithiocarbamate (EBDC) moiety was formed out of BAS 222 F and extracted from the specimen with a buffer solution consisting of EDTA, cysteine, methanol and sodium hydroxide adjusted to pH 11.0. The formed ethylene-bisdithiocarbamate (EBDC) analyte was methylated with iodmethane prior to C18 SPE clean up. Specimens were quantified by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).
The limit of quantitation (LOQ) was 0.05 mg/kg.

BASF method L0234/01 was used to determine Metiram as CS_2
Metiram was transformed to CS_2 by means of orthophosphoric acid. Subsequently, the CS_2 was transferred to isooctane with a flow of nitrogen. The quantification was carried out using GC-MS. The limit of quantitation (LOQ) was 0.10 mg/kg.

BASF method L0176/01 was used to determine ETU:
ETU was extracted from the specimen material with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol-water. After centrifugation an aliquot was taken and the methanol was evaporated from the extract. The pH was adjusted to 8 before evaporating (in some cases after evaporation). The remaining water phase was cleaned by liquid/liquid (water/dichloromethane) partition on an Extrelut column. After concentration of the eluate, the residue was determined by LC-MS/MS.
The limit of quantitation (LOQ) was 0.01 mg/kg.

EU was determined according to BASF method L0233/01:
The extraction from EU was performed with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol-water and was followed by a clean-up by liquid-liquid partition on an Extrelut column where ethyl acetate was used for the elution.
Water was added prior the evaporation of the specimen extract. For the final reconstitution of the specimen extract a solution of H_2O/MeOH was used. The determination was performed by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

EBIS was determined according to BASF method L0233/01:
The extraction from EBIS was performed with a mixture of acetonitrile/formic acid in combination with a thiourea solution and was followed by a clean-up step utilizing C18-EC. Specimens were diluted with a solution of acetonitrile/H_2O/formic acid before being determined by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

The results of procedural recovery experiments are summarized in Table 6.3.1-34.

Table 6.3.1-34: Summary of recoveries

Matrix	Fortification Level (mg/kg)	Summary Recoveries			
		n	Mean (%)	SD (+/-)	RSD (%)
Method No. L0089/01		**Metiram (by EBDC)**			
Grapes / Fruit	0.05, 0.5, 1.0	13	89	14	16
Method No. L0234/01		**Metiram (by CS_2)**			
Grapes / Fruit	0.1, 1.0, 10.0	14	84	6.7	8.0
Method No. L0176/01		**ETU**			
Grapes / Fruit	0.01, 0.1	6	90	7.8	8.6
Method No. L0233/01		**EU**			
Grapes / Fruit	0.01, 0.1	6	72	6.9	9.5
Method No. L0233/01		**EBIS**			
Grapes / Fruit	0.01, 0.05, 0.1	10	93	9.1	9.8

II. RESULTS AND DISCUSSION

Residue data of the field trials is summarized in Table 6.3.1-35 and Table 6.3.1-36. Detailed information is shown in Table 6.3.1-37 to Table 6.3.1-40.

Samples from untreated control plots did not contain apparent residues of CS_2, EBDC, ETU, EU and EBIS, i.e. residues were below the corresponding LOQ. The maximum storage interval of deep frozen samples from harvest until extraction was 444 days for metiram (by EBDC), 318 days for metiram (by CS_2) and 311 days for ETU, 431 days for EU and 444 days for EBIS.
The mean of the concurrent recoveries was within the acceptable range of 70 – 110% for CS_2, EBDC, ETU, EU and EBIS.

Table 6.3.1-35: Summary of metiram level in BAS 222 28 F treated wine grapes

Region	Matrix	DALA [1)]	BBCH	Range of CS_2 Residues [2)] [mg/kg]	Range of Metiram Residues (by CS_2) [mg/kg]	Range of Metiram Residues (by EBDC) [mg/kg]
EU North & South	Grapes (fruit)	0	75 - 81	0.68 - 4.57	1.22 - 8.19	2.33 - 11.10
		49 - 50	77 - 87	<0.056 - 0.97	< 0.1 - 1.74	0.21 - 2.82
		55 - 57	85 - 89	0.059 - 0.57	0.11 - 1.01	0.21 - 1.87
		62 - 63	89	0.062 - 0.88	0.11 - 1.57	0.23 - 3.08

[1)] DALA = Days after Last Application
[2)] the conversion factor from CS_2 to metiram is 1.79

Table 6.3.1-36: Summary of ETU, EU, and EBIS level in BAS 222 28 F treated wine grapes

Region	Matrix	DALA [1)]	BBCH	Range of ETU Residues [mg/kg]	Range of EU Residues [mg/kg]	Range of EBIS Residues [mg/kg]
EU North & South	Grapes (fruit)	0	75 - 81	0.013 - 0.074	< 0.01 - 0.015	0.063 - 0.27
		49 - 50	77 - 87	< 0.01 - 0.019	< 0.01 - 0.013	< 0.01 - 0.036
		55 - 57	85 - 89	< 0.01 - 0.026	< 0.01 - 0.012	< 0.01 - 0.025
		62 - 63	89	< 0.01 - 0.025	< 0.01 - 0.016	< 0.01

1) DALA = Days after Last Application

Table 6.3.1-37: Level of metiram in BAS 222 28 F treated wine grapes (N-EU)

Study details		Crop	Country	Formulation Application rate	GS[3] at last appl. (BBCH)	DALA[2]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	CS_2[1]	Metiram (by CS_2)	Metiram (by EBDC)
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389449 2012/1272626 L120532 yes 2012	Grapes	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	77	0 50 56 62	fruit fruit fruit fruit	2.50 0.43 0.38 0.30	4.48 0.76 0.68 0.53	6.61 1.52 1.12 1.12
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389449 2012/1272626 L120533 yes 2012	Grapes	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	81	0 49 56 63	fruit fruit fruit fruit	4.57 0.97 0.57 0.88	8.19 1.74 1.01 1.57	11.10 2.82 1.87 3.08
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389449 2012/1272626 L120535 yes 2012	Grapes	United Kingdom	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	75	0 49 57 63	fruit fruit fruit fruit	1.94 0.11 0.26 0.09	3.47 0.20 0.46 0.15	5.74 0.33 0.85 0.29

1) the conversion factor from metiram to CS_2 is 1.79
2) Days after last application
3) Growth stage
_ underlined values were used for MRL calculation

Table 6.3.1-38: Level of metiram in BAS 222 28 F treated wine grapes (S-EU)

Study details		Crop	Country	Formulation Application rate	GS[3] at last appl. (BBCH)	DA LA[2]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	CS_2[1]	Metiram (by CS_2)	Metiram (by EBDC)
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389449 2012/1272626 L120536 yes 2012	Grapes	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	77	0 49 55 62	fruit fruit fruit fruit	2.91 0.50 0.51 0.61	5.20 0.90 0.91 1.09	8.07 1.76 1.85 2.29
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389449 2012/1272626 L120537 yes 2012	Grapes	Greece	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	75-77	0 49 56 63	fruit fruit fruit fruit	1.39 <0.056 0.059 0.062	2.48 <0.1 0.11 0.11	3.94 0.21 0.21 0.23
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389449 2012/1272626 L120539 yes 2012	Grapes	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	80	0 49 55 62	fruit fruit fruit fruit	0.68 0.12 0.10 0.082	1.22 0.21 0.18 0.15	2.33 0.47 0.34 0.28

1) the conversion factor from metiram to CS_2 is 1.79
2) Days after last application
3) Growth stage
For calculation purposes for example < 0.056 was set to 0.056
_ underlined values were used for MRL calculation

Table 6.3.1-39: Level of ETU, EU, EBIS in BAS 222 28 F treated wine grapes (N-EU)

Study details		Crop	Country	Formulation Application rate	GS[2] at last appl. (BBCH)	DA LA[1]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	ETU	EU	EBIS
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389449 2012/1272626 L120532 yes 2012	Grapes	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	77	0 50 56 62	fruit fruit fruit fruit	0.053 <0.01 <0.01 <0.01	<0.01 <0.01 0.011 <0.01	0.19 0.022 0.015 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389449 2012/1272626 L120533 yes 2012	Grapes	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	81	0 49 56 63	fruit fruit fruit fruit	0.074 0.013 0.010 0.025	0.015 0.013 0.012 0.017	0.27 0.036 0.025 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389449 2012/1272626 L120535 yes 2012	Grapes	United Kingdom	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	75	0 49 57 63	fruit fruit fruit fruit	0.046 <0.01 <0.01 <0.01	0.012 <0.01 <0.01 <0.01	0.17 <0.01 0.014 <0.01

1) Days after last application
2) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

Table 6.3.1-40: Level of ETU, EU, EBIS in BAS 222 28 F treated wine grapes (S-EU)

Study details		Crop	Country	Formulation Application rate	GS[2] at last appl. (BBCH)	DALA[1]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	ETU	EU	EBIS
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389449 2012/1272626 L120536 yes 2012	Grapes	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	77	0 49 55 62	fruit fruit fruit fruit	0.044 0.019 0.026 0.022	<0.01 0.012 0.012 0.016	0.19 0.024 0.025 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389449 2012/1272626 L120537 yes 2012	Grapes	Greece	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	75-77	0 49 56 63	fruit fruit fruit fruit	0.020 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	0.10 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389449 2012/1272626 L120539 yes 2012	Grapes	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 800 L/ha	80	0 49 55 62	fruit fruit fruit fruit	0.013 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	0.063 <0.01 <0.01 <0.01

1) Days after last application
2) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

In specimens taken at 0 DALA residues of metiram (by EBDC) ranged from 2.33 – 11.10 mg/kg. They decreased to 0.21 – 2.82 mg/kg in the specimens taken 49 - 50 DALA and further to 0.21 – 1.87 mg/kg (55 - 57 DALA). In the specimens of the final sampling (62 - 63 DALA) 0.23 – 3.08 mg/kg were found.
No residues above the limit of quantitation were found in any of the analyzed untreated specimens.

The specimens taken 0 DALA showed residues of CS_2 (Metiram by CS_2) in a range of 0.68 – 4.57 mg/kg (1.22 – 8.19 mg/kg expressed as metiram). In the 49 - 50 DALA specimens they decreased to < 0.056 – 0.97 mg/kg (< 0.1 – 1.74 mg/kg expressed as metiram), a further decrease to 0.059 – 0.57 mg/kg (0.11 – 1.01 mg/kg expressed as metiram) was observed in the specimens taken 55 - 57 DALA. 0.062 – 0.88 mg/kg (0.11 – 1.57 mg/kg expressed as metiram) were found the specimens of the final sampling (62 - 63 DALA).
No residues of metiram were found above the limit of quantitation in any of the analyzed untreated specimens, except for the 0 DALA specimens taken from trial L120537, where 0.13 mg/kg (0.22 mg/kg expressed as metiram) were found

In specimens taken at 0 DALA residues of ETU ranged from 0.013 – 0.074 mg/kg and showed a decline in the 49 - 50 DALA specimens (< 0.01 – 0.019 mg/kg). They remained at this level in the specimens taken 55 - 57 DALA (< 0.01 – 0.026 mg/kg), as well as the last sampling (62 - 63 DALA) (< 0.01 – 0.025 mg/kg).
No residues of ETU above the limit of quantitation were found in any of the analyzed untreated specimens.

In specimens taken at 0 DALA residues of EU ranged < 0.01 – 0.016 mg/kg for all four sampling events (< 0.01 – 0.015 (0 DALA), < 0.01 – 0.013 (49 - 50 DALA), < 0.01 – 0.012 mg/kg (55 - 57 DALA) and < 0.01 – 0.016 mg/kg (62 - 63 DALA)).
No residues of EU above the limit of quantitation were found in any of the analyzed untreated specimens.

In specimens taken at 0 DALA residues of EBIS ranged from 0.063 – 0.27 mg/kg and showed a decline over time (< 0.01 – 0.036 mg/kg (49 - 50 DALA), < 0.01 – 0.025 mg/kg (55 - 57 DALA) and < 0.01 mg/kg (62 - 63 DALA)).
No residues of EBIS above the limit of quantitation were found in any of the analyzed untreated specimens.

III. CONCLUSION

Six field trials with wine grapes were conducted in Europe (North and South) in season 2012. BAS 222 28 F was applied three times at single rates of 1.4 kg/ha BAS 222 F (2.0 kg/ha of WG formulation BAS 222 28 F) in a spray volume of 800 L/ha. Residue analysis of samples taken at different time intervals after the last application (0, 49-50, 55-57, and 62-63 DALA) showed a decline of residue levels.
At the recommended PHI of 56 days (when higher residues were detected in specimens sampled at a later sampling event then these residue values were taken), residues of metiram ranged between 0.11 - 1.57 mg/kg (determination as CS_2) and 0.21 – 3.08 mg/kg (determination as EBDC). Residues of CS_2 ranged between 0.059 - 0.88 mg/kg. Residues of ETU ranged between <0.010 – 0.026 mg/kg. Residues of EU ranged between <0.01 – 0.016 mg/kg and residues of EBIS ranged between <0.01 – 0.025 mg/kg.
Samples from untreated control plots did not contain apparent residues of CS_2, EBDC, ETU, EU and EBIS, i.e. residues were below the corresponding LOQ. The maximum storage interval of deep frozen samples from harvest until extraction was 444 days for metiram (by EBDC), 318 days for metiram (by CS_2) and 311 days for ETU, 431 days for EU and 444 days for EBIS. The mean of the concurrent recoveries was within the acceptable range of 70 – 110% for CS_2, EBDC, ETU, EU and EBIS.

CA 6.3.2 Potatoes

In support of the representative use in potatoes, a total of 23 cGAP compliant new field trials on potatoes were conducted. The cGAP is given in Table 6.3.2-1. The residue trials were performed in various European Member States in N-EU and S-EU during the growing seasons 2012, 2013 and 2014 and thereby fulfill the requirements of seasonal and geographical distribution (see Table 6.3.2-2).

Table 6.3.2-1: Summary of the critical GAP for the proposed use in potatoes for BAS 222 28 F

Crop	Outdoor/ Protected	Growth stage (BBCH)	Maximum number of applications	Minimum application interval (days)	Maximum		Minimum PHI (days)
					Rate (kg as/ha)	Water (L/ha)	
Potatoes	Outdoor	21 - 89	3	7	1.26	100 - 1000	14

Table 6.3.2-2: Number of residue trials conducted per geographical region and vegetation period - BAS 222 28 F

Crop	Vegetation Period	Number of Trials					Reference
		EU North	Country	EU South	Country	Total	
Potato	2014	4	DE, NL, UK	4	ES, IT, GR	8	6.3.2/1
Potato	2013	4	DE, FR, UK	4	ES, IT, FR, GR	8	6.3.2/2
Potato	2012	4	DE, UK	4	ES, GR	8	6.3.2/3
Total number of trials per Region		**12**		**12**	**Total number of trials**	**24**	

Table 6.3.2-3: Overall summary of residue data for metiram from potato residue trials

Crop	Region	RAC	n	Residue level (mg/kg)								
				CS_2			Metiram, determined as CS_2			Metiram, determined as EBDC		
				Min.	HR	STMR	Min.	HR	STMR	Min.	HR	STMR
Potato	N-EU	tuber	11	<0.056	0.056	0.056	<0.1	<0.1	<0.1	<0.05	<0.05	<0.05
	S-EU	tuber	12	<0.056	0.056	0.056	<0.1	<0.1	<0.1	<0.05	<0.05	<0.05

Table 6.3.2-4: Overall summary of residue data for ETU, EU and EBIS from potato residue trials

Crop	Region	RAC	n	Residue level (mg/kg)								
				ETU			EU			EBIS		
				Min.	HR	STMR	Min.	HR	STMR	Min.	HR	STMR
Potato	N-EU	tuber	11	<0.01	0.038	0.01	<0.01	0.03	0.01	<0.01	<0.01	<0.01
	S-EU	tuber	12	<0.01	0.023	0.01	<0.01	0.01	0.01	<0.01	<0.01	<0.01

The study reports are summarized below.

Table 6.3.2-5: Potato residue data: summary of maximum storage interval and storage stability

Crop	DocID	Study No.	Maximum storage interval (days)*					Storage stability (days)				
			Metiram by CS_2	Metiram by EBDC	ETU	EU	EBIS	Metiram by CS_2	Metiram by EBDC	ETU	EU	EBIS
Potatoes	2015/1000321	731171	135	83	48 Δ (17 for PHI specimens) Δ	48 (17 for PHI specimens)	48 Δ (17 for PHI specimens) Δ	373	373	0	365	0
	2014/1000222	389461	350	431 Δ	263 Δ	263	263 Δ					
	2012/1272625	389454	318	428 Δ	310 Δ	448 Δ	452 Δ					

* of deep frozen samples from harvest until extraction
Δ freezer storage stability not confirmed (see section MCA 6.1)

Table 6.3.2-6: Potato residue data: summary of storage intervals of PHI samples

DocID	Study No.	PHI Specimen No.*	Storage interval of PHI specimens* (days)				
			Metiram by CS_2	Metiram by EBDC	ETU	EU	EBIS
2015/1000321	731171	L1403820007	92	66	16 Δ	16	16 Δ
		L1403830007	28	23	16 Δ	16	16 Δ
		L1403840007	43	38	9 Δ	9	9 Δ
		L1403850007	33	62	13 Δ	13	13 Δ
		L1403860007	121	39	10 Δ	10	10 Δ
		L1403870007	113	31		17	17 Δ
		L1403870008			11 Δ		
		L1403880007	13	48	13 Δ	13	13 Δ
		L1403890007	43	18	11 Δ	11	11 Δ
2014/1000222	389461	L1301180007	259	238	189 Δ		190 Δ
		L1301180008				186	
		L1301190007	257	215	166 Δ		
		L1301190008				163	164 Δ
		L1301200007	249	225	176 Δ	176	177 Δ
		L1301210007	239	215	166 Δ	166	167 Δ
		L1301220007	211	182	135 Δ	135	134 Δ
		L1301230007	284	263	216 Δ	216	215 Δ
		L1301240007	243	221	174 Δ	174	173 Δ
		L1301250007	324	295	248 Δ	248	247 Δ
2012/1272625	389454	L1205240007	364	457Δ	357 Δ	350	504 Δ
		L1205250007	273	366	266 Δ	259	413 Δ
		L1205260007	223	335	216 Δ	209	364 Δ
		L1205270007	207	319	200 Δ	193	348 Δ
		L1205280007	302	414Δ	295 Δ	288	443 Δ
		L1205290007	270	382Δ	263 Δ	256	411 Δ
		L1205300007	239	349	230 Δ	223	378 Δ
		L1205310007	232	342	223 Δ	216	371 Δ

* PHI samples (when higher residues were detected in specimens sampled at a later sampling event then these residue values were taken)
Δ freezer storage stability not confirmed (see section MCA 6.1)

Report: CA 6.3.2/1
Meyer M.,Gabriel E.J., 2015a
Study on the residue behaviour of Metiram (BAS 222 F) in potato after treatment with BAS 222 28 F under field conditions in Germany, the Netherlands, the United Kingdom, Italy, Spain and Greece, 2014
2015/1000321

Guidelines: EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, EU Regulation Regulation 544/2011 (10 June 2011) implementing Regulation No 1107/2009, EEC 7029/VI/95 rev. 5, EEC 7525/VI/95 rev. 9 (March 2011), SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000)

GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
   Description: BAS 222 28 F: 700 g/kg of BAS 222 F (metiram), WG formulation
   Lot/Batch #: FRE-000977
   Purity: Not relevant
   CAS#: Metiram (BAS 222 F): 9006-42-2
   Development code: Metiram: BAS 222 F
   ETU: M222F002
   EU: M222F003
   EBIS: M222F004
   Spiking levels: BAS 222 F (by EBDC): 0.05, 0.5 mg/kg
   BAS 222 F (by CS_2): 0.1, 1.0 mg/kg
   ETU, EU, EBIS: 0.01, 0.1 mg/kg

2. **Test Commodity:** root and tuber vegetables
   Crop: potato
   Type: *Solanum tuberosum* L.
   Variety: Musica, Toscana, Home guard, Lady Claire, Carlita, Jaerla, Kennebeck
   Crop parts(s) or processed Commodity: potato / tuber
   Sample size: 2.0 kg (24 tubers)

B. STUDY DESIGN AND METHODS

1. Test procedure

During the growing season of 2014, eight field trials with potatoes were conducted in Germany, the United Kingdom, the Netherlands, Greece, Italy and Spain, in order to determine the magnitude of the residues of metiram (BAS 222 F) after application of BAS 222 28 F.
The WG formulation BAS 222 28 F was applied three times at single rates of 1.4 kg as/ha for BAS 222 F in a spray volume of 200 L/ha in order to determine the magnitude of the residues of the active ingredient on Raw Agricultural Commodities (RAC). The first application took place 26 - 29 DBH (days before harvest), the second application 20 - 22 DBH and the third application 13 - 15 DBH, except for trial L140385, where only two applications at 21 and 14 DBH were performed. Specimens were collected at 0, 6 - 8, 13 - 15 and 20 - 22 days after the last application (DALA).

Table 6.3.2-7 Application and sampling details for trials conducted in 2014

Region	No. of trials	No. of Appl.	F, G, I[2]	Method	Test Item	Active Substance	Application		Target Timing	
							Rate (kg a.s./ha)	Water vol. (L/ha)	Appl. (DBH)[3]	Sampl. (DALA)[1]
EU North & South	8	3	F	-	BAS 222 28 F (WG)	Metiram BAS 222 F	1.4	200	1st appl.: 26 - 29 2nd appl.: 20 - 22 3rd appl.: 13 - 15	0 6 - 8 13 - 15 20 - 22

1) Days after last application,
2) Field, Glasshouse or Indoor,
3) Days before harvest

2. Description of analytical procedures

The specimens were analyzed for residues of metiram according to BASF method No. L0089/01 and as carbondisulfide according to BASF method L0234/01. The residues of ETU were determined according to the BASF method No. L0176/01. The residues of EU and EBIS were determined with BASF method L0233/01.
The limit of quantitation (LOQ) of the method L0089/01 was 0.05 mg/kg and for metiram as carbondisulfide it was 0.10 mg/kg. The LOQ of the method L0176/01 was 0.01 mg/kg. The LOQ for EU and EBIS were 0.01 mg/kg with the applied method.
The results of procedural recovery experiments averaged at about 100% for BAS 222 F (by EBDC) at fortification levels between 0.05 and 0.5 mg/kg, at 80.2% for BAS 222 F (by CS_2) at fortification levels between 0.1 and 1.0 mg/kg, at 87.4% for ETU, at 83.0% for EU and at 92.4% for EBIS at fortification levels between 0.01 and 0.1 mg/kg.

BASF method L0089/01was used to determine Metiram as EBDC:
The ethylene-bisdithiocarbamate (EBDC) moiety was formed out of BAS 222 F and extracted from the specimen with a buffer solution consisting of EDTA, cysteine, methanol and sodium hydroxide adjusted to pH 11.0. The formed ethylene-bisdithiocarbamate (EBDC) analyte was methylated with iodmethane prior to C18 SPE clean up. Specimens were quantified by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). The limit of quantitation (LOQ) was 0.05 mg/kg.

BASF method L0234/01 was used to determine Metiram as CS_2
Metiram was transformed to CS_2 by means of orthophosphoric acid. Subsequently, the CS_2 was transferred to isooctane with a flow of nitrogen. The quantification was carried out using GC-MS. The limit of quantitation (LOQ) was 0.10 mg/kg.

BASF method L0176/01 was used to determine ETU:
ETU was extracted from the specimen material with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol-water. After centrifugation an aliquot was taken and the methanol was evaporated from the extract. The pH was adjusted to 8 before evaporating (in some cases after evaporation). The remaining water phase was cleaned by liquid/liquid (water/dichloromethane) partition on an Extrelut column. After concentration of the eluate, the residue was determined by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

EU was determined according to BASF method L0233/01:
The extraction from EU was performed with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol-water and was followed by a clean-up by liquid-liquid partition on an Extrelut column where ethyl acetate was used for the elution.
Water was added prior the evaporation of the specimen extract. For the final reconstitution of the specimen extract a solution of H_2O/MeOH was used. The determination was performed by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

EBIS was determined according to BASF method L0233/01:
The extraction from EBIS was performed with a mixture of acetonitrile/formic acid in combination with a thiourea solution and was followed by a clean-up step utilizing C18-EC. Specimens were diluted with a solution of acetonitrile/H_2O/formic acid before being determined by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

The results of procedural recovery experiments are summarized in Table 6.3.2-8.

Table 6.3.2-8 Summary of recoveries

Matrix	Fortification Level (mg/kg)	Summary Recoveries			
		n	Mean (%)	SD (+/-)	RSD (%)
Method No. L0089/01		**Metiram (by EBDC)**			
Potato / Tuber	0.05, 0.5	8	100	7.3	7.3
Method No. L0234/01		**Metiram (by CS_2)**			
Potato / Tuber	0.1, 1.0	12	80.2	8.6	11
Method No. L0176/01		**ETU**			
Potato / Tuber	0.01, 0.1	16	87.4	9.6	11
Method No. L0233/01		**EU**			
Potato / Tuber	0.01, 0.1	15	83.0	11	13
Method No. L0233/01		**EBIS**			
Potato / Tuber	0.01, 0.1	16	92.4	10	11

II. RESULTS AND DISCUSSION

Residue data of the field trials is summarized in Table 6.3.2-9 and Table 6.3.2-10. Detailed information is shown in Table 6.3.2-13 to Table 6.3.2-16.

Samples from untreated control plots did not contain apparent residues of CS_2, EBDC, ETU, EU and EBIS, i.e. residues were below the corresponding LOQ. The maximum storage interval of deep frozen samples from harvest until extraction was 83 days for metiram (by EBDC), 135 days for metiram (by CS_2) and 48 days for ETU (17 days for PHI specimens), 48 days for EU (17 days for PHI specimens) and 48 days for EBIS (17 days for PHI specimens). The mean of the concurrent recoveries was within the acceptable range of 70 – 110% for CS_2, EBDC, ETU, EU and EBIS.

Table 6.3.2-9 Summary of metiram level in BAS 222 28 F treated potato (Trials L140382 – L140384 and L140386 – L140389)

Region	Matrix	DALA [1]	BBCH	Range of CS_2 Residues [2] [mg/kg]	Range of metiram Residues (by CS_2) [mg/kg]	Range of metiram Residues (by EBDC) [mg/kg]
EU North & South	Potato (tuber)	0	45 – 48	< 0.056	< 0.1	< 0.05 - 0.064
		6 – 8	46 – 48	< 0.056	< 0.1	< 0.05
		13 – 15	48 – 49	< 0.056	< 0.1	< 0.05
		20 – 22	48 - 49	< 0.056	< 0.1	< 0.05

[1] DALA = Days after Last Application

[2] the conversion factor from CS_2 to metiram is 1.79

Table 6.3.2-10 Summary of ETU, EU, and EBIS level in BAS 222 28 F treated potato (Trials L140382 – L140384 and L140386 – L140389)

Portion analysed	No. of specimens	DALA [1]	BBCH	Range of ETU Residues [mg/kg]	Range of EU Residues [mg/kg]	Range of EBIS Residues [mg/kg]
EU North & South	Potato (tuber)	0	45 – 48	< 0.01	< 0.01	< 0.01
		6 – 8	46 – 48	< 0.01	< 0.01	< 0.01
		13 – 15	48 – 49	< 0.01 – 0.01	< 0.01	< 0.01
		20 – 22	48 - 49	< 0.01 – 0.01	< 0.01	< 0.01

[1] DALA = Days after Last Application

Table 6.3.2-11 Summary of metiram level in BAS 222 28 F treated potato (Trial L140385)

Region	Matrix	DALA [1]	BBCH	Range of CS_2 Residues [2] [mg/kg]	Range of metiram Residues (by CS_2) [mg/kg]	Range of metiram Residues (by EBDC) [mg/kg]
EU North	Potato (tuber)	7	48	< 0.056	< 0.1	< 0.05
		14	48	< 0.056	< 0.1	< 0.05
		20	49	< 0.056	< 0.1	< 0.05

[1] DALA = Days after Last Application
[2] the conversion factor from CS_2 to metiram is 1.79

Table 6.3.2-12 Summary of ETU, EU, and EBIS level in BAS 222 28 F treated potato (Trial L140385)

Portion analysed	No. of specimens	DALA [1]	BBCH	Range of ETU Residues [mg/kg]	Range of EU Residues [mg/kg]	Range of EBIS Residues [mg/kg]
EU North	Potato (tuber)	7	48	< 0.01	< 0.01	< 0.01
		14	48	< 0.01	< 0.01	< 0.01
		20	49	< 0.01	< 0.01	< 0.01

[1] DALA = Days after Last Application

Table 6.3.2-13 Level of metiram in BAS 222 28 F treated potato (N-EU)

Study details		Crop	Country	Formulation Application rate	GS[3] at last appl. (BBCH)	DA LA[2]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	CS_2[1]	Metiram (by CS_2)	Metiram (by EBDC)
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140382 yes 2014	Potato	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	46	0 8 14 22	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140383 yes 2014	Potato	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	47	0 6 13 20	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140384 yes 2014	Potato	United Kingdom	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	45	0 8 13 22	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140385 yes 2014	Potato	The Netherlands	**Note: trial underdosed** BAS 222 28 F: 2x 1.4 kg metiram/ha Spray volume: 200 L/ha	47	7 14 20	tuber tuber tuber	<0.056 <0.056 <0.056	<0.1 <0.1 <0.1	<0.05 <0.05 <0.05

1) the conversion factor from metiram to CS_2 is 1.79
2) Days after last application
3) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

Table 6.3.2-14 Level of metiram in BAS 222 28 F treated potato (S-EU)

Study details		Crop	Country	Formulation Application rate	GS[3] at last appl. (BBCH)	DALA[2]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	CS_2[1]	Metiram (by CS_2)	Metiram (by EBDC)
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140386 yes 2014	Potato	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	47	0 7 14 21	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140387 yes 2014	Potato	Greece	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	45/47	0 6 15 21	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140388 yes 2014	Potato	Italy	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	48	0 7 14 21	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	0.064 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140389 yes 2014	Potato	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	47	0 7 14 21	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05

1) the conversion factor from metiram to CS_2 is 1.79
2) Days after last application
3) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

Table 6.3.2-15 Level of ETU, EU, EBIS in BAS 222 28 F treated potato (N-EU)

Study details		Crop	Country	Formulation Application rate	GS[2] at last appl. (BBCH)	DALA[1]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	ETU	EU	EBIS
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140382 yes 2014	Potato	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	46	0 8 14 22	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140383 yes 2014	Potato	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	47	0 6 13 20	tuber tuber tuber tuber	<0.01 <0.01 0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140384 yes 2014	Potato	United Kingdom	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	45	0 8 13 22	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140385 yes 2014	Potato	The Netherlands	**Note: trial underdosed** BAS 222 28 F: 2x 1.4 kg metiram/ha Spray volume: 200 L/ha	47	7 14 20	tuber tuber tuber	<0.01 <0.01 <0.01	<0.01 <0.01 <0.01	<0.01 <0.01 <0.01

1) Days after last application
2) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

Table 6.3.2-16 Level of ETU, EU, EBIS in BAS 222 28 F treated potato (S-EU)

Study details		Crop	Country	Formulation Application rate	GS[2] at last appl. (BBCH)	DALA[1]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	ETU	EU	EBIS
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140386 yes 2014	Potato	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	47	0 7 14 21	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140387 yes 2014	Potato	Greece	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	45/47	0 6 15 21	tuber tuber tuber tuber	<0.01 <0.01 <0.01 0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140388 yes 2014	Potato	Italy	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	48	0 7 14 21	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	731171 2015/1000321 L140389 yes 2014	Potato	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	47	0 7 14 21	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01

1) Days after last application
2) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

Trials L140382 – L140384 and L140386 – L140389

No residues of metiram (by EBDC) above the limit of quantitation (< 0.05 mg/kg) were found in any of the analysed treated specimens, except for the 0 DALA specimen of trial L140388, where 0.064 mg/kg were found.
No residues of metiram were found above the limit of quantitation in any of the analysed untreated specimens.

No residues of CS_2 above the limit of quantitation (< 0.056 mg/kg (< 0.1 mg/kg expressed as metiram)) were found in any of the analysed treated specimens.
No residues of CS_2 were found above the limit of quantitation in any of the analysed untreated specimens.

No residues of ETU above the limit of quantitation (< 0.01 mg/kg) were found in any of the analysed treated specimens, except for the specimens L1403830007 (13 DALA) and L1403870008 (21 DALA), where 0.010 mg/kg were found each.
No residues of metiram were found above the limit of quantitation in any of the analysed untreated specimens.

No residues of EU above the limit of quantitation (< 0.01 mg/kg) were found in any of the analysed treated specimens.
No residues of EU were found above the limit of quantitation in any of the analysed untreated specimens.

No residues of EBIS were found above the limit of quantitation (< 0.01 mg/kg) in any of the analysed treated specimens.
No residues of EBIS were found above the limit of quantitation in any of the analysed untreated specimens.

Trial L140385

Trial L140385 will be reported separately as due to an infection with phythophtora infestans only two applications could be made and not all samplings were available. For trial L140385 plot 2 was treated two times at 21 and 14 DBH with 2.0 kg/ha of BAS 222 28 F (1.4 kg/ha of metiram (BAS 222 F), WG formulation, with a spray volume of 200 L/ha. 3 samplings were carried out (no 0 DALA sampling).

No residues of metiram (by EBDC) above the limit of quantitation (< 0.05 mg/kg) were found in any of the analysed treated specimens of trial L140385.
No residues of metiram were found above the limit of quantitation in any of the analysed untreated specimens of trial L140385.

No residues of CS_2 above the limit of quantitation (< 0.056 mg/kg (< 0.1 mg/kg expressed as metiram)) were found in any of the analysed treated specimens of trial L140385.
No residues of CS_2 were found above the limit of quantitation in any of the analysed untreated specimens of trial L140385.

No residues of ETU above the limit of quantitation (< 0.01 mg/kg) were found in any of the analysed treated specimens of trial L140385.
No residues of ETU were found above the limit of quantitation in any of the analysed untreated specimens of trial L140385.

No residues of EU above the limit of quantitation (< 0.01 mg/kg) were found in any of the analysed treated specimens of trial L140385.
No residues of EU were found above the limit of quantitation in any of the analysed untreated specimens of trial L140385.

No residues of EBIS above the limit of quantitation (< 0.01 mg/kg) were found in any of the analysed treated specimens of trial L140385.
No residues of EBIS were found above the limit of quantitation in any of the analysed untreated specimens of trial L140385.

III. CONCLUSION

Eight field trials with potatoes were conducted in Europe (North and South) in season 2014. BAS 222 28 F was applied three times at single rates of 1.4 kg/ha BAS 222 F (2.0 kg/ha of WG formulation BAS 222 28 F) in a spray volume of 200 L/ha. (Note, seven field trials are considered further, omitting one trial L140385 due to deviations from the GAP). Residue analysis of samples taken at different time intervals after the last application (0, 6 - 8, 13 - 15 and 20 - 22 DALA) showed a decline of residue levels.
At the recommended PHI of 14 days, residues of metiram (determination as CS_2 and EBDC), CS_2, EU and EBIS were below the limit of quantitation (LOQ <0.056, <0.05 mg/kg, <0.10. <0.01 mg/kg). Residues of ETU were between <0.01 - 0.01 mg/kg.
Samples from untreated control plots did not contain apparent residues of CS_2, EBDC, ETU, EU and EBIS, i.e. residues were below the corresponding LOQ. The maximum storage interval of deep frozen samples from harvest until extraction was 83 days for metiram (by EBDC), 135 days for metiram (by CS_2) and 48 days for ETU (17 days for PHI specimens), 48 days for EU (17 days for PHI specimens) and 48 days for EBIS (17 days for PHI specimens). The mean of the concurrent recoveries was within the acceptable range of 70 – 110% for CS_2, EBDC, ETU, EU and EBIS.

Report: CA 6.3.2/2
Meyer M., 2014d
Study on the residue behaviour of Metiram (BAS 222 F) in potato after treatment with BAS 222 28 F under field conditions in Germany, Northern France, the United Kingdom, Southern France, Greece, Italy and Spain, 2013
2014/1000222

Guidelines: EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, EEC 79/117, EEC 91/414, EU Regulation 1107/2009 with Regulation 554/2011, EEC 7029/VI/95 rev. 5 Appendix B (July 22 1997), EEC 7525/VI/95 rev. 9 (March 2011), SANCO/825/00 rev. 8.1 (16 November 2010)

GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
   Description: BAS 222 28 F: 700 g/kg of BAS 222 F (metiram), WG formulation
   Lot/Batch #: FRE-000825
   Purity: Not relevant
   CAS#: Metiram (BAS 222 F): 9006-42-2
   Development code: Metiram: BAS 222 F
   ETU: M222F002
   EU: M222F003
   EBIS: M222F004
   Spiking levels:

BAS 222 F (by EBDC):	0.05, 0.5, 5.0 mg/kg
BAS 222 F (by CS_2):	0.1, 1.0 mg/kg
ETU and EU:	0.01, 0.1 mg/kg
EBIS:	0.01, 0.1 mg/kg

2. **Test Commodity:** root and tuber vegetables
   Crop: potato
   Type: *Solanum tuberosum* L.
   Variety: Marabell, Allians, Spunta, Harmony, Jaerla, Primura, Condor
   Crop parts(s) or processed Commodity: potato / tuber
   Sample size: 2.0 kg (12 tubers)

B. STUDY DESIGN AND METHODS

1. Test procedure

During the growing season of 2013, eight field trials with potatoes (field conditions) were conducted in Germany, Northern France, the United Kingdom, Southern France, Greece, Italy and Spain, in order to determine the magnitude of the residues of metiram (BAS 222 F) after application of BAS 222 28 F.
The WG formulation BAS 222 28 F was applied three times (27 - 29, 20 - 22 and 13 - 14 days before harvest) at single rates of 1.4 kg as/ha for BAS 222 F in a spray volume of 200 L/ha in order to determine the magnitude of the residues of the active ingredient on Raw Agricultural Commodities (RAC).
Specimens were collected at 0, 6 - 8, 13 - 14 and 20 - 22 days after the last application (DALA).

Table 6.3.2-17 Application and sampling details for trials conducted in 2013

Region	No. of trials	No. of Appl.	F, G, I[2]	Method	Test Item	Active Substance	Application		Target Timing	
							Rate (kg a.s./ha)	Water vol. (L/ha)	Appl. (DBH)[3]	Sampl. (DALA)[1]
EU North & South	8	3	F	-	BAS 222 28 F (WG)	Metiram BAS 222 F	1.4	200	1st appl.: 27 - 29 2nd appl.: 20 - 22 3rd appl.: 13 - 14	0 6 - 8 13 - 14 20 - 22

1) Days after last application,
2) Field, Glasshouse or Indoor,
3) Days before harvest

2. Description of analytical procedures

The specimens were analyzed for residues of metiram according to BASF method No. L0089/01 and as carbondisulfide according to BASF method L0234/01. The residues of ETU were determined according to the BASF method No. L0176/01. The residues of EU and EBIS were determined with BASF method L0233/01.
The limit of quantitation (LOQ) of the method L0089/01 was 0.05 mg/kg and for metiram as carbondisulfide it was 0.10 mg/kg. The LOQ of the method L0176/01 was 0.01 mg/kg. The LOQ for EU and EBIS were 0.01 mg/kg with the applied method.
The results of procedural recovery experiments averaged at about 79.8% for BAS 222 F (by EBDC) at fortification levels between 0.05 and 5.0 mg/kg, at 73.9% for BAS 222 F (by CS_2) at fortification levels between 0.1 and 1.0 mg/kg, at 90.3% for ETU, at 80.3% for EU and at 88.9% for EBIS at fortification levels between 0.01 and 0.1 mg/kg.

BASF method L0089/01was used to determine Metiram as EBDC:
The ethylene-bisdithiocarbamate (EBDC) moiety was formed out of BAS 222 F and extracted from the specimen with a buffer solution consisting of EDTA, cysteine, methanol and sodium hydroxide adjusted to pH 11.0. The formed ethylene-bisdithiocarbamate (EBDC) analyte was methylated with iodmethane prior to C18 SPE clean up. Specimens were quantified by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). The limit of quantitation (LOQ) was 0.05 mg/kg.

BASF method L0234/01 was used to determine Metiram as CS_2
Metiram was transformed to CS_2 by means of orthophosphoric acid. Subsequently, the CS_2 was transferred to isooctane with a flow of nitrogen. The quantification was carried out using GC-MS. The limit of quantitation (LOQ) was 0.10 mg/kg.

BASF method L0176/01 was used to determine ETU:
ETU was extracted from the specimen material with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol-water. After centrifugation an aliquot was taken and the methanol was evaporated from the extract. The pH was adjusted to 8 before evaporating (in some cases after evaporation). The remaining water phase was cleaned by liquid/liquid (water/dichloromethane) partition on an Extrelut column. After concentration of the eluate, the residue was determined by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

EU was determined according to BASF method L0233/01:
The extraction from EU was performed with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol-water and was followed by a clean-up by liquid-liquid partition on an Extrelut column where ethyl acetate was used for the elution.
Water was added prior the evaporation of the specimen extract. For the final reconstitution of the specimen extract a solution of H_2O/MeOH was used. The determination was performed by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

EBIS was determined according to BASF method L0233/01:
The extraction from EBIS was performed with a mixture of acetonitrile/formic acid in combination with a thiourea solution and was followed by a clean-up step utilizing C18-EC. Specimens were diluted with a solution of acetonitrile/H_2O/formic acid before being determined by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

The results of procedural recovery experiments are summarized in Table 6.3.2-18.

Table 6.3.2-18 Summary of recoveries

Matrix	Fortification Level (mg/kg)	Summary Recoveries			
		n	Mean (%)	SD (+/-)	RSD (%)
Method No. L0089/01		**Metiram (by EBDC)**			
Potato / Tuber	0.05, 0.5, 5.0	6	79.8	9.4	11.8
Method No. L0234/01		**Metiram (by CS_2)**			
Potato / Tuber	0.1, 1.0	12	73.9	13	17
Method No. L0176/01		**ETU**			
Potato / Tuber	0.01, 0.1	12	90.3	6.3	6.9
Method No. L0233/01		**EU**			
Potato / Tuber	0.01, 0.1	12	80.3	8.1	10.1
Method No. L0233/01		**EBIS**			
Potato / Tuber	0.01, 0.1	12	88.9	7.8	8.7

II. RESULTS AND DISCUSSION

Residue data of the field trials is summarized in Table 6.3.2-19 and Table 6.3.2-20. Detailed information is shown in Table 6.3.2-21 to Table 6.3.2-24.

Samples from untreated control plots did not contain apparent residues of CS_2, EBDC, ETU, EU and EBIS, i.e. residues were below the corresponding LOQ. The maximum storage interval of deep frozen samples from harvest until extraction was 431 days for metiram (by EBDC), 350 days for metiram (by CS_2) and 263 days for ETU, EU and EBIS. The mean of the concurrent recoveries was within the acceptable range of 70 – 110% for CS_2, EBDC, ETU, EU and EBIS.

Table 6.3.2-19 Summary of metiram level in BAS 222 28 F treated potato

Region	Matrix	DALA [1)]	BBCH	Range of CS_2 Residues [2)] [mg/kg]	Range of metiram Residues (by CS_2) [mg/kg]	Range of metiram Residues (by EBDC) [mg/kg]
EU North & South	Potato (tuber)	0	42 – 48	< 0.056	< 0.1	< 0.05 - 0.07
		6 – 8	45 – 48	< 0.056	< 0.1	< 0.05
		13 – 14	45 – 49	< 0.056	< 0.1	< 0.05
		20 – 22	49	< 0.056	< 0.1	< 0.05

[1)] DALA = Days after Last Application
[2)] the conversion factor from CS_2 to metiram is 1.79

Table 6.3.2-20 Summary of ETU, EU, and EBIS level in BAS 222 28 F treated potato

Portion analysed	No. of specimens	DALA [1)]	BBCH	Range of ETU Residues [mg/kg]	Range of EU Residues [mg/kg]	Range of EBIS Residues [mg/kg]
EU North & South	Potato (tuber)	0	42 – 48	< 0.01 – 0.037	< 0.01	< 0.01
		6 – 8	45 – 48	< 0.01 – 0.028	< 0.01	< 0.01
		13 – 14	45 – 49	< 0.01 – 0.038	< 0.01 - 0.01	< 0.01
		20 – 22	49	< 0.01 – 0.031	< 0.01 - 0.01	< 0.01

[1)] DALA = Days after Last Application

Table 6.3.2-21 Level of metiram in BAS 222 28 F treated potato (N-EU)

Study details		Crop	Country	Formulation Application rate	GS[3] at last appl. (BBCH)	DALA[2]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	CS_2[1]	Metiram (by CS_2)	Metiram (by EBDC)
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130118 yes 2013	Potato	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	47	0 8 14 21	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130119 yes 2013	Potato	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	48	0 7 14 21	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130120 yes 2013	Potato	France (N)	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	45	0 7 14 22	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130121 yes 2013	Potato	United Kingdom	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	47	0 6 13 20	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05

1) the conversion factor from metiram to CS_2 is 1.79
2) Days after last application
3) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

Table 6.3.2-22 Level of metiram in BAS 222 28 F treated potato (S-EU)

Study details		Crop	Country	Formulation Application rate	GS[3] at last appl. (BBCH)	DALA[2]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	CS_2[1]	Metiram (by CS_2)	Metiram (by EBDC)
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130122 yes 2013	Potato	France (S)	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	44	0 8 14 21	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130123 yes 2013	Potato	Greece	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	42-45	0 7 14 21	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130124 yes 2013	Potato	Italy	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	47-48	0 7 14 21	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	0.07 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130125 yes 2013	Potato	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	45	0 6 14 21	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05

1) the conversion factor from metiram to CS_2 is 1.79
2) Days after last application
3) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

Table 6.3.2-23 Level of ETU, EU, EBIS in BAS 222 28 F treated potato (N-EU)

Study details		Crop	Country	Formulation Application rate	GS[2] at last appl. (BBCH)	DA LA[1]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	ETU	EU	EBIS
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130118 yes 2013	Potato	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	47	0 8 14 21	tuber tuber tuber tuber	0.037 0.028 0.038 0.031	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130119 yes 2013	Potato	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	48	0 7 14 21	tuber tuber tuber tuber	0.011 0.020 0.017 0.013	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130120 yes 2013	Potato	France (N)	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	45	0 7 14 22	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130121 yes 2013	Potato	United Kingdom	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	47	0 6 13 20	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01

1) Days after last application
2) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

Table 6.3.2-24 Level of ETU, EU, EBIS in BAS 222 28 F treated potato (S-EU)

Study details		Crop	Country	Formulation Application rate	GS[2] at last appl. (BBCH)	DALA[1]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	ETU	EU	EBIS
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130122 yes 2013	Potato	France (S)	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	44	0 8 14 21	tuber tuber tuber tuber	<0.01 <0.01 0.011 0.013	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130123 yes 2013	Potato	Greece	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	42-45	0 7 14 21	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130124 yes 2013	Potato	Italy	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	47-48	0 7 14 21	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389461 2014/1000222 L130125 yes 2013	Potato	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	45	0 6 14 21	tuber tuber tuber tuber	<0.01 0.018 0.023 0.023	<0.01 <0.01 0.010 0.010	<0.01 <0.01 <0.01 <0.01

1) Days after last application
2) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

No residues of Metiram (by EBDC) were found above the limit of quantitation (< 0.05 mg/kg), except for trial no. L130124 with 0.070 mg/kg for 0 DALA sampling.
No residues of metiram were found above the limit of quantitation in any of the analysed untreated specimens.

No residues of CS_2 (Metiram by CS_2) were found above the limit of quantitation (< 0.056 mg/kg (< 0.1 mg/kg expressed as metiram) in any of the analysed treated specimens.
No residues of CS_2 were found above the limit of quantitation in any of the analysed untreated specimens.

In specimens taken at 0 DALA residues of ETU ranged from < 0.01 – 0.037 mg/kg. They remained at this level in the specimens taken 6 - 8 DALA (< 0.01 – 0.028 mg/kg), 13 - 14 DALA (< 0.01 – 0.038 mg/kg) as well as 20 - 22 DALA (< 0.01 – 0.031) mg/kg.
No residues of ETU were found above the limit of quantitation in any of the analysed untreated specimens, apart from the specimens taken in trial L130118. The following residue concentrations were found: 0.018 mg/kg (L1301180001), 0.019 mg/kg (L1301180002), 0.016 mg/kg (L1301180003) and 0.017 mg/kg (L1301180004).

No residues of EU were found above the limit of quantitation (< 0.01 mg/kg) in any of the analysed treated specimens, apart from the specimens taken 14 and 21 DALA in trial L130125 (L1301250007 and L1301250008) where 0.010 mg/kg were determined.
No residues of EU were found above the limit of quantitation in any of the analysed untreated specimens.

No residues of EBIS were found above the limit of quantitation (< 0.01 mg/kg) in any of the analysed treated specimens.
No residues of EBIS were found above the limit of quantitation in any of the analysed untreated specimens.

III. CONCLUSION

Eight field trials with potatoes were conducted in Europe (North and South) in season 2013. BAS 222 28 F was applied three times at single rates of 1.4 kg/ha BAS 222 F (2.0 kg/ha of WG formulation BAS 222 28 F) in a spray volume of 200 L/ha. Residue analysis of samples taken at different time intervals after the last application (0, 6 - 8, 13 - 14 and 20 - 22 DALA) showed a decline of residue levels.
At the recommended PHI of 14 days, residues of metiram (determination as CS_2 and EBDC) and CS_2 were below the limit of quantitation (LOQ <0.056, <0.05 mg/kg, <0.10 mg/kg). Residues of ETU were between <0.01 - 0.038 mg/kg and for EU between <0.01 - 0.01 mg/kg. Residues of EBIS were below the limit of quantitation (LOQ <0.01 mg/kg). Samples from untreated control plots did not contain apparent residues of CS_2, EBDC, ETU, EU and EBIS, i.e. residues were below the corresponding LOQ (except for trial L130118, residues of ETU of 0.016 mg/kg and 0.017 mg/kg were found at DALA 13 - 22). The maximum storage interval of deep frozen samples from harvest until extraction was 431 days for metiram (by EBDC), 350 days for metiram (by CS_2) and 263 days for ETU, EU and EBIS. The mean of the concurrent recoveries was within the acceptable range of 70 – 110% for CS_2, EBDC, ETU, EU and EBIS.

Report: CA 6.3.2/3
Meyer M., 2014c
Study on the residue behaviour of Metiram (BAS 222 F) in potato after treatment with BAS 222 28 F under field conditions in Germany, the United Kingdom, Greece and Spain, 2012
2012/1272625

Guidelines: EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, EEC 79/117, EEC 91/414, EU Regulation Regulation 544/2011 (10 June 2011) implementing Regulation No 1107/2009, EEC 7029/VI/95 rev. 5 Appendix B (July 22 1997), EEC 7525/VI/95 rev. 9 (March 2011), SANCO/825/00 rev. 8.1 (16 November 2010), SANCO/3029/99 rev. 4 (11 July 2000)

GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
Description: BAS 222 28 F: 700 g/kg of BAS 222 F (metiram), WG formulation
Lot/Batch #: 80449975L0
Purity: Not relevant
CAS#: Metiram (BAS 222 F): 9006-42-2
Development code: Metiram: BAS 222 F
ETU: M222F002
EU: M222F003
EBIS: M222F004
Spiking levels:
BAS 222 F (by EBDC): 0.05, 0.5, 1.0 mg/kg
BAS 222 F (by CS_2): 0.1, 1.0 mg/kg
ETU and EU: 0.01, 0.1 mg/kg
EBIS: 0.01, 0.05, 0.1 mg/kg

2. **Test Commodity:** root and tuber vegetables
Crop: potato
Type: *Solanum tuberosum* L.
Variety: Marabell, Quarta, Markies, Agria, Kennebec
Crop parts(s) or processed Commodity: potato / tuber
Sample size: 2.0 kg (12 tubers)

B. STUDY DESIGN AND METHODS

1. Test procedure

During the growing season of 2012, eight field trials with potatoes (field conditions) were conducted in Germany, the United Kingdom and Spain, in order to determine the magnitude of the residues of metiram (BAS 222 F) after application of BAS 222 28 F.
The WG formulation BAS 222 28 F was applied three times (27 - 30, 20 - 22 and 13 - 15 days before harvest) at single rates of 1.4 kg as/ha for BAS 222 F in a spray volume of 200 - 400 L/ha in order to determine the magnitude of the residues of the active ingredient on Raw Agricultural Commodities (RAC).
Specimens were collected at 0, 6 - 8, 13 - 15 and 20 - 22 days after the last application (DALA).

Table 6.3.2-25 Application and sampling details for trials conducted in 2012

Region	No. of trials	No. of Appl.	F, G, I[2]	Method	Test Item	Active Substance	Application		Target Timing	
							Rate (kg a.s./ha)	Water vol. (L/ha)	Appl. (DBH)[3]	Sampl. (DALA)[1]
EU North & South	8	3	F	-	BAS 222 28 F (WG)	Metiram BAS 222 F	1.4	200-400	1st appl.: 27 -30 2nd appl.: 20 - 22 3rd appl.: 13 - 15	0 6 - 8 13 - 15 20 - 22

1) Days after last application,
2) Field, Glasshouse or Indoor,
3) Days before harvest

2. Description of analytical procedures

The specimens were analyzed for residues of metiram according to BASF method No. L0089/01 and as carbondisulfide according to BASF method L0234/01. The residues of ETU were determined according to the BASF method No. L0176/01. The residues of EU and EBIS were determined with BASF method L0233/01.
The limit of quantitation (LOQ) of the method L0089/01 was 0.05 mg/kg and for metiram as carbondisulfide it was 0.10 mg/kg. The LOQ of the method L0176/01 was 0.01 mg/kg. The LOQ for EU and EBIS were 0.01 mg/kg with the applied method.
The results of procedural recovery experiments averaged at about 92% for BAS 222 F (by EBDC) at fortification levels between 0.05 and 1.0 mg/kg, at 84% for BAS 222 F (by CS_2) at fortification levels between 0.1 and 1.0 mg/kg, at 87% for ETU, at 74% for EU and at 89% for EBIS at fortification levels between 0.01 and 0.1 mg/kg.

<u>BASF method L0089/01was used to determine Metiram as EBDC:</u>
The ethylene-bisdithiocarbamate (EBDC) moiety was formed out of BAS 222 F and extracted from the specimen with a buffer solution consisting of EDTA, cysteine, methanol and sodium hydroxide adjusted to pH 11.0. The formed ethylene-bisdithiocarbamate (EBDC) analyte was methylated with iodmethane prior to C18 SPE clean up. Specimens were quantified by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). The limit of quantitation (LOQ) was 0.05 mg/kg.

<u>BASF method L0234/01 was used to determine Metiram as CS_2</u>
Metiram was transformed to CS_2 by means of orthophosphoric acid. Subsequently, the CS_2 was transferred to isooctane with a flow of nitrogen. The quantification was carried out using GC-MS. The limit of quantitation (LOQ) was 0.10 mg/kg.

<u>BASF method L0176/01 was used to determine ETU:</u>
ETU was extracted from the specimen material with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol-water. After centrifugation an aliquot was taken and the methanol was evaporated from the extract. The pH was adjusted to 8 before evaporating (in some cases after evaporation). The remaining water phase was cleaned by liquid/liquid (water/dichloromethane) partition on an Extrelut column. After concentration of the eluate, the residue was determined by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

<u>EU was determined according to BASF method L0233/01:</u>
The extraction from EU was performed with a mixture of sodium ascorbate, thiourea, ammonium chloride and methanol-water and was followed by a clean-up by liquid-liquid partition on an Extrelut column where ethyl acetate was used for the elution.
Water was added prior the evaporation of the specimen extract. For the final reconstitution of the specimen extract a solution of H_2O/MeOH was used. The determination was performed by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

<u>EBIS was determined according to BASF method L0233/01:</u>
The extraction from EBIS was performed with a mixture of acetonitrile/formic acid in combination with a thiourea solution and was followed by a clean-up step utilizing C18-EC. Specimens were diluted with a solution of acetonitrile/H_2O/formic acid before being determined by LC-MS/MS. The limit of quantitation (LOQ) was 0.01 mg/kg.

The results of procedural recovery experiments are summarized in Table 6.3.2-26.

Table 6.3.2-26 Summary of recoveries

Matrix	Fortification Level (mg/kg)	Summary Recoveries			
		n	Mean (%)	SD (+/-)	RSD (%)
Method No. L0089/01		**Metiram (by EBDC)**			
Potato / Tuber	0.05, 0.5, 1.0	9	92	7.9	8.5
Method No. L0234/01		**Metiram (by CS_2)**			
Potato / Tuber	0.1, 1.0	14	84	10.9	13.0
Method No. L0176/01		**ETU**			
Potato / Tuber	0.01, 0.1	8	87	5.0	5.8
Method No. L0233/01		**EU**			
Potato / Tuber	0.01, 0.1	8	74	4.1	5.6
Method No. L0233/01		**EBIS**			
Potato / Tuber	0.01, 0.05, 0.1	12	89	13.9	15.6

II. RESULTS AND DISCUSSION

Residue data of the field trials is summarized in Table 6.3.2-27 and Table 6.3.2-28. Detailed information is shown in Table 6.3.2-29 to Table 6.3.2-32.

Samples from untreated control plots did not contain apparent residues of CS_2, EBDC, ETU, EU and EBIS, i.e. residues were below the corresponding LOQ. The maximum storage interval of deep frozen samples from harvest until extraction was 428 days for metiram (by EBDC), 318 days for metiram (by CS_2) and 310 days for ETU, 448 days for EU and 452 days for EBIS.
The mean of the concurrent recoveries was within the acceptable range of 70 – 110% for CS_2, EBDC, ETU, EU and EBIS.

Table 6.3.2-27 Summary of metiram level in BAS 222 28 F treated potato

Region	Matrix	DALA [1]	BBCH	Range of CS_2 Residues [2] [mg/kg]	Range of metiram Residues (by CS_2) [mg/kg]	Range of metiram Residues (by EBDC) [mg/kg]
EU North & South	Potato (tuber)	0	45 – 48	< 0.056	< 0.1	< 0.05
		6 – 8	47 – 49	< 0.056	< 0.1	< 0.05
		13 – 15	48 – 49	< 0.056	< 0.1	< 0.05
		20 – 22	49	< 0.056	< 0.1	< 0.05

[1] DALA = Days after Last Application
[2] the conversion factor from CS_2 to metiram is 1.79

Table 6.3.2-28 Summary of ETU, EU, and EBIS level in BAS 222 28 F treated potato

Portion analysed	No. of specimens	DALA [1)]	BBCH	Range of ETU Residues [mg/kg]	Range of EU Residues [mg/kg]	Range of EBIS Residues [mg/kg]
EU North & South	Potato (tuber)	0	45 – 48	< 0.01 – 0.019	< 0.01	< 0.01
		6 – 8	47 – 49	< 0.01 – 0.022	< 0.01	< 0.01
		13 – 15	48 – 49	< 0.01 – 0.026	< 0.01	< 0.01
		20 – 22	49	< 0.01 – 0.022	< 0.01	< 0.01

[1)] DALA = Days after Last Application

Table 6.3.2-29 Level of metiram in BAS 222 28 F treated potato (N-EU)

Study details		Crop	Country	Formulation Application rate	GS[3] at last appl. (BBCH)	DALA[2]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	CS_2[1]	Metiram (by CS_2)	Metiram (by EBDC)
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120524 yes 2012	Potato	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	47	0 7 14 22	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120525 yes 2012	Potato	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	48	0 8 14 21	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120526 yes 2012	Potato	United Kingdom	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 250 L/ha	45	0 7 13 20	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120527 yes 2012	Potato	United Kingdom	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 250 L/ha	47	0 8 15 22	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05

1) the conversion factor from metiram to CS_2 is 1.79
2) Days after last application
3) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

Table 6.3.2-30 Level of metiram in BAS 222 28 F treated potato (S-EU)

Study details		Crop	Country	Formulation Application rate	GS[3] at last appl. (BBCH)	DALA[2]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	CS_2[1]	Metiram (by CS_2)	Metiram (by EBDC)
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120528 yes 2012	Potato	Greece	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 300 L/ha	45-47	0 7 14 22	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120529 yes 2012	Potato	Greece	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 400 L/ha	45-47	0 7 14 20	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120530 yes 2012	Potato	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 250 L/ha	46	0 6 14 20	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120531 yes 2012	Potato	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 250 L/ha	46	0 8 13 21	tuber tuber tuber tuber	<0.056 <0.056 <0.056 <0.056	<0.1 <0.1 <0.1 <0.1	<0.05 <0.05 <0.05 <0.05

1) the conversion factor from metiram to CS_2 is 1.79
2) Days after last application
3) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

Table 6.3.2-31 Level of ETU, EU, EBIS in BAS 222 28 F treated potato (N-EU)

Study details		Crop	Country	Formulation Application rate	GS[2] at last appl. (BBCH)	DA LA[1]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	ETU	EU	EBIS
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120524 yes 2012	Potato	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	47	0 7 14 22	tuber tuber tuber tuber	0.019 0.022 0.026 0.021	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120525 yes 2012	Potato	Germany	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 200 L/ha	48	0 8 14 21	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120526 yes 2012	Potato	United Kingdom	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 250 L/ha	45	0 7 13 20	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120527 yes 2012	Potato	United Kingdom	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 250 L/ha	47	0 8 15 22	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01

1) Days after last application
2) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

Table 6.3.2-32 Level of ETU, EU, EBIS in BAS 222 28 F treated potato (S-EU)

Study details		Crop	Country	Formulation Application rate	GS[2] at last appl. (BBCH)	DALA[1]	Residues found[3] (mg/kg parent equiv.)			
							Matrix	ETU	EU	EBIS
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120528 yes 2012	Potato	Greece	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 300 L/ha	45-47	0 7 14 22	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120529 yes 2012	Potato	Greece	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 400 L/ha	45-47	0 7 14 20	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120530 yes 2012	Potato	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 250 L/ha	46	0 6 14 20	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01
Study code: **Doc ID:** **Trial No.** **GLP:** **Year:**	389454 2012/1272625 L120531 yes 2012	Potato	Spain	BAS 222 28 F: 3x 1.4 kg metiram/ha Spray volume: 250 L/ha	46	0 8 13 21	tuber tuber tuber tuber	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01	<0.01 <0.01 <0.01 <0.01

1) Days after last application
2) Growth stage
For calculation purposes for example < 0.01 was set to 0.01

No residues of Metiram (by EBDC) above the limit of quantitation (0.05 mg/kg) were found in any of the analyzed treated and untreated specimens.

No residues of CS_2 (Metiram by CS_2) above the limit of quantitation (0.056 mg/kg) (0.1 mg/kg expressed as metiram) were found in any of the analyzed treated specimens.
In the untreated specimens L1205260001 and L1205260004 the residue concentrations were determined as 0.067 mg/kg and 0.073 mg/kg (0.12 mg/kg and 0.13 mg/kg expressed as metiram), respectively and hence were above the LOQ of 0.056 mg/kg (0.1 mg/kg expressed as metiram). In all the other untreated specimens, no residues above the limit of quantitation were found at any sampling occasion.

In the treated specimens taken from trial L120524 residues of ETU remained relatively stable (0.02 – 0.03 mg/kg) over the sampling occasions. In all the other treated specimens, residues of ETU were below the limit of quantitation (< 0.01 mg/kg).
No residues of ETU above the limit of quantitation were found in any of the analyzed untreated specimens.

No residues of EU above the limit of quantitation were found in any of the analyzed treated and untreated specimens.

No residues of EBIS above the limit of quantitation were found in any of the analyzed treated and untreated specimens.

III. CONCLUSION

Eight field trials with potatoes were conducted in Europe (North and South) in season 2012. BAS 222 28 F was applied three times at single rates of 1.4 kg/ha BAS 222 F (2.0 kg/ha of WG formulation BAS 222 28 F) in a spray volume of 200-400 L/ha. Residue analysis of samples taken at different time intervals after the last application (0, 6 - 8, 13 - 15 and 20 - 22 DALA) showed a decline of residue levels.
At the recommended PHI of 14 days, residues of metiram (determination as CS_2 and EBDC), CS_2, EU and EBIS were below the limit of quantitation (<0.056, <0.05 mg/kg, <0.10. <0.01 mg/kg). Residues of ETU were between <0.01 - 0.026 mg/kg.
Samples from untreated control plots did not contain apparent residues of CS_2, EBDC, ETU, EU and EBIS, i.e. residues were below the corresponding LOQ (except for trial L120526 residues of CS_2 of 0.073 mg/kg (0.13 mg/kg for metiram determination as CS_2) were found at DALA 20 - 22). The maximum storage interval of deep frozen samples from harvest until extraction was 428 days for metiram (by EBDC), 318 days for metiram (by CS_2) and 310 days for ETU, 448 days for EU and 452 days for EBIS. The mean of the concurrent recoveries was within the acceptable range of 70 – 110% for CS_2, EBDC, ETU, EU and EBIS.

CA 6.4 Feeding studies

Annex II Dossier:
Livestock feeding studies with metiram (poultry and lactating ruminants) have previously been reviewed during the Annex I inclusion process. They were considered to be acceptable.

AIR3 Dossier:
No new studies were conducted.

In support of the representative uses supported in the present dossier, the dietary exposure of livestock to metiram residues resulting from the use in potato and grapes needs to be considered. In particular, potential intake of the parent compound metiram and the metabolite ETU has to be addressed.

Regarding metiram intake, the available livestock feeding studies with metiram, allow to conclude that the estimated the residues in commodities of animal origin are below the LOQ for both metiram and ETU. For potato, field residue trials show that residue level of metiram and ETU are below the LOQ. For grape, residues do not contribute to the feed burden as grapes are normally not fed to livestock. In conclusion, the contribution to the livestock feed burden is insignificant. Therefore, the uses supported in the present dossier are covered by previous estimations covering a broader range of metiram uses. A new estimation in the context of the present dossier is therefore not required. These previous estimations conclude that if the intake is <0.1 mg/kg/day, no residues in animal commodities are expected (i.e. metiram and ETU below the corresponding LOQ, see below).

Regarding ETU intake, the available residue data allows to conclude that livestock feeding studies with metabolite ETU are not required (see DocID 2002/1006209). In brief, the contribution of ETU to the intake is very low. Taking ETU residue in processed feed items into account, assuming worst case scenarios the following estimations are obtained:

Dairy cow	0.07 mg/animal/d
Beef cattle	0.17 mg/animal/d
Pigs	0.08 mg/animal/d
Chicken	0.003 mg/animal/d

Moreover, livestock metabolism studies with metiram show that the ETU formed in animal is rapidly eliminated either by excretion or by further transformation to natural products. In consequence, ETU levels in commodities of animal origin are insignificant and thus do not need to be included in a definition of the residue (see section 6.7). In conclusion, for ETU additional livestock studies (nature of residue, magnitude of residue) are not appropriate, considering both limited knowledge gain as well as animal welfare perspective).

For information, result from previous evaluations is provided below.

Information from Annex I inclusion process

The following table is copied from the Monograph prepared by Italy: based on an estimated feed burden below 0.1 mg/kg/day, the residues of metiram and ETU in animal commodities are expected to be below the corresponding LOQ:

Residues from livestock feeding studies

Estimated intakes by livestock ≤ 0.1 mg/kg/day:	Ruminant: yes/no	Poultry: yes/no	Pig: yes/no
Muscle	<0.05*/<0.02**		
Liver	<0.05*/<0.02**		
Kidney	<0.05*/<0.02**		
Fat	<0.05*/<0.02**		
Milk	<0.05*/<0.02**		
Eggs	<0.05*/<0.02**		

*LOD for metiram; **LOD for ETU
After normalization to a 1x dose level

Information from EU MRL compilation dossier (BASF DocID 2008/1042839)

Furthermore, in 2008 and in context of the re-evaluation of MRLs according to Reg. 396/2005, Art. 12, BASF SE provided an EU MRL compilation dossier (BASF DocID 2008/1042839) to Italy as the evaluating Member State (Italy, 2010: Evaluation report on the setting of MRLs for metiram in plant commodities prepared by the evaluating Member State Italy under Article 8 of Regulation (EC) No 396/2005, 15 November 2010, 174p.). The following table is copied from this dossier. It confirms the previous conclusion that based on an estimated feed burden below 0.1 mg/kg/day, the residues of metiram and ETU in animal commodities are expected to be below the corresponding LOQ.

Residues from livestock feeding studies (OECD data point number IIA 6.4 and IIIA 8.4)

Intakes by livestock ≥ 0.1 mg/kg diet/day:	Ruminant: Yes/no	Poultry: Yes/no	Pig: Not assessed
Muscle	<0.05*	<0.05*	no pig feeding study conducted; metabolism in rat and ruminant similar
Liver	<0.05*	<0.05*	
Kidney	<0.05*	<0.05*	
Fat	<0.05*	<0.05*	
Milk	<0.05*		
Eggs		<0.05*	

*LOQ for metiram (mg/kg)

CA 6.4.1 Poultry

Data/information on poultry feeding studies for metiram were reviewed during the Annex I inclusion process and was considered to be acceptable (see section MCA 6.4). No further data has been generated.

In summary, a residue transfer study was conducted in chicken. The dose levels tested were highly exaggerated compared to the dietary feed burden expected after feeding metiram-treated plant commodities (the animals were dosed with 0.3, 1.5, 3.0 and 150 mg/animal/d).
Egg samples and tissue samples were analysed for both metiram and ETU. In eggs, no residues were detected in samples from the three lower dose groups (0.3, 1.5, 3.0 mg/animal/d). In tissues, residue levels were below the LOQ of 0.05 mg/kg.
Residues of metiram and of ETU residues are expected to be below the respective LOQ (see see section MCA 6.4) after normalization to the estimated feed burden (1X dose level), both if the uses supported in the present dossier are considered as well as if all EU registered uses of metiram are considered.

Report: CA 6.4.1/1
[redacted] 1987a
The determination of residues of Metiram in the eggs and tissues of the laying hen following oral gavage of Metiram complex
1987/10156
Guidelines: none
GLP: yes
(certified by Department of Health and Social Security of the Government of the United Kingdom, United Kingdom)

Report: CA 6.4.1/2
[redacted] 1987b
The determination of Ethylenethiourea (ETU) residues in the eggs and tissues of the laying hen following oral administration of Metiram complex
1987/10157
Guidelines: none
GLP: yes
(certified by Department of Health and Social Security of the Government of the United Kingdom, United Kingdom)

CA 6.4.2 Ruminants

Data/information on cow/goat feeding studies for metiram was reviewed as part of the Annex I inclusion process and was considered to be acceptable (see section MCA 6.4). No further data has been generated.

In summary, a residue transfer study was conducted in goat. The dose levels tested were highly exaggerated compared to the dietary feed burden expected after feeding metiram-treated plant commodities (the animals were dosed with 40, 200, 400 and 20 000 mg/animal/d).
Milk samples and tissue samples were analysed for both metiram and ETU. In milk, no residues were detected in samples from the three lower dose groups (0.3, 1.5, 3.0 mg/animal/d). In goat tissues, residue levels were below the LOQ of 0.05 mg/kg except for liver and fat (one subsample).
Residues of metiram and of ETU residues are expected to be below the respective LOQ (see section MCA 6.4) after normalization to the estimated feed burden (1X dose level), both if the uses supported in the present dossier are considered as well as if all EU registered uses of metiram are considered.

Report: CA 6.4.2/1
[redacted], 1986a
Residues of Metiram in milk and tissues of dairy cows
1986/10206
Guidelines: none
GLP: yes
(certified by Department of Health and Social Security, London, United Kingdom)

Report: CA 6.4.2/2
[redacted] 1986a
Residues of Metiram in milk and tissues of dairy cows
1986/10205
Guidelines: none
GLP: yes
(certified by Department of Health and Social Security of the Government of the United Kingdom, United Kingdom)

Report: CA 6.4.2/3
[redacted] 1986a
Residues of ETU (Ethylene thiourea) in milk and tissues of dairy cows following oral adminstration of Metiram
1986/10184
Guidelines: none
GLP: yes
(certified by Department of Health and Social Security of the Government of the United Kingdom, United Kingdom)

CA 6.4.3 Pigs

A feeding study in pig is not required since the metabolism of metiram has been shown to be similar in rats and in ruminants.

CA 6.4.4 Fish

A feeding study in fish is not required considering the properties of metiram both the low logPow as well as the chemical properties as a “solvolytic” metal complex (see section 6.2).

In addition, currently no test method or guidance document is available. As a consequence waiving of this particular data requirement is considered acceptable according to the “guidance document for applicants on preparing dossiers for the approval of a chemical new active substance and the renewal of approval of the chemical active substance according to regulation (EU) No. 283/2013 and regulation (EU) No. 284/2013” (SANCO/10181/2013-rev.2 of 2-May-2013).

CA 6.5 Effects of Processing

CA 6.5.1 Nature of the residue

Annex II Dossier :
A high temperature hydrolysis study for metiram (14C-BAS 222 F) was reviewed during the Annex I inclusion process and was considered acceptable.

AIR3 Dossier :
No further studies have been performed.

In support of the representative uses supported in the present dossier, the stability of the parent compound metiram under standard test conditions has to be taken into account. This study shows that metiram is effectively transformed to ETU when suspended and heated under the standard conditions simulating processing, notably representative for pasteurisation (pH 4, 90°C, 20 min), for baking/boiling/brewing (pH 5, 100°C, 60 min) and for sterilisation (pH 6, 120°C, 20 min).

In conclusion, the nature of the metiram residue in processed commodities is considered sufficiently elucidated. For processed commodities the residue definition for risk assessment is ETU (see section MCA 6.7).

Report:	*CA 6.5.1/1* *Hassink J., 2002a* *Hydrolysis of BAS 222 F at 90 C, 100 C, and 120 C* *2002/1005307*
Guidelines:	*EEC 94/37, EEC Method C7, FAO Revised Guidelines on Environmental Criteria for the Registration of Pesticides Revision 3 (28 August 1993), EPA 161-1*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

CA 6.5.2 Distribution of the residue in inedible peel and pulp

Data on the distribution of residues between peel and pulp is not required for the representative uses supported in the present dossier (grape, potato).

CA 6.5.3 Magnitude of residues in processed commodities

Annex II Dossier :
Processing studies in apple, cherry, plum, grape, tomato, potato lettuce have previously been reviewed during the Annex I inclusion process. They were considered to be acceptable.

AIR3 Dossier :
New processing studies were conducted in apple, onion, and gherkin.

In support of the representative uses supported in the present dossier, studies addressing grape processed commodities have to be taken into account. For potato residue levels in the RAC are below the LOQ, therefore a processing study is not required. However, to provide a comprehensive overview over available data for metiram, summaries of processing studies are provided in this section encompassing apple, cherry, plums, grape, onion, tomato, gherkin, lettuce (no summary is provided for potato processing studies).

Table 6.5.3-1: Number of residue trials conducted

Region	Crop	Trial number	Report Number	Dossier reference
N-EU	apple	4	DocID 2009/1098978	CA 6.5.3/1
N-EU	cherry	4	DocID 2002/1004073	CA 6.5.3/2
N-EU	plum	4	DocID 2002/1004074	CA 6.5.3/3
N-EU	grape	4	DocID 2002/1004093	CA 6.5.3/4
S-EU	grape	2	DocID 1998/11226	CA 6.5.3/5
N-EU/S-EU	grape	4	DocID 1998/11302	CA 6.5.3/6
S-EU	grape	2	DocID 1994/10693	CA 6.5.3/7
N-EU	onion	4	DocID 2007/1017185	CA 6.5.3/8
N-EU	tomato	4	DocID 2002/1004075	CA 6.5.3/9
N-EU	gherkins	4	DocID 2007/1017186	CA 6.5.3/10
N-EU	lettuce, head	2	DocID 2002/1004092	CA 6.5.3/11

Note, that for calculating the processing factors of ETU, the following formula is used. This equation considers that ETU is exclusively formed from the parent compound metiram (or dithiocarbamate fungicides). In the absence of metiram residues (or other dithiocarbamate fungicide), therefore ETU residues are absent.

$$PF_{ETU} = \frac{\text{Conc. } ETU\ in\ process\ fraction}{\text{Conc. m}etiram\ (as\ CS2)\ in\ raw\ com\text{mod}ity}$$

In 2008 and in context of the re-evaluation of MRLs according to Reg. 396/2005, Art. 12, BASF SE has provided an EU MRL compilation dossier (BASF DocID 2008/1042839) to Italy as the evaluating Member State (Italy, 2010: Evaluation report on the setting of MRLs for metiram in plant commodities prepared by the evaluating Member State Italy under Article 8 of Regulation (EC) No 396/2005, 15 November 2010, 174p.). In this EU MRL compilation dossier, both a table listing metiram processing factors (see Table 6.5.3-2) and a table listing ETU processing factors (see Table 6.5.3-3) were included. These values were used as basis for the dietary exposure assessment for metiram and its transformation products (see section MCA 6.7).

Table 6.5.3-2: Processing factors: Metiram (OECD data point number IIA 6.5 and IIIA 8.5)

Crop/Processed Crop	Number of Studies	Mean Transfer Factor	% Transference
Apples - fresh juice	1	0.05	
Apples - cooked juice	1	0.05	
Apples - wet pomace	1	4.64	
Apples - dry pomace	1	12.95	
Apples - sauce	1	0.09	
Apples - baby food	1	0.05	
Cherries - washed cherries	4	0.25	
Cherries - wash water	4	0.38	
Cherries - juice	4	0.40	
Cherries - canned cherries	4	0.15	
Cherries - fruit syrup	4	0.18	
Plums - wash water	4	0.18	
Plums - washed plums	4	0.19	
Plums - remainder of the staining process	4	0.17	
Plums - condensed water	4	0.01	
Plums - purree	4	0.68	
Plums - dipping water	4	0.02	
Plums - prunes	4	0.75	
Grapes - wine (thermo-vinification)	10	0.03	
Grapes - wine	10	0.03	
Grapes - juice	10	0.55	
Grapes - pomace (thermo-vinification)	10	1.32	
Grapes - pomace	10	1.9	
Grapes - raisins	4	3.3	
Banana - pulp*	12	<0.19	
Banana - peel	12	1.4	
Tomatoes - washed tomatoes	4	0.12	
Tomatoes - wash water	4	0.32	
Tomatoes - cooking water	4	0.04	
Tomatoes - peeled tomatoes	4	0.04	
Tomatoes - peel	4	3.76	
Tomatoes - canned tomatoes	4	0.04	
Tomatoes - vegetable stock	4	0.04	
Tomato - puree	4	0.36	
Tomato - condensed water	4	0.04	
Tomato - tomato juice	4	0.05	
Tomato - remainder	4	0.10	
Onions - onions peel	4	9.6	
Onions -peeled onions	4	0.7	
Onions - wash water	4	1.5	
Onions - dried onions	4	2.0	
Gherkins - wash water	4	0.74	
Gherkins - washed gherkins	4	<0.42	
Gherkins -canned gherkins	4	<0.42	
Gherkins -vegetable stock	4	<0.42	

* in seven trials CS_2 residues below LOQ of 0.03 mg/kg

Table 6.5.3-3: Processing factors: ETU (OECD data point number IIA 6.5 and IIIA 8.5)

Crop/Processed Crop	Number of Studies	Mean Transfer Factor	% Transference
Apples - fresh juice	1	0.07	
Apples - cooked juice	1	0.06	
Apples - wet pomace	1	0.18	
Apples - dry pomace	1	0.85	
Apples - sauce	1	0.06	
Apples - baby food	1	0.06	
Cherries - washed cherries	4	0.03	
Cherries - wash water	4	0.03	
Cherries - juice	4	0.03	
Cherries - canned cherries	4	0.04	
Cherries - fruit syrup	4	0.05	
Plums - wash water	4	0.01	
Plums - washed plums	4	0.02	
Plums - remainder of the staining process	4	0.02	
Plums - condensed water	4	0.01	
Plums - purree	4	0.08	
Plums - dipping water	4	0.01	
Plums - prunes	4	0.01	
Grapes - wine (thermo-vinification)	10	0.22	
Grapes - wine	10	0.06	
Grapes - juice	10	0.20	
Grapes - pomace (thermo-vinification)	10	0.08	
Grapes - pomace	10	0.02	
Grapes - raisins	4	0.05	
Banana - pulp*	12	<0.2	
Banana - peel*	12	<0.2	
Tomatoes - washed tomatoes	4	0.01	
Tomatoes - wash water	4	0.01	
Tomatoes - cooking water	4	0.01	
Tomatoes - peeled tomatoes	4	0.01	
Tomatoes - peel	4	0.05	
Tomatoes - canned tomatoes	4	0.02	
Tomatoes - vegetable stock	4	0.04	
Tomato puree	4	0.24	
Tomato - condensed water	4	0.01	
Tomato - tomato juice	4	0.07	
Tomato - remainder	4	0.03	
Onions - onions peel	4	0.2	
Onions -peeled onions	4	0.0	
Onions - wash water	4	0.0	
Onions - dried onions	4	0.043	
Gherkins - wash water	4	0.04	
Gherkins - washed gherkins	4	0.18	
Gherkins -canned gherkins	4	0.08	
Gherkins -vegetable stock	4	0.04	

* all ETU residues below LOQ of 0.05 mg/kg

Report: CA 6.5.3/1
Braun D., 2009a
Determination of residues of BAS 222 F in apples and their processed products after five applications of BAS 222 28 F in Germany
2009/1098978

Guidelines: EEC 7029/VI/95 rev. 5, Working document of the Commission of the European Communities Directorate General for Agriculture VI B II-1 Appendix B, BBA IV 3-3, IVA Guideline IA-II (1992)

GLP: yes
(certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
   Description: BAS 222 28 F
   Lot/Batch #: FRE-000595
   Purity: BAS 222 28 F (BAS 222 F 70%, nominal)
   CAS#: 9006-42-2
   Development code:
   Metiram: BAS 222 F
   ETU: M222F002
   Spiking levels: BAS 222 F (0.1, 5.0, 10.0 and 30.0 mg/kg)
   ETU (0.01, 0.1, 0.5 and 1.0 mg/kg)

2. **Test Commodity:**
   Crop: apples
   Type: not applicable
   Variety: Jonagold, Shampion, Jonamac, Elstar
   Botanical name: *Malus domestica*
   Crop parts(s) or processed commodity: fruit (RAC), washed apples, wash water, apple sauce, juice, wet pomace, dried pomace, dried apples and canned apples
   Sample size: 0.2-1.5 kg

B. STUDY DESIGN

1. Test procedure

During the 2008 growing season, four field trials were conducted in order to investigate the residue behavior of metiram (BAS 222 F) in apples and their processed products previously treated with BAS 222 28 F. The following field trials were located in representative apple growing areas in Germany. Each field trial consisted of a treated plot. Two field trials (L080111 and L080113) also included a control plot. The test item BAS 222 28 F, a WG formulation of BAS 222 F was foliar applied five times at an exaggerated target rate of 7.5 kg product/ha (nominal a.s./ha: 5.25 kg BAS 222 F) for each application. This corresponds to a three times higher rate as normal for each application. The applications were made 49±1 days, 42±1 days, 35±1 days, 28±1 days and 21±1 days before harvest using a spray volume of 800 L/ha.
Apple specimens for analysis were sampled on the day of last application and 21±1 days after last application. All processed fractions fulfill the requirements for a balance study. The processing of apples was conducted with apples taken at the last sampling. After processing, eight different fractions of apple products or intermediates were collected for analysis, namely washed apples, wash water, apple sauce, juice, wet pomace, dried pomace, dried apples and canned apples.

2. Description of analytical procedures

The specimens were analyzed for residues of metiram (expressed as metiram and as CS_2) and ETU. Metiram was analyzed according to the validated method IF-05/00449055 (BASF DocID 2006/1021409) which quantifies the residues with a limit of quantitation (LOQ) of 0.1 mg/kg related to metiram or 0.056 mg/kg of CS_2. ETU was analyzed according to the BASF method No. 373/3 which quantifies the residues with an LOO of 0.01 mg/kg.

The result of the procedural recovery experiment was about 80.9% at fortification levels between 0.1 and 30.0 mg/kg for metiram and 83.6% at a fortification level between 0.01 and 1.0 mg/kg for ETU.

Principle of the method IF-05/00449055: Dithiocarbamate is degraded to carbon disulfide by means of orthophosphoric acid and passed into isooctane by a stream of nitrogen. The final determination is carried out using GC-MS detection. The ion fragment m/z 76 is used for evaluation. The limit of quantitation of the method for apples is 0.1 mg/kg, related to metiram and 0.056 mg/kg, related to CS_2.

Principle of the method no. 373/2: ETU is extracted from the plant material with a mixture of sodium ascorbate, ethylene urea, ammonium chloride and methanol-water. After centrifugation, an aliquot is taken and the methanol is evaporated from the extract. The pH is adjusted to 8 before evaporating (in some cases after evaporation). The remaining water phase is cleaned by liquid/liquid partition (water/dichloromethane) on an Extrelut/Al_2O_3 column. After concentration of the eluate, the residue is determined by HPLC using pulsed amperometric detection. In this study, the final determination is carried out using LC-MS/MS detection. The fragment ion 103 -> 44 is used for evaluation. The limit of quantitation in plant material is 0.01 mg/kg.

II. RESULTS AND DISCUSSION

The residues of BAS 222 F and ETU in apple processed commodities as well as transfer factors are provided in Table 6.5.3-4 and Table 6.5.3-5

Table 6.5.3-4: Residues of BAS 222 F and ETU in apples and process fraction thereof after four applications with BAS 222 28 F.

Trial No.	Portion analyzed	DALA (nominal)	Residues (mg/kg)		
			BAS 222 F	carbon disulfide	ETU
L080111 (Germany)	apple, fruit	0	12.65	7.07	0.39
	apple, fruit (RAC)	20	3.10	1.73	0.034
	washed apples	n/a	3.35	1.87	0.024
	wash water	n/a	1.21	0.68	0.35
	apple sauce	n/a	0.2	0.11	0.19
	juice	n/a	< 0.1	< 0.056	< 0.01
	wet pomace	n/a	5.10	2.85	0.023
	dried pomace	n/a	21.45	11.98	0.31
	dried apples	n/a	10.69	5.97	0.046
	canned apples	n/a	< 0.1	< 0.056	0.014
L080112 (Germany)	apple, fruit	0	6.70	3.74	0.071
	apple, fruit (RAC)	21	3.71	2.07	0.027
	washed apples	n/a	2.24	1.25	0.022
	wash water	n/a	0.96	0.54	0.12
	apple sauce	n/a	0.20	0.11	0.27
	juice	n/a	< 0.1	< 0.056	0.018
	wet pomace	n/a	7.43	4.15	0.024
	dried pomace	n/a	26.39	14.74	0.77
	dried apples	n/a	10.71	5.98	0.36
	canned apples	n/a	< 0.1	< 0.056	0.017
L080113 (Germany)	apple, fruit	0	11.55	6.45	0.40
	apple, fruit (RAC)	22	2.40	1.34	0.030
	washed apples	n/a	1.92	1.07	< 0.01
	wash water	n/a	0.78	0.44	0.078
	apple sauce	n/a	0.32	0.18	0.10
	juice	n/a	< 0.1	< 0.056	0.012
	wet pomace	n/a	4.21	2.35	0.015
	dried pomace	n/a	12.75	7.12	0.059
	dried apples	n/a	4.68	2.61	0.068
	canned apples	n/a	< 0.1	< 0.056	0.012
L080114 (Germany)	apple, fruit	0	6.13	3.42	0.035
	apple, fruit (RAC)	21	2.27	1.27	0.022
	washed apples	n/a	1.21	0.68	0.025
	wash water	n/a	0.89	0.50	0.11
	apple sauce	n/a	0.10	0.056	0.13
	juice	n/a	< 0.1	< 0.056	0.013
	wet pomace	n/a	7.45	4.16	0.028
	dried pomace	n/a	29.07	16.24	0.93
	dried apples	n/a	6.71	3.75	0.36
	canned apples	n/a	< 0.1	< 0.056	0.026

DALA = days after last treatment
n/a = not applicable
for calculation purposes < 0.1 / < 0.01 is set 0.1 / 0.01

The residues of metiram in the treated apple specimens ranged from 6.13 - 12.65 mg/kg at 0 DALA and decreased to 2.27 - 3.71 mg/kg at 20 - 22 DALA. In the treated processed specimens, the residues were highest in the "dried pomace" and "dried apples" specimens with respective ranges of 12.75 - 29.07 mg/kg and 4.68 - 10.71 mg/kg. In the specimens relevant for consumer consumption "juice" and "canned apples", no residues of metiram above the limit of quantitation were detected. The residues in the "washed apples" ranged from 1.21 - 3.35 mg/kg, in the "wash water" from 0.78 - 1.21 mg/kg in the "wet pomace" from 4.21- 7.45 mg/kg and in "apple sauce" from 0.10 - 0.32 mg/kg.

The residues of ETU in the treated apple specimens ranged from 0.035 - 0.40 mg/kg at 0 DALA and decreased to 0.022 - 0.034 mg/kg at 20 - 22 DALA. The highest residues of ETU were detected in the specimens of "dried pomace" (0.059 - 0.93 mg/kg), "dried apples" (0.046 - 0.36.mg/kg), "wash water" (0.078 - 0.35 mg/kg) and "apple sauce" (0.10 - 0.27 mg/kg). The lowest residues were found in "juice" (< 0.01 - 0.018 mg/kg), "washed apples" (< 0.01 - 0.025 mg/kg), "canned apples" (0.012 - 0.026 mg/kg) and "wet pomace" (0.015 - 0.028 mg/kg).

No residues above the limit of quantitation (0.01 mg/kg) were found in the untreated specimens.

Table 6.5.3-5: Transfer factors of BAS 222 F (metiram) and ETU in apples and process fractions thereof after five applications with BAS 222 28 F.

Location	Portion analyzed	Residues (mg/kg)			
		Metiram	Transfer factor	ETU	Transfer factor
L080111 (Germany)	apple, fruit (RAC)	3.10	1	0.034	1
	washed apples	3.35	1.08	0.024	0.71
	wash water	1.21	0.39	0.35	-
	apple sauce	0.2	0.06	0.19	5.59
	juice	< 0.1	0.03	< 0.01	0.29
	wet pomace	5.10	1.65	0.023	0.68
	dried pomace	21.45	6.92	0.31	9.12
	dried apples	10.69	3.45	0.046	1.35
	canned apples	< 0.1	0.03	0.014	0.41
L080112 (Germany)	apple, fruit (RAC)	3.71	1	0.027	1
	washed apples	2.24	0.60	0.022	0.81
	wash water	0.96	0.26	0.12	-
	apple sauce	0.20	0.05	0.27	10.00
	juice	< 0.1	0.03	0.018	0.67
	wet pomace	7.43	2.00	0.024	0.89
	dried pomace	26.39	7.11	0.77	28.52
	dried apples	10.71	2.89	0.36	13.33
	canned apples	< 0.1	0.03	0.017	0.63
L080113 (Germany)	apple, fruit (RAC)	2.40	1	0.030	1
	washed apples	1.92	0.80	< 0.01	0.33
	wash water	0.78	0.33	0.078	-
	apple sauce	0.32	0.13	0.10	3.33
	juice	< 0.1	0.04	0.012	0.40
	wet pomace	4.21	1.75	0.015	0.50
	dried pomace	12.75	5.31	0.059	1.97
	dried apples	4.68	1.95	0.068	2.27
	canned apples	< 0.1	0.04	0.012	0.40

Location	Portion analyzed	Residues (mg/kg)			
		Metiram	Transfer factor	ETU	Transfer factor
L080114 (Germany)	apple, fruit (RAC)	2.27	1	0.022	1
	washed apples	1.21	0.53	0.025	1.14
	wash water	0.89	0.39	0.11	-
	apple sauce	0.10	0.04	0.13	5.91
	juice	< 0.1	0.04	0.013	0.59
	wet pomace	7.45	3.28	0.028	1.27
	dried pomace	29.07	12.81	0.93	42.27
	dried apples	6.71	2.96	0.36	16.36
	canned apples	< 0.1	0.04	0.026	1.18

The mean transfer factors representing the different processing steps were below 1 for Metiram in washed apples, wash water, apple sauce, juice and canned apples. Therefore it can be concluded, that metiram is not being accumulated in these processed fractions. The result show that during juice production the metiram residues stayed on the waste products Wet and Dried Pomace. The transfer factor of metiram in dried apples indicates a concentration of the parent compound because of water loss during drying.

The mean transfer factors representing the different processing steps were below 1 for ETU in washed apples, juice, wet pomace and canned apples. Therefore it can be concluded, that ETU is not being accumulated in these processed fractions. The transfer factor of wash water, apple sauce, dried pomace and dried apples indicates that the ETU residues are mainly located on these processed fractions. The transfer factor of the intermediate fraction wash water will not be reported. Due to the fact that metiram is present in the water, most likely metiram is transferred to ETU. The referenced to the original values in apple fruit (RAC) is not suitable. The transfer factors of apple sauce, dried pomace and dried apples indicate a concentration of ETU because of water loss.

III. CONCLUSION

This supervised field trial study in apples showed that the residues of metiram in the treated apple specimens ranged from 6.13 - 12.65 mg/kg at 0 DALA and decreased to 2.27 - 3.71 mg/kg at 20 - 22 DALA. In the treated processed specimens; the residues were highest in the "dried pomace" and "dried apples" specimens with respective ranges of 12.75 - 29.07 mg/kg and 4.68 - 10.71 mg/kg. In the specimens relevant for consumer consumption "juice" and "canned apples", no residues of metiram above the limit of quantitation were detected. The residues in the "washed apples" ranged from 1.21 - 3.35 mg/kg, in the "wash water" from 0.78 - 1.21 mg/kg and in the "wet pomace" from 4.21 - 7.45 mg/kg.
The residues of ETU in the treated apple specimens ranged from 0.035 - 0.40 mg/kg at 0 DALA and decreased to 0:022 - 0.034 mg/kg at 20 - 22 DALA. The highest residues of ETU were detected in the specimens of "dried pomace" (0.059 - 0.93 mg/kg), "dried apples" (0.046 - 0.36 mg/kg), "wash water" (0.078 - 0.35 mg/kg) and "apple sauce" (0.10 - 0.27 mg/kg). The lowest residues were found in "juice" (< 0.01 - 0.018 mg/kg), "washed apples" (< 0.01 - 0.025 mg/kg), "canned apples" (0.012 - 0.026 mg/kg) and "wet pomace" (0.015 - 0.028 mg/kg). .
No residues of either metiram or ETU above the respective limits of quantitation were found in any of the analyzed untreated specimens.

Report:	*CA 6.5.3/2* *Schulz H., 2002a* *Determination of the residues of Metiram and ETU in cherries and processed products following treatment with BAS 222 28 F under field conditions in Germany 2001* *2002/1004073*
Guidelines:	*BBA IV 3-3, BBA IV 3-4, IVA Guidelines for Residue Studies, Sections IA and IB, 2nd edition 1992*
GLP:	*yes* *(certified by Hessisches Ministerium fuer Umwelt, Landwirtschaft und Forsten, Wiesbaden)*

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
   Description: BAS 222 28 F
   Lot/Batch #: 2001-1
   Purity: BAS 222 28 F (BAS 222 F 70%, nominal)
   CAS#: 9006-42-2
   Development code:
   Metiram: BAS 222 F
   ETU: M222F002
   Spiking levels: BAS 222 F (0.05, 0.5 and 5.0 mg/kg)
   ETU (0.01, 0.1, 0.05 and 0.5 mg/kg)

2. **Test Commodity:**
   Crop: Cherry
   Type: Sour cherry, Sweet cherry
   Variety: Karina, Schattenmorelle, Koröser
   Botanical name: *Prunus cerasus, Prunus avium*
   Crop parts(s) or processed commodity: fruit, cherries without stems (RAC), washed cherries (de-stemmed and washed cherries), wash water, cherry juice, stones (not analysed), canned cherries, fruit syrup
   Sample size: 1.0-12.0 kg

B. STUDY DESIGN

1. Test procedure

During the 2001 growing season, four field trials were conducted in different representative cherry growing areas in Germany to determine the residue levels of BAS 222 F and ETU in cherries and processed fractions thereof. The fungicidal test substance BAS 222 28 F (700 g/kg BAS 222 F, WG) was applied four times with application rates of 2.0 kg/ha (corresponding to 1.6 kg a.s./ha) in a spray volume of 500 L/ha (per meter of crown height) beginning 42±1 days before expected harvest with a spray interval of 7 day. For the analysis of cherry fruits, samples were collected 2 times: directly after the last application and at harvest time which was about 21 days thereafter. These cherries were used for processing canned cherries and cherry juice. Specimen were shipped and stored frozen at ≤-18°C.

2. Description of analytical procedures

The samples were analyzed for metiram with BASF method no. 135/4 which determines the active ingredient as CS_2 The limit of quantitation is 0.05 mg/kg metiram. The result of the procedural recovery experiment was about 100% at fortification levels between 0.05 and 5.0 mg/kg. In addition, the samples were analyzed for ethylenethiourea (ETU), a common metabolite of all ethylenebisdithiocarbamates, using BASF method No.373/1 with some adjustments. These adjustments were made in order to lower the LOQ of the method from 0.05 mg/kg to the required LOQ of 0.01 mg/kg, and are incorporated into the revised method 373/2. The average result of the procedural recovery experiments was 73% at a fortification level between 0.01 and 0.5 mg/kg.

Principle of the method no. 135/4: Carbon disulphide was released from metiram and comparable substances by heating with hydrochloric acid and tin chloride. Reaction with the anion of methanol formed by KOH lead to the formation of a xanthogenate which was determined photometrically at 302 nm.

Principle of the method no. 373/2: ETU is extracted from the plant material with a mixture of sodium ascorbate, ethylene urea, ammonium chloride and methanol-water. After centrifugation of an aliquot the methanol is evaporated from the extract. The remaining water phase is cleaned by liquid/liquid(water/dichloromethane) partition on an Extrelut/Al_2O_3 column. After concentration of the eluate, the residue is determined by HPLC using pulsed amperometric detection.

II. RESULTS AND DISCUSSION

The residues of BAS 222 F and ETU in cherry processed commodities as well as transfer factors are provided in Table 6.5.3-6 and Table 6.5.3-7.

Table 6.5.3-6: Residues of BAS 222 F and ETU in cherries and process fraction thereof after four applications with BAS 222 28 F.

Trial No.	Portion analyzed	DALA	Residues (mg/kg)		
			BAS 222 F	carbon disulfide	ETU
AT-01/023-1	fruit	0	3.95	2.21	0.012
	fruit	21	0.55	0.31	<0.01
	cherries w/o stems	n/a	0.57	0.32	<0.01
	wash water	n/a	0.24	0.13	<0.01
	washed cherries	n/a	0.10	0.06	<0.01
	cherry juice	n/a	0.29	0.16	<0.01
	canned cherries	n/a	0.06	0.03	<0.01
	fruit syrup	n/a	0.10	0.06	0.018
AT-01/023-2	fruit	0	10.71	5.99	0.026
	fruit	26	0.21	0.12	<0.01
	cherries w/o stems	n/a	0.15	0.08	<0.01
	wash water	n/a	0.07	0.04	<0.01
	washed cherries	n/a	0.10	0.06	<0.01
	cherry juice	n/a	0.05	0.03	<0.01
	canned cherries	n/a	<0.05	<0.03	<0.01
	fruit syrup	n/a	<0.05	<0.03	<0.01
AT-01/023-3	fruit	0	13.02	7.28	0.020
	fruit	21	1.08	0.60	<0.01
	cherries w/o stems	n/a	1.44	0.81	<0.01
	wash water	n/a	0.44	0.25	<0.01
	washed cherries	n/a	0.22	0.12	<0.01
	cherry juice	n/a	0.37	0.21	<0.01
	canned cherries	n/a	0.11	0.06	0.014
	fruit syrup	n/a	<0.05	<0.03	0.023
AT-01/023-4	fruit	0	7.83	4.38	0.039
	fruit	21	2.65	1.48	<0.01
	cherries w/o stems	n/a	1.35	0.76	<0.01
	wash water	n/a	0.94	0.53	<0.01
	washed cherries	n/a	0.21	0.12	<0.01
	cherry juice	n/a	1.08	0.60	<0.01
	canned cherries	n/a	0.21	0.12	<0.01
	fruit syrup	n/a	<0.05	<0.03	0.020

DALA = days after last treatment
n/a = not applicable

Directly after the application, BAS 222 F found to range between 3.95 and 13.02 mg/kg in cherry fruits corresponding to 2.21 to 7.28 mg/kg carbon disulfide and after 21 days the residues were between 0.21 to 2.65 mg/kg BAS 222 F (0.12 to 1.48 mg/kg carbon disulfide). After washing, the cherries contained 0.10 to 0.22 mg/kg of BAS 222 F (0.06 to 0.12 mg/kg carbon disulfide). In the final product cherry juice, BAS 222 F was found between 0.05 to 1.08 mg/kg (0.03 to 0.60 mg/kg carbon disulfide), in canned cherries between <0.05 and 0.21 mg/kg (< 0.03 to 0.12 mg/kg carbon disulfide). The starting residue level of ETU was 0.012 to 0.039 mg/kg on DALA 0. After 21 days, no residues of ETU above the limit of quantitation were found in cherry fruit. Washed cherries as well as cherry juice did not contain ETU above the limit of quantitation. In canned cherries, one out of four samples contained 0.014 m/kg ETU.

Table 6.5.3-7: The BAS 222 F and ETU residue in cherries and corresponding transfer factor of process fractions thereof after ten applications with BAS 222 28 F. The transfer factors were re-calculated based on the initial CS_2 values for metiram and ETU.

RAC	residue level CS_2	residue level Metiram	PF	residue level CS_2	PF CS_2	Mean PF CS_2	residue level ETU	PF ETU	Mean PF
cherries	0.31	0.55	cherries (retain)	0.32	1.0	0.90	0.01	0.03	0.06
cherries	0.12	0.21	cherries (retain)	0.08	0.7		0.01	0.08	
cherries	0.6	1.07	cherries (retain)	0.81	1.4			0.00	
cherries	1.48	2.65	cherries (retain)	0.76	0.5			0.00	
cherries	0.31	0.55	washed cherries	0.06	0.2	0.25	0.01	0.03	0.03
cherries	0.12	0.21	washed cherries	0.06	0.5		0.01	0.08	
cherries	0.6	1.07	washed cherries	0.12	0.2		0.01	0.02	
cherries	1.48	2.65	washed cherries	0.12	0.1		0.01	0.01	
cherries	0.31	0.55	wash water	0.13	0.4	0.38	0.01	0.03	0.03
cherries	0.12	0.21	wash water	0.04	0.3		0.01	0.08	
cherries	0.6	1.07	wash water	0.25	0.4		0.01	0.02	
cherries	1.48	2.65	wash water	0.53	0.4		0.01	0.01	
cherries	0.31	0.55	cherry juice	0.16	0.5	0.40	0.01	0.03	0.03
cherries	0.12	0.21	cherry juice	0.03	0.3		0.01	0.08	
cherries	0.6	1.07	cherry juice	0.21	0.4		0.01	0.02	
cherries	1.48	2.65	cherry juice	0.6	0.4		0.01	0.01	
cherries	0.31	0.55	canned cherries	0.03	0.1	0.15	0.01	0.03	0.04
cherries	0.12	0.21	canned cherries	0.03	0.3		0.01	0.08	
cherries	0.6	1.07	canned cherries	0.06	0.1		0.014	0.02	
cherries	1.48	2.65	canned cherries	0.12	0.1		0.01	0.01	
cherries	0.31	0.55	fruit syrup	0.06	0.2	0.18	0.018	0.06	0.05
cherries	0.12	0.21	fruit syrup	0.03	0.3		0.01	0.08	
cherries	0.6	1.07	fruit syrup	0.03	0.1		0.023	0.04	
cherries	1.48	2.65	fruit syrup	0.03	0.1		0.02	0.01	

Directly after the last application, the residues of carbon disulfide in cherries ranged between 2.21 and 7.28 mg/kg. Within the next 21 days, the residues declined to 0.12 to 1.48 mg/kg, which were the starting concentrations in the RAC material used for processing. In the retain samples, which were taken at the processing facility immediately before processing was started, the carbon disulfide residues ranged between 0.08 and 0.81 mg/kg, showing that the carbon disulfide residues between harvesting and processing start were in the sample order of magnitude. Regarding the processed fractions, the carbon disulfide residues in washed cherries were between 0.06 and 0.12 mg/kg and in wash water between 0.04 and 0.53 mg/kg, which resulted in mean transfer factors of 0.25 and 0.38, respectively. In cherry juice (0.03 and 0.21 mg/kg), a mean transfer factors of 0.40 was calculated. The carbon disulfide residues in canned cherries were lower than the residues in the RAC samples and a mean transfer factor of 0.15 was determined. In the fruit syrup samples, the mean transfer factor was 0.18. Directly after the last application, the ETU residues in de-stemmed cherries ranged from 0.012 to 0.039 mg/kg. Within the next 21 days, the residues declined to <0.01 mg/kg. Regarding the processed fractions, no ETU residues above the limit of quantitation were obtained in washed cherries, wash water and cherry juice (mean transfer factor for each processed fraction was 0.03). In one out of four canned cherry samples, ETU residue above the LOQ was found (0.014 mg/kg); the mean transfer factor was determined to be 0.04. In fruit syrup, the ETU residues ranged between < 0.01 and 0.023 mg/kg, the corresponding mean transfer factor was 0.5.

III. CONCLUSION

This supervised field trial study in sweet and sour cherries showed that the residues of BAS 222 F after four applications of BAS 222 28 F ranged from 0.12-1.48 mg/kg in unwashed cherries (RAC), 0.06-0.12 mg/kg in washed cherries, 0.03-0.60 mg/kg in cherry juice, <0.03-0.12 mg/kg in canned cherries and <0.03-0.06 mg/kg in fruit syrup. After processing, declining residue levels of BAS 222 F were observed in all processed fractions of cherries (mean transfer factors ranged from 0.25 to 0.90). No significant enrichment was found in regard to ETU residues in the different processed fraction of cherries with mean transfer factors between 0.03 and 0.06.

Report:	*CA 6.5.3/3* *Schulz H., 2002b* *Determination of the residues of Metiram and ETU in plums and processed products following treatment with BAS 222 28 F under field conditions in Germany 2001* *2002/1004074*
Guidelines:	*BBA IV 3-3, BBA IV 3-4, IVA Guidelines for Residue Studies, Sections IA and IB, 2nd edition 1992*
GLP:	*yes* *(certified by Hessisches Ministerium fuer Umwelt, Landwirtschaft und Forsten, Wiesbaden)*

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** Polyram DF
   Description: BAS 222 28 F
   Lot/Batch #: 2001-1 (BAS 222 28 F)
   Purity: BAS 222 28 F (BAS 222 F 70%, nominal)
   CAS#: 9000-42-2
   Development code:
   Metiram: BAS 222 F
   ETU: M222F002
   Spiking levels: BAS 222 F (0.05, 0.1, 0.5 and 5.0 mg/kg)
   ETU (0.01, 0.10 and 0.20 mg/kg)

2. **Test Commodity:**
   Crop: plum
   Type: not applicable
   Variety: Valjerka, Eilena
   Botanical name: *Prunus domestica*
   Crop parts(s) or processed commodity: de-stemmed plums (RAC), wash water, stones (not used for residue analyses), washed plums (i.e. de-stemmed, washed and de-stoned plums), remainder of the straining process, condensed water, plum puree, dipping water and prunes
   Sample size: 1.5 - 13.0 kg

B. STUDY DESIGN

1. Test procedure

During the 2001 growing season, four field trials were conducted in two representative plum growing regions in Germany to determine the residue levels of BAS 222 F and ETU in plums as well as the process fractions involved in the production of plum puree and prunes. The fungicidal test substance BAS 222 28 F (70% BAS 222 F, WG) was applied 4 times by spray application rates of 6.0 kg/ha (corresponding to 4.2 kg a.s./ha) in a spray volume of 1500 I/ha beginning 42 days before expected harvest with a spray interval of 7 days. For the analysis of plum RACs, samples were collected directly after the last application as well as on a second sampling 19 to 22 days later. The later plums were additionally used for the following products: de-stemmed plums (RAC), wash water, stones (not used for residue analyses), washed plums (i.e. de-stemmed, washed and de-stoned plums), remainder of the straining process, condensed water, plum puree, dipping water and prunes. All processing steps were carried out according to commercial procedures. Specimens were shipped frozen and remained at temperatures below -18°C until analysis.

2. Description of analytical procedures

The samples were analyzed according to BASF method no. 135/4 and BASF method no. 373/2 for BAS 222 F and ETU, respectively. Carbon disulphide was released from Metiram and comparable substances by heating with hydrochloric acid and tin chloride. Reaction with the anion of methanol formed by KOH led to the formation of a xanthogenate which was determined photometrically at 302 nm.

The mean procedural recovery were about 92.1±18.9% (n=18; fortification level 0.05, 0.1, 0.5 and 5.0 mg/kg) and the limit of quantification was 0.05 mg/kg (0.03 mg/kg carbon disulfide). The ETU was extracted from the plant material with a mixture of sodium ascorbate, ethyleneurea, ammonium chloride and methanol-water. After centrifugation an aliquot was taken and the methanol was evaporated from the extract. The pH was adjusted to 8 before evaporating. The remaining water phase was cleaned by liquid/liquid (water/dichloromethane) partition on an Extrelut/aluminium oxide column. After concentration of the eluate, the residue was determined by HPLC using pulsed amperometric detection. The mean procedural recovery were about 73.8±15.1% (n=19; fortification level between 0.01, 0.10 and 0.20 mg/kg) and the limit of quantification was 0.01 mg/kg.

II. RESULTS AND DISCUSSION

The residues of BAS 222 F and ETU in plums, process fractions thereof and correspondent transfer factors are provided in Table 6.5.3-8 and Table 6.5.3-9.

Table 6.5.3-8: Residues of BAS 222 F and ETU in plums and process fractions thereof after four applications with BAS 222 28 F

Trial No.	Portion analyzed	DALA (nominal)	Residues (mg/kg)	
			carbon disulfide	ETU
AT-01/ 024-1	plum, fruit	0	5.71	0.045
	plum, fruit (RAC)	19	4.35	0.112
	plums, fruit (retain samples)	n/a	3.43	0.085
	wash water	n/a	0.95	0.019
	washed plums	n/a	1.23	0.019
	remainder of the straining process	n/a	0.05	0.085
	condensed water	n/a	<0.03	<0.01
	plum puree	n/a	2.47	0.281
	dipping water	n/a	0.60	0.018
	prunes	n/a	3.30	0.038
AT-01/ 024-1/3	plum, fruit	0	5.71	0.045
	plum, fruit (RAC)	19	4.35	0.112
	plums, fruit (retain samples)	n/a	1.80	0.064
	wash water	n/a	1.46	0.028
	washed plums	n/a	1.29	0.020
	remainder of the straining process	n/a	0.80	0.102
	condensed water	n/a	<0.03	<0.01
	plum puree	n/a	3.87	0.207
	dipping water	n/a	0.04	<0.01
	prunes	n/a	4.16	0.037
AT-01/ 024-2	plum, fruit	0	4.92	0.043
	plum, fruit (RAC)	22	1.26	0.041
	plums, fruit (retain samples)	n/a	0.74	0.010
	wash water	n/a	0.16	<0.01
	washed plums	n/a	0.10	<0.01
	remainder of the straining process	n/a	0.10	0.035
	condensed water	n/a	<0.03	<0.01
	plum puree	n/a	0.79	0.141
	dipping water	n/a	0.03	<0.01
	prunes	n/a	0.65	0.011

Trial No.	Portion analyzed	DALA (nominal)	Residues (mg/kg)	
			carbon disulfide	ETU
AT-01/ 024-2/3	plum, fruit	0	4.92	0.043
	plum, fruit (RAC)	22	1.26	0.041
	plums, fruit (retain samples)	n/a	0.91	0.012
	wash water	n/a	0.15	<0.01
	washed plums	n/a	0.09	<0.01
	remainder of the straining process	n/a	0.40	0.032
	condensed water	n/a	<0.03	<0.01
	plum puree	n/a	0.69	0.121
	dipping water	n/a	0.04	<0.01
	prunes	n/a	0.93	0.014

DALA = days after last treatment
n/a = not applicable

Directly after the last application, the residues of carbon disulfide in plums ranged between 4.92 and 5.71 mg/kg. Within the next 19 - 22 days, the residues declined to 1.26 and 4.35 mg/kg, which were the starting concentrations in the RAC material used for processing. In the retain samples, which were taken at the processing facility immediately before processing was started, the carbon disulfide residues were between 0.74 and 3.43 mg/kg. Regarding the processed fractions, the carbon disulfide, residues in washed plums were between 0.09 and 1.29 mg/kg and in wash water between 0.1 and 1.46 mg/kg. In condensed water, no carbon disulfide above the limit of quantification was found. In plum puree, carbon disulfide residues between 0.79 and 3.87 mg/kg were detected. Small residues between 0.03 and 0.06 mg/kg were found in dipping water. In prunes, the carbon disulfide residues ranged between 0.65 to 4.16 mg/kg. The residues of ETU in plums sampled directly after the last application, ranged from 0.043 to 0.045 mg/kg. Within the next 19-22 days, the residues ranged between 0.041 and 0.112 mg/kg, which were the starting concentrations in the RAC material used for processing. In the retain samples, which were taken at the processing facility immediately before processing was started, the ETU residues were between 0.010 and 0.085 mg/kg. Regarding the processed fractions, the ETU residues in washed plums were between <0.01 and 0.020 mg/kg and in wash water between <0.01 and 0.028 mg/kg. In condensed water, no -ETU residues above the limit of quantification were found. In plum puree, ETU residues concentrated to 0.121-0.281 mg/kg, which could be caused by ETU formation due to heating treatment. In dipping water, the ETU residues were below the limit of quantification and 0.018 mg/kg. Compared with the starting ETU concentrations in the RAC samples, the residues in prunes declined to 0.011 to 0.038 mg/kg.

Table 6.5.3-9: The BAS 222 F and ETU residue in plums and corresponding transfer factor of process fractions thereof after applications with BAS 222 28 F. The transfer factors were re-calculated based on the initial CS_2 values for metiram and ETU.

RAC	residue level CS_2	residue level Metiram	PF	residue level CS_2	PF CS_2	Mean PF CS_2	residue level ETU	PF ETU	Mean PF
plums	4.35	7.79	plums (retain)	3.43	0.8	0.63	0.085	0.02	0.01
plums	4.35	7.79	plums (retain)	1.8	0.4		0.064	0.01	
plums	1.26	2.26	plums (retain)	0.74	0.6		0.01	0.01	
plums	1.26	2.26	plums (retain)	0.91	0.7		0.012	0.01	
plums	4.35	7.79	wash water	0.95	0.2	0.18	0.019	0.00	0.01
plums	4.35	7.79	wash water	1.46	0.3		0.028	0.01	
plums	1.26	2.26	wash water	0.16	0.1		0.01	0.01	
plums	1.26	2.26	wash water	0.15	0.1		0.01	0.01	
plums	4.35	7.79	washed plums	1.23	0.3	0.19	0.19	0.04	0.03
plums	4.35	7.79	washed plums	1.29	0.3		0.2	0.05	
plums	1.26	2.26	washed plums	0.1	0.1		0.01	0.01	
plums	1.26	2.26	washed plums	0.09	0.1		0.01	0.01	
plums	4.35	7.79	remainder of the straining process	0.5	0.1	0.17	0.085	0.02	0.02
plums	4.35	7.79	remainder of the straining process	0.8	0.2		0.102	0.02	
plums	1.26	2.26	remainder of the straining process	0.1	0.1		0.035	0.03	
plums	1.26	2.26	remainder of the straining process	0.4	0.3		0.032	0.03	
plums	4.35	7.79	condensed water	0.03	0.0	0.01	0.01	0.00	0.01
plums	4.35	7.79	condensed water	0.03	0.0		0.01	0.00	
plums	1.26	2.26	condensed water	0.03	0.0		0.01	0.01	
plums	1.26	2.26	condensed water	0.03	0.0		0.01	0.01	
plums	4.35	7.79	plum puree	2.47	0.6	0.68	0.281	0.06	0.08
plums	4.35	7.79	plum puree	3.87	0.9		0.207	0.05	
plums	1.26	2.26	plum puree	0.79	0.6		0.141	0.11	
plums	1.26	2.26	plum puree	0.69	0.6		0.121	0.10	
plums	4.35	7.79	dipping water	0.06	0.0	0.02	0.018	0.00	0.01
plums	4.35	7.79	dipping water	0.04	0.0		0.01	0.00	
plums	1.26	2.26	dipping water	0.03	0.0		0.01	0.01	
plums	1.26	2.26	dipping water	0.04	0.0		0.01	0.01	
plums	4.35	7.79	prunes	3.3	0.8	0.75	0.038	0.01	0.01
plums	4.35	7.79	prunes	4.16	1.0		0.037	0.01	
plums	1.26	2.26	prunes	0.65	0.5		0.011	0.01	
plums	1.26	2.26	prunes	0.93	0.7		0.014	0.01	

For calculation purposes, <0.03 mg/kg were set at 0.03 mg/kg of carbon disulfide and <0.01 mg/kg were set at 0.01 mg/kg of ETU

No significant accumulation of metiram in the investigated processed fractions could be observed. The transfer factor were well below 1 and constituted 0.63, 0.19, 0.01, 0.68 and 0.75 for retained plums, washed plums, plum puree and prunes, respectively. Similar results were obtained in regard to transfer factors calculated for ETU. Again, the transfer factor were well below 1.0, namely 0.01, 0.03, 0.08 and 0.01 for retained plums, washed plums, plum puree and prunes, respectively.

III. CONCLUSION

This processing study showed that the residues of BAS 222 F (expressed as carbon disulfide) in plum RACs after four applications of BAS 222 28 F ranged from 4.92 to 5.71 mg/kg on DALA 0 and from 1.26 to 4.35 mg/kg on DALA 19-22, which were the starting concentrations for the processing. After processing, no significant enrichment of BAS 222 F (indicated by a transfer factor >1.0) in any of the processed plum fractions was observed. Rather, the mean transfer factors were consistently ≤1.0, namely in plums washed (residue: 0.15-1.29 mg/kg; mean transfer factor: 0.18), plum puree (residue: 0.69-3.87 mg/kg; mean transfer factor: 0.68) and prunes (residue: 0.65-4.16 mg/kg; mean transfer factor: 0.75). The residues of ETU in plum RACs ranged from 0.043 to 0.045 mg/kg on DALA 0 and from 0.041 to 0.112 mg/kg on DALA 19-22, which were the starting concentrations for the processing. In regard to ETU, no significant enrichment of BAS 222 F during processing was observed in plum puree (residue: 0.121-0.281 mg/kg; mean transfer factor: 0.08), only plums washed (residue: <0.01-0.020 mg/kg; mean transfer factor: 0.03) and prunes (residue: 0.011-0.038 mg/kg; mean transfer factor: 0.01).

Report:	*CA 6.5.3/4* *Raunft E. et al., 2002a* *Study on the residue behaviour of Metiram in grapes and grape process fractions after application of BAS 222 28 F under field conditions in Germany, 2001* *2002/1004093*
Guidelines:	*BBA IV 3-3.4, EEC 7029/VI/95 rev. 5, EEC 7525/VI/95 rev. 2, EEC 7035/VI/95 rev. 5*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
   Description: BAS 222 28 F
   Lot/Batch #: 2001-1, 2000-2
   Purity: BAS 222 28 F (BAS 222 F 70%, nominal)
   CAS#: 9006-42-2
   Development code:
   Metiram: BAS 222 F
   ETU: M222F002
   Spiking levels: BAS 222 F (0.05 and 20.0 mg/kg)
   ETU (between 0.01 and 1.0 mg/kg)

2. **Test Commodity:**
   Crop: grape/wine
   Type: red wine
   Variety: Spätburgunder, Portugieser
   Botanical name: *Vitis vinifera*
   Crop parts(s) or processed commodity: rose pomace, rosé must, rose wine, red pomace, red must, red wine, red stalks
   Sample size: 1.0 - 200.0 kg

B. STUDY DESIGN

1. Test procedure

During the 2001 growing season, four field trials were conducted in different representative wine-producing regions in Germany to determine the residue levels of BAS 222 F and ETU in grapes and processed wine fractions thereof. The fungicidal test substance BAS 222 28 F (70% BAS 222 F, WG) was applied six times by spray application rates between 1.8 and 4.8 kg/ha (corresponding to 1.26 to 3.36 kg a.i./ha) in a spray volume of 300-800 I/ha beginning 106 days before expected harvest with a spray interval of 10 day. For the analysis of wine grapes, samples were collected directly after the last application as well as on a second sampling 55 to 56 days later. These wine grapes were used for production rosé and red wine resulting in the fractions must, pomace, wine and stalk. Additionally, grapes were dried to produce raisins. All processing steps were carried out according to commercial procedures. Specimens were frozen within 24h and remained at temperatures below -18°C until analysis.

2. Description of analytical procedures

The samples were analyzed according to BASF method no. 135/4 and BASF method no. 373/2 for BAS 222 F and ETU, respectively. The active substance was converted into carbon disulfide by treating the sample with a refluxing aqueous solution of hydrochloric acid in the presence of tin(II) chloride. The carbon disulfide was trapped in a methanol solution of potassium hydroxide and was determined by UV detection at 302 nm of the xanthogenate formed. The mean procedural recovery were about 81±20.4% (n=29; fortification level between 0.05 and 20.0 mg/kg) and the limit of quantification was 0.05 mg/kg. The ETU was extracted from the plant material with a mixture of sodium ascorbate, ethyleneurea, ammonium chloride and methanol-water. After centrifugation an aliquot was taken and the methanol was evaporated from the extract. The pH was adjusted to 8 before evaporating. The remaining water phase was cleaned by liquid/liquid (water/dichloromethane) partition on an Extrelut/aluminium oxide column. After concentration of the eluate, the residue was determined by HPLC using pulsed amperometric detection. The mean procedural recovery were about 90±16.4% (n=26; fortification level between 0.01 and 1.0 mg/kg) and the limit of quantification was 0.01 mg/kg.

II. RESULTS AND DISCUSSION

The residues of BAS 222 F and ETU in grapes and process fractions thereof as well as transfer factors are provided in Table 6.5.3-10 and Table 6.5.3-11.

Table 6.5.3-10: Residues of BAS 222 F and ETU in grapes and process fractions thereof after six applications with BAS 222 28 F

Trial No.	Portion analyzed	DALA	Residues (mg/kg)		
			BAS 222 F	carbon disulfide	ETU
DU4/02/01	grape, fruit	0	10.83	6.06	0.307
	grape, fruit	55	2.05	1.15	0.040
	raisin	n/a	1.46	0.82	0.133
	rosé must	n/a	0.13	0.07	0.010
	red must	n/a	0.39	0.22	0.337
	red stalks	n/a	6.73	3.77	0.047
	rosé pomace	n/a	3.73	2.09	0.016
	red pomace	n/a	2.90	1.62	0.177
	red wine	n/a	0.07	0.04	0.349
	rosé wine	n/a	0.06	0.03	0.019
DU4/03/01	grape, fruit	0	14.45	8.08	0.515
	grape, fruit	56	4.45	2.49	0.041
	raisin	n/a	19.29	10.79	0.055
	rosé must	n/a	0.19	0.11	<0.01
	red must	n/a	0.32	0.18	0.570
	red stalks	n/a	11.18	6.26	0.048
	rosé pomace	n/a	16.64	9.31	0.034
	red pomace	n/a	6.00	3.36	0.298
	red wine	n/a	0.07	0.04	0.567
	rosé wine	n/a	0.07	0.04	0.025
DU2/03/01	grape, fruit	0	19.44	10.88	0.437
	grape, fruit	56	2.22	1.24	0.050
	raisin	n/a	11.02	6.17	0.040
	rosé must	n/a	0.45	0.25	0.011
	red must	n/a	0.15	0.08	0.353
	red stalks	n/a	4.42	2.47	0.064
	rosé pomace	n/a	5.63	3.15	0.020
	red pomace	n/a	4.22	2.36	0.199
	red wine	n/a	0.08	0.04	0.364
	rosé wine	n/a	0.08	0.04	0.028
DU2/02/01	grape, fruit	0	10.04	5.62	0.228
	grape, fruit	56	1.98	1.11	0.023
	raisin	n/a	6.53	3.65	0.028
	rosé must	n/a	0.27	0.15	<0.01
	red must	n/a	0.11	0.06	0.284
	red stalks	n/a	5.04	2.82	0.061
	rosé pomace	n/a	7.08	3.96	0.021
	red pomace	n/a	2.91	1.63	0.136
	red wine	n/a	0.1	0.06	0.300
	rosé wine	n/a	0.07	0.04	0.015

DALA = days after last treatment
n/a = not applicable
Concentrations measured on DALA 55-56 were set 1

Directly after the last application, BAS 222 F was found to range between 10.04 and 19.44 mg/kg in grapes corresponding to 5.62 to 10.88 mg/kg carbon disulfide. These residues degraded during the course of the study ranging between 1.98 to 4.45 mg/kg of BAS 222 F (1.11 to 2.49 mg/kg carbon disulfide) after about eight weeks. The starting residue level of ETU was 0.228 to 0.515 mg/kg on DALA 0. After about eight weeks, ETU ranged between 0.023 and 0.050 mg/kg in grape RACs. The final product raisins contained BAS 222 F between 1.46 and 19.29 mg/kg (0.82 to 10.79 mg/kg carbon disulfide), ETU was found between 0.028 and 0.133 mg/kg. In the wine making processes, the following results were obtained: looking at BAS 222 F, no considerable differences between the rose and the red wine procedure were observed. The must leading to rosé wine contained 0.13 to 0.45 mg/kg BAS 222 F which is comparable to 0.11 to 0.39 mg/kg of BAS 222 F in the must for red wine. Also the levels of BAS 222 F found in pomace were similar: 3.73 to 16.64 mg/kg were in the rose wine pomace, 2.90 to 6.00 mg/kg in the red wine pomace. Finally, the wines contained 0.07 to 0.10 mg/kg in case of red wine and 0.06 to 0.08 mg/kg of BAS 222 F in case of rose wine. However, in case of the metabolite ETU which is gradually formed from the parent compound, the procedures led to different results: the rose must, which was obtained by cold pressing, contained < 0.01 to 0.011 mg/kg of ETU. In the red must, which is produced by pressing after heating overnight contained ETU residues between 0.284 to 0.570 mg/kg? The pomace from rose wine making has 0.016 to 0.034 mg/kg, the red wine pomace, however, 0.136 to 0.298 mg/kg. Also, the final product wine shows these differences: 0.015 to 0.028 mg/kg of ETU was found in rose wine, whereas 0.300 to 0.567 mg/kg was measured in red wine.

The mean transfer factor for raisins in regard to BAS 222 F was calculated to be 3.33 and is most likely linked to the considerable loss of water during the drying process. Slight indications of BAS 222 F enrichment was additionally observed in red stalk (mean transfer factors: 2.58), rosé pomace (mean transfer factors: 2.92) and red pomace (mean transfer factors: 1.53). These fractions, however, are not final consumer products. In pomace, which is the solid left-over material from the pressing, a loss of water and therefore a concentration is explainable. In the remaining fractions (rosé/red must and red/rosé wine), the mean transfer factors were low and ranged from 0.03 to 0.11. In regard to the metabolite ETU, the mean transfer factors are quite low and ranged from 0.01 in rosé must to 0.27 in red must and red wine.

III. CONCLUSION

This supervised field trial study showed that the residues of BAS 222 F in wine grape RACs after six applications of BAS 222 28 F ranged from 10.04 to 19.44 mg/kg on DALA 0 and from 1.98 to 4.45 mg/kg on DALA 55-56. After processing, significant enrichment of BAS 222 F was observed in raisins (residue: 1.46 to 19.29 mg/kg; mean transfer factor 3.33), red stalks (residue: 4.42 to 11.18 mg/kg; mean transfer factor 2.58), red and rosé pomace (residue: 2.90 to 16.64 mg/kg; mean transfer factor 2.92 and 1.53), whereas low levels of BAS 222 F were measured in red and rosé must (residue: 0.11 to 0.45 mg/kg; mean transfer factors: 0.10 to 0.11) and red and rosé wine (residue: 0.07 to 0.08 mg/kg; mean transfer factors: 0.03). The residues of ETU in wine grape RACs ranged from 0.23 to 0.52 mg/kg on DALA 0, which declined to concentrations of 0.023 to 0.050 mg/kg on DALA 55-56. The mean transfer factors are quite low and ranged from 0.01 in rosé must to 0.27 in red must and red wine.

Table 6.5.3-11: The BAS 222 F and ETU residues in grapes and corresponding transfer factors of process fractions thereof after six applications with BAS 222 28 F. The transfer factors were re-calculated based on the initial CS_2 values for metiram and ETU.

RAC	Trial location	residue level CS_2	residue level Metiram	PF	residue level CS_2	PF CS_2	Mean PF CS_2	residue level ETU	PF ETU (based on CS_2)	Mean PF ETU (in % CS_2)
grape	Germany	1.15	2.05	raisin	0.82	0.71	3.33	0.13	0.12	0.05
grape	Germany	2.49	4.45	raisin	10.80	4.34		0.06	0.02	
grape	Germany	1.24	2.22	raisin	6.17	4.98		0.04	0.03	
grape	Germany	1.11	1.98	raisin	3.66	3.29		0.03	0.03	
grape	Germany	1.15	2.05	rosé must	0.07	0.06	0.11	0.01	0.01	0.01
grape	Germany	2.49	4.45	rosé must	0.11	0.04		0.01	0.00	
grape	Germany	1.24	2.22	rosé must	0.25	0.20		0.01	0.01	
grape	Germany	1.11	1.98	rosé must	0.15	0.14		0.01	0.01	
grape	Germany	1.15	2.05	red must	0.22	0.19	0.10	0.34	0.29	0.27
grape	Germany	2.49	4.45	red must	0.18	0.07		0.57	0.23	
grape	Germany	1.24	2.22	red must	0.08	0.07		0.35	0.28	
grape	Germany	1.11	1.98	red must	0.06	0.06		0.28	0.26	
grape	Germany	1.15	2.05	red stalk	3.77	3.28	2.58	0.05	0.04	0.04
grape	Germany	2.49	4.45	red stalk	6.26	2.51		0.05	0.02	
grape	Germany	1.24	2.22	red stalk	2.48	2.00		0.06	0.05	
grape	Germany	1.11	1.98	red stalk	2.82	2.54		0.06	0.05	
grape	Germany	1.15	2.05	rosé pomace	2.09	1.82	2.92	0.02	0.01	0.02
grape	Germany	2.49	4.45	rosé pomace	9.32	3.74		0.03	0.01	
grape	Germany	1.24	2.22	rosé pomace	3.15	2.54		0.02	0.02	
grape	Germany	1.11	1.98	rosé pomace	3.96	3.57		0.02	0.02	
grape	Germany	1.15	2.05	red pomace	1.62	1.41	1.53	0.18	0.15	0.14
grape	Germany	2.49	4.45	red pomace	3.36	1.35		0.30	0.12	
grape	Germany	1.24	2.22	red pomace	2.36	1.91		0.20	0.16	
grape	Germany	1.11	1.98	red pomace	1.63	1.47		0.14	0.12	
grape	Germany	1.15	2.05	red wine	0.04	0.03	0.03	0.35	0.30	0.27
grape	Germany	2.49	4.45	red wine	0.04	0.02		0.57	0.23	
grape	Germany	1.24	2.22	red wine	0.04	0.04		0.36	0.29	
grape	Germany	1.11	1.98	red wine	0.06	0.05		0.30	0.27	
grape	Germany	1.15	2.05	rosé wine	0.03	0.03	0.03	0.02	0.02	0.02
grape	Germany	2.49	4.45	rosé wine	0.04	0.02		0.03	0.01	
grape	Germany	1.24	2.22	rosé wine	0.04	0.04		0.03	0.02	
grape	Germany	1.11	1.98	rosé wine	0.04	0.04		0.02	0.01	

Report:	*CA 6.5.3/5* *Schulz H., 1998a* *Determination of the residues of Metiram and ETU in grapes and in processing products following treatment with BAS 222 28 F under field conditions in Spain 1997* *1998/11226*
Guidelines:	*FAO Guidelines Rome 1990, BBA IV 3-3, BBA IV 3-4, IVA Guidelines for Residue Studies, Sections IA and IB, 2nd edition 1992*
GLP:	*yes* *(certified by Hessisches Ministerium fuer Umwelt, Energie, Jugend, Familie und Gesundheit, Wiesbaden)*

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
   Description: Polyram DF
   Lot/Batch #: 96-1
   Purity: BAS 222 28 F (BAS 222 F 70%, nominal)
   CAS#: 9006-42-2
   Development code:
   Metiram: BAS 222 F
   ETU: M222F002
   Spiking levels: BAS 222 F (0.1, 0.5, 10.0 and 20.0 mg/kg)
   ETU (0.05, 0.1, 0.5, 5.0 and 10.0 mg/kg)

2. **Test Commodity:**
   Crop: grape/wine
   Type: white wine
   Variety: Macabeo, Malvasia
   Botanical name: *Vitis vinifera*
   Crop parts(s) or processed commodity: pomace, pasteurized and unpasteurized must, pasteurized and unpasteurized young wine, pasteurized and unpasteurized mature wine
   Sample size: 125 - 126.2 kg

B. STUDY DESIGN

1. Test procedure

During the 1997 growing season, two field trials were conducted in different representative wine-producing regions in Spain to determine the residue levels of BAS 222 F and ETU in white wine grapes and processed wine fractions thereof. The fungicidal test substance BAS 222 28 F (70% BAS 222 F, WG) was applied ten times by spray application rates of 3.5 kg/ha (corresponding to 2.45 kg a.i./ha) in a spray volume of 800-1500 I/ha beginning 94/89 days before expected harvest with a spray interval of 8 day. For the analysis of wine grapes, samples were collected 22/17 days after the last application. These wine grapes were used for production of pomace, pasteurized and unpasteurized must, pasteurized and unpasteurized young wine as well as , pasteurized and unpasteurized mature wine. Specimens were stored frozen until further analysis.

2. Description of analytical procedures

The samples were analyzed according to method IF-95/22962-00 and F-95/24090-00 for BAS 222 F and ETU, respectively. A mixture of grapes, tin chloride and hydrochloric acid was brought to the boil. The liberated carbon disulphide was distilled, then purified and trapped by dissolving in ethanolic solution containing copper (II) acetate and diethanolamine. Two copper dithiocarbamate complexes were formed and subsequently measured together using spectral photometry at 435 nm. For the analyses of the wine samples, the liberated carbon disulphide was trapped by dissolving in a methanolic potassium hydroxide solution. The xanthogenate formed was subsequently measured using UV spectral photometry at a wavelength of 302 nm. The mean procedural recovery were about 79.0±8.7% (n=6; fortification level 0.1, 0.5, 10.0 and 20.0 mg/kg) and the limit of quantification was 0.01 mg/kg. The ETU was extracted from grapes with water/methanol. Methanol was evaporated of the extract. An aliquot of the remaining aqueous phase was cleaned-up by liquid/liquid partition (water/dichloromethane) on an Extrelut column followed by a further clean up step on an aluminium oxide column. After concentration of the eluate, the ETU residues were determined by HPLC with UV detection. The mean procedural recovery were about 90.9% (n=16; fortification level 0.05, 0.1, 0.5, 5.0 and 10.0 mg/kg and the limit of quantification was 0.05 mg/kg.

II. RESULTS AND DISCUSSION

The residues of BAS 222 F and ETU in grapes and process fractions thereof as well as transfer factors are provided in Table 6.5.3-12 and Table 6.5.3-13.

Table 6.5.3-12: Residues of BAS 222 F and ETU in grapes and process fractions thereof after ten applications with BAS 222 28 F

Trial	Portion analyzed	Residues (mg/kg)		
		BAS 222 F	carbon disulfide	ETU
No.1	grapes	5.50	3.07	0.13
	pomace	6.61	3.69	0.14
	must, unpasteurized	10.17	5.68	0.06
	must, pasteurized	9.08	5.07	1.15
	young wine, unpasteurized	0.18	0.10	0.52
	young wine, pasteurized	0.16	0.09	1.25
	mature wine, unpasteurized	0.39	0.22	0.49
	mature wine, pasteurized	0.14	0.08	1.24
No.2	grapes	8.32	4.65	0.08
	pomace	17.65	9.86	0.22
	must, unpasteurized	14.98	8.37	0.07
	must, pasteurized	11.76	6.57	1.54
	young wine, unpasteurized	0.21	0.12	0.84
	young wine, pasteurized	0.13	0.07	1.23
	mature wine, unpasteurized	0.20	0.11	0.69
	mature wine, pasteurized	0.18	0.10	1.57

Twenty-one days after the last application, the residues of BAS 222 F in grape RACs were found to be between 5.50 and 8.32 mg/kg, corresponding to 3.07 and 4.65 mg/kg carbon disulfide. Considerable residues of BAS 222 F were detected in both pomace and must with residual concentrations ranging from 6.61 to 17.65 mg/kg (pomace) and 9.08 to 14.98 mg/kg (must). Low levels of BAS 222 F were found in young and mature wine products (ranging from 0.13 to 0.39 mg/kg). The starting residue levels of ETU were 0.08 to 0.13 mg/kg which were in a similar concentration range found in pomace (0.14 and 0.22 mg/kg) and unpasteurized must (0.06 and 0.07 mg/kg). After pasteurization, the ETU residues increased considerably (1.15 and 1.54 mg/kg). In unpasteurized young and mature wine, the residues ranged from 0.49 and 0.84 mg/kg, whereas after pasteurization the ETU residues were between 1.23 and 1.57 mg/kg.

Table 6.5.3-13: The BAS 222 F and ETU residues in grapes and corresponding transfer factors of process fractions thereof after ten applications withn BAS 222 28 F. The transfer factors were re-calculated based on the initial CS_2 values for metiram and ETU

RAC	Trial location	residue level CS_2	residue level Metiram	PF	residue level CS_2	PF CS_2	Mean PF CS_2	residue level ETU	PF ETU (based on CS_2)	Mean PF ETU (in % CS_2)
grape	Spain	3.07	5.50	pomace	3.69	1.20	1.66	0.14	0.05	0.05
grape	Spain	4.65	8.32	pomace	9.86	2.12		0.22	0.05	
grape	Spain	3.07	5.50	must, unpasteurized	5.68	1.85	1.83	0.06	0.02	0.02
grape	Spain	4.65	8.32	must, unpasteurized	8.37	1.80		0.07	0.02	
grape	Spain	3.07	5.50	must, pasteurized	5.07	1.65	1.53	1.15	0.37	0.35
grape	Spain	4.65	8.32	must, pasteurized	6.57	1.41		1.54	0.33	
grape	Spain	3.07	5.50	young wine, unpasteurized	0.1	0.03	0.03	0.52	0.17	0.18
grape	Spain	4.65	8.32	young wine, unpasteurized	0.12	0.03		0.84	0.18	
grape	Spain	3.07	5.50	young wine, pasteurized	0.09	0.03	0.02	1.25	0.41	0.34
grape	Spain	4.65	8.32	young wine, pasteurized	0.07	0.02		1.23	0.26	
grape	Spain	3.07	5.50	mature wine, unpasteurized	0.22	0.07	0.05	0.49	0.16	0.15
grape	Spain	4.65	8.32	mature wine, unpasteurized	0.11	0.02		0.69	0.15	
grape	Spain	3.07	5.50	mature wine, pasteurized	0.08	0.03	0.02	1.24	0.40	0.37
grape	Spain	4.65	8.32	mature wine, pasteurized	0.1	0.02		1.57	0.34	

Residues of carbon disulfide in grape RAC material used for processing were at concentrations ranging from 3.07 to 4.65 mg/kg. The transfer factors of pomace (mean transfer factor: 1.66), unpasteurized must (mean transfer factor: 1.83) and in pasteurized must (mean transfer factor: 1.53), which indicates small enrichment of BAS 222 F in early process steps. However, in young and mature wine the transfer factors were low (range from 0.08 to 0.22).. The mean transfer factors of ETU for the different processing fractions were even after pasteurization low and ranged from 0.15 to 0.37.

III. CONCLUSION

This supervised field trial study showed that the residues of BAS 222 F after ten applications of BAS 222 28 F ranged from 5.50 to 8.32 mg/kg in wine grape RACs. After processing, minor enrichment of BAS 222 F was found in pomace (mean transfer factor: 1.66), unpasteurized must (mean transfer factor: 1.83) and in pasteurized must (mean transfer factor: 1.53). In young and mature wine the transfer factors were low and ranged from 0.08 to 0.22). Processing did not resulted in a significant enrichment of ETU and the transfer factors ranged from 0.15 to 0.37.

Report: *CA 6.5.3/6*
Schulz H., 1998b
Determination of the residues of Metiram and ETU in red wine grapes and in processing products following treatment with BAS 222 28 F under field conditions in France 1997
1998/11302

Guidelines: *FAO Guidelines Rome 1990, BBA IV 3-3, BBA IV 3-4, IVA Guidelines for Residue Studies Sections IA and IB 2nd edition 1992*

GLP: *yes*
(certified by Hessisches Ministerium fuer Umwelt, Energie, Jugend, Familie und Gesundheit, Wiesbaden)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
   Description: Polyram DF
   Lot/Batch #: 96-1
   Purity: BAS 222 28 F (BAS 222 F 70%, nominal)
   CAS#: 9006-42-2
   Development code:
   Metiram: BAS 222 F
   ETU: M222F002
   Spiking levels: BAS 222 F (0.01 and 20.0 mg/kg)
   ETU (between 0.05 and 5.0 mg/kg)

2. **Test Commodity:**
   Crop: grape/wine
   Type: red wine
   Variety: Pinot noir, Cabernet, Grenache
   Botanical name: *Vitis vinifera*
   Crop parts(s) or processed commodity: rose pomace, rosé must, young rose wine, mature rose wine, red pomace, red must, young red wine, mature red wine
   Sample size: 120 - 128 kg

B. STUDY DESIGN

1. Test procedure

During the 1997 growing season, four field trials were conducted in different representative wine-producing regions in France to determine the residue levels of BAS 222 F and ETU in grapes and processed wine fractions thereof. The fungicidal test substance BAS 222 28 F (70% BAS 222 F, WG) was applied ten times by spray application rates of 3.5 kg/ha (corresponding to 2.45 kg a.i./ha) in a spray volume of 300-500 I/ha beginning 98±1 days before expected harvest with a spray interval of 8 day. For the analysis of wine grapes, samples were collected 21 days after the last application. These wine grapes were used for production rosé and red pomace, must, young wine as well as mature wine. All processing steps were carried out according to commercial procedures. Specimens were shipped chilled and stored frozen at ≤-18°C until further analysis.

2. Description of analytical procedures

The samples were analyzed according to method IF-95/22962-00 and F-95/24090-00 for BAS 222 F and ETU, respectively. A mixture of grapes, tin chloride and hydrochloric acid was brought to the boil. The liberated carbon disulphide was distilled, then purified and trapped by dissolving in ethanolic solution containing copper (II) acetate and diethanolamine. Two copper dithiocarbamate complexes were formed and subsequently measured together using spectral photometry at 435 nm. For the analyses of the wine samples, the liberated carbon disulphide was trapped by dissolving in a methanolic potassium hydroxide solution. The xanthogenate formed was subsequently measured using UV spectral photometry at a wavelength of 302 nm. The mean procedural recovery were about 80.9±8.5% (n=13; fortification level between 0.01 and 20.0 mg/kg) and the limit of quantification was 0.01 mg/kg. The ETU was extracted from grapes with water/methanol. Methanol was evaporated of the extract. An aliquot of the remaining aqueous phase was cleaned-up by liquid/liquid partition (water/dichloromethane) on an Extrelut column followed by a further clean up step on an aluminium oxide column. After concentration of the eluate, the ETU residues were determined by HPLC with UV detection. The mean procedural recovery were about 91.2±6.0% (n=18; fortification level between 0.05 and 5.0 mg/kg) and the limit of quantification was 0.05 mg/kg.

II. RESULTS AND DISCUSSION

The residues of BAS 222 F and ETU in grapes and process fractions thereof as well as transfer factors are provided in Table 6.5.3-14 and Table 6.5.3-15.

Table 6.5.3-14: Residues of BAS 222 F and ETU in grapes and process fractions thereof after ten applications with BAS 222 28 F

Trial No.	Portion analyzed	DALA	Residues (mg/kg)		
			BAS 222 F	carbon disulfide	ETU
X976251	grapes	21	7.92	4.02	<0.05
	rosé pomace	n/a	6.68	3.39	<0.05
	rosé must	n/a	2.72	1.38	<0.05
	young rosé wine	n/a	<0.10	0.05	0.17
	mature rosé wine	n/a	<0.10	0.05	0.16
	red pomace	n/a	5.48	2.78	0.10
	red must	n/a	3.84	1.95	0.19
	young red wine	n/a	0.10	0.05	0.24
	mature red wine	n/a	0.10	0.05	0.22
X976252	grapes	21	5.22	2.65	0.062
	rosé pomace	n/a	6.07	3.08	0.076
	rosé must	n/a	2.90	1.47	<0.05
	young rosé wine	n/a	<0.10	0.05	0.25
	mature rosé wine	n/a	<0.10	0.05	0.025
	red pomace	n/a	6.86	3.48	0.065
	red must	n/a	2.99	1.52	0.064
	young red wine	n/a	<0.10	0.05	0.20
	mature red wine	n/a	<0.10	0.05	0.19
X976253	grapes	21	3.43	1.74	<0.05
	rosé pomace	n/a	4.65	2.36	<0.05
	rosé must	n/a	3.15	1.6	<0.05
	young rosé wine	n/a	<0.10	0.05	0.16
	mature rosé wine	n/a	<0.10	0.05	0.15
	red pomace	n/a	5.59	2.84	0.084
	red must	n/a	1.93	0.98	0.16
	young red wine	n/a	<0.10	0.05	0.24
	mature red wine	n/a	<0.10	0.05	0.22
X976254	grapes	21	11.13	5.65	0.21
	rosé pomace	n/a	6.54	3.32	0.074
	rosé must	n/a	5.28	2.68	0.088
	young rosé wine	n/a	0.18	0.09	0.28
	mature rosé wine	n/a	0.18	0.09	0.29
	red pomace	n/a	8.94	4.54	0.12
	red must	n/a	3.53	1.79	0.41
	young red wine	n/a	0.33	0.17	0.44
	mature red wine	n/a	0.39	0.2	0.43

Twenty-one days after the last application, the residues of BAS 222 F in grape RACs were found to range between 3.43 and 11.13 mg/kg corresponding to 2.03 to 6.59 mg/kg carbon disulfide. Considerable residues of BAS 222 F were detected in both pomace and must with residual concentrations ranging from 4.65 to 8.94 mg/kg (pomace) and 1.93 to 5.28 mg/kg (must). Low levels of BAS 222 F were found in young and mature wine products (ranging from <0.10 to 0.39 mg/kg). The starting residue levels of ETU were <0.05 to 0.21 mg/kg, which were in a similar concentration range found in pomace and must (<0.05 to 0.41 mg/kg). In young and mature wine, residues of ETU were detected with concentrations between 0.15 and 0.43 mg/kg. However, residues in red wine samples were slightly higher than in rosé wine samples - a difference which is probably associated with heating during processing.

Table 6.5.3-15: The BAS 222 F and ETU residues in grapes and corresponding transfer factors of process fractions thereof after ten applications with BAS 222 28 F. The transfer factors were re-calculated based on the initial CS_2 values for metiram and ETU

RAC	Trial location	residue level CS_2	residue level Metiram	PF	residue level CS_2	PF CS_2	Mean PF CS_2	residue level ETU	PF ETU (based on CS_2)	Mean PF ETU (in % CS_2)
grape	France	4.02	7.92	rose pomace	3.39	0.84	0.99	0.05	0.01	0.07
grape	France	2.65	5.22	rose pomace	3.08	1.16		0.76	0.25	
grape	France	1.74	3.43	rose pomace	2.36	1.35		0.05	0.02	
grape	France	5.65	11.13	rose pomace	3.32	0.59		0.07	0.01	
grape	France	4.02	7.92	rose must	1.38	0.34	0.57	0.05	0.01	0.02
grape	France	2.65	5.22	rose must	1.47	0.55		0.05	0.02	
grape	France	1.74	3.43	rose must	1.60	0.92		0.05	0.02	
grape	France	5.65	11.13	rose must	2.68	0.47		0.09	0.01	
grape	France	4.02	7.92	young rose wine	<0.05	0.01	0.02	0.17	0.04	0.06
grape	France	2.65	5.22	young rose wine	<0.05	0.02		0.25	0.08	
grape	France	1.74	3.43	young rose wine	<0.05	0.03		0.16	0.08	
grape	France	5.65	11.13	young rose wine	0.09	0.02		0.28	0.04	
grape	France	4.02	7.92	mature rose wine	<0.05	0.01	0.02	0.16	0.03	0.04
grape	France	2.65	5.22	mature rose wine	<0.05	0.02		0.03	0.01	
grape	France	1.74	3.43	mature rose wine	<0.05	0.03		0.15	0.07	
grape	France	5.65	11.13	mature rose wine	0.09	0.02		0.29	0.04	
grape	France	4.02	7.92	red pomace	2.78	0.69	1.11	0.10	0.02	0.03
grape	France	2.65	5.22	red pomace	3.48	1.31		0.07	0.02	
grape	France	1.74	3.43	red pomace	1.84	1.63		0.08	0.04	
grape	France	5.65	11.13	red pomace	4.54	0.80		0.12	0.02	
grape	France	4.02	7.92	red must	1.95	0.48	0.48	0.19	0.04	0.05
grape	France	2.65	5.22	red must	1.52	0.58		0.06	0.02	
grape	France	1.74	3.43	red must	0.98	0.56		0.16	0.08	
grape	France	5.65	11.13	red must	1.79	0.32		0.41	0.06	

RAC	Trial location	residue level CS_2	residue level Metiram	PF	residue level CS_2	PF CS_2	Mean PF CS_2	residue level ETU	PF ETU (based on CS_2)	Mean PF ETU (in % CS_2)
grape	France	4.02	7.92	young red wine	<0.05	0.01	0.02	0.24	0.05	0.08
grape	France	2.65	5.22	young red wine	<0.05	0.02		0.20	0.06	
grape	France	1.74	3.43	young red wine	<0.05	0.03		0.24	0.12	
grape	France	5.65	11.13	young red wine	0.17	0.03		0.44	0.07	
grape	France	4.02	7.92	mature red wine	<0.05	0.01	0.02	0.22	0.05	0.07
grape	France	2.65	5.22	mature red wine	<0.05	0.02		0.19	0.06	
grape	France	1.74	3.43	mature red wine	<0.05	0.03		0.22	0.11	
grape	France	5.65	11.13	mature red wine	0.20	0.03		0.43	0.07	

Residues of carbon disulfide in grape RAC material used for processing were at concentrations ranging from 1.74 to 5.65 mg/kg on DALA 21. The transfer factors of pomace (mean transfer factor: 0.99-1.11) and in must (mean transfer factor: 0.48-0.57), but also in young (mean transfer factor: 0.02) and mature wine (mean transfer factor: 0.02) clearly evidenced that no significant enrichment of BAS 222 F occurred during processing. The mean transfer factors of ETU, for the different processing fractions were quite low and ranged from 0.02 in rosé must and 0.08 in young red wine.

III. CONCLUSION

This supervised field trial study showed that the residues of BAS 222 F after ten applications of BAS 222 28 F ranged from 4.65 to 8.94 mg/kg in wine grape RACs. After processing, no significant enrichment of BAS 222 F in any of the investigated processing fractions was determined. Generally, the transfer factors for BAS 222 F were below 1. Highest transfer factor was calculated for rose pomace (mean transfer factor: 0.99) and red pomace (mean transfer factor: 1.11). The ETU concentrations in grapes, pomace and must were below or close to the limit of quantification and increased slightly in young and mature wine samples. Nevertheless, the mean transfer factors were quite low and ranged from 0.02 to 0.08.

Report:	*CA 6.5.3/7* *Mamouni A., 1994a* *Determination of residues of Metiram (CS2 and ETU) in grapes following treatment with Polyram DF (=BAS 222 28 F) under field conditions in Italy, 1992, and in industrial grape processing products* *1994/10693*
Guidelines:	*BBA IV 3-1, BBA IV 3-3, BBA IV 3-4*
GLP:	*yes* *(certified by Eidgenoessisches Departement des Innern, Bern, Schweiz)*

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
   Description: Polyram DF WG
   Lot/Batch #: 92-2
   Purity: BAS 222 28 F (BAS 222 F 80%, nominal)
   CAS#: 9006-42-2
   Development code:
   Metiram: BAS 222 F
   ETU: M222F002
   Spiking levels: BAS 222 F (between 0.05 and 3445.0 mg/kg)
   ETU (between 0.02 and 0.30 mg/kg)

2. **Test Commodity:**
   Crop: grapes
   Type: not applicable
   Variety: Traminer-Aromatico, Barbera
   Botanical name: *Vitis vinifera*
   Crop parts(s) or processed commodity: grape (fruit), must cold, must heated, maturing wine, mature wine
   Sample size: 2.0 - 100.0 kg

B. STUDY DESIGN

1. Test procedure

During the 1992 growing season, two field trials were conducted at two locations (namely Cormons and San Brizio) in representative wine grape growing regions in Italy to determine the residue levels of BAS 222 F and ETU in grapes and processed wine fractions thereof. The fungicidal test substance Polyram DF (80% BAS 222 F, WG) was applied 8 times by spray application rates of 2.0 to 4.5 kg/ha (corresponding to 1.6 to 3.6 kg a.i./ha) in a spray volume of 300-400 l/ha beginning at growing stage 7-9 (BBCH). For the analysis of wine grape RACs, samples were collected either directly after the last application as well as on DALA 28 and DALA 35 (± 1 day). The wine grapes were used for production of wine using different processing techniques resulting in the following fractions: fruits (RAC), must cold before heating, must heated, must (mash fermented), must (mash heated), maturing wine and mature wine. All processing steps were carried out according to commercial procedures.

2. Description of analytical procedures

The samples were analyzed for BAS 222 F and ETU by means of gas chromatography according to analytical method supplied by RCC UMWELTCHEMIE AG. To the analytical material which contains BAS 222 F, a solution of stannous chloride and hydrochloric acid was added and heated to yield carbon disulfide. The carbon disulfide was distilled, purified and trapped by dissolution in ethanol. Following gas chromatographic separation, the generated carbon disulfide was determined with a flame photo-metric detector (S-FPD, specific for sulfur). The mean procedural recovery were about 95.8±17.9% (n=14; fortification level between 0.05 and 3445.0 mg/kg) and the limit of quantification was 0.03 mg/kg (expressed as carbon disulfide). ETU was extracted from the matrix with methanol. The methanol was evaporated and the remaining water phase was extracted with n-hexane and cleaned up on an aluminium oxide column. The aqueous eluate was concentrated and extracted by partitioning with dichloromethane. The dichloromethane extract was concentrated and cleaned up on an aluminium oxide column. After concentration of the eluate, the residue was determined by gas chromatography using a sulfur specific flame photometric detector. The mean procedural recovery were about 90.4±15.6% (n=12; fortification level between 0.02 and 0.30 mg/kg) and the limit of quantification was 0.02 mg/kg.

II. RESULTS AND DISCUSSION

The residues of BAS 222 F and ETU in grapes and process fractions thereof as well as transfer factors are provided in Table 6.5.3-16 and Table 6.5.3-17.

Table 6.5.3-16: Residues of BAS 222 F (as carbon disulfide) and ETU in grapes and process fractions thereof after eight applications with BAS 222 28 F

Location	Portion analyzed	Residues (mg/kg)		
		carbon disulfide	corrected carbon disulfide*	ETU
Cormons (Italy)	grapes (DALA 0)	4.25	3.98	0.22
	grapes (DALA 28)	2.91	2.55	<0.02
	must cold before heating	0.54	0.46	0.03
	must heated	1.64	1.64	0.05
	maturing wine (A)	0.05	0.01	0.07
	maturing wine (B)	0.05	0.00	0.07
	wine (A)	0.04	0.00	0.07
	wine (B)	0.04	0.00	0.05
	grapes (DALA 45)	0.14	0.00	<0.02
San Brizio (Italy)	grapes (DALA 0)	23.73	23.36	0.57
	grapes (DALA 29)	4.09	3.97	<0.02
	must cold (A)	1.95	1.88	<0.02
	must mash fermented (B2)	1.04	1.00	<0.02
	must mash heated (C2)	2.31	2.23	0.03
	maturing wine (A2)	<0.03	<0.03	0.07
	maturing wine (B2)	<0.03	<0.03	0.04
	maturing wine (C)	<0.03	<0.03	0.06
	wine (A2)	0.04	0.04	0.04
	wine (B2)	0.04	0.04	0.02
	wine (C)	0.03	0.03	0.05
	grapes (DALA 45)	2.06	1.96	<0.02

Cormons: control samples contained 0.27 mg/kg BAS 222 F (DALA 0), 0.36 mg/kg BAS 222 F on DALA 28 and 0.19 mg/kg BAS 222 F on DALA 35. ETU was below <0.02 mg/kg at each harvesting
San Brizio: control samples contained 0.37 mg/kg BAS 222 F (DALA 0), 0.12 mg/kg BAS 222 F on DALA 29 and 0.10 mg/kg BAS 222 F on DALA 35. ETU was below <0.02 mg/kg at each harvesting.
* calculated as treated minus control
(A) = wine from cold must; (B) wine from heated must; (B2) wine from fermented must and mash; (C) wine from heated must and mash

The carbon disulfide residue found in the grapes harvested immediately after the eighth treatment amounted to 3.98 and 23.36 mg/kg in grapes from Cormons and San Brizio fields, respectively. Thereafter, the carbon disulfide residues decreased rapidly. After 35 days from the last treatment, no carbon disulfide residues in Cormons grapes and 1.96 mg/kg in San Brizio grapes were detected. In the control samples carbon disulfide residues up to 0.37 mg/kg grapes were detected. The treated samples were corrected by subtraction of the amounts found in the control samples. The grapes harvested 28 and 29 days from Cormons (2.55 mg carbon disulfide/kg grapes) and San Brizio fields (3.97 carbon disulfide/kg grapes), respectively, were further processed to wine. In the must obtained from Cormons grapes, the carbon disulfide residues detected amounted to 0.46 and 1.64 mg/kg must before and after heating, respectively. A decrease in carbon disulfide concentration was observed after fermentation of the must by yeast. The carbon disulfide residues as result from the eight treatments in maturing wine and wine were very low and amounted to a maximum to 0.01 mg/kg wine. Three sorts of must or wine were obtained from San Brizio grapes: by pressing directly the grapes (A), after fermentation of the must with the mash for 4 days (B) or after heating the must with the mash (C).

The carbon disulfide residues in the must A, B and C amounted to 1.88, 1.00 and 2.23 mg/kg. After fermentation the carbon disulfide residues decreased rapidly and amounted <0.03 mg/kg in maturing wine and up to 0.04 mg/kg in wine. In the must from processed control samples from both fields, the amounts of carbon disulfide residues amounted to a maximum of 0.08 mg/kg. Approximately, the same residues were detected in the treated and control wine samples and did not exceed 0.05mg carbon disulfide/kg wine (Cormons). These results showed that BAS 222 F was completely degraded during the first days of fermentation. In the analysed whole grapes, ETU residues were detected only in the treated samples harvested immediately after the eighth treatment and amounted to 0.22 and 0.57 mg/kg grapes from Cormons and San Brizio fields, respectively. Thereafter, all analysed samples showed ETU concentration lower than 0.02 mg/kg. However, ETU residues were observed in the treated processed products and amounted up to 0.05 and 0.07 mg ETU/kg in the must and wine obtained from Cormons grapes, respectively. The corresponding values in the must, maturing wine and wine obtained from San Brizio grapes were 0.03, 0.07 and 0.05 mg ETU /kg, respectively.

Table 6.5.3-17: Transfer factors of BAS 222 F (carbon disulfide) and ETU in grapes and process fractions thereof after eight applications with BAS 222 28 F. The transfer factors were re-calculated based on the initial CS_2 values for metiram and ETU.

Location	Portion analyzed	Residues (mg/kg)			
		carbon disulfide	Transfer factor	ETU	Transfer factor
Cormons (Italy)	grapes (DALA 28)	2.91	n/a	<0.02	n/a
	must cold before heating	0.54	0.19	0.03	0.01
	must heated	1.64	0.56	0.05	0.02
	maturing wine (A)	0.05	0.02	0.07	0.02
	maturing wine (B)	0.05	0.02	0.07	0.02
	wine (A)	0.04	0.01	0.07	0.02
	wine (B)	0.04	0.01	0.05	0.02
San Brizio (Italy)	grapes (DALA 29)	4.09	n/a	<0.02	n/a
	must cold (A)	1.95	0.48	<0.02	0.01
	must mash fermented (B2)	1.04	0.25	<0.02	0.01
	must mash heated (C2)	2.31	0.56	0.03	0.01
	maturing wine (A2)	0.01	<0.01	0.07	0.02
	maturing wine (B2)	0.01	<0.01	0.04	0.01
	maturing wine (C)	0.01	<0.01	0.06	0.01
	wine (A2)	0.01	0.01	0.04	0.01
	wine (B2)	0.01	0.01	0.02	0.01
	wine (C)	0.01	0.01	0.05	0.01

(A) = wine from cold must; (B) wine from heated must; (B2) wine from fermented must and mash; (C) wine from heated must and mash

During processing, no considerable enrichment of BAS 222 F was observed; the transfer factors were below 1 in each of the processed wine fractions. The individual transfer factors ranged from 0.01 in maturing and matured wine fractions up to 0.56 in wine must samples. Low transfer factors were also determined in regard to ETU; the individual transfer factors were below 0.02 in each process fraction.

III. CONCLUSION

This supervised field trial study showed that the residues of BAS 222 F in wine grape RACs after eight applications of BAS 222 28 F ranged from 9.98 to 23.36 mg/kg on DALA 0 and from 2.55 to 3.97 mg/kg on DALA 28-29. After processing, no significant enrichment of BAS 222 F was observed, rather the residual concentrations declined to values slightly above or below the limit of detection. The individual transfer factors ranged from 0.01 in maturing and matured wine fractions up to 0.56 in wine must samples. No considerable enrichment of ETU (indicated by transfer factors >1.0) were determined in any of the processed fractions. The individual transfer factors were below 0.02 in each process fraction.

Report: *CA 6.5.3/8*
Schulz H., 2007a
Study on the residue behaviour of Metiram in onions and processed products after treatment with BAS 222 28 F under field conditions in Germany, 2005
2007/1017185

Guidelines: *EEC 91/414 Annex II (Part A Section 6), EEC 91/414 Annex III (Part A Section 8), EEC 1607/VI/97 rev. 2 10.06.1999, EEC 7029/VI/95 rev. 5, EEC 7525/VI/95 rev. 7, EEC 7035/VI/95 rev. 5, EEC 96/46, SANCO/825/00 rev. 7 (17 March 2004)*

GLP: *yes*
(certified by Hessisches Ministerium fuer Umwelt, Laendlichen Raum und Verbraucherschutz)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
   Description: BAS 222 28 F
   Lot/Batch #: 18792 (BAS 222 28 F)
   Purity: BAS 222 28 F (BAS 222 F 70%, nominal)
   CAS#: 9000-42-2
   Development code:
   Metiram: BAS 222 F
   ETU: M222F002
   Spiking levels: BAS 222 F (0.3, 1.0, 3.0, 5.0, 9.0, 15.0 and 30.0 mg/kg)
   ETU (0.01, 0.02, 0.1 and 1.0 mg/kg)

2. **Test Commodity:**
   Crop: onion
   Type: not applicable
   Variety: Bravo, Hystzin, Stuttgarter Riesen, Copra F1
   Botanical name: *Allium cepa*
   Crop parts(s) or processed commodity: peel, peeled onions, wash water, dried onions
   Sample size: 1.0 - 25.0 kg

B. STUDY DESIGN

1. Test procedure

During the 2005 growing season, four field trials were conducted in representative plum growing regions in Germany to determine the residue levels of BAS 222 F and ETU in onion as well as the process fractions involved in the production of dried onions. The fungicidal test substance BAS 222 28 F (70% BAS 222 F, WG) was applied six times by spray application rates of 5.4 kg/ha (corresponding to 3.78 kg a.i./ha) in a spray volume of 300 I/ha (first 3 applications) and 500 I/ha (last 3 applications) beginning 42 days before expected harvest with a spray interval of 7 days. For the residue analysis of onions, whole plants (without roots) were collected directly after the last application and bulbs were sampled 6 to 7 days later. The bulbs were processed to the following products: peel, peeled onions, wash water, dried onions. All processing steps were carried out according to commercial procedures. Specimens were shipped frozen and remained at temperatures ≤-18°C until analytical measurements.

2. Description of analytical procedures

The active substance BAS 222 F was analyzed according to Institute Fresenius method no. IF-05/00449055. The active substance was degraded to carbon disulfide by means of orthophosphoric acid and passed into isooctane by a stream of nitrogen. The final determination was carried out using GC-MS detection. The ion fragment m/z 76 was used for evaluation. The mean procedural recovery was about 76.2±10.5% (n=14; fortification level 0.3, 1.0, 3.0, 5.0, 9.0, 15.0 and 30.0 mg/kg) and the limit of detection was 0.3 mg/kg BAS 222 F (corresponding to 0.168 mg/kg carbon disulfide). The ETU was extracted from the plant material with a mixture of sodium ascorbate, ethylene urea, ammonium chloride and methanol-water. After centrifugation, an aliquot was taken and the methanol was evaporated from the extract. The pH is adjusted to 8 before evaporating (in some cases after evaporation).

The remaining water phase was cleaned by liquid/liquid partition (water/dichloromethane) on an Extrelut/AI_2O_3 column. After concentration of the eluate, the residue was determined by HPLC using pulsed amperometric detection. In this study, the final determination is carried out using LC-MS/MS detection. The fragment ion 103 → 44 was used for evaluation (BASF method No. 373/3). The mean procedural recovery was about 97.2±10.1% (n=12; fortification level 0.01, 0.02, 0.1 and 1.0 mg/kg) and the limit of detection was 0.01 mg/kg.

II. RESULTS AND DISCUSSION

The residues of BAS 222 F and ETU in onions, process fractions thereof and correspondent transfer factors are provided in Table 6.5.3-18 to Table 6.5.3-21.
The processing factors included in both tables which are also presented in the report were calculated based on the individual residue levels in the RAC. In order to take the formation of the metabolite ETU from parent residues into account, a different equation has to be used for dithiocarbamate fungicides.

Table 6.5.3-18: Residues and transfer factors of BAS 222 F in onions and process fractions thereof after four applications with BAS 222 28 F

	Residues (mg/kg) Carbon disulfide				Transfer factor				
Trial No.	**ACK/ 02/05/**	**ACK/ 03/05/**	**AGR/ 10/05/**	**AGR/ 11/05/**	**ACK/ 02/05/**	**/ACK/ 03/05**	**AGR/ 10/05/**	**AGR/ 11/05/**	**mean**
whole plant, no roots (DALA 0)	4.71	10.09	6.08	10.95	n.a.	n.a.	n.a.	n.a.	n.a.
bulb RAC (DALA 6-7)	0.783	0.482	<0.3	0.491	1	1	n.a.	1	1
peel	7.65	2.74	3.59	5.48	9.77	5.68	n.c.	11.16	8.87
peeled onion	<0.3	<0.3	<0.3	<0.3	<0.38	<0.62	n.c.	<0.61	0.54
wash water	0.545	0.785	0.630	0.695	0.70	1.36	n.c.	1.42	1.25
dried onion	0.529	1.21	0.780	1.12	0.68	2.51	n.c.	2.28	1.82

DALA = days after last treatment
n.c. = not calculated; n.a. = not applicable
The transfer factors <0.38, <0.62 and <0.61 were calculated as 0.38, 0.62 and 0.61

Table 6.5.3-19: Residues and transfer factors of BAS 222 F in onions and process fractions thereof after four applications with BAS 222 28 F

	Residues (mg/kg) ETU				Transfer factor				
Trial No.	**ACK/ 02/05/**	**ACK/ 03/05/**	**AGR/ 10/05/**	**AGR/ 11/05/**	**ACK/ 02/05/**	**/ACK/ 03/05**	**AGR/ 10/05/**	**AGR/ 11/05/**	**mean**
whole plant, no roots (DALA 0)	0.060	0.084	0.042	0.079	n.a.	n.a.	n.a.	n.a.	n.a.
bulb RAC (DALA 6-7)	0.014	0.014	<0.01	<0.01	1	1	n.a.	1	n.c.
peel	0.238	0.075	0.040	0.081	16.86	5.36	n.c.	n.c.	11.11
peeled onion	<0.01	<0.01	<0.01	<0.01	<0.71	<0.71	n.c.	n.c.	n.c.
wash water	<0.01	<0.01	<0.01	<0.01	<0.71	<0.71	n.c.	n.c.	n.c.
dried onion	0.019	0.018	0.028	0.032	1.36	1.29	n.c.	n.c.	1.33

DALA = days after last treatment
n.c. = not calculated; n.a. = not applicable

In the treated whole plant (no roots) specimens at 0 DALA, the residues of metiram ranged from 4.71-10.95 mg/kg. In the bulbs specimens at 6-7 DALA (7 = PHI), the residues had dropped to <0.3-0.783 mg/kg. Out of the processed specimens, only peeled onions had residues below the limit of quantification. The residues of metiram in the remaining specimens were highest in peel (2.74-7.65 mg/kg), followed by dried onions (0.529-1.21 mg/kg) and wash water (0.545-0.785 mg/kg). In peeled onions and in one out of four trials of the wash water and the dried onions, the transfer factors were below 1. The residues of ETU in the treated whole plant (no roots) specimens at 0 DALA were 0.042-0.084 mg/kg. At 6-7 DALA (7=PHI), they had decreased to <0.01-0.014 mg/kg. In the processed specimens peeled onions and wash water, no residues above the limit of quantification were analysed. In the specimens peel and dried onions, residues of ETU were found in the range of 0.040-0.236 mg/kg and 0.018-0.032 mg/kg, respectively. In peeled onions and wash water, the transfer factors were below 1.

The processing recoveries of metiram and ETU in the processed fractions were as follows, whereby the initial amounts of metiram in the RAC specimens were set as 100 %: The summary of the recoveries for the different processing steps is presented in the following table. Since the calculation of the mass balance is not relevant for the dietary exposure assessment, the table is copied directly from the report.

Table 6.5.3-20: Distribution of BAS 222 F (expressed as carbon disulfide) and ETU residues in the processed products related to onion RAC

Trial No.	Percent recovery of BAS 222 F and ETU							
	Carbon disulfide				ETU			
	ACK/ 02/05/	ACK/ 03/05/	AGR/ 10/05/	AGR/ 11/05/	ACK/ 02/05/	ACK/ 03/05/	AGR/ 10/05/	AGR/ 11/05/
bulbs RAC (DALA 6-7))	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
peel	133.3	88.7	n.c.	202.1	229.9	83.6	n.c.	n.c.
peeled onion	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
wash water	32.4	64.7	n.c.	63.1	n.c.	n.c.	n.c.	n.c.
dried onion	5.8	28.5	n.c.	26.3	11.6	14.6	n.c.	n.c.

DALA = days after last treatment
n.a. = not applicable

Based on the residue levels found in onions at 7 DAT, the processing factors were recalculated using the following equations:

$$PF_{metiram} = \frac{\text{Conc. metiram (or } CS_2\text{) in processed fraction}}{\text{Conc. metiram (or } CS_2\text{) in raw agricultural commodity}}$$

$$PF_{ETU} = \frac{\text{Conc. ETU in process fraction}}{\text{Conc. metiram (as } CS2\text{) in raw commodity}}$$

For calculation purposes, residue levels at the LOQ were set at 0.3 mg/kg and 0.01 mg/kg, respectively.

Table 6.5.3-21: Onions: Re-Calculation of processing factors for metiram and ETU

RAC	residue level CS_2	residue level Metiram	PF	residue level CS_2	PF CS_2	Mean PF CS_2	residue level ETU	PF ETU	Mean PF ETU
onion	0.78	1.402	peel	7.65	9.8	9.6	0.238	0.3	0.2
onion	0.48	0.863	peel	2.74	5.7		0.075	0.2	
onion	0.30	0.537	peel	3.59	12.0		0.04	0.1	
onion	0.49	0.879	peel	5.48	11.2		0.081	0.2	
onion	0.78	1.402	peeled onion	0.3	0.4	0.7	0.01	0.0	0.0
onion	0.48	0.863	peeled onion	0.3	0.6		0.01	0.0	
onion	0.30	0.537	peeled onion	0.3	1.0		0.01	0.0	
onion	0.49	0.879	peeled onion	0.3	0.6		0.01	0.0	
onion	0.78	1.402	wash water	0.545	0.7	1.5	0.01	0.0	0.0
onion	0.48	0.863	wash water	0.785	1.6		0.01	0.0	
onion	0.30	0.537	wash water	0.630	2.1		0.01	0.0	
onion	0.49	0.879	wash water	0.695	1.4		0.01	0.0	
onion	0.78	1.402	dried onion	0.529	0.7	2.0	0.019	0.0	0.043
onion	0.48	0.863	dried onion	1.21	2.5		0.018	0.0	
onion	0.30	0.537	dried onion	0.780	2.6		0.028	0.1	
onion	0.49	0.879	dried onion	1.12	2.3		0.032	0.1	

III. CONCLUSION

This processing study showed that the residues of BAS 222 F (expressed as carbon disulfide) in onions after six applications of BAS 222 28 F ranged from 4.71 to 10.95 mg/kg (whole plant w/o roots) on DALA 0 and from <0.3 to 0.783 mg/kg on DALA 6-7, which were the starting concentrations for the processing. After processing, no enrichment of BAS 222 F (indicated by a mean transfer factor <1.0) was observed in peeled onions residue: <0.3 mg/kg; mean transfer factor 0.54). In the remaining processed fractions, the transfer factor was >1.0, namely in peel (residue: 2.74-7.65 mg/kg; mean transfer factor 8.87) and in dried onions residue: (0.529-1.21 mg/kg; mean transfer factor 1.82). In terms of ETU, significant enrichment was observed in peel (residue: 0.040-0.283 mg/kg; mean transfer factor 11.11), whereas in dried onion the ETU enrichment was comparatively low (residue: 1.29-1.36 mg/kg; mean transfer factor 1.33). In the remaining processed fraction, the peeled onion, the transfer factors were <1.0; (residue: <0.01 mg/kg; transfer factor <0.71).

Report:	*CA 6.5.3/9* *Schulz H.,Scharm M., 2002a* *Determination of the residues of Metiram and ETU in tomatoes and processed products following treatment with BAS 222 28 F in greenhouses in Germany, the Netherlands and Belgium 2001* *2002/1004075*
Guidelines:	*BBA IV 3-3, BBA IV 3-4, IVA Guidelines for Residue Studies, Sections IA and IB, 2nd edition 1992*
GLP:	*yes* *(certified by Hessisches Ministerium fuer Umwelt, Landwirtschaft und Forsten, Wiesbaden)*

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
   Description: Polyram DF
   Lot/Batch #: 2000-2
   Purity: BAS 222 28 F (BAS 222 F 70%, nominal)
   CAS#: 9006-42-2
   Development code:
   Metiram: BAS 222 F
   ETU: M222F002
   Spiking levels: BAS 222 F (0.05 and 5.0 mg/kg)
   ETU (between 0.01 and 0.20 mg/kg)

2. **Test Commodity:**
   Crop: tomato
   Type: not applicable
   Variety: Cederico R2, Delicimo, Centiro, Cheers
   Botanical name: *Solanum lycopersicum*
   Crop parts(s) or processed commodity: washed tomatoes, wash water, cooking water, peeled tomatoes, peels, canned tomatoes, vegetable stock, tomato puree, condensed water, tomato juice, remainder of the straining process
   Sample size: 1.0 - 20.0 kg

B. STUDY DESIGN

1. Test procedure

During the 2001 growing season, four field trials were conducted in four representative tomato growing regions in Germany, Belgium and the Netherlands to determine the residue levels of BAS 222 F and ETU in tomatoes as well as the process fractions involved in the production of canned tomato, tomato puree and tomato juice. The fungicidal test substance BAS 222 28 F (70% BAS 222 F, WG) was applied 8 times by spray application rates of 2.0 kg/ha (corresponding to 1.4 kg a.i./ha) in a spray volume of 800-1000 l/ha beginning 63 days before expected harvest with a spray interval of 7 days. For the analysis of tomatoes RAC, samples were collected directly after the last application as well as on a second sampling 13 to 15 days later. The later tomatoes were used for the following products: washed tomatoes, wash water, cooking water, peeled tomatoes, peels, canned tomatoes, vegetable stock, tomato puree, condensed water, tomato juice, remainder of the straining process. All processing steps were carried out according to commercial procedures. Specimens were shipped frozen and remained at temperatures below -18°C until analysis.

2. Description of analytical procedures

The samples were analyzed according to BASF method no. 135/4 and BASF method no. 373/2 for BAS 222 F and ETU, respectively. The active substance was converted into carbon disulfide by treating the sample with a refluxing aqueous solution of hydrochloric acid in the presence of tin(II) chloride. The carbon disulfide was trapped in a methanol solution of potassium hydroxide and was determined by UV detection at 302 nm of the xanthogenate formed. The mean procedural recovery were about 78.6±20.5% (n=31; fortification level between 0.05 and 5.0 mg/kg) and the limit of quantification was 0.05 mg/kg. The ETU was extracted from the plant material with a mixture of sodium ascorbate, ethyleneurea, ammonium chloride and methanol-water. After centrifugation an aliquot was taken and the methanol was evaporated from the extract. The pH was adjusted to 8 before evaporating. The remaining water phase was cleaned by liquid/liquid (water/dichloromethane) partition on an Extrelut/aluminium oxide column. After concentration of the eluate, the residue was determined by HPLC using pulsed amperometric detection. The mean procedural recovery were about 78.3±13.3% (n=48; fortification level between 0.01 and 0.20 mg/kg) and the limit of quantification was 0.01 mg/kg.

II. RESULTS AND DISCUSSION

The residues of BAS 222 F and ETU in tomatoes and process fractions thereof as well as transfer factors are provided in Table 6.5.3-22 and Table 6.5.3-23.

Table 6.5.3-22: Residues of BAS 222 F and ETU in tomatoes and process fractions thereof after eight applications with BAS 222 28 F

Trial No.	Portion analyzed	DALA (nominal)	Residues (mg/kg)		
			BAS 222 F	carbon disulfide	ETU
AGR 50	tomato, fruit	0	2.44	1.36	0.026
	tomato, fruit	14	2.46	1.38	0.037
	tomato, fruit*	14*	1.39	0.78	0.026
	fruit, washed	n/a	0.16	0.09	0.017
	wash water	n/a	0.87	0.49	<0.01
	cooking water	n/a	<0.05	<0.03	<0.01
	fruit, peeled	n/a	0.07	0.04	0.020
	peel	n/a	8.20	4.59	0.059
	fruit, canned	n/a	0.07	0.04	0.034
	vegetable stock	n/a	0.05	0.03	0.066
	tomato puree	n/a	0.74	0.41	0.351
	remainder	n/a	0.24	0.13	0.047
	condensed water	n/a	0.05	0.03	<0.01
	tomato juice	n/a	0.09	0.05	0.108
AGR 51	tomato, fruit	0	2.33	1.30	0.033
	tomato, fruit	14	3.12	1.75	0.028
	tomato, fruit*	14*	1.01	0.57	0.021
	fruit, washed	n/a	0.22	0.12	0.012
	wash water	n/a	1.34	0.75	<0.01
	cooking water	n/a	0.06	0.03	0.025
	fruit, peeled	n/a	0.07	0.04	<0.01
	peel	n/a	8.84	4.95	0.074
	fruit, canned	n/a	0.06	0.04	0.033
	vegetable stock	n/a	0.06	0.03	0.067
	tomato puree	n/a	0.65	0.36	0.388
	remainder	n/a	0.22	0.12	0.046
	condensed water	n/a	0.06	0.04	<0.01
	tomato juice	n/a	0.08	0.04	0.102
AGR 52	tomato, fruit	0	1.73	0.97	0.023
	tomato, fruit	14	0.82	0.46	<0.01
	tomato, fruit*	14*	0.42	0.24	0.014
	fruit, washed	n/a	0.16	0.09	<0.01
	wash water	n/a	0.06	0.03	<0.01
	cooking water	n/a	<0.05	<0.03	<0.01
	fruit, peeled	n/a	<0.05	<0.03	<0.01
	peel	n/a	5.15	2.88	0.032
	fruit, canned	n/a	<0.05	<0.03	0.011
	vegetable stock	n/a	<0.05	<0.03	0.029
	tomato puree	n/a	0.59	0.33	0.122
	remainder	n/a	0.12	0.07	0.018
	condensed water	n/a	<0.05	<0.03	<0.01
	tomato juice	n/a	0.07	0.04	0.041

Trial No.	Portion analyzed	DALA (nominal)	Residues (mg/kg)		
			BAS 222 F	carbon disulfide	ETU
AGR 53	tomato, fruit	0	3.03	1.70	0.021
	tomato, fruit	14	1.83	1.03	0.026
	tomato, fruit*	14*	1.18	0.66	0.022
	fruit, washed	n/a	0.27	0.15	0.013
	wash water	n/a	0.75	0.42	<0.01
	cooking water	n/a	<0.05	<0.03	<0.01
	fruit, peeled	n/a	0.06	0.04	0.012
	peel	n/a	4.79	2.68	0.053
	fruit, canned	n/a	0.06	0.03	0.026
	vegetable stock	n/a	0.06	0.03	0.036
	tomato puree	n/a	0.39	0.22	0.216
	remainder	n/a	0.12	0.07	0.026
	condensed water	n/a	0.05	0.03	<0.01
	tomato juice	n/a	0.06	0.03	0.070

DALA = days after last treatment
n/a = not applicable
* used for processing (retained samples)

Directly after the last application, BAS 222 F was found to range between 1.73 and 3.03 mg/kg in tomatoes corresponding to 0.97 to 1.70 mg/kg carbon disulfide. These residues degraded during the course of the study ranging between 0.82 to 3.12 mg/kg of BAS 222 F (0.46 to 1.75 mg/kg carbon disulfide) after about two weeks. Retained tomato samples, which were used for processing, contained 0.42 to 1.39 mg/kg BAS 222 F (corresponding to 0.24 to 0.78 mg/kg carbon disulfide). Residual concentrations higher than 1.0 mg/kg were detected in particular in the peel (range: 4.79 to 8.84 mg/kg; corresponding to 2.68 to 4.95 mg/kg carbon disulfide), but also in wash water (range: 0.06 to 1.34 mg/kg; corresponding to 0.03 to 0.75 mg/kg carbon disulfide). In the other processed fractions, the levels of BAS 222 F were either below the limit of detection (<0.05 mg/kg) or 0.75 mg/kg (0.42 mg/kg carbon disulfide) at the most.

Table 6.5.3-23: The BAS 222 F and ETU residues in tomatoes and corresponding transfer factors of process fractions thereof after eight applications with BAS 222 28 F. The transfer factors were re-calculated based on the initial CS_2 values for metiram and ETU

RAC	Trial location	residue level CS_2	residue level Metiram	PF	residue level CS_2	PF CS_2	Mean PF CS_2	residue level ETU	PF ETU (based on CS_2)	Mean PF ETU (in %CS_2)
tomato	Germany	1.38	1.39	washed fruit	0.09	0.07	0.11	0.02	0.01	0.01
tomato	Germany	1.75	1.01	washed fruit	0.12	0.05		0.01	0.01	
tomato	Belgium	0.46	0.42	washed fruit	0.09	0.20		<0.01	0.02	
tomato	The Netherland	1.03	1.18	washed fruit	0.15	0.15		0.01	0.01	
tomato	Germany	1.38	1.39	wash water	0.49	0.36	0.31	<0.01	0.04	0.04
tomato	Germany	1.75	1.01	wash water	0.75	0.43		<0.01	0.04	
tomato	Belgium	0.46	0.42	wash water	0.03	0.07		<0.01	0.04	
tomato	The Netherland	1.03	1.18	wash water	0.42	0.41		<0.01	0.04	
tomato	Germany	1.38	1.39	cooking water	0.03	0.02	0.03	<0.01	0.04	0.03
tomato	Germany	1.75	1.01	cooking water	0.03	0.02		0.03	0.01	
tomato	Belgium	0.46	0.42	cooking water	0.03	0.07		<0.01	0.04	
tomato	The Netherland	1.03	1.18	cooking water	0.03	0.03		<0.01	0.04	

RAC	Trial location	residue level CS_2	residue level Metiram	PF	residue level CS_2	PF CS_2	Mean PF CS_2	residue level ETU	PF ETU (based on CS_2)	Mean PF ETU (in %CS_2)
tomato	Germany	1.38	1.39	fruit peeled	0.04	0.03	0.04	0.02	0.01	0.03
tomato	Germany	1.75	1.01	fruit peeled	0.04	0.02		<0.01	0.04	
tomato	Belgium	0.46	0.42	fruit peeled	0.03	0.07		<0.01	0.04	
tomato	The Netherland	1.03	1.18	fruit peeled	0.04	0.04		0.01	0.01	
tomato	Germany	1.38	1.39	peel	4.59	3.33	3.75	0.06	0.04	0.05
tomato	Germany	1.75	1.01	peel	4.95	2.83		0.07	0.04	
tomato	Belgium	0.46	0.42	peel	2.88	6.26		0.03	0.07	
tomato	The Netherland	1.03	1.18	peel	2.68	2.60		0.05	0.05	
tomato	Germany	1.38	1.39	fruit canned	0.04	0.03	0.04	0.03	0.02	0.02
tomato	Germany	1.75	1.01	fruit canned	0.04	0.02		0.03	0.02	
tomato	Belgium	0.46	0.42	fruit canned	0.03	0.07		0.01	0.02	
tomato	The Netherland	1.03	1.18	fruit canned	0.03	0.03		0.03	0.03	
tomato	Germany	1.38	1.39	vegetable stock	0.03	0.02	0.03	0.07	0.05	0.05
tomato	Germany	1.75	1.01	vegetable stock	0.03	0.02		0.07	0.04	
tomato	Belgium	0.46	0.42	vegetable stock	0.03	0.07		0.03	0.06	
tomato	The Netherland	1.03	1.18	vegetable stock	0.03	0.03		0.04	0.03	
tomato	Germany	1.38	1.39	tomato puree	0.41	0.30	0.36	0.35	0.25	0.24
tomato	Germany	1.75	1.01	tomato puree	0.36	0.21		0.39	0.22	
tomato	Belgium	0.46	0.42	tomato puree	0.33	0.72		0.12	0.27	
tomato	The Netherland	1.03	1.18	tomato puree	0.22	0.21		0.22	0.21	
tomato	Germany	1.38	1.39	remainder	0.13	0.09	0.10	0.05	0.03	0.03
tomato	Germany	1.75	1.01	remainder	0.12	0.07		0.05	0.03	
tomato	Belgium	0.46	0.42	remainder	0.07	0.15		0.02	0.04	
tomato	The Netherland	1.03	1.18	remainder	0.07	0.07		0.03	0.03	
tomato	Germany	1.38	1.39	condensed water	0.03	0.02	0.03	<0.01	0.01	0.01
tomato	Germany	1.75	1.01	condensed water	0.04	0.02		<0.01	0.01	
tomato	Belgium	0.46	0.42	condensed water	0.03	0.07		<0.01	0.01	
tomato	The Netherland	1.03	1.18	condensed water	0.03	0.03		<0.01	0.01	
tomato	Germany	1.38	1.39	tomato juice	0.05	0.04	0.04	0.07	0.05	0.10
tomato	Germany	1.75	1.01	tomato juice	0.04	0.02		0.04	0.02	
tomato	Belgium	0.46	0.42	tomato juice	0.04	0.09		0.10	0.22	
tomato	The Netherland	1.03	1.18	tomato juice	0.03	0.03		0.11	0.10	

For calculation purposes, residues <0.03 mg/kg were set at 0.03 mg/kg of carbon disulfide and <0.01 mg/kg were set at 0.01 mg/kg of ETU. For the calculation of the transfer factor, the residue concentration in the processed sample was divided by the carbon disulfide residue concentration in the corresponding RAC sample. Directly after the last application, the carbon disulfide residues were between 0.97 and 1.70 mg/kg. After 13-15 days, the residues declined to 0.46 - 1.75 mg/kg, which were the starting concentrations for processing. In peel, a mean transfer factor was calculated to be 3.75, showing that the main part of carbon disulfide was concentrated on the tomato surface. In the other products, the transfer factors were below 1. Directly after the last application, the ETU residues were between 0.021 and 0.033 mg/kg. On DALA 13-15, the ETU residues were between <0.01 and 0.037 mg/kg, which were the starting concentrations for processing. In each of the processed tomato fractions the transfer factor was below 1 (range of mean transfer factors were 0.01 to 0.24)

III. CONCLUSION

This supervised field trial study showed that the residues of BAS 222 F in tomato RACs after eight applications of BAS 222 28 F ranged from 0.97 to 1.70 mg/kg on DALA 0 and from 0.46 to 1.75 mg/kg on DALA 13-15, which was the starting concentration for processing. After processing, significant enrichment of BAS 222 F was observed in tomato peel (residue: 2.68 - 4.95 mg/kg; mean transfer factor 3.75) only, whereas comparatively low levels of BAS 222 F were measured in canned tomatoes (residue: <0.03 to 0.04 mg/kg; mean transfer factor 0.04), in tomato puree (residue: 0.22 to 0.41 mg/kg; mean transfer factor 0.24), and in tomato juice (residue: 0.03 to 0.05 mg/kg; transfer factor 0.04). The transfer factors of ETU determined in peels (residue: 0.032 to 0.074 mg/kg), canned tomatoes (residue: 0.011 to 0.034 mg/kg, tomato puree (residue: 0.122 to 0.388 mg/kg and tomato juice (residue: 2.69 to 4.10 mg/kg) were calculated to be 0.05, 0.02, 0.24 and 0.10, respectively.

Report: *CA 6.5.3/10*
Schulz H., 2007b
Study on the residue behaviour of Metiram in gherkins and processed products after treatment with BAS 222 28 F under field conditions in Germany, 2005
2007/1017186

Guidelines: *EEC 91/414 Annex II (Part A Section 6), EEC 91/414 Annex III (Part A Section 8), EEC 1607/VI/97 rev. 2 10.06.1999, EEC 7029/VI/95 rev. 5, EEC 7525/VI/95 rev. 7, EEC 7035/VI/95 rev. 5, EEC 96/46, SANCO/825/00 rev. 7 (17 March 2004)*

GLP: *yes*
(certified by Hessisches Ministerium fuer Umwelt, Laendlichen Raum und Verbraucherschutz)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
   Description: BAS 222 28 F
   Lot/Batch #: WF18792 (BAS 222 28 F)
   Purity: BAS 222 28 F (BAS 222 F 70%, nominal)
   CAS#: 9000-42-2
   Development code:
   Metiram: BAS 222 F
   ETU: M222F002
   Spiking levels: BAS 222 F (0.1, 1.0, 2.0 and 10.0 mg/kg)
   ETU (0.01, 0.1 and 1.0 mg/kg)

2. **Test Commodity:**
   Crop: gherkins
   Type: not applicable
   Variety: Peggy, Nadine, Profi
   Botanical name: *Cucumis sativus*
   Crop parts(s) or processed commodity: washed gherkins, wash water, canned gherkins and vegetable stock
   Sample size: 1.0 - 4.0 kg

B. STUDY DESIGN

1. Test procedure

During the 2005 growing season, four field trials were conducted in representative gherkins growing regions in Germany to determine the residue levels of BAS 222 F and ETU in gherkins as well as the process fractions involved in the production of dried gherkins and vegetable stock. The fungicidal test substance BAS 222 28 F (70% BAS 222 F, WG) was applied six times by spray application rates of 5.4 kg/ha (corresponding to 3.78 kg a.i./ha) in a spray volume of 500 I/ha beginning 16-18 days before expected harvest and subsequent applications on day 7-10 and 3-4. For the residue analysis of gherkins, fruits were collected directly after the last application as well as 3-4 days later. The fruits were processed to the following products: washed gherkins, wash water, canned gherkins and vegetable stock. All processing steps were carried out according to commercial procedures. Specimens were shipped frozen and remained at temperatures ≤-18°C until analytical measurements.

2. Description of analytical procedures

The active substance BAS 222 F was analyzed according to Institute Fresenius method no. IF-05/00449055. The active substance was degraded to carbon disulfide by means of orthophosphoric acid and passed into isooctane by a stream of nitrogen. The final determination was carried out using GC-MS detection. The ion fragment m/z 76 was used for evaluation. The mean procedural recovery was about 78.0±6.5% (n=13; fortification level 0.1, 1.0, 2.0 and 10.0 mg/kg) and the limit of detection was 0.1 mg/kg BAS 222 F (corresponding to 0.056 mg/kg carbon disulfide). The ETU was extracted from the plant material with a mixture of sodium ascorbate, ethylene urea, ammonium chloride and methanol-water. After centrifugation, an aliquot was taken and the methanol was evaporated from the extract. The pH is adjusted to 8 before evaporating (in some cases after evaporation). The remaining water phase was cleaned by liquid/liquid partition (water/dichloromethane) on an Extrelut/AI_2O_3 column.

After concentration of the eluate, the residue was determined by HPLC using pulsed amperometric detection. In this study, the final determination is carried out using LC-MS/MS detection. The fragment ion 103 → 44 was used for evaluation (BASF method No. 373/3). The mean procedural recovery was about 89.3±11.7% (n=10; fortification level 0.01, 0.1 and 1.0 mg/kg) and the limit of detection was 0.01 mg/kg.

II. RESULTS AND DISCUSSION

The residues of BAS 222 F and ETU in gherkins, process fractions thereof and correspondent transfer factors are provided in Table 6.5.3-24 to Table 6.5.3-27.

Table 6.5.3-24: Residues and transfer factors of BAS 222 F in gherkins and process fractions thereof after four applications with BAS 222 28 F

	Residues (mg/kg) Carbon disulfide				Transfer factor				
Trial No.	**ACK/ 04/05/**	**ACK/ 10/05/**	**AGR/ 12/05/**	**AGR/ 13/05/**	**ACK/ 04/05/**	**ACK/ 10/05/**	**AGR/ 12/05/**	**AGR/ 13/05/**	**mean**
fruit (DALA 0)	0.577	0.495	0.532	0.907	n.a.	n.a.	n.a.	n.a.	n.a.
fruit RAC (DALA 3-4)	0.185	0.143	0.293	1.033	1	1	1	1	1
wash water	0.155	0.162	0.199	0.326	0.84	1.14	0.68	0.32	0.75
washed gherkins	<0.1	<0.1	<0.1	<0.1	<0.54	<0.70	<0.34	<0.10	<0.42
canned gherkins	<0.1	<0.1	<0.1	<0.1	<0.54	<0.70	<0.34	<0.10	<0.42
vegetable stock	<0.1	<0.1	<0.1	<0.1	<0.54	<0.70	<0.34	<0.10	<0.42

DALA = days after last treatment; n.a. = not applicable

Table 6.5.3-25: Residues and transfer factors of ETU in gherkins and process fractions thereof after four applications with BAS 222 28

	Residues (mg/kg) ETU				Transfer factor				
Trial No.	**ACK/ 04/05/**	**ACK/ 10/05/**	**AGR/ 12/05/**	**AGR/ 13/05/**	**ACK/ 04/05/**	**ACK/ 10/05/**	**AGR/ 12/05/**	**AGR/ 13/05/**	**mean**
fruit (DALA 0)	0.016	0.021	0.038	0.031	n.a.	n.a.	n.a.	n.a.	n.a.
fruit RAC (DALA 3-4)	0.018	0.022	0.035	0.033	1	1	1	1	1
wash water	<0.01	<0.01	<0.01	<0.01	<0.55	<0.45	<0.29	<0.30	<0.40
washed gherkins	0.042	0.036	0.056	0.036	2.33	1.64	1.60	1.09	1.67
canned gherkins	0.020	0.018	0.023	0.021	1.11	0.82	0.66	0.64	0.81
vegetable stock	0.047	0.018	0.020	0.014	2.61	0.82	0.57	0.42	1.11

DALA = days after last treatment; n.a. = not applicable

In the treated fruit specimens at 0 and 3-4 DALA, the residues of Metiram were 0.495-0.907 mg/kg and 0.143-1.033 mg/kg, respectively. In the processed specimens "washed gherkins", "canned gherkins" and "vegetable stock", no residues above the limit of quantification were found, whereas the residues of Metiram in the processed specimen "wash water" ranged from 0.155-0.326 mg/kg. In "washed gherkins", "canned gherkins" and "vegetable stock", the transfer factors were below 1. The residues of ETU in the treated fruit specimens at 0 DALA ranged from 0.016-0.038 mg/kg and remained at that level in the specimens sampled at the PHI. The only processed specimen with no residues of ETU above the limit of quantification was "wash water".

Residues of ETU were analysed in the "washed gherkins" (0.036-0.056 mg/kg), as well as in the "canned gherkins"(0.018-0.023 mg/kg) and in the "vegetable stock" (0.014-0.047 mg/kg). In "wash water" and in three out of four trials of the "canned gherkins" and the "vegetable stock", the transfer factors were below 1. No residues of the Metiram above the limit of quantitation were found in the analysed control specimens, apart from the fruit specimen at 0 DALA of the field trial AGR/12/05, where 0.172 mg/kg were found.

The processing recoveries of Metiram and ETU in the processed fractions were as follows, whereby the initial amounts of Metiram in the RAC specimens were set as 100 %: The summary of the recoveries for the different processing steps is presented in the following table. Since the calculation of the mass balance is not relevant for the dietary exposure assessment, the table is copied directly from the report.

Table 6.5.3-26: Distribution of BAS 222 F (expressed as carbon disulfide) and ETU residues in the processed products related to gherkin RACs.

	Percent recovery of BAS 222 F and ETU							
	Carbon disulfide				**ETU**			
Trial No.	**ACK/ 04/05/**	**ACK/ 10/05/**	**AGR/ 12/05/**	**AGR/ 13/05/**	**ACK/ 04/05/**	**ACK/ 10/05/**	**AGR/ 12/05/**	**AGR/ 13/05/**
gherkin RAC (DALA 3-4))	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
wash water	156.4	219.0	130.0	59.9	n.c.	n.c.	n.c.	n.c.
washed gherkins	n.c.	n.c.	n.c.	n.c.	233.3	163.6	160.0	190.1
canned gherkins	n.c.	n.c.	n.c.	n.c.	103.4	76.5	59.2	56.8
vegetable stock	n.c.	n.c.	n.c.	n.c.	242.5	83.9	62.6	46.4

DALA = days after last treatment
n.c. = not calculated

Based on the residue levels found in gherkins at 3 DAT, the processing factors were recalculated using the following equations:

$$PF_{metiram} = \frac{\text{Conc. m}etiram\,(or\,CS_2)\ \text{in processed fraction}}{\text{Conc.metiram (or CS}_2\text{) in } raw\,agricultural\,com\text{mod}ity}$$

$$PF_{ETU} = \frac{\text{Conc. } ETU\ in\ process\ fraction}{\text{Conc. m}etiram\ (as\,CS2)\,in\ raw\,com\text{mod}ity}$$

For calculation purposes, residue levels at the LOQ were set at 0.1 mg/kg and 0.01 mg/kg, respectively.

Table 6.5.3-27: Gherkins: Calculation of processing factors for metiram and ETU

RAC	residue level CS_2	residue level Metiram	PF	residue level CS_2	PF CS_2	Mean PF CS_2	residue level ETU	PF ETU	Mean PF ETU
gherkins	0.190	0.331	wash water	0.155	0.84	0.74	0.01	0.1	0.04
gherkins	0.140	0.256	wash water	0.162	1.13		0.01	0.1	
gherkins	0.293	0.524	wash water	0.199	0.68		0.01	0.0	
gherkins	1.033	1.849	wash water	0.326	0.32		0.01	0.0	
gherkins	0.190	0.331	washed gherkins	0.1	0.54	0.42	0.042	0.2	0.18
gherkins	0.140	0.256	washed gherkins	0.1	0.70		0.036	0.3	
gherkins	0.293	0.524	washed gherkins	0.1	0.34		0.056	0.2	
gherkins	1.033	1.849	washed gherkins	0.1	0.10		0.036	0.0	
gherkins	0.190	0.331	canned gherkins	0.1	0.54	0.42	0.020	0.1	0.08
gherkins	0.140	0.256	canned gherkins	0.1	0.70		0.018	0.1	
gherkins	0.293	0.524	canned gherkins	0.1	0.34		0.023	0.1	
gherkins	1.033	1.849	canned gherkins	0.1	0.10		0.021	0.0	
gherkins	0.190	0.331	vegetable stock	0.1	0.54	0.42	0.047	0.3	0.04
gherkins	0.140	0.256	vegetable stock	0.1	0.70		0.018	0.1	
gherkins	0.293	0.524	vegetable stock	0.1	0.34		0.020	0.1	
gherkins	1.033	1.849	vegetable stock	0.1	0.10		0.014	0.0	

III. CONCLUSION

This processing study showed that the residues of BAS 222 F in gherkin fruits after six applications of BAS 222 28 F ranged from 0.495 to 0.907 mg/kg on DALA 0 and from 0.143 to 1.033 mg/kg on DALA 3-4, which were the starting concentrations for the processing. After processing, no enrichment of BAS 222 F (indicated by a mean transfer factor <1.0) was observed in any of the analysed processed products, namely washed gherkins (residue: <0.1 mg/kg; mean transfer factor <0.42), canned gherkins (residue: <0.1 mg/kg; mean transfer factor <0.42) and vegetable stock (residue: <0.1 mg/kg; mean transfer factor <0.42). In terms of ETU, significant enrichment was observed in washed gherkins (residue: 0.036-0.056 mg/kg; mean transfer factor 1.67), canned gherkins (residue: 0.018-0.023 mg/kg; mean transfer factor 0.81) as well as to a lower extent in vegetable stock (residue: 0.014-0.047 mg/kg; mean transfer factor 1.11).

Report: *CA 6.5.3/11*
Raunft E. et al., 2002b
Study on the residue behaviour of Metiram in head lettuce and its processing products after application of BAS 222 28 F under field conditions in Germany and the Netherlands, 2001
2002/1004092
Guidelines: *EEC 7029/VI/95 rev. 5, EEC 7525/VI/95 rev. 2*
GLP: *yes*
(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** BAS 222 28 F
   Description: BAS 222 28 F
   Lot/Batch #: 2001-1
   Purity: BAS 222 28 F (BAS 222 F 700 g/kg, nominal)
   CAS#: 9006-42-2
   Development code:
   Metiram: BAS 222 F
   ETU: M222F002
   Spiking levels: BAS 222 F (0.5 to 10.0 mg/kg)
   ETU (0.01 to 0.5 mg/kg)

2. **Test Commodity:**
   Crop: head lettuce
   Type: not applicable
   Variety: Sylvesta, Nadin
   Botanical name: *Lactuca sativa*
   Crop parts(s) or processed commodity: head, leaves (interior and exterior), washed leaves (interior and exterior)
   Sample size: 0.5 - 1.0 kg

B. STUDY DESIGN

1. Test procedure

During the 2001 growing season, two field trials were conducted at two locations in representative head lettuce growing areas in Germany and the Netherlands to determine the residue levels of BAS 222 F and ETU in lettuce and processed fractions thereof. The fungicidal test substance BAS 222 28 F (700 g/kg BAS 222 F, WG) was applied 2 times by spray application rates of 1.6 kg/ha (corresponding to 1.12 kg a.i./ha) in a spray volume of 400 I/ha. First application was done 21 days before harvest, followed by a second application 7 days later. Head lettuce specimens were collected immediately after the second application on DALA 0). Further head lettuce specimens were collected on DALA 6-8, 13-15 and 20-21. Lettuce sampled on DALA 13-15 were separated into exterior and interior leaves and were measured as washed and unwashed specimens. Specimens were stored at temperatures ≤-18°C until analysis.

2. Description of analytical procedures

The samples were analyzed for BAS 222 F and ETU by means of gas chromatography according to analytical method supplied by RCC UMWELTCHEMIE AG. To the analytical material which contains BAS 222 F, a solution of stannous chloride and hydrochloric acid was added and heated to yield carbon disulfide. The carbon disulfide was distilled, purified and trapped by dissolution in ethanol. Following gas chromatographic separation, the generated carbon disulfide was determined with a flame photo-metric detector (S-FPD, specific for sulfur). The mean procedural recovery were about 95.4±10.2% (n=18; fortification level between 0.05 and 1826.0 mg/kg) and the limit of quantification was 0.03 mg/kg (expressed as carbon disulfide). ETU was extracted from the matrix with methanol. The methanol was evaporated and the remaining water phase was extracted with n-hexane and cleaned up on an aluminium oxide column. The aqueous eluate was concentrated and extracted by partitioning with dichloromethane. The dichloromethane extract was concentrated and cleaned up on an aluminium oxide column. After concentration of the eluate, the residue was determined by gas chromatography using a sulfur specific flame photometric detector. The mean procedural recovery were about 97.8±7.7% (n=21; fortification level between 0.02 and 0.20 mg/kg) and the limit of quantification was 0.02 mg/kg

II. RESULTS AND DISCUSSION

The residues of BAS 222 F and ETU in head lettuce and process fractions thereof as well as transfer factors are provided in Table 6.5.3-28 and Table 6.5.3-29.

Table 6.5.3-28: Residues of BAS 222 F and ETU in head lettuce and process fractions thereof after two applications of BAS 222 28 F.

Trial No.	Portion analyzed	DALA (nominal)	Residues (mg/kg)		
			BAS 222 F	carbon disulfide	ETU
AGR/04/01	heads	0	44.18	24.72	0.061
	heads	6	4.32	2.42	0.020
	heads	13	0.58	0.32	<0.01
	leaves (exterior)	13	0.99	0.55	<0.01
	leaves (interior)	13	0.34	0.19	<0.01
	washed (exterior)	13	0.42	0.23	<0.01
	washed (interior)	13	0.14	0.08	<0.01
	heads	20	0.18	0.10	<0.01
DU4/05/01	heads	0	41.02	22.95	0.109
	heads	8	2.98	1.67	<0.05
	heads	15	0.72	0.40	<0.05
	leaves (exterior)	15	1.26	0.71	<0.05
	leaves (interior)	15	0.06	0.04	<0.05
	washed (exterior)	15	0.30	0.17	<0.05
	washed (interior)	15	0.06	0.03	<0.05
	heads	21	<0.05	<0.03	<0.05

DALA = days after last treatment
Concentrations measured on DALA 13 were set 1
n/a = not applicable

Directly after the last application, BAS 222 F was found to range between 41.02 and 44.18 mg/kg in head lettuce corresponding to 22.95 to 24.72 mg/kg carbon disulfide. These residues degraded during the course of the study ranging between 0.58 to 0.72 mg/kg of BAS 222 F (0.32 to 0.40 mg/kg carbon disulfide) after about two weeks and between <0.05 to 0.18 mg/kg (<0.05 to 0.10 mg/kg carbon disulfide) after 3 weeks. The lettuce heads collected after about two weeks were separated in exterior and interior leaves. As expected, the exterior leaves contained higher residues (0.99 to 1.26 mg/kg of BAS 222 F) compared to the interior ones (0.06 to 0.34 mg/kg of BAS 222 F). Washing of these fractions lead to further reductions of the residues found: the washed exterior leaves contained 0.30 to 0.42 mg/kg of BAS 222 F, in the washed interior ones, 0.06 to 0.14 mg/kg of BAS 222 F were found. The starting residue level of ETU was 0.061 to 0.109 mg/kg at day 0. After about one week, ETU ranged between <0.05 and 0.020 mg/kg in lettuce. In all lettuce specimen collected later including the separated or washed fractions, no ETU residues above the limit of quantification was seen.

Table 6.5.3-29: The BAS 222 F and ETU residues in head lettuce and corresponding transfer factors of process fractions thereof after eight applications with BAS 222 28 F. The transfer factors were re-calculated based on the initial CS_2 values for metiram and ETU.

RAC	residue level CS_2	residue level Metiram	PF	residue level CS_2	PF CS_2	Mean PF CS_2	residue level ETU	PF ETU	Mean PF ETU
lettuce	0.32	0.57	leaves ext.	0.55	0.96	0.98	0.01	0.03	0.03
lettuce	0.4	0.72	leaves ext.	0.71	0.99		0.01	0.03	
lettuce	0.32	0.57	leaves int.	0.19	0.33	0.19	0.01	0.03	0.03
lettuce	0.4	0.72	leaves int.	0.04	0.06		0.01	0.03	
lettuce	0.32	0.57	leaves ext. washed	0.23	0.40	0.32	0.05	0.16	0.14
lettuce	0.4	0.72	leaves ext. washed	0.17	0.24		0.05	0.13	
lettuce	0.32	0.57	leaves int. washed	0.08	0.14	0.09	0.05	0.16	0.14
lettuce	0.4	0.72	leaves int. washed	0.03	0.04		0.05	0.13	

No significant enrichment of BAS 222 F in any of the investigated lettuce fraction was observed. The mean transfer factors were 0.98 in the exterior leaves, 0.19 in the interior leaves, 0.32 in exterior leaves (washed) and 0.19 in the interior leaves (washed). The transfer factors of ETU were quite low and ranged from 0.03 in exterior and interior leaves to 0.14 in exterior leaves (washed) and interior leaves (washed).

III. CONCLUSION

This supervised field trial study showed that the residues of BAS 222 F head lettuce RACs after two applications of BAS 222 28 F ranged from 41.02 to 44.18 mg/kg on DALA 0, 2.98 to 4.32 mg/kg on DALA 6-8 and <0.05 to 0.18 mg/kg on DALA 20-21. No significant enrichment (indicated by transfer factors >1.0) of BAS 222 F was observed in any of the investigated fractions (exterior lettuce leaves; range 0.99 to 1.26 mg/kg, mean transfer factor 0.98; interior leaves, range 0.06 to 0.34 mg/kg; mean transfer factor 0.19. After leaf washing, the residues of BAS 222 F were ≤0.42 mg/kg and the mean transfer factors were 0.32 and 0.09 for exterior and interior leaves, respectively. The residue levels of ETU were 0.061 to 0.109 mg/kg on DALA 0 and <0.05 to 0.18 mg/kg on DALA 6-8. In all lettuce specimen collected later including the separated or washed fractions, no ETU residues above the limit of quantification were seen. The mean transfer factors of ETU were quite low and ranged from 0.03 in exterior and interior leaves to 0.14 in exterior leaves (washed) and interior leaves (washed).

CA 6.6 Residues in Rotational Crops

Annex II Dossier:

Metabolism in rotational crop study (wheat, beet and kale) with metiram has previously been reviewed in the Annex I inclusion process.
The total radioactive residues in the edible parts of succeeding crops destined for human consumption were <0.2 mg/kg in kale, <0.5 mg/kg in beet (tuber) and <1.5 mg/kg in wheat (grain). As already observed in the plant metabolism studies, the metabolites of BAS 222 F were extensively incorporated into the carbon pool and hence, into the natural products of the plants. It was concluded that the exposure of the consumers to residues in rotational crops grown on plots treated with metiram is negligible. The following table was copied from the Monograph prepared by Italy.

Residues in succeeding crops

Based on the results on rotational crops and on the fast degradation of the a.i. it can be concluded that the application of Metiram will not lead to uptake of any residue of concern from soil by succeeding crops

AIR3 Dossier:

In addition, a new metabolism in rotational crop study (wheat, radish, lettuce) with metiram was conducted in 2009. The findings confirm the previous conclusion that application of metiram does not result in significant uptake of residue of concern into the succeeeding crop. In contrast, metiram degradation products taken up from soil are effectively entering primary metabolism followed by incorporation into naturally occurring plant constituents. In conclusion, exposure of the consumers to metiram and its transformation products from rotational crops grown on soil treated with metiram is negligible. A magnitude of the residue study in rotational crop study is not required.

CA 6.6.1 Metabolism in rotational crops

Report: CA 6.6.1/1
Bross M. et al., 2010a
Confined rotational crop study with 14C-BAS 222 F
2009/1017248

Guidelines: EPA 860.1000: Background - PMRA Section 97.13 (Canada): Residue Chemistry Guidelines Confined Accumulation in Rotational Crops (June 1997), EPA 860.1000: EPA Residue Chemistry Test Guidelines, EPA 860.1850: Confined Accumulation in Rotational Crops, EEC 7524/VI/95 rev. 2 (July 22 1997)

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

I. MATERIAL AND METHODS

A. MATERIALS

1. **Test Material:** ^{14}C-BAS 222 F (Metiram)
   Description: ^{14}C- BAS 222 F (radiolabeled in the ethylene bridge) (Specific radioactivity: 4.76 MBq/mg)
   Lot/Batch #: 153-3101
   Purity: 88.2% (chemical purity; Jodometry, Non-GLP)
   CAS#: 9006-42-2
   Stability of test Compound: The residues in lettuce, treated with BAS 222 F were stable under the chosen experimental conditions (-18°C, 30 months)

2. **Test Commodity:**
   Crop: lettuce, white radish, spring wheat
   Type: not reported
   Variety: lettuce: Matilda, Estelle; white radish: April Cross; spring wheat: Thassos
   Botanical name: *Lactuca sativa L., Rhaphanus sativus, Triticum L.*
   Crop parts(s) or processed commodity: lettuce (unripe leaf and ripe head)
   white radish (immature plant, mature top and root)
   spring wheat (forage, straw, chaff and grain)

   Sample size: Lettuce leaf: > 0.49 kg, Lettuce head: < 14.74 kg, White radish top: < 0.34 kg, White radish root:< 25.07 kg, Spring wheat forage: < 0.69 kg, Spring wheat straw: < 0.48 kg, Spring wheat chaff: < 0.85 kg, Spring wheat grain: > 0.154 kg.

3. **Soil:** Loamy sand soil was used. The soil physicochemical properties are described below (see Table 6.6.1-1).

Table 6.6.1-1: Soil physicochemical properties

USDA classification	
clay	5.5
silt	15.2
sand	79.3
soil class	loamy sand
DIN classification	
clay	5.5
silt	16.7
sand	77.7
soil class	loamy sand (DIN 4220)
Other soil parameters	
total nitrogen	0.07 %
total organic carbon	0.55 %
total carbon	0.55 %
pH ($CaCl_2$)	6.4
pH (H_2O)	7.1
effective cation exchange capacity	7.0 cmol/kg
max. water holding capacity	22.3 g/100 g dry soil
microbial biomass	9.9 mg C/100 g dry soil
microbial C / organic C	1.8 %
bulk density	1614 g/L
dry matter	88.3 %

B. STUDY DESIGN AND METHODS

1. Test procedure

A confined rotational crop study was conducted with ^{14}C-BAS 222 F (Metiram, Reg. No. 250284) radiolabeled in the ethylene bridge and blended with unlabelled test item. The active substance was suspended in acetone and applied to bare loamy sand soil in plastic containers at an application rate of 1 x 12.500 kg as/ha (approximately 11.16 lb/A). The nature and the level of radioactive residues were investigated in lettuce (unripe leaf and ripe head), white radish (immature plant, mature top and root) and spring wheat (forage, straw, chaff and grain) after plant back intervals of 30, 121 and 365 days. Plant samples were harvested at maturity, and additional immature lettuce leaf samples as well as immature white radish plant (plant back interval of 365 days only) and spring wheat forage samples were taken 29 to 41 days after planting, 13 days after sowing and 49 to 67 days after sowing, respectively. Soil samples were taken after plowing and after harvest of the mature crops for each plant back interval. The harvested material was stored in a freezer.

2. Description of analytical procedures

All plant samples and soil samples were homogenized and combusted to $^{14}C0_2$ which was trapped by an absorption and scintillation liquid and analyzed by LSC for the determination of the total radioactive residues.

Aliquots of homogenized plant material were extracted three times with methanol and two times with water. The methanol and water extracts were measured by LSC.

The results of the methanol extraction and the water extraction were summarized and referred to as extractable radioactive residues (ERR). The residue after solvent extraction of each sample was dried and homogenized. Aliquots were combusted for the determination of the residual radioactive residue (RRR).

In order to characterize the methanol extractable radioactive residues as organosoluble or water soluble fractions, liquid / liquid partition was carried out using isohexane, dichloromethane and ethyl acetate as organic solvents. Aliquots of the liquid phases were analyzed by LSC measurement.

Solubilization treatments of the Residual Radioactive Residues comprised treatments with aqueous ammonia, enzymes and sodium hydroxide.

After the extraction and partition procedures and the various solubilization treatments of the Residual Radioactive Residues after solvent extraction (RRR), HPLC analyses were carried out for the extracts, partition phases and solubilizates with a sufficient level of radioactivity.

For quantitation, HPLC method LC02, LC12 or LC08 with a Phenomenex Gemini C18 column was used. Confirmatory HPLC analyses were carried out using HPLC method LC05 with a YMC Polyamine II column.

<u>HPLC method LC02/LC12</u>
The eluent system consisted of 2 mobile phases (Eluent A: Water / cone. NH_4OH (1000 + 2.5, v/v), Eluent B: Acetonitrile) which were used applying gradient elution.

<u>HPLC method LC08:</u>
The eluent system consisted of 2 mobile phases (Eluent A: Water / cone. NH_4OH (1000 + 2.5, v/v), Eluent B: Acetonitrile + THF (550 + 450, v/v)) which were used applying gradient elution.

<u>HPLC method LC05:</u>
The eluent system consisted of 2 mobile phases (Eluent A: Water + 25 mM NH_4H_2 $P0_4$ + 25 mM $(NH_4)_2HP0_4$, Eluent B: Acetonitrile) which were used applying gradient elution.

3. Identification of metabolites

Identification of the metabolites was based on analyses of purified fractions (seven fractions) isolated from methanol extracts of spring wheat straw (30 DAT) by HPLC-MS and co-chromatography experiments with reference items. Peak assignment in the other samples was done by comparison of the HPLC retention times and the elution profiles / metabolite patterns with those of the fractions investigated by HPLC-MS or co-chromatography experiments and with the reference items.

II. RESULTS AND DISCUSSION

A. TOTAL RADIOACTIVE RESIDUES (TRRs)

The calculated TRR (ERR +RRR) for unripe lettuce leaf accounted for 0.766 mg/kg after the plant back interval of 30 days. Lower levels of 0.180 and 0.112 mg/kg were found after the longer periods of soil aging (121 Days after Treatment and 365 DAT).

The total residues in ripe lettuce head reached comparable values of 0.656, 0.221 and 0.091 mg/kg after plant back intervals of 30, 121 and 365 days, respectively.

The total radioactive residues in unripe white radish plant (365 DAT) amounted to 0.094 mg/kg. The residue concentrations in mature white radish top also showed decreasing levels of 0.906, 0.283 and 0.093 mg/kg after increasing soil aging periods of 30, 121 and 365 days, respectively. In mature white radish root, lower TRR levels of 0.393 mg/kg (30 DAT), 0.136 mg/kg (121 DAT) and 0.059 mg/kg (365 DAT) were detected.

In spring wheat, the highest residue levels were measured in straw at all plant back intervals (4.245 mg/kg at 30 DAT, 1.739 mg/kg at 121 DAT and 1.424 mg/kg at 365 DAT, respectively). The TRR levels in wheat chaff accounted for 3.775 mg/kg, 1.023 mg/kg and 1.396 mg/kg after the soil aging periods of 30, 121 and 365 days. Somewhat lower residue levels of 2.752 mg/kg (30 DAT), 0.530 mg/kg (121 DAT) and 1.181 mg/kg (365 DAT) were observed in wheat grain. In spring wheat forage, residue levels of 1.690 mg/kg (30 DAT), 0.416 mg/kg (121 DAT) and 0.200 mg/kg (365 DAT) were detected.

After soil aging and plowing, the total radioactive residues in soil accounted for 2.510 mg/kg at 30 DAT, 1.402 mg/kg at 121 DAT and 1.599 mg/kg after a plant back interval of 356 days.

After harvest of individual mature crops, soil residue levels of 2.004 to 2.681 mg/kg (30 DAT), 1.744 to 1.769 mg/kg (121 DAT) and 1.367 to 1.473 mg/kg (365 DAT) were observed.

The results of the Total Radioactive Residues (TRR) after ^{14}C-BAS 222 F treatment in lettuce, white radish and spring wheat are provided in Table 6.6.1-2, the results of the soil samples are shown in Table 6.6.1-3.

Table 6.6.1-2: Total Radioactive Residues in Rotational Crops Samples after Treatment with ^{14}C-BAS 222 F

Matrix	Days After Sowing / Planting DAP	TRR measured[1)] [mg/kg]	TRR calculated[2)] [mg/kg]
Plant back interval: 30 DAT			
Lettuce leaf	38	0.839	0.766
Lettuce head	59	0.785	0.656
White radish top	88	1.091	0.906
White radish root	88	0.377	0.393
Spring wheat forage	54	1.905	1.690
Spring wheat straw	131	5.096	4.245
Spring wheat chaff	131	3.890	3.775
Spring wheat grain	131	2.819	2.752
Plant back interval: 121 DAT			
Lettuce leaf	41	0.216	0.180
Lettuce head	55	0.225	0.221
White radish top	109	0.384	0.283
White radish root	109	0.159	0.136
Spring wheat forage	67	0.467	0.416
Spring wheat straw	146	1.856	1.739
Spring wheat chaff	146	1.007	1.023
Spring wheat grain	146	0.458	0.530
Plant back interval: 365 DAT			
Lettuce leaf	29	0.147	0.112
Lettuce head	56	0.100	0.091
White radish plant	13	0.087	0.094
White radish top	77	0.123	0.093
White radish root	77	0.081	0.059
Spring wheat forage	49	0.207	0.200
Spring wheat straw	109	1.370	1.424
Spring wheat chaff	109	1.426	1.396
Spring wheat grain	109	1.216	1.181

1) TRR was determined by direct combustion
2) TRR was calculated as the sum of ERR (extraction with methanol and water) + RRR
DAT = Days After Treatment

Table 6.6.1-3: Total Radioactive Residues in Soil Samples after Treatment with ^{14}C-BAS 222 F

Soil samples (Days After Treatment DAT)	Days After Sowing DAP	TRR measured[1)] [mg/kg]
Plant back interval: 30 DAT		
After ploughing		
30 DAT	6	2.510
After harvest of mature crops		
Lettuce (89 DAT)	59	2.004
White radish (118 DAT)	88	2.681
Spring wheat (161 DAT)	131	2.137
Plant back interval: 121 DAT		
After ploughing		
121 DAT	0	1.402
After harvest of mature crops		
White radish (232 DAT)	111	1.744
Spring wheat (267 DAT)	146	1.769
Plant back interval: 365 DAT		
After ploughing		
365 DAT	0	1.599
After harvest of mature crops		
Lettuce (421 DAT)	56	1.380
White radish (442 DAT)	77	1.367
Spring wheat (474 DAT)	109	1.473

1) TRR was determined by direct combustion

B. EXTRACTION, CHARACTERIZATION AND IDENTIFICATION OF RESIDUES

1. Extraction of residues

The extractability of radioactive residues with methanol and water was above 50 % of the TRR for most of the lettuce samples (54.4 % to 65.2 % TRR; 46.1 % TRR for unripe lettuce leaf after 365 days) and around 50 % for mature white radish top and root (48.8 % to 61.0 % TRR; 39.4 % TRR for unripe white radish plant, 365 DAT). For spring wheat forage (37.0 % to 49.4 % TRR), straw (38.0 % to 50.8 % TRR) and chaff (25.6 % to 45.0 % TRR), the extraction efficiency was around or below 50 %. In the case of spring wheat grain, the extractability was lower with 13.6% to 19.0% TRR. The main part of the radioactive residues was generally extracted with methanol, except for wheat grain where water was more effective as extracting solvent.

The extractabilities with methanol and water are summarised in Table 6.6.1-4.

Table 6.6.1-4: Extractability of Radioactive Residues in Rotational Crops Samples after Treatment with ^{14}C-BAS 222 F T

Matrix	DAP	TRR calculated[1)]	Methanol Extract		Water Extract		ERR[2)]		RRR[3)]	
		[mg/kg]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]
Plant back interval: 30 DAT										
Lettuce leaf	38	0.766	0.438	57.2	0.061	8.0	0.499	65.2	0.267	34.8
Lettuce head	59	0.656	0.349	53.2	0.070	10.7	0.419	63.9	0.237	36.1
White radish top	88	0.906	0.359	39.6	0.140	15.5	0.499	55.0	0.407	45.0
White radish root	88	0.393	0.188	48.0	0.015	3.7	0.203	51.7	0.190	48.3
Spring wheat forage	54	1.690	0.699	41.4	0.135	8.0	0.834	49.4	0.856	50.6
Spring wheat straw	131	4.245	1.151	27.1	0.463	10.9	1.614	38.0	2.631	62.0
Spring wheat chaff	131	3.775	0.666	17.6	0.302	8.0	0.967	25.6	2.808	74.4
Spring wheat grain	131	2.752	0.175	6.4	0.278	10.1	0.453	16.5	2.299	83.5
Plant back interval: 121 DAT										
Lettuce leaf	41	0.180	0.087	48.2	0.011	6.2	0.098	54.4	0.082	45.6
Lettuce head	55	0.221	0.114	51.5	0.020	9.0	0.134	60.5	0.087	39.5
White radish top	109	0.283	0.097	34.2	0.044	15.6	0.141	49.8	0.142	50.2
White radish root	109	0.136	0.061	44.5	0.006	4.3	0.066	48.8	0.070	51.2
Spring wheat forage	67	0.416	0.132	31.8	0.022	5.2	0.154	37.0	0.262	63.0
Spring wheat straw	146	1.739	0.511	29.4	0.208	12.0	0.720	41.4	1.019	58.6
Spring wheat chaff	146	1.023	0.271	26.5	0.093	9.0	0.364	35.5	0.659	64.5
Spring wheat grain	146	0.530	0.037	7.0	0.064	12.0	0.101	19.0	0.429	81.0
Plant back interval: 365 DAT										
Lettuce leaf	29	0.112	0.043	38.0	0.009	8.1	0.052	46.1	0.060	53.9
Lettuce head	56	0.091	0.050	54.5	0.008	9.0	0.058	63.5	0.033	36.5
White radish plant	13	0.094	0.031	32.9	0.006	6.5	0.037	39.4	0.057	60.6
White radish top	77	0.093	0.042	44.7	0.008	8.0	0.049	52.8	0.044	47.2
White radish root	77	0.059	0.034	56.7	0.003	4.2	0.036	61.0	0.023	39.0
Spring wheat forage	49	0.200	0.064	31.8	0.011	5.5	0.075	37.3	0.126	62.7
Spring wheat straw	109	1.424	0.482	33.9	0.242	17.0	0.724	50.8	0.700	49.2
Spring wheat chaff	109	1.396	0.371	26.6	0.258	18.5	0.629	45.0	0.768	55.0
Spring wheat grain	109	1.181	0.059	5.0	0.101	8.6	0.160	13.6	1.021	86.4

1) TRR was calculated as the sum of ERR (extraction with methanol and water) + RRR
2) ERR = Extractable Radioactive Residue
3) RRR = Residual Radioactive Residue (after solvent extraction)
DAP = Days After Planting (or sowing, respectively)
DAT = Days After Treatment

The residual radioactive residues of metiram after solvent extraction ranged from a minimum of 34.8 % TRR (0.267 mg/kg, lettuce leaf 30 DAT) to a maximum of 86.4 % TRR (1.021 mg/kg, spring wheat grain 365 DAT).

2. Partition behaviour

In most cases, the major portions of the radioactive residues extracted with methanol were water soluble, and only lower portions were found in the organic fractions. In the cases of spring wheat chaff (30 DAT and 121 DAT) and grain (all plant back intervals), comparable portions were found in the organic phases (sum) and in the water phase. Results are summarised in Table 6.6.1-5.

Table 6.6.1-5: Partition Characteristics of Radioactive Residues extracted with Methanol from Rotational Crop Samples

Matrix	DAP	Methanol extract		Organosoluble						Sum organosoluble		Water phase		Recovery[1)]
				Isohexane phase		Dichloromethane phase		Ethyl acetate phase						
		[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[%]
Plant back interval: 30 DAT														
Lettuce leaf	38	0.438	57.2	0.017	2.3	0.011	1.5	0.012	1.5	0.041	5.3	0.368	48.0	93.1
Lettuce head	59	0.349	53.2	0.020	3.0	0.006	0.9	0.009	1.3	0.034	5.2	0.285	43.5	91.6
White radish top	88	0.359	39.6	0.009	1.0	0.022	2.5	0.027	3.0	0.059	6.5	0.279	30.8	94.1
White radish root	88	0.188	48.0	0.007	1.7	0.004	1.0	0.005	1.2	0.015	3.9	0.168	42.7	97.2
Spring wheat forage	54	0.699	41.4	0.017	1.0	0.030	1.8	0.054	3.2	0.101	6.0	0.514	30.4	88.0
Spring wheat straw	131	1.151	27.1	0.052	1.2	0.145	3.4	0.129	3.0	0.326	7.7	0.599	14.1	80.3
Spring wheat chaff	131	0.666	17.6	0.106	2.8	0.143	3.8	0.080	2.1	0.329	8.7	0.300	8.0	94.5
Spring wheat grain	131	0.175	6.4	0.074	2.7	0.002	0.1	0.003	0.1	0.078	2.8	0.082	3.0	91.9
Plant back interval: 121 DAT														
Lettuce leaf	41	0.087	48.2	0.008	4.3	0.002	1.1	0.003	1.4	0.012	6.8	0.064	35.2	67.2
Lettuce head	55	0.114	51.5	0.007	3.1	0.002	1.0	0.003	1.5	0.013	5.7	0.094	42.4	93.4
White radish top	109	0.097	34.2	0.004	1.5	0.008	2.7	0.009	3.1	0.021	7.3	0.070	24.6	93.1
White radish root	109	0.061	44.5	0.003	2.0	0.001	0.9	0.001	1.0	0.005	3.9	0.052	38.3	94.7
Spring wheat forage	67	0.132	31.8	0.004	1.0	0.010	2.4	0.014	3.3	0.028	6.6	0.096	23.2	93.7
Spring wheat straw	146	0.511	29.4	0.031	1.8	0.104	6.0	0.060	3.5	0.194	11.2	0.283	16.3	93.3
Spring wheat chaff	146	0.271	26.5	0.018	1.7	0.094	9.2	0.031	3.0	0.143	14.0	0.155	15.2	110.1
Spring wheat grain	146	0.037	7.0	0.012	2.3	0.013	2.4	0.002	0.3	0.027	5.0	0.021	3.9	128.3

Matrix	DAP	Methanol extract		Organosoluble						Sum organosoluble		Water phase		Recovery[1)]
				Isohexane phase		Dichloromethane phase		Ethyl acetate phase						
		[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[%]
Plant back interval: 365 DAT														
Lettuce leaf	29	0.043	38.0	0.002	1.9	0.002	1.6	0.002	1.4	0.005	4.8	0.031	27.4	84.9
Lettuce head	56	0.050	54.5	0.003	2.9	0.002	2.0	0.001	1.4	0.006	6.3	0.042	45.8	95.6
White radish plant	13	0.031	32.9	0.001	1.2	0.003	3.2	0.002	2.1	0.006	6.5	0.022	23.4	90.9
White radish top	77	0.042	44.7	0.001	1.2	0.003	2.8	0.003	3.0	0.007	7.0	0.030	31.8	86.7
White radish root	77	0.034	56.7	0.001	1.2	0.001	2.2	0.001	1.3	0.003	4.7	0.029	48.9	94.5
Spring wheat forage	49	0.064	31.8	0.000	0.2	0.003	1.3	0.004	2.0	0.007	3.5	0.061	30.5	107.1
Spring wheat straw	109	0.482	33.9	0.031	2.2	0.033	2.3	0.031	2.2	0.096	6.7	0.349	24.5	92.2
Spring wheat chaff	109	0.371	26.6	0.024	1.7	0.020	1.4	0.024	1.7	0.067	4.8	0.268	19.2	90.4
Spring wheat grain	109	0.059	5.0	0.036	3.0	0.003	0.3	0.003	0.3	0.042	3.6	0.044	3.7	145.2

DAP = Days After Planting (or sowing, respectively)
DAT = Days After Treatment
1) Recovery calculated as (Isohexane phase + Dichloromethane phase + Ethyl acetate phase + Water phase) [mg/kg] o 100 / Methanol extract [mg/kg]

3. Identification, characterization and quantitation of extractable residues

The identification of metabolites was mainly based on fractionation of a wheat straw methanol extract (30 DAT) and analysis of the purified fractions (seven fractions) by HPLC-MS and / or co-chromatography experiments with reference items. In fraction 1 a group of three sugars (glucose, fructose and sucrose) was identified. In other samples the sugars (glucose, fructose and sucrose) were always collectively assigned, because they were not consistently separated upon quantitative HPLC analysis. The metabolite M222F001 of metiram was identified in fraction 5 by HPLC-MS. In the remaining fractions no further components were identified. No evidence was obtained for the occurrence of ETU (imidazolidine-2-thione) in the rotational crop samples.

In lettuce leaf the carbohydrates (glucose, fructose, sucrose) represented the main components in the extractable radioactive residues (0.295 mg/kg or 38.5 % TRR at 30 DAT, 0.060 mg/kg or 33.1 % TRR at 121 DAT and 0.011 mg/kg or 10.0 % TRR at 365 DAT). The metabolite M222F001 was the second most abundant component at 30 DAT and 121 DAT (0.123 mg/kg or 16.0% TRR and 0.011 mg/kg or 6.1 % TRR) and was also detected at 365 DAT (0.002 mg/kg or 2.2 % TRR). The carbohydrates (glucose, fructose, sucrose) and the metabolite M222F001 were also identified in the ammonia solubilizate after solvent extraction of 30 DAT (0.012 mg/kg or 1.6% TRR and 0.014 mg/kg or 1.9% TRR, respectively).

In lettuce head the carbohydrates (glucose, fructose, sucrose) were the main constituents in the extractable radioactive residues (0.273 mg/kg or 41.7 % TRR at 30 DAT, 0.085 mg/kg or 38.3% TRR at 121 DAT and 0.044 mg/kg or 47.9 % TRR at 365 DAT). The metabolite M222F001 was the second most abundant component at 30 DAT and 121 DAT (0.101 mg/kg or 15.3% TRR and 0.022 mg/kg or 9.8% TRR) and was also detected at 365 DAT (0.002 mg/kg and 2.0 mg/kg). The carbohydrates (glucose, fructose, sucrose) and the metabolite M222F001 were also identified in the solubilizates after solvent extraction of 30 DAT (0.055 mg/kg or 8.4 % TRR and 0.023 mg/kg or 3.7 % TRR, respectively).

In white radish plant (365 DAT) the carbohydrates (glucose, fructose, sucrose) were identified as the main components in the extractable radioactive residues (0.008 mg/kg or 8.7 % TRR) and metabolite M222F001 was also present (0.001 mg/kg or 0.8 % TRR).

The carbohydrates (glucose, fructose, sucrose) were the most abundant components in the extractable radioactive residues of white radish top (0.187 mg/kg or 20.6 % TRR at 30 DAT, 0.046 mg/kg or 16.4 % TRR at 121 DAT and 0.011 mg/kg or 12.1 % TRR at 365 DAT). The metabolite M222F001 was the second most abundant component at 30 DAT and 121 DAT (0.040 mg/kg or 4.4 % TRR and 0.008 mg/kg or 2.8 % TRR) and was also identified at 365 DAT (0.002 mg/kg or 2.3 % TRR). In the solubilizates after solvent extraction of 30 DAT the carbohydrates (glucose, fructose, sucrose) were the main constituents (0.099 mg/kg or 11.0% TRR) and metabolite M222F001 was the second most abundant component and (0.042 mg/kg or 4.6 % TRR).

In white radish root the carbohydrates (glucose, fructose, sucrose) represented the main constituents in the extractable radioactive residues (0.168 mg/kg or 42.8 % TRR at 30 DAT, 0.052 mg/kg or 38.4 % TRR at 121 DAT and 0.020 mg/kg or 33.5 % TRR at 365 DAT). The metabolite M222F001 was the second most abundant component at 30 DAT and 121 DAT (0.011 mg/kg or 2.9% TRR and 0.004 mg/kg or 2.7 % TRR) and was also detected at 365 DAT (0.002 mg/kg or 3.0 % TRR). The carbohydrates (glucose, fructose, sucrose) were also the main constituents in the solubilizates after solvent extract of 30 DAT (0.068 mg/kg or 17.3% TRR) and metabolite M222F001 was also the second most abundant component (0.011 mg/kg or 2.8% TRR).

In spring wheat forage the carbohydrates (glucose, fructose, sucrose) were the main constituents in the extractable radioactive residues (0.450 mg/kg or 26.6 % TRR at 30 DAT, 0.091 mg/kg or 21.9 % TRR at 121 DAT and 0.045 mg/kg or 22.3 % TRR at 365 DAT). The metabolite M222F001 was the second most abundant component at 30 DAT and 121 DAT (0.062 mg/kg or 3.6% TRR and 0.014 mg/kg or 3.3 % TRR) and was also identified at 365 DAT (0.002 mg/kg or 1.0 % TRR). In the solubilizates after solvent extraction of 30 DAT the carbohydrates (glucose, fructose, sucrose) were also the main constituents (0.101 mg/kg or 5.9 % TRR) and metabolite M222F001 was the second most abundant component (0.076 mg/kg or 4.5 % TRR).

The carbohydrates (glucose, fructose, sucrose) were the main components in the extractable radioactive residues of spring wheat straw (0.847 mg/kg or 20.0 % TRR at 30 DAT, 0.279 mg/kg or 16.1 % TRR at 121 DAT and 0.373 mg/kg or 26.2 % TRR at 365 DAT). The metabolite M222F001 was identified as the second most abundant component (0.298 mg/kg or 7.0% TRR at 30 DAT, 0.075 mg/kg or 4.3% TRR at 121 DAT and 0.054 mg/kg or 3.8 % TRR at 365 DAT). In the solubilizates after solvent extraction of 30 DAT and 121 DAT the carbohydrates (glucose, fructose, sucrose) were also the main constituents (0.204 mg/kg or 4.8 % TRR and 0.099 mg/kg or 5.7 % TRR) and metabolite M222F001 was the second most abundant component at 30 DAT and (0.119 mg/kg or 2.8 % TRR).

In spring wheat chaff the carbohydrates (glucose, fructose, sucrose) were the main constituents in the extractable radioactive residues (0.488 mg/kg or 12.9 % TRR at 30 DAT, 0.208 mg/kg or 20.4 % TRR at 121 DAT and 0.360 mg/kg or 25.8 % TRR at 365 DAT). The metabolite M222F001 was the second most abundant component (0.088 mg/kg or 2.3% TRR at 30 DAT, 0.019 mg/kg or 1.8% TRR at 121 DAT and 0.039 mg/kg or 2.8 % TRR at 365 DAT). In the solubilizates after solvent extraction of 30 DAT the carbohydrates (glucose, fructose, sucrose) were also the main constituents (0.513 mg/kg or 13.6% TRR) and metabolite M222F001 was the second most abundant component (0.130 mg/kg or 3.4 % TRR).

In spring wheat grain the carbohydrates (glucose, fructose, sucrose) were the main constituents in the extractable radioactive residues and the solubilizates after solvent extraction (1.726 mg/kg or 62.7 % TRR at 30 DAT, 0.328 mg/kg or 61.9 % TRR at 121 DAT and 0.586 mg/kg or 49.6 % TRR at 365 DAT). The metabolite M222F001 was also identified (0.123 mg/kg or 4.5 % TRR at 30 DAT, 0.007 mg/kg or 1.3% TRR at 121 DAT and 0.046 mg/kg or 3.9 % TRR at 365 DAT).

The results of the carbohydrates (glucose, fructose, sucrose) and the metabolite M222F001 identified in the ammonia solubilizate after solvent extraction and in the extractable radioactive residues of 30 DAT are summarized in Table 6.6.1-6.

Table 6.6.1-6: Summary of identified components and portions characterized in rotational crop matrices after treatment with ^{14}C-BAS 222 F (^{14}C-Metiram; 1x12.500 kg as/ha) and plant back intervals of 30, 121 and 365 days

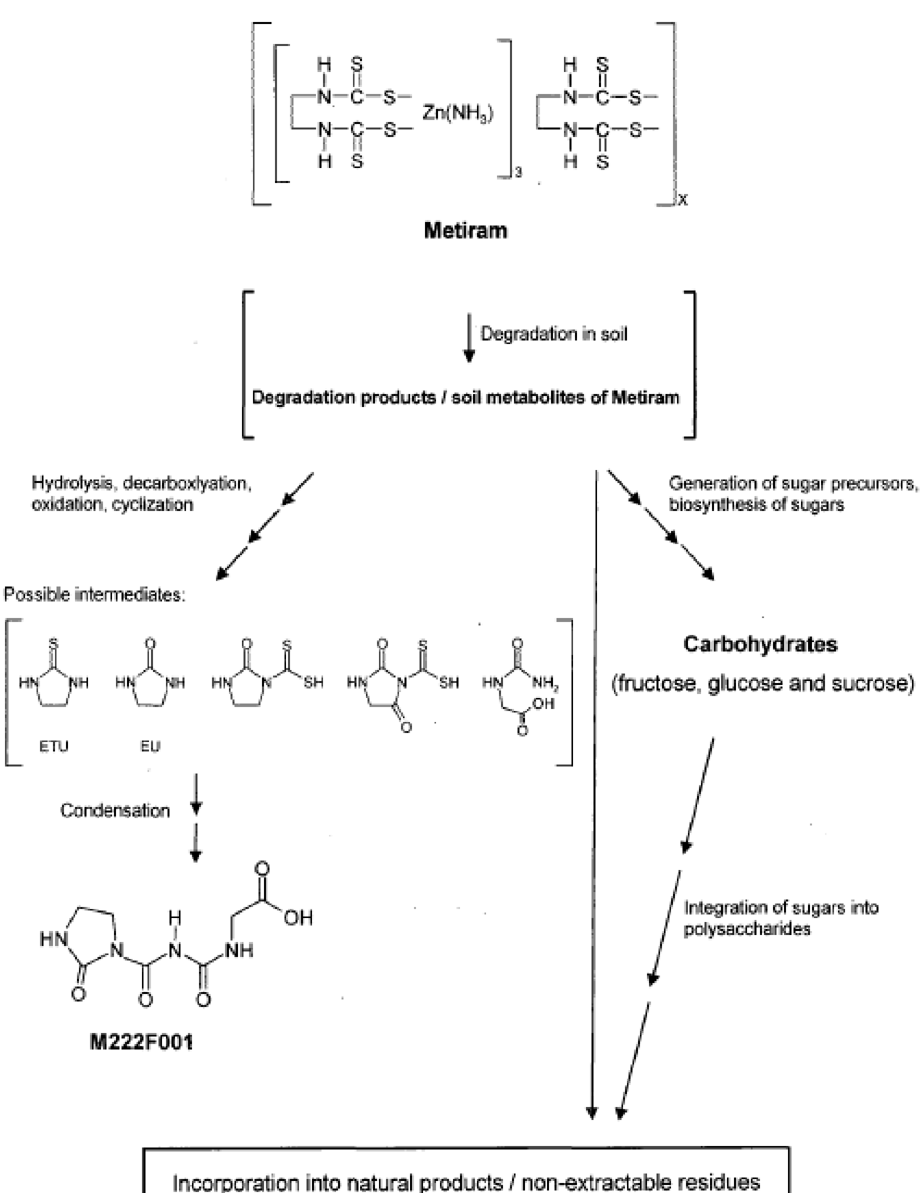
Matrix	DAP	Carbohydrates (glucose, fructose, sucrose)		M222F001		Sum of identified components		Total characterized	
		[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]	[mg/kg]	[% TRR]
Plant back interval: 30 DAT*									
Lettuce leaf	38	0.307	40.1	0.137	17.9	0.444	58.0	0.197	25.7
Lettuce head	59	0.328	50.0	0.125	19.0	0.453	69.0	0.074	11.3
White radish top	88	0.286	31.6	0.082	9.0	0.368	40.7	0.356	39.4
White radish root	88	0.236	60.1	0.022	5.7	0.258	65.8	0.054	13.7
Spring wheat forage	54	0.550	32.6	0.138	8.2	0.688	40.7	0.565	33.4
Spring wheat straw	131	1.051	24.8	0.417	9.8	1.468	34.6	1.416	33.4
Spring wheat chaff	131	1.000	26,5	0.218	5.8	1.218	32.3	1.158	30.7
Spring wheat grain	131	1.726	62.7	0.123	4.5	1.849	67.2	0.701	25.5
Plant back interval: 121 DAT									
Lettuce leaf	41	0.060	33.1	0.011	6.1	0.071	39.2	0.075	41.3
Lettuce head	55	0.085	38.3	0.022	9.8	0.107	48.2	0.072	32.7
White radish top	109	0.046	16.4	0.008	2.8	0.054	19.2	0.156	55.2
White radish root	109	0.052	38.4	0.004	2.7	0.056	41.0	0.053	38.8
Spring wheat forage	67	0.091	21.9	0.014	3.3	0.104	25.1	0.198	47.8
Spring wheat straw	146	0.378	21.7	0.075	4.3	0.452	26.0	0.688	39.6
Spring wheat chaff	146	0.208	20.4	0.019	1.8	0.227	22.2	0.456	44.6
Spring wheat grain	146	0.328	61.9	0.007	1.3	0.335	63.2	0.151	28.5
Plant back interval: 365 DAT									
Lettuce leaf	29	0.011	10.0	0.002	2.2	0.014	12.1	0.076	67.8
Lettuce head	56	0.044	47.9	0.002	2.0	0.045	49.9	0.032	35.2
White radish top	77	0.011	12.1	0.002	2.3	0.013	14.4	0.081	86.8
White radish root	77	0.020	33.5	0.002	3.0	0.022	36.5	0.027	45.1
White radish plant	13	0.008	8.7	0.001	0.8	0.009	9.4	0.070	73.8
Spring wheat forage	49	0.045	22.3	0.002	1.0	0.047	23.3	0.102	50.9
Spring wheat straw	109	0.373	26.2	0.054	3.8	0.427	30.0	0.720	50.5
Spring wheat chaff	109	0.360	25.8	0.039	2.8	0.399	28.6	0.690	49.4
Spring wheat grain	109	0.586	49.6	0.046	3.9	0.632	53.5	0.454	38.4

* Sum of ammonia solubilizate after solvent extraction residues and extractable radioactive residues

4. Metabolic pathway

Since metiram is a water-insoluble complex, the metabolism in crops implies the decomposition of the complex and formation of soluble degradation products in soil prior plant uptake. The soil metabolites of metiram are taken up and transformed in rotational crops primarily into carbohydrates (fructose, glucose and sucrose) and to a lower extend to metabolite M222F001. Incorporation of metiram moieties into sugar molecules requires the transformation of the soil metabolites into suitable compounds for the biosynthesis of carbohydrates. As a consequence of the incorporation of metiram moieties into sugars, they are also incorporated into biopolymers (e.g. amylopectin and cellulose). Another transformation path of metiram leads to the condensation product M222F001. The formation of M222F001 from metiram presumes a series of transformation steps, which include hydrolysis, decarboxylation, cyclization, oxidation and condensation. In the present study neither ETU nor any other known degradation product of metiram was found.

Figure 6.6.1-1: Metabolic Pathway of BAS 222 F in Rotational Crops



5. Storage stability

All samples were stored in a freezer at approximately -18 °C or below during the course of the study. A comparison of the extractabilities and of the metabolite patterns (HPLC analyses) obtained at the beginning and at the end of the investigation period (representative samples of lettuce head 30 DAT and spring wheat straw 30 DAT) showed that there was no relevant change in the nature of the radioactive residues during storage of the plant samples over a period of at least three years. The stability in stored extracts was demonstrated for at least 30 months.

III. CONCLUSION

The metabolism of ^{14}C-BAS 222 F (metiram) in representative succeeding crops was investigated after application to bare soil at a single rate of 1 x 12.500 kg as/ha. After ageing of the soil for 30, 121 and 365 days, the crops lettuce, white radish and spring wheat were cultivated. In brief, the parent metiram was not detected in the crop plants. Metiram is a metal ion complex which desintegrates upon contact with water and therefore cannot be taken up from soil into the plant. Furthermore, none of the known degradation products of metiram were found in the plant. In contrast, the radioactive residue consisted predominantly of natural plant constituents such as sugars indicating that radioactive residue taken up from the soil into the plant is quantitatively entering primary metabolism followed by incorporation of the radiocarbon into plant constituents such as polysaccharides. In addition, the metabolite M222F001 was identified in all matrices. Considering its molecular structure this compound is a condensation product of various low molecular weight intermediates formed by degradation in soil. As-yet this condensation compound has not been detected in any other metiram metabolism study, and therefore is considered an incidential finding due to the laboratory conditions of this present study (an indicative assessment of exposure is provided in section 6.10 of the present dossier).

Regarding total radioactive residues, the TRR in unripe lettuce leaf did not exceed 0.766 mg/kg for all plant back intervals. The total residues in ripe lettuce head were comparable and reached up to 0.656 mg/kg. The TRR in immature white radish plant and mature white radish top ranged from 0.093 to 0.906 mg/kg. In mature white radish root, the residue levels ranged from 0.059 to 0.393 mg/kg. In spring wheat, the highest residue levels were measured in straw (ranging from 1.424 to 4.245 mg/kg) and chaff (1.023 to 3.775 mg/kg). The total radioactive residues in grain were somewhat lower and accounted for 0.530 to 2.752 mg/kg, and concentrations of 0.200 to 1.690 mg/kg were found in forage. After aging and ploughing, the residue concentrations in the soil decreased from the first plant back interval to the second and third plant back interval, respectively.

Regarding extractability of the radioactive residues with methanol and water generally above 50 % of the TRR were obtained for the lettuce samples, around 50 % for white radish top and root and up to 50 % or below for unripe white radish plant, spring wheat forage, straw and chaff. The extractability of spring wheat grain was approximately 10 to 20 %. With the exception of spring wheat grain, the main part of the radioactive residue was extracted with methanol.

Regarding composition of the residue, the parent compound metiram was not detected by HPLC analyses of the extracts or solubilizates. Also, neither ETU nor any other known degradation product of metiram were found. Uptake of metiram by crops implies the decomposition of the metiram complex and formation of soluble degradation products in soil, as it was previously described (Staudenmaier H.: Aerobic Metabolism of BAS 222 F (Metiram) in Soil, BASF Doc ID 2002/1012954 and Staudenmaier H.: Aerobic Metabolism of BAS 222 F (Metiram) in Cashmere Soil, BASF Doc ID 2002/1011913). The soil metabolites of metiram were taken up and transformed in the rotational crops primarily into sugars (glucose, fructose and sucrose), which were without exception the most abundant components in all matrices (in methanol/ water extracts and solubilizates after solvent extraction). Due to the incorporation of metiram moieties into sugars, they were finally also integrated into polysaccharides (e.g. amylopectin and cellulose). The incorporation of radioactive residues into polysaccharides was proven by treatment of the spring wheat grain (30 DAT) extraction residues with macerozyme and amylase / amyloglucosidase, whereby virtually all released components were identified as sugars (a smaller portion was identified as metabolite M222F001). The metabolite M222F001 was identified in all matrices and represented in the majority of cases the second most abundant component. Noteworthy, the metabolite M222F001 was not identified in previous investigations on metabolism of metiram.

CA 6.6.2 Magnitude of residues in rotational crops

According to Reg. 283/2013, studies on the magnitude of residues in rotational crops are required under the following circumstances:

If the metabolism studies indicate that residues of the active substance or of relevant metabolites or breakdown products either from plant or soil metabolism may occur (> 0.01 mg/kg), limited field studies and, if necessary, field trials shall be carried out.

Studies shall not be required in the following cases:
- no metabolism studies on rotational crops are to be performed, or
- metabolism studies on rotational crops show that no residues of concern are to be expected in rotational crops

A magnitude of the residue study is not required for metiram, based on results of the metabolism study on rotational crops for metiram (see section 6.6.1)
In brief, the parent metiram was not detected in the plant. Metiram is a metal ion complex which desintegrates upon contact with water and therefore cannot be taken up from soil into the plant. Furthermore, none of the known degradation products of metiram were found in the plant. In contrast, the radioactive residue consisted predominantly of natural plant constituents such as sugars indicating that radioactive residue taken up from the soil into the plant is quantitatively entering primary metabolism followed by incorporation of the radiocarbon into plant constituents such as polysaccharides. In addition, the metabolite M222F001 was identified in all matrices. Considering its molecular structure this compound is a condensation product of various low molecular weight intermediates formed by degradation in soil (for example see Staudenmaier H.: Aerobic Metabolism of BAS 222 F (Metiram) in Soil, BASF Doc ID 2002/1012954 and Staudenmaier H.: Aerobic Metabolism of BAS 222 F (Metiram) in Cashmere Soil, BASF Doc ID 2002/1011913). As-yet this condensation compound has not been detected in any other metiram metabolism study, and therefore is considered an incidential finding due to the laboratory conditions of this present study. An indicative assessment of exposure is provided in section 6.10 of the present dossier.

Considering the metabolism on rotation crop study has been conducted under worst case conditions, notably at an overdosed rate (factor >3) to bare soil under laboratory conditions, it can be concluded that in practice the application of metiram does not lead to uptake of residue of concern from soil by succeeding crops.

CA 6.7 Proposed residue definitions and maximum residue levels

CA 6.7.1 Proposed residue definitions

CA 6.7.1.1 Plant commodities

The definition of the relevant residue in commodities of plant origin both for MRL establisment as well as risk assessment has been extensively discussed within the peer review under Directive 91/414/EEC based on metabolism of metiram in the two crop groups of fruit/fruiting vegetable and root/tuber. This residue definition, as established by the peer review, is further supported by metabolism in leafy vegetables (additional new data submitted as part of the present dossier).

Residue definition for establishment of MRLs and enforcement: In the European Union, MRLs are established only for the raw agricultural commodities of crops (RAC, as opposed to the processed fractions thereof). In plant RAC, the parent compound metiram is the predominant portion of the residue and therefore a suitable marker compound. The terminal residue is the result of significant incorporation of metiram transformation products into various naturally occurring plant constituents which as such are not relevant for a residue definition. In the RAC, metiram transformation products notably EBIS, ETU, EU and others, are detected only in low amounts. They are dynamic intermediates leading to other metabolites of low or no toxicological concern.
Due to these facts, metiram is regarded as the only relevant component of the residue in plants (RAC) for MRL setting and enforcement purposes

MRLs in the European Union (and internationally) are not set specifically for metiram, but for the group of dithiocarbamates including also maneb, mancozeb, propineb, thiram, and ziram.
Metiram as a metal ion complex cannot be detected directly, e.g. by chromatographic means. However, determination is feasible with a multi-residue method analysing residues of dithiocarbamates measured as CS_2.
In addition, metiram can be determined with an “EBDC specific method” which is based on hydrolysis to ethylene diamine, derivatisation and final HPLC/MS-MS quantitation (see section MCA 4.3). This method, as it allows to distinguish EBDC fungicides from other CS_2 sources, is suitable as a higher tier analytical method.
Regarding enforcement of metiram the following definition of the relevant residue has been established by the peer review. As part of the present dossier it is proposed to maintain the established definition of the relevant residue for establishment of MRLs and enforcement:

Metiram, expressed as CS_2

Residue definition for data generation and risk assessment: In plant RAC, the parent compound metiram is the predominant portion of the residue (see above). The terminal residue is the result of significant incorporation of metiram transformation products into various naturally occurring plant constituents which as such are not relevant for a residue definition. Metiram transformation products noteably EBIS, ETU, EU and others, are detected only in low amounts. A potential relevance of these transformation products can be ruled out based on a detailed assessment (see sections MCA 6.7.1.3 and MCA 6.10.1).
Under food processing conditions metiram can rapidly degrade to ETU. Considering its toxicological properties, ETU has to be considered in consumer dietary risk assessment as far as processed commodities are concerned. Regarding the risk assessment of metiram the following definition of the relevant residue has been established by the peer review. As part of the present dossier it is proposed to maintain the established residue definition:

Metiram, expressed as CS_2 *ETU (only for processed commodities)*

CA 6.7.1.2 Animal commodities

The definition of the relevant residue in commodities of animal origin both for MRL establisment as well as risk assessment has been extensively discussed within the peer review under Directive 91/414/EEC based on metabolism of metiram in poultry and ruminants. The definition of the relevant residue established by the peer review is further supported by metabolism in rat (notably additional new data submitted as part of the present dossier).

The metabolism studies indicate that the parent compound metiram, as a metal ion complex, is not resorbed in the gastrointestinal tract of rat, goat, or hen. Bioavailable are only the desintegration products : dynamic intermediates formed upon solvolysis of the metal ion complex. Their significant incorporation results in various naturally occurring cell constituents as the terminal residue (these product fractions as such are not relevant for a residue definition). These dynamic intermediates are occurring at variable levels. Under realistic conditions, levels are expected to be below the quantitation limit and therefore not suitable for a residue definition. A potential relevance of these transformation products can be ruled out based on a detailed exposure assessment (see sections MCA 6.7.1.3 and MCA 6.10.1).

By default, the parent compound is considered suitable for the residue definition. A multi-residue method is available which allows the determination of metiram as well as metiram transformation products which release CS_2 (notably EBIS). Under food processing conditions metiram can rapidly degrade to ETU. Considering its toxicological properties, ETU has to be considered in consumer dietary risk assessment as far as processed commodities are concerned.

Regarding enforcement of metiram the following definition of the relevant residue has been established by the peer review. As part of the present dossier it is proposed to maintain the established residue definition:

Metiram, expressed as CS_2

Regarding risk assessment of metiram the following definition of the relevant residue has been established previously. As part of the present dossier it is proposed to maintain the established residue definition:

Metiram, expressed as CS_2 *ETU (only for processed commodities)*

The list of agreed EU endpoints is summarised below.

Table 6.7.1.2-1: EU End-points - Metiram

End-Point	Active Substance: Metiram	
	EU Agreed Endpoints (SANCO/4059/2001 - rev. 3-3, Monograph as of July 2000)	**Endpoints Used in Risk Assessment**
Residue definition in plant matrices for risk assessment	Metiram, expressed as CS_2 , ETU (only for processed commodities)	Metiram, expressed as CS_2 ETU (only for processed commodities)
Residue definition in plant matrices for monitoring	Metiram, expressed as CS_2	Metiram, expressed as CS_2
Residue definition in animal matrices for risk assessment	Metiram, expressed as CS_2 ETU (only for processed commodities)	Metiram, expressed as CS_2 ETU (only for processed commodities)
Residue definition in animal matrices for monitoring	Metiram, expressed as CS_2	Metiram, expressed as CS_2
Conversion factor between both residue definitions in animal matrices	Not applicable	Not applicable

CA 6.7.1.3 Relevance assessment of further transformation products of metiram

As part of the current metiram re-registration process, the dietary risk assessment for the consumer resulting from the use of metiram has to take into account the actual toxicological burden of the components of the residue. Therefore, the establishment of the residue definition for risk assessment purposes involves a decision on which transformation products of metiram are of toxicological concern. To achieve robustness of such a residue definition, the data base considered should be reasonably broad. Thus, first, any metiram transformation product identified in a *nature of the residue* study is to be included (i.e. crop metabolism, rotational crop metabolism, livestock metabolism, high temperature hydrolysis). And second, dietary exposure is assessed for two crop scopes, first scope, the representative uses supported in the AIR3 dossier (grape, potato) and second scope, all uses registered in EU including import tolerances.

For an initial evaluation of relevance of transformation products, the estimated human exposure can be compared with a safe threshold derived using the assumptions of the TTC concept and information on the transformation product (i.e. molecular structure and genotoxic potential). To this end, a stepwise approach is envisaged (see decision tree in *Scientific Opinion on Evaluation of the Toxicological Relevance of Pesticide Metabolites for Dietary Risk Assessment, EFSA Journal 2012;10(07):2799*). The threshold of toxicological concern (TTC) is based on the concept that exposure levels can be defined (pending the molecular structure) below which human exposure to the chemical results in “no appreciable risk to human health”.
A further refined evaluation is to be done for transformation products with calculated exposure exceeding the corresponding TTC or specific reference values. The initial estimations generally result in considerable overestimations of exposure since typically based on various worst case assumptions, in particular in the absence of data from *magnitude of the residue* studies. (In general, all intake for the crop considered is from treated crop, which is an overestimation as market share is well below 100%. For a certain diet all included food items are assumed to have residues at the upper limit which is an overestimation as most crops have residues well below the MRL. Data generation such as crop field trials are conducted to represent the worst case condition as far as residues are concerned.) Therefore, on a case-by-case basis further detailed considerations allow to refine to more realistic exposure scenarios.

For metiram, a total of eleven potentially relevant transformation products has been identified in *nature of the residue* studies (M222F004=EBIS, M222F003=EU, M222F002=ETU, M222F001, M222F007, M222F008, M222F013, M222F021, M222F022, M222F023, and glycine).

For two of these transformation products, no evaluation of relevance is provided in the context of the present assessment: first, glycine is a naturally occuring amino acid. Second, M222F002 (=ETU) is already defined a relevant metabolite and thus, is included in the existing definition of the residue for risk assessment (processed commodities).

For nine of these transformation products, dietary exposure has been estimated and expressed in percentage of the corresponding toxicological reference value (TTC or specific value, see Table 6.7.1.3-2). To allow to reproduce the exposure calculations for each of the metiram transformation products, the detailed steps are provided in a separate document (DocID 2015/1087922, see section MCA6.10.1 for a summary). For all of them, the indicative exposure calculations both chronic and acute is below (or at) the corresponding toxicological reference value (see Table 6.7.1.3-3and Table 6.7.1.3-4) indicating non-significant contribution to the overall toxicological burden.
Detailed considerations for each of the eleven transformation products are provided below. In brief, the available information, noteably molecular structure, genotoxicological investigations, occurrence in food/feed commodities, exposure estimations etc was evaluated. For each transformation product sufficient information is available to exclude relevance the compond for the purpose of dietary risk assessment. Moreover, they are not suitable as marker compounds. Thus, none of them has to be included in the existing definition of the relevant residue both for for risk assessment as wellas MRL setting.

In conclusion, this relevance assessment confirms the definition of the relevant residue for risk assessment, presently established, is sufficiently protective for dietary risk assessment (see section 6.7.1 and 6.7.2).

Table 6.7.1.3-1: Overview metabolites: occurrence, molecular structure and molecular mass

Parent			
Name	**Occurrence**	**Structure**	**Molecular mass [g/mol]**
Metiram (BAS 222 F)	not relevant		1088.7
Metabolites			
Name	**Occurrence**	**Structure**	**Molecular mass [g/mol]**
M222F004 (EBIS)	crop (lettuce, apple, potato)* goat** poultry***		176.3
M222F003 (EU)	crop (lettuce, apple, potato)* goat** poultry***		86.1
M222F002 (ETU)	crop (lettuce, apple, potato)* goat** poultry***		102.2

M222F023 (EDA)	crop (apple)* goat** poultry***		60.1
M222F021 (N-AcEDA)	crop (apple)* goat** poultry***		102.1
M222F022 (Jaffe`s Base)	crop (apple)* goat** poultry***		170.2
M222F007 (TDIT)	crop (lettuce)*		212.3
M222F001	rotational crop****		230.2
Glycine	crop (apples, potato)* goat** poultry***		75.1
M222F008 (Hydantoin)	crop (apple)* goat** poultry***		100.1
M222F013	crop (lettuce)*		114.1

* 'Metabolism of 14C-Metiram (14C-BAS 222 F) in lettuce', BASF DocID 2009/1049027
'Metiram: Nature of Residues in Apples', BASF DocID 1990/10669
'Metabolism of 14C-Metiram Complex in Potatoes', BASF DocID 1990/10668
** 'Metabolism of 14C-Metiram Complex in Lactating Goats', BASF DocID 1989/10487
*** 'Metabolism of 14C-Metiram Complex in Laying Hens', BASF DocID 1990/5080
**** 'Confined rotational crop study with 14C-BAS 222 F', BASF DocID 2009/1017248

The following toxicological reference values were used for chronic assessments (ADI) and acute assessments (ARfD).

Table 6.7.1.3-2: Toxicological reference values used

Name	End-Point		Study	Safety factor	Reference
	Acceptable Daily Intake (ADI)	**Acute Reference Dose (ARfD)**			
Metiram	0.03 mg/kg bw/d		2-year study in rats	100	SANCO/4059/2001-rev 3.3 (03.06.2005)
		not necessary - not allocated	-	-	SANCO/4059/2001-rev 3.3 (03.06.2005)
ETU	0.002 mg/kg bw/d		2-year study in rats	100	SANCO/4059/2001-rev 3.3 (03.06.2005)
		0.05 mg/kg bw/d	-	-	SANCO/4059/2001-rev 3.3 (03.06.2005)
EU	0.06 mg/kg bw/d		90 day study, rat	200	M-CA 5.8
		0.06 mg/kg bw/d	90 day study, rat	200	M-CA 5.8
EBIS	0.02 mg/kg bw/d		90 day study, rat	200	M-CA 5.8
		0.15 mg/kg bw/d	90 day study, rat	200	M-CA 5.8
EDA / NAcEDA	0.2 mg/kg bw/d		90 day study, rat	200	M-CA 5.8
		0.5 mg/kg bw/d	90 day study, rat	200	M-CA 5.8
TTC approach	0.0015 mg/kg bw/d		none	not relevant	TTC Cramer class III*
		0.005 mg/kg bw/d	none	not relevant	recommended by EFSA PPR*

* Scientific Opinion on Evaluation of the Toxicological Relevance of Pesticide Metabolites for Dietary Risk Assessment (EFSA Journal 2012;10(07):2799), page 32

Consideration on M222F004 (EBIS)

M222F004 is a dynamic intermediate which is formed from metiram when this metal ion complex is exposed to water (solvolysis). EBIS itself is unstable and breaks down into compounds such as EU and ETU (as indicated by results of freezer storage stability investigations, see section MCA6.1). The transient formation of EBIS from metiram can occur under abiotic conditions as well as does occur generally in metabolism studies with metiram in plants and animals (livestock, rat). Noteworthy, it is a metabolite in common with fungicides of the dithiocarbamate group.

In rat, EBIS can be considered as being present in significant amounts based on the finding of a methylated derivative of EBIS, M222F024. Levels in bile amounted to 5.1% of applied dose, lower levels were found in faeces and urine (section MCA5.1). Thus, the toxicity of EBIS can be considered as covered by studies with the parent metiram. As a more conservative alternative to applying the metiram toxicological reference values, EBIS specific toxicological reference can be derived based on the available toxicological data (section MCA5.8, 0.02 mg/kg bw/d (ADI) and 0.15 mg/kg bw (ARfD).

In order to provide an indicative assessment of dietary exposure, the chronic and acute exposure was calculated based on residue data calculated from nature of the residue studies. EBIS levels determined in crop residue trials are not covered by storage stability (instability even under freezer storage conditions, section MCA6.1). Therefore, residue levels were estimated based on EBIS/metiram ratio (*nature of the residue* studies) and metiram field residue data. For animal commodities, EBIS levels (*nature of the residue* studies) were normalized to a realistic feed burden.
These calculations confirmed that the exposure is below the toxicological reference values, not only for (a) the uses supported in the present dossier but also when (b) all registered uses of metiram including import tolerances are included:

chronic	(a) up to 3.9 % ADI,	(b) up to 8.6% ADI,
acute	(a) up to 17.8 %ARfD,	(b) up to 33.7% ARfD.

Considering that conservative assumptions were used both for estimating dietary exposure as well as for deriving a toxicological reference value (see above), these results indicate, with a large margin of safety, the absence of a health concern.
It should be noted that metiram residue data was generated using a common moiety method detecting CS_2. In addition to metiram, CS_2 is also released from EBIS. Therefore, transient amounts of EBIS can be considered included in the residue of metiram, expressed as CS_2.
In conclusion, absence of a health concern under very conservative assumptions as well as instability of the molecule show that the transformation product EBIS is not relevant for the residue definition, both regarding risk assessment as well as enforcement.
Result: no further consideration for M222F004 (EBIS) is necessary.

Consideration on M222F003 (EU)

EU is a dynamic intermediate which is formed from metiram when this metal ion complex is exposed to water (solvolysis). EU is converted further to ETU. Stability investigations indicated that EU also has limited stability (up to 90d under freezer storage conditions, section MCA6.1). The transient formation of EU from metiram can occur under abiotic conditions as well as is does occur generally in metabolism studies with metiram in plants and animals (livestock, rat). Noteworthy, it is a metabolite in common with fungicides of the dithiocarbamate group.

In rat, EU can be considered as being present in significant amounts based on the finding levels in urine up to 5.8% of applied dose. EU was also found in faeces, albeit at lower level (section MCA5.1). As a polar low molecular weight molecule, EU is rapidly excreted in addition to effective elimination by incorporation into natural cell constituents (rat metabolism, section MCA5.1 and livestock metabolism, section MCA6.2). Based on the available toxicological data EU specific toxicological reference can be derived (section MCA5.8, 0.02 mg/kg bw/d (ADI) and 0.15 mg/kg bw (ARfD).

In order to provide an indicative assessment of dietary exposure, the chronic and acute exposure was calculated based on residue data calculated from nature of the residue studies. For two crops grape and potato, EU levels determined in crop residue trials are available. For other crops, residue levels were estimated based on EU/metiram ratio (*nature of the residu*e studies) and metiram field residue data. For animal commodities, EU levels (*nature of the residue* studies) were normalized to a realistic feed burden.
These calculations confirmed that the exposure is below the toxicological reference values, not only for (a) the uses supported in the present dossier but also when (b) all registered uses of metiram including import tolerances are included:
chronic (a) up to 0.1 % ADI, (b) up to 1.4% ADI,
acute (a) up to 7.7 %ARfD, (b) up to 73.6% ARfD).
Considering that conservative assumptions were used (see above), these results indicate absence of a health concern.

For enforcement purpose in commodities of plant origin, the parent metiram can be determined using a common moiety method measuring CS_2 as well as an "EBDC specific method" (section MCA4.3). Determination of EU is not possible with the analytical method for metiram, thus requiring application of a separate method. However, the available data shows that residue levels of metiram are generally severalfold higher than EU, and therefore better suited as a marker compound for enforcement. For enforcement purpose in commodities of animal origin, EU is not a suitable marker compound as residue levels resulting from the use of metiram are at the LOQ of 0.01 mg/kg if quantifyable at all. In light of the fact that conservative exposure estimations indicate absence of a health concern for EU, the metiram transformation product EU is considered non-relevant for the residue definition for MRL setting.

For risk assessment purpose, the parent metiram is generally the predominant component of the residue in crops. Under practical conditions, levels of EU are considerably lower than metiram levels as exemplarily shown by analysis of metiram as well as metabolite EU in crop field trials with grape. Extrapolation of EU residue levels in other crops (based on EU/metiram ratios observed in metabolism studies) are including very conservative assumptions. In commodities of animal origin, residue levels resulting from the use of metiram are at the LOQ of 0.01 mg/kg if quantifyable at all. Taken together, the data shows that under realistic conditions, the metabolite EU does not contribute significantly to the toxicological burden. In conclusion, the metiram transformation product EU is considered non-relevant for the residue definition for risk assessment.
Result: no further consideration for M222F003 (EU) is necessary.

Consideration on M222F002 (ETU)

M222F002 (ETU) is a transformation product found in plant and livestock metabolism studies. It is formed in significant amounts from metiram under food processing conditions. Consequently, ETU has been included in the residue definition for processed commodities. A detailed dietary exposure assessment is provided in section MCA 6.9 of the present dossier.
Result: no further consideration for M222F002 (ETU) is necessary.

Consideration on M222F023 / M222F021 (EDA / N-AcEDA)

The compound M222F023 and its acetylated derivative M222F021 are dynamic intermediates formed after desintegration of the metiram metal ion complex, which is then further transformed into glycine thereby channeling metiram derived carbon into metabolic carbon pool. This pair of compounds has structural characteristics similar to the ligand of metiram complex. It is found in metiram metabolism studies in plant and livestock (tissues, milk, egg).
To provide an indicative assessment of dietary exposure, the chronic and acute exposure scenarios were calculated and compared with compound specific toxicological reference values (see MCA 5.8, Table 6.7.1.3-1). For chronic assessment an ADI of 0.02 mg/kg bw/d was used as toxicological reference value and for acute assessment 0.5 mg/kg bw was used. Calculations are provided for two crop scopes: first, the uses supported in the present dossier (grape, potato) and second, the entirety of all registered EU uses of metiram. For all scenarios exposure was less than 1% of the toxicological reference value.

In conclusion, the contribution of M222F023/M222F021 to the toxicological burden resulting from the uses of metiram is insignificant. Therefore, M222F023/M222F021 is not relevant for the definition of the relevant residue both regarding MRL setting and dietary risk assessment.
Result: no further consideration for both M222F023 and M222F021 (EDA / NAcEDA) is necessary.

Consideration on M222F022 (Jaffes Base)

This compound has structural characteristics similar to ETU with an addition of an ethylene diamine moiety. M222F022 is found in metiram metabolism studies in plant and livestock (tissues, milk, egg).
To provide an indicative assessment of dietary exposure, the chronic and acute exposure scenarios were calculated and compared with default toxicological reference values according to the TTC approach. For chronic assessment the Cramer class III trigger of 0.0015 mg/kg bw/d was used as toxicological reference value and for acute assessment 0.005 mg/kg bw was used. Both genotox studies as well as a QSAR (DEREK) evaluation did not indicate any carcinogenic, genotoxic or neurotoxic effect (see section MCA 5.1), thereby confirming the applicability of the default toxicological reference values. Considering the uses supported in the present dossier (and in addition, all registered uses of EU metiram) for the chronic scenario exposures 1.9 (and 9.0 %) of the toxicological reference value were obtained. For the acute scenario exposures of 1.7% (and 14.3 %) of the toxicoloical reference value were obtained.
In conclusion, the contribution of M222F022 to the toxicological burden resulting from the uses of metiram is insignificant. Therefore, M222F022 is not relevant for the definition of the relevant residue both regarding MRL setting and dietary risk assessment.
Result: no further consideration for M222F022 (Jaffes base) is necessary.

Consideration on M222F007 (TDIT)

The compound M222F007 was identified in only one of the nature of the residue studies of metiram metabolism in lettuce). In the only matrix analysed in this study, M222F007 was found at 1.4% TRR, thus at only low proportion of the overall residue. Considering the artificial conditions of the laboratory study, it can be assumed that under realistic conditions the proportion of M222F007 would be even lower. While this compound has not been identified in other nature of the residue studies (apple, potato, goat, hen), it has previously been reported in soil and is known to undergo fast degradation. In addition, it was identified in low amounts in a rat excretion study (see section MCA 5.1). Taken together, the incidental occurrence in the metiram studies available, indicates that M222F007 is not a ubiquitous transformation product of metiram.
To provide an indicative assessment of dietary exposure, the chronic and acute exposure scenarios were calculated and compared with default toxicological reference values according to the TTC approach. For chronic assessment the Cramer class III trigger of 0.0015 mg/kg bw/d was used as toxicological reference value and for acute assessment 0.005 mg/kg bw was used. Both genotox studies as well as a QSAR (DEREK) evaluation did not indicate any carcinogenic, genotoxic or neurotoxic effect (see MCA 5.1), thereby confirming the applicability of the default toxicological reference values.

Chronic exposure calculations resulted in a value of 0% of the toxicological reference value (for both crop scopes, representative uses and all registered uses). The acute exposure calculations for grape and potato resulted in a value of 0% of the toxicological reference value. If all EU registered uses, i.e. including leafy vegetables, were considered values at the toxicological threshold were obtained (100.9% for scarole, children, 31.0% for lettuce). This however is an overestimation since considering these artificial conditions of the laboratory study, M222F007 levels under realistic conditions are expected to be much lower. Therefore, these calculations indicate absence of an appreciable dietary risk (chronic and acute) to human health. The contribution to the toxicological burden is insignificant. In conclusion, M222F007 is not relevant for the definition of the relevant residue for dietary risk assessment as well as for MRL setting.
Result: no further consideration for M222F007 (TDIT) is necessary.

Consideration on M222F001

This transformation product of metiram was identified only in one of the nature of the residue studies with metiram hinting at the likelihood of only incidental occurrence (rotational crop metabolism. After bare soil treatment at 3X (dose 12.5 kg ai/ha compared with 4.2 kg ai/ha maximal seasonal application rate) M222F001 was found in edible commodities at levels up to 0.137 mg/kg (lettuce leaf).
Based on its molecular structure this compound is a condensation product of EU (M222F003) with various low molecular weight intermediates formed by degradation in soil (see section MCA 6.6.2). The rotational crop study was conducted under laboratory conditions with plants cultivated in plastic boxes where elimination from root zone of such low molecular weight molecules is restricted resulting in an artificial concentration in the root zone.

Radiolabelled metiram at a dose of 3X of the seasonal application rate was applied to bare soil. The purpose is to study qualitatively the nature of the residue. It only provides limited knowledge on the quantity of components of the residue. Even if an overdosing factor of 3 is considered, this study design does not account for the effect of crop interception (study design: bare soil application) as well as the effect of degradation between the applications (study design: simultaneous application) which both would result in reduction of residue levels under realistic conditions. Considering these artificial conditions of the laboratory study, it can be concluded that under realistic conditions M222F001 residue would be much lower if present at all.
However, in order to provide an indicative assessment of dietary exposure, the chronic and acute exposure scenarios were calculated and compared with default toxicological reference values according to the TTC approach. For chronic assessment the Cramer class III trigger of 0.0015 mg/kg bw/d was used as toxicological reference value and for acute assessment 0.005 mg/kg bw was used. QSAR (DEREK) evaluation did not indicate any carcinogenic, genotoxic or neurotoxic effect (see MCA 5.1), thereby confirming the applicability of the default toxicological reference values.
Taking into account the representative uses supported in the present dossier (or, in addition, all registered uses of EU metiram) for the chronic scenario, exposures up to 2.8% (or 5.3%) of the toxicological reference value were obtained. For the acute scenario, exposures up to 21.5% (or 80.4%) of the toxicological reference value were obtained.
Considering the significant overestimation of estimated residue levels used in these calculations, the contribution of M222F001 to the dietary burden is insignificant. Therefore, M222F001 is not relevant for the definition of the relevant residue both regarding MRL setting and dietary risk assessment.
Result: no further consideration for M222F001 is necessary.

Consideration on glycine

Glycine is an amino acide normally present in the diet. Therefore further consideration of this compound is not required. The detection of glycine in the radioactive residue in metabolism studies with radiolabeled metiram indicates that the radiocarbon is eventually entering the metabolic carbon pool which is in accordance with naturally occurring cell constituents as the terminal residue of metiram.
Result: no further consideration for glycine is necessary.

Consideration on M222F008

This compound was identified in metabolism studies conducted in plant and livestock, however only in few matrices and in low proportions (goat liver <1% TRR, goat muscle 3.7% TRR, hen muscle 8.1% TRR, hen skin 2.7%). It can be considered a transient intermediate derived by oxidation from EU towards glycine and thus entry into the metabolic carbon pool. Considering that M222F008 was found in livestock studies which were considerably overdosed, levels under realistic conditions can be expected to be insignificant (see TTC position paper, DocID201571087922, table 18 provides overdosing factors, tables 21 and 22 list input values).
To provide an indicative assessment of dietary exposure, the chronic and acute exposure scenarios were calculated and expressed as toxicological reference values according to the TTC approach both considering the uses supported in the present dossier as well as considering all registered uses of EU metiram. For all scenarios exposure was less than 1% of the toxicological reference value.
In conclusion, the contribution of M222F008 to the dietary burden resulting from the uses of metiram is insignificant. Therefore, M222F008 is not relevant for the definition of the relevant residue both regarding MRL setting and dietary risk assessment.
Result: no further consideration for M222F008 is necessary.

Consideration on M222F013 (Formamid derivative)

This compound was identified only in one study (DocID 2009/1049027). As stated in the study report (page 53, footnote 4) this compound is "*probably an artefact formed of EU*" (formamide of EU). With 0.4 % TRR, the proportion of formamide derivative detected in this study is very low, noteably when compared to the level of EU (upper level at 14.7 % TRR, common EU/ETU fraction).
Based on its incidental occurrence, M222F013 does not need to be considered a metabolite of metiram. However, in order to be comprehensive in the context of the present dossier, an indicative assessment of exposure to this compound was calculated. In a first scenario, when grouping the formamid-derivative of EU (M222F013) with EU (M222F003), the exposure to M222F013 can be considered as covered by the exposure estimation for EU alone, as EU levels are much higher compared with the M222F013 levels (in particular as the estimate for EU is already an overestimation by using the 14.7% TRR value for EU). In a second scenario (separate assessment for M222F013), an indicative assessment for the representative uses (potato, grape) results in exposures of 0.8 % of the chronic toxicological reference point (TTC Cramer class III) and 7.8 % of the default ARfD.

Similar levels well below the corresponding toxicological reference points are obtained if all registered uses of metiram are considered (chronic 1.7%, acute 32.6%). Therefore, also this calculation using the TTC approach indicates that a hypothetical exposure to M222F013 would be insignificant.
Note, for chronic assessment the Cramer class III trigger of 0.0015 mg/kg bw/d was used as toxicological reference value and for acute assessment 0.005 mg/kg bw was used. QSAR (DEREK) evaluation did not indicate any carcinogenic, genotoxic or neurotoxic effect (see MCA 5.1), thereby confirming the applicability of the default toxicological reference values.
In conclusion, M222F013 (which most likely is an artefact limited to one metabolism study) is not relevant for the definition of the relevant residue both regarding MRL setting and dietary risk assessment.

Result: no further consideration for M222F013 is necessary.

Table 6.7.1.3-3: Overview: chronic exposure assessments [1)]

Metabolite	Dietary exposure [% reference value], most critical diet		Commodity with highest contribution [% reference value]	
EBIS	8.6	DE child	Pome fruit	[6.1]
EU	1.4	DE child	Pome fruit	[1.0]
EDA/N-AcEDA	0.1	DE child	Pome fruit	[0.1]
Jaffe`s Base	9.1	FR toddler	Milk and cream	[8.7]
TDIT	0.0	IT adult	Lettuce and other salad	[0.0]
M222F001	5.3	WHO Cluster diet B	Tomatoes	[1.4]
M222F008	3.1	DE child	Pome fruit	[2.3]
M222F013	0.1	DK child	Cucurbits – edible peel	[0.0]

1) scope: uses registered in EU, including import tolerances

Table 6.7.1.3-4: Overview: acute exposure assessments

Metabolite	Commodity with highest contribution [% reference value]	Commodity with 2nd highest contribution [% reference value]	Commodity with 3rd highest contribution [% reference value]
EBIS	33.7 (Melons, children)	27.1 (Watermelons, children)	17.8 (Table grapes, children)
EU	73.6 (Scarole, children)	57.0 (Melons, children)	45.9 (Watermelons, children)
EDA/N-AcEDA	1.2 (Melons, children)	1.0 (Watermelons, children)	0.6 (Table grapes, children)
Jaffe`s Base	14.3 (Cattle milk and milk products, children)	12.2 (Bovine kidney, children)	5.5 (Bovine kidney, adults)
TDIT	100.9 (Scarole, children)	31.0 (Lettuce, children)	12.7 (Lettuce, adults)
M222F001	80.4 (Scarole, children)	24.8 (Lettuce, children)	21.5 (Potatoes, children)
M222F008	28.7 (Melons, children)	23.2 (Watermelons, children)	15.2 (Table grapes, children)
M222F013	32.6 (Scarole, children)	14.7 (Melons, children)	11.8 (Watermelons, children)

1) scope: uses registered in EU, including import tolerances

Conclusion

The relevance assessment performed for the eleven metiram transformation products identified in *nature of the residue* studies, shows that for all componds no further consideration is necessary. This result confirms that the present definition of the relevant residue for risk assessment, is sufficiently protective for dietary risk assessment (see section 6.7.1 and 6.7.2). In addition, the none of these transformation products is suitable to be included in the residue definition for enforcement. In summary, this relevance assessment supports the proposal to maintain the currently established residue definitions for metiram (section MCA6.7.1.1 and MCA6.7.1.2).

CA 6.7.2 Proposed maximum residue levels (MRLs) and justification of the acceptability of the levels proposed

In the European Union MRLs are not set specifically for metiram, but for the group of dithiocarbamates comprising maneb, mancozeb, propineb, thiram, ziram as well as metiram. The residues are expressed as carbon disulfide CS_2, which is the common moiety generated during the analytical procedure. Dithiocarbamate MRLs have been established in various commodities of plant and animal origin. An overview over the established EU MRLs for dithiocarbamates is given below (
Table **6.7.3-1**).

For the representative uses of metiram supported by the present dossier, the OECD calculator[1] was used to calculate MRLs based on the residue data (see section MCA 6.3) both for commodities of plant origin as well as commodities of animal origin. The calculated MRL proposals were below the established dithiocarbamate MRLs. Therefore, a change of existing MRLs for commodities of plant and animal origin is not required.

Commodities of plant origin

Grape (wine)

In support of the representative use in grapes, a total 24 GAP compliant field residue trials on wine grapes were conducted. The residue trials were conducted in various European Member States in S-EU and N-EU during the growing seasons 2012 to 2014 and thereby fulfill the requirements for seasonal and geographical distribution. The number of residue trials is sufficient to derive an MRL proposal. The MRL proposal for wine grapes is extrapolated to table grapes based on current guidelines (SANCO 7525/VI/95 – rev.9 of March 2011).

The samples were stored under conditions for which integrity of the samples was demonstrated. The analytical methods used have been validated. All samples were analysed for metiram via the CS_2 method as well as via the EBDC specific method. Residues in untreated control samples were below the limit of quantitation (LOQ).

Expressed as CS_2 equivalent, residue levels were in a range of <0.056 mg/kg - 0.88 mg/kg, resulting in a MRL_{OECD} value for grapes of 1.5 mg/kg. The existing EU MRL for dithiocarbamates in grapes is established at 5.0 mg/kg. Therefore, there is no need to modify the existing EU MRL in grapes.

The overview over the residue trials data, the related risk assessment input values (HR, STMR) and the MRL proposals are given below (
Table **6.7.2-1**).

[1] OECD calculator (OECD calculator spreadsheet, taken from the OECD page: http://www.oecd.org/document/34/0,3746,en_2649_37465_48447010_1_1_1_37465,00.html

In the meantime, specific methods exist which allow a differentiation between the EBDC fungicides and other plant protection products generating CS_2. The analytical method selective for the EBDC fungicides used (see section MCA 4.3) is based on hydrolysis to ethylene diamine, derivatisation and final HPLC/MS-MS quantitation. To facilitate future application as confirmatory method (higher tier analysis), field residue trials supporting the representive uses in the present dossier do include analytical results obtained with the EBDC method:
Expressed as EBDC equivalent, residue levels were in a range of 0.053 mg/kg - 3.08 mg/kg.

The metiram concentration calculated based on EBDC detection and the metiram concentration calculated based on CS_2 detection and the calculated differ by a range of 29 - 94%. It is noted that both analytical methods have been fully validated (see section MCA 4.3). Therefore, the metiram concentration can be expressed either as CS_2 equivalents or as EBDC equivalents.

Potato

In support of the representative use in grapes, a total 24 GAP compliant field residue trials on potato were conducted. The residue trials were conducted in various European Member States in S-EU and N-EU during the growing seasons 2012 to 2014 and thereby fulfill the requirements for seasonal and geographical distribution. The number of residue trials is sufficient to derive a MRL proposal.

The samples were stored under conditions for which integrity of the samples was demonstrated. The analytical methods used have been validated. All samples were analysed for metiram via the CS_2 method as well as via the EBDC specific method. Residues in untreated control samples were below the limit of quantitation (LOQ).

Expressed as CS_2 equivalent, residue levels were all ≤0.056 mg/kg, resulting in a MRL_{OECD} value for grapes of 0.06 mg/kg. The existing EU MRL for dithiocarbamates in potato is established at 0.3 mg/kg. Therefore, there is no need to modify the existing EU MRL in potato.

The overview over the residue trials data, the related risk assessment input values (HR, STMR) and the MRL proposals are provided below (
Table **6.7.2-3**).

In the meantime, specific methods exist which allow a differentiation between the EBDC fungicides and other plant protection products generating CS_2. The analytical method selective for the EBDC fungicides used (see section MCA 4.3) is based on hydrolysis to ethylene diamine, derivatisation and final HPLC/MS-MS quantitation. To facilitate future applicability as confirmatory method (higher tier analysis), field residue trials supporting the representive uses in the present dossier do include analytical results obtained with the EBDC method: Expressed as EBDC equivalent, residue levels were all <0.05 mg/kg.

Commodities of animal origin

Estimation of residues in livestock feed

The previous estimation of residues in livestock feed (*Italy, 2010: Evaluation report on the setting of MRLs for metiram in plant commodities prepared by the evaluating Member State Italy under Article 8 of Regulation (EC) No 396/2005, 15 November 2010, 174p.)* is covering the uses supported in the present dossier. A new estimation is therefore not required. The rationale is as follows:
Residues resulting from the use of metiram in grapes do not contribute to the livestock feed burden as grapes are not fed to livestock. Residues in potato are below the LOQ if metiram is applied according to the cGAP supported in the present dossier. Therefore, a contribution to the livestock feed burden is insignificant.

Estimation of residues in livestock products

Since the previous estimation of residues in livestock feed is covering the uses supported in the present dossier, the established EU MRLs for commodities of animal origin are covering the use of BAS 222 28 F. These MRLs are established at the limit of quantitation of the relevant enforcement method (0.05 mg/kg).

Table 6.7.2-1: MRL calculation for grapes (calculation based on CS_2 detection)

Commodity	Region	Individual trial results [mg/kg][1, 3]		Median residue [mg/kg]	Highest residue [mg/kg]	MRL calculated [mg/kg]	MRL established [mg/kg]
		Enforcement (metiram, expressed as CS_2)	**Risk assessment** (metiram, expressed as CS_2)				
Grapes (table and wine)	NEU	Samples analysed as CS_2, **residues expressed as CS_2** 2x <0.056, 0.11, 0.26, 0.38, 0.41, 0.44, 0.45, 0.49, 0.52, 0.88	Samples analysed as CS_2, **residues expressed as CS_2** 2x <0.056, 0.11, 0.26, 0.38, 0.41, 0.44, 0.45, 0.49, 0.52, 0.88 Samples analysed as CS_2, **residues recalculated to metiram[4]** 2x <0.1, 0.21, 0.46, 0.68, 0.74, 0.78, 0.81, 0.88, 0.94, 1.57	0.41	0.88	1.5	5.0
	SEU	Samples analysed as CS_2, **residues expressed as CS_2** 0.062, 2x 0.10, 0.13, 2x 0.29, 0.32, 0.35, 0.54, 0.58, 0.61, 0.65	Samples analysed as CS_2, **residues expressed as CS_2** 0.062, 2x 0.10, 0.13, 2x 0.29, 0.32, 0.35, 0.54, 0.58, 0.61, 0.65 Samples analysed as CS_2, **residues recalculated to metiram[4]** 0.11, 2x 0.18, 0.24, 2x 0.52, 0.58, 0.62, 0.97, 1.03, 1.09, 1.2	0.305	0.65	1.5	

1. If higher residues were determined at harvest intervals exceeding the PHI these values were used for the MRL calculation
2. Indicates that the MRL is set at the limit of analytical quantitation.
3. Individual residue levels considered for MRL calculation are reported in ascending order.
4. Metiram concentration derived by re-calculating the CS_2 to metiram applying the molecular weight conversion factor of 1.79

Table 6.7.2-2: MRL calculation for grapes (calculation based on EBDC detection)

Commodity	Region	Individual trial results [mg/kg][1, 3]		Median residue [mg/kg]	Highest residue [mg/kg]	MRL calculated [mg/kg]
		Enforcement (metiram, expressed as EBDC)	**Risk assessment** (metiram, expressed as EBDC)			
Grapes (table and wine)	NEU	Samples analysed as EBDC, **residues of metiram** 0.088, 0.16, 0.34, 0.85, 1.12, 1.3, 1.8, 1.93, 2.05, 2.4, 3.08	Samples analysed as EBDC, **residues of metiram** 0.088, 0.16, 0.34, 0.85, 1.12, 1.3, 1.8, 1.93, 2.05, 2.4, 3.08	1.30	3.08	6.0
	SEU	Samples analysed as EBDC, **residues of metiram** 0.23, 0.34, 0.32, 0.52, 0.81, 1.2, 2x 1.41, 2.03, 2.29, 2x 2.7	Samples analysed as EBDC, **residues of metiram** 0.23, 0.34, 0.32, 0.52, 0.81, 1.2, 2x 1.41, 2.03, 2.29, 2x 2.7	1.305	2.70	5.0

1. If higher residues were determined at harvest intervals exceeding the PHI these values were used for the MRL calculation
2. Indicates that the MRL is set at the limit of analytical quantitation.
3. Individual residue levels considered for MRL calculation are reported in ascending order.

Table 6.7.2-3: MRL calculation for potatoes (calculation based on CS_2 detection)

<table>
<tr><th rowspan="2">Commodity</th><th rowspan="2">Region</th><th colspan="2">Individual trial results [mg/kg][1, 2, 3]</th><th rowspan="2">Median residue [mg/kg]</th><th rowspan="2">Highest residue [mg/kg]</th><th rowspan="2">MRL calculated [mg/kg]</th><th rowspan="2">MRL established [mg/kg]</th></tr>
<tr><th>Enforcement
(metiram, expressed as CS_2)</th><th>Risk assessment
(metiram, expressed as CS_2)</th></tr>
<tr><td rowspan="2">Potatoes</td><td>NEU</td><td>Samples analysed as CS_2, residues expressed as CS_2

All residues below LOQ
(< 0.056 mg/kg)</td><td>Samples analysed as CS_2, residues expressed as CS_2

All residues below LOQ
(< 0.056 mg/kg)

Samples analysed as CS_2, residues recalculated to metiram[4]

All residues below LOQ
(< 0.1 mg/kg)</td><td>0.056</td><td>0.056</td><td>0.06</td><td rowspan="2">0.3</td></tr>
<tr><td>SEU</td><td>Samples analysed as CS_2, residues expressed as CS_2

All residues below LOQ
(< 0.056 mg/kg)</td><td>Samples analysed as CS_2, residues expressed as CS_2

All residues below LOQ
(< 0.056 mg/kg)

Samples analysed as CS_2, residues recalculated to metiram[4]

All residues below LOQ
(< 0.1 mg/kg)</td><td>0.056</td><td>0.056</td><td>0.06</td></tr>
</table>

1. if higher residues were determined at harvest intervals exceeding the PHI these values were used for the MRL calculation
2. Indicates that the MRL is set at the limit of analytical quantitation.
3. Individual residue levels considered for MRL calculation are reported in ascending order.
4. Metiram concentration derived by recalculating the CS_2 to metiram applying the molecular weight conversion factor of 1.79

Table 6.7.2-4: MRL calculation for potatoes (calculation based on EBDC detection)

Commodity	Region	Individual trial results [mg/kg][1, 2, 3]		Median residue [mg/kg]	Highest residue [mg/kg]	MRL calculated [mg/kg]
		Enforcement (metiram, expressed as EBDC)	**Risk assessment** (metiram, expressed as EBDC)			
Potatoes	NEU	Samples analysed as EBDC, **residues of metiram** All residues below LOQ (< 0.05 mg/kg)	Samples analysed as EBDC, **residues of metiram** All residues below LOQ (< 0.05 mg/kg)	0.05	0.05	0.05
	SEU	Samples analysed as EBDC, **residues of metiram** All residues below LOQ (< 0.05 mg/kg)	Samples analysed as EBDC, **residues of metiram** All residues below LOQ (< 0.05 mg/kg)	0.05	0.05	0.05

1. If higher residues were determined at harvest intervals exceeding the PHI these values were used for the MRL calculation
2. Indicates that the MRL is set at the limit of analytical quantitation.
3. Individual residue levels considered for MRL calculation are reported in ascending order.

CA 6.7.3 Proposed maximum residue levels (MRLs) and justification of the acceptability of the levels proposed for imported products (import tolerance)

Not applicable for uses supported in the present dossier.

APPENDIX

Table 6.7.3-1: EU MRLs set for the uses of metiram (BAS 222 F)

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)

Code number	Groups and examples of individual products to which the MRLs apply (a)	Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)
100000	. FRUITS, FRESH or FROZEN; TREE NUTS	
110000	. Citrus fruits	5
110010	. Grapefruits	5
110020	. Oranges	5
110030	. Lemons	5
110040	. Limes	5
110050	. Mandarins	5
110990	. Others (2)	5
120000	. Tree nuts	
120010	. Almonds	0.05*
120020	. Brazil nuts	0.05*
120030	. Cashew nuts	0.05*
120040	. Chestnuts	0.05*
120050	. Coconuts	0.05*
120060	. Hazelnuts/cobnuts	0.05*
120070	. Macadamias	0.05*
120080	. Pecans	0.05*
120090	. Pine nut kernels	0.05*
120100	. Pistachios	0.05*
120110	. Walnuts	0.1
120990	. Others (2)	0.05*
130000	. Pome fruits	5
130010	. Apples	5
130020	. Pears	5
130030	. Quinces	5
130040	. Medlars	5
130050	. Loquats/Japanese medlars	5
130990	. Others (2)	5
140000	. Stone fruits	
140010	. Apricots	2 (ft)
140020	. Cherries (sweet)	2 (ft)
140030	. Peaches	2 (ft)
140040	. Plums	2 (ft)
140990	. Others (2)	0.05*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
150000	. Berries and small fruits	
151000	. (a) grapes	5 (ft)
151010	. Table grapes	5
151020	. Wine grapes	5
152000	. (b) strawberries	10 (ft)
153000	. (c) cane fruits	0.05*
153010	. Blackberries	0.05*
153020	. Dewberries	0.05*
153030	. Raspberries (red and yellow)	0.05*
153990	. Others (2)	0.05*
154000	. (d) other small fruits and berries	
154010	. Blueberries	5
154020	. Cranberries	5
154030	. Currants (black, red and white)	5 (ft)
154040	. Gooseberries (green, red and yellow)	5
154050	. Rose hips	0.05*
154060	. Mulberries (black and white)	0.05*
154070	. Azaroles/Mediterranean medlars	0.05*
154080	. Elderberries	0.05*
154990	. Others (2)	5
160000	. Miscellaneous fruits with	
161000	. (a) edible peel	
161010	. Dates	0.05*
161020	. Figs	0.05*
161030	. Table olives	5 (ft)
161040	. Kumquats	0.05*
161050	. Carambolas	0.05*
161060	. Kaki/Japanese persimmons	0.05*
161070	. Jambuls/jambolans	0.05*
161990	. Others (2)	0.05*
162000	. (b) inedible peel, small	0.05*
162010	. Kiwi fruits (green, red, yellow)	0.05*
162020	. Litchis/lychees	0.05*
162030	. Passionfruits/maracujas	0.05*
162040	. Prickly pears/cactus fruits	0.05*
162050	. Star apples/cainitos	0.05*
162060	. American persimmons/Virginia kaki	0.05*
162990	. Others (2)	0.05*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
163000	. (c) inedible peel, large	
163010	. Avocados	0.05*
163020	. Bananas	2 (ft)
163030	. Mangoes	2 (ft)
163040	. Papayas	7 (ft)
163050	. Granate apples/pomegranates	0.05*
163060	. Cherimoyas	0.05*
163070	. Guavas	0.05*
163080	. Pineapples	0.05*
163090	. Breadfruits	0.05*
163100	. Durians	0.05*
163110	. Soursops/guanabanas	0.05*
163990	. Others (2)	0.05*
200000	. VEGETABLES, FRESH or FROZEN	
210000	. Root and tuber vegetables	
211000	. (a) potatoes	0.3 (ft)
212000	. (b) tropical root and tuber vegetables	0.05*
212010	. Cassava roots/manioc	0.05*
212020	. Sweet potatoes	0.05*
212030	. Yams	0.05*
212040	. Arrowroots	0.05*
212990	. Others (2)	0.05*
213000	. (c) other root and tuber vegetables except sugar beets	
213010	. Beetroots	0.5 (ft)
213020	. Carrots	0.2 (ft)
213030	. Celeriacs/turnip rooted celeries	0.3 (ft)
213040	. Horseradishes	0.2 (ft)
213050	. Jerusalem artichokes	0.05*
213060	. Parsnips	0.2 (ft)
213070	. Parsley roots/Hamburg roots parsley	0.2 (ft)
213080	. Radishes	2 (ft)
213090	. Salsifies	0.2 (ft)
213100	. Swedes/rutabagas	0.05*
213110	. Turnips	0.05*
213990	. Others (2)	0.05*
220000	. Bulb vegetables	
220010	. Garlic	0.6 (ft)
220020	. Onions	1 (ft)
220030	. Shallots	1 (ft)
220040	. Spring onions/green onions and Welsh onions	1 (ft)
220990	. Others (2)	0.05*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
230000	. Fruiting vegetables	
231000	. (a) solanacea	
231010	. Tomatoes	3 (ft)
231020	. Sweet peppers/bell peppers	5 (ft)
231030	. Aubergines/eggplants	3 (ft)
231040	. Okra/lady's fingers	0.5 (ft)
231990	. Others (2)	0.05*
232000	. (b) cucurbits with edible peel	2 (ft)
232010	. Cucumbers	2
232020	. Gherkins	2
232030	. Courgettes	2
232990	. Others (2)	2
233000	. (c) cucurbits with inedible peel	1.5 (ft)
233010	. Melons	1.5 (ft)
233020	. Pumpkins	1.5 (ft)
233030	. Watermelons	1.5 (ft)
233990	. Others (2)	1.5 (ft)
234000	. (d) sweet corn	0.05*
239000	. (e) other fruiting vegetables	0.05*
240000	. Brassica vegetables (excluding brassica roots and brassica baby leaf crops)	
241000	. (a) flowering brassica	1 (ft)
241010	. Broccoli	1
241020	. Cauliflowers	1
241990	. Others (2)	1
242000	. (b) head brassica	
242010	. Brussels sprouts	2 (ft)
242020	. Head cabbages	3 (ft)
242990	. Others (2)	0.05*
243000	. (c) leafy brassica	0.5 (ft)
243010	. Chinese cabbages/pe-tsai	0.5
243020	. Kales	0.5
243990	. Others (2)	0.5
244000	. (d) kohlrabies	1 (ft)

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
250000	. Leaf vegetables, herbs and edible flowers	
251000	. (a) lettuces and salad plants	5 (ft)
251010	. Lamb's lettuces/corn salads	5
251020	. Lettuces	5
251030	. Escaroles/broad-leaved endives	5
251040	. Cresses and other sprouts and shoots	5
251050	. Land cresses	5
251060	. Roman rocket/rucola	5
251070	. Red mustards	5
251080	. Baby leaf crops (including brassica species)	5
251990	. Others (2)	5
252000	. (b) spinaches and similar leaves	
252010	. Spinaches	0.05*
252020	. Purslanes	5
252030	. Chards/beet leaves	0.05*
252990	. Others (2)	0.05*
253000	. (c) grape leaves and similar species	0.05*
254000	. (d) watercresses	0.3 (ft)
255000	. (e) witloofs/Belgian endives	0.5 (ft)
256000	. (f) herbs and edible flowers	5 (ft)
256010	. Chervil	5
256020	. Chives	5
256030	. Celery leaves	5
256040	. Parsley	5
256050	. Sage	5
256060	. Rosemary	5
256070	. Thyme	5
256080	. Basil and edible flowers	5
256090	. Laurel/bay leave	5
256100	. Tarragon	5
256990	. Others (2)	5
260000	. Legume vegetables	
260010	. Beans (with pods)	1 (ft)
260020	. Beans (without pods)	0.1 (ft)
260030	. Peas (with pods)	1 (ft)
260040	. Peas (without pods)	0.2 (ft)
260050	. Lentils	0.05*
260990	. Others (2)	0.05*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
270000	. Stem vegetables	
270010	. Asparagus	0.5 (ft)
270020	. Cardoons	0.05*
270030	. Celeries	0.05*
270040	. Florence fennels	0.05*
270050	. Globe artichokes	0.05*
270060	. Leeks	3 (ft)
270070	. Rhubarbs	0.5 (ft)
270080	. Bamboo shoots	0.05*
270090	. Palm hearts	0.05*
270990	. Others (2)	0.05*
280000	. Fungi, mosses and lichens	0.05*
280010	. Cultivated fungi	0.05*
280020	. Wild fungi	0.05*
280990	. Mosses and lichens	0.05*
290000	. Algae and prokaryotes organisms	0.05*
300000	. PULSES	
300010	. Beans	0.1 (ft)
300020	. Lentils	0.05*
300030	. Peas	0.1 (ft)
300040	. Lupins/lupini beans	0.05*
300990	. Others (2)	0.05*
400000	. OILSEEDS AND OIL FRUITS	
401000	. Oilseeds	
401010	. Linseeds	0.1*
401020	. Peanuts/groundnuts	0.1*
401030	. Poppy seeds	0.1*
401040	. Sesame seeds	0.1*
401050	. Sunflower seeds	0.1*
401060	. Rapeseeds/canola seeds	0.5 (ft)
401070	. Soyabeans	0.1*
401080	. Mustard seeds	0.1*
401090	. Cotton seeds	0.1*
401100	. Pumpkin seeds	0.1*
401110	. Safflower seeds	0.1*
401120	. Borage seeds	0.1*
401130	. Gold of pleasure seeds	0.1*
401140	. Hemp seeds	0.1*
401150	. Castor beans	0.1*
401990	. Others (2)	0.1*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	Groups and examples of individual products to which the MRLs apply (a)	Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)
402000	. Oil fruits	
402010	. Olives for oil production	5 (ft)
402020	. Oil palms kernels	0.1*
402030	. Oil palms fruits	0.1*
402040	. Kapok	0.1*
402990	. Others (2)	0.1*
500000	. CEREALS	
500010	. Barley	2 (ft)
500020	. Buckwheat and other pseudo-cereals	0.05*
500030	. Maize/corn	0.05*
500040	. Common millet/proso millet	0.05*
500050	. Oat	2 (ft)
500060	. Rice	0.05*
500070	. Rye	1 (ft)
500080	. Sorghum	0.05*
500090	. Wheat	1 (ft)
500990	. Others (2)	0.05*
600000	. TEAS, COFFEE, HERBAL INFUSIONS, COCOA AND CAROBS	0.1*
610000	. Teas	0.1*
620000	. Coffee beans	0.1*
630000	. Herbal infusions from	0.1*
631000	. (a) flowers	0.1*
631010	. Chamomile	0.1*
631020	. Hibiscus/roselle	0.1*
631030	. Rose	0.1*
631040	. Jasmine	0.1*
631050	. Lime/linden	0.1*
631990	. Others (2)	0.1*
632000	. (b) leaves and herbs	0.1*
632010	. Strawberry	0.1*
632020	. Rooibos	0.1*
632030	. Mate/maté	0.1*
632990	. Others (2)	0.1*
633000	. (c) roots	0.1*
633010	. Valerian	0.1*
633020	. Ginseng	0.1*
633990	. Others (2)	0.1*
639000	. (d) any other parts of the plant	0.1*
640000	. Cocoa beans	0.1*
650000	. Carobs/Saint John's breads	0.1*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
700000	. HOPS	25 (ft)
800000	. SPICES	
810000	. Seed spices	0.1*
810010	. Anise/aniseed	0.1*
810020	. Black caraway/black cumin	0.1*
810030	. Celery	0.1*
810040	. Coriander	0.1*
810050	. Cumin	0.1*
810060	. Dill	0.1*
810070	. Fennel	0.1*
810080	. Fenugreek	0.1*
810090	. Nutmeg	0.1*
810990	. Others (2)	0.1*
820000	. Fruit spices	0.1*
820010	. Allspice/pimento	0.1*
820020	. Sichuan pepper	0.1*
820030	. Caraway	0.1*
820040	. Cardamom	0.1*
820050	. Juniper berry	0.1*
820060	. Peppercorn (black, green and white)	0.1*
820070	. Vanilla	0.1*
820080	. Tamarind	0.1*
820990	. Others (2)	0.1*
830000	. Bark spices	0.1*
830010	. Cinnamon	0.1*
830990	. Others (2)	0.1*
840000	. Root and rhizome spices	0.1*
840010	. Liquorice	0.1*
840020	. Ginger	0.1*
840030	. Turmeric/curcuma	0.1*
840040	. Horseradish	0.1*
840990	. Others (2)	0.1*
850000	. Bud spices	
850010	. Cloves	0.1*
850020	. Capers	25
850990	. Others (2)	0.1*
860000	. Flower pistil spices	0.1*
860010	. Saffron	0.1*
860990	. Others (2)	0.1*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
870000	. Aril spices	0.1*
870010	. Mace	0.1*
870990	. Others (2)	0.1*
900000	. SUGAR PLANTS	
900010	. Sugar beet roots	2
900020	. Sugar canes	0.05*
900030	. Chicory roots	0.05*
900990	. Others (2)	0.05*
1000000	. PRODUCTS OF ANIMAL ORIGIN - TERRESTRIAL ANIMALS	0.05*
1010000	. Tissues from	0.05*
1011000	. (a) swine	0.05*
1011010	. Muscle	0.05*
1011020	. Fat tissue	0.05*
1011030	. Liver	0.05*
1011040	. Kidney	0.05*
1011050	. Edible offals (other than liver and kidney)	0.05*
1011990	. Others (2)	0.05*
1012000	. (b) bovine	0.05*
1012010	. Muscle	0.05*
1012020	. Fat tissue	0.05*
1012030	. Liver	0.05*
1012040	. Kidney	0.05*
1012050	. Edible offals (other than liver and kidney)	0.05*
1012990	. Others (2)	0.05*
1013000	. (c) sheep	0.05*
1013010	. Muscle	0.05*
1013020	. Fat tissue	0.05*
1013030	. Liver	0.05*
1013040	. Kidney	0.05*
1013050	. Edible offals (other than liver and kidney)	0.05*
1013990	. Others (2)	0.05*
1014000	. d) goat	0.05*
1014010	. Muscle	0.05*
1014020	. Fat tissue	0.05*
1014030	. Liver	0.05*
1014040	. Kidney	0.05*
1014050	. Edible offals (other than liver and kidney)	0.05*
1014990	. Others (2)	0.05*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
1015000	. (e) equine	0.05*
1015010	. Muscle	0.05*
1015020	. Fat tissue	0.05*
1015030	. Liver	0.05*
1015040	. Kidney	0.05*
1015050	. Edible offals (other than liver and kidney)	0.05*
1015990	. Others (2)	0.05*
1016000	. (f) poultry	0.05*
1016010	. Muscle	0.05*
1016020	. Fat tissue	0.05*
1016030	. Liver	0.05*
1016040	. Kidney	0.05*
1016050	. Edible offals (other than liver and kidney)	0.05*
1016990	. Others (2)	0.05*
1017000	. (g) other farmed terrestrial animals	0.05*
1017010	. Muscle	0.05*
1017020	. Fat tissue	0.05*
1017030	. Liver	0.05*
1017040	. Kidney	0.05*
1017050	. Edible offals (other than liver and kidney)	0.05*
1017990	. Others (2)	0.05*
1020000	. Milk	0.05*
1020010	. Cattle	0.05*
1020020	. Sheep	0.05*
1020030	. Goat	0.05*
1020040	. Horse	0.05*
1020990	. Others (2)	0.05*
1030000	. Birds eggs	0.05*
1030010	. Chicken	0.05*
1030020	. Duck	0.05*
1030030	. Geese	0.05*
1030040	. Quail	0.05*
1030990	. Others (2)	0.05*
1040000	. Honey and other apiculture products	0.05*
1050000	. Amphibians and Reptiles	0.05*
1060000	. Terrestrial invertebrate animals	0.05*
1070000	. Wild terrestrial vertebrate animals	0.05*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
Pesticide residue	**Legislation**	**Entry in to force**
Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)		
	Reg. (EU) No 34/2013	26.01.2013

Pesticide residues and maximum residue levels (mg/kg)
*** indicates lower limit of analytical determination**

The MRLs expressed as CS_2 can arise from different dithiocarbamates and therefore they do not reflect a single Good Agricultural Practice (GAP). It is therefore not appropriate to use these MRLs to check compliance with a GAP.
(ft) Footnotes
In brackets the origin of the residue (ma: maneb mz: mancozeb me: metiram pr: propineb t: thiram z: ziram).

0140020 : Cherries (sweet) (mz, me, pr, t, z)
0140030 : Peaches (mz, t)
0140040 : Plums (mz, me, t, z)
0151000 : (a) grapes (ma, mz, me, pr, t)
0152000 : (b) strawberries (t)
0154030 : Currants (black, red and white) (mz)
0161030 : Table olives (mz, pr)
0163020 : Bananas (mz, me, t)
0163030 : Mangoes (mz)
0163040 : Papayas (mz)
0211000 : (a) potatoes (ma, mz, me, pr)
0213010 : Beetroots (mz)
0213020 : Carrots (mz)
0213030 : Celeriacs/turnip rooted celeries (ma, me, pr, t)
0213040 : Horseradishes (mz)
0213060 : Parsnips (mz)
0213070 : Parsley roots/Hamburg roots parsley (mz)
0213080 : Radishes (mz)
0213090 : Salsifies (mz)
0220010 : Garlic (me)
0220020 : Onions (ma, mz)
0220030 : Shallots (ma, mz)
0220040 : Spring onions/green onions and Welsh onions (mz)
0231010 : Tomatoes (ma, mz, me, pr)
0231020 : Sweet peppers/bell peppers (mz, pr)
0231030 : Aubergines/eggplants (mz, me)
0231040 : Okra/lady's fingers (mz)
0232000 : (b) cucurbits with edible peel (mz, pr)
0233000 : (c) cucurbits with inedible peel (me)
0233010 : Melons (me)
0233020 : Pumpkins (me)
0233030 : Watermelons (me)
0233990 : Others (2) (me)
0241000 : (a) flowering brassica (mz)
0242010 : Brussels sprouts (mz)
0242020 : Head cabbages (mz)
0243000 : (c) leafy brassica (mz)
0244000 : (d) kohlrabies (mz)
0251000 : (a) lettuces and salad plants (mz, me, t)
0254000 : (d) watercresses (mz)
0255000 : (e) witloofs/Belgian endives (mz)
0256000 : (f) herbs and edible flowers (mz, me)
0260010 : Beans (with pods) (mz)
0260020 : Beans (without pods) (mz)
0260030 : Peas (with pods) (ma, mz)
0260040 : Peas (without pods) (mz)
0270010 : Asparagus (mz)
0270060 : Leeks (ma, mz)
0270070 : Rhubarbs (mz)
0300010 : Beans (mz)
0300030 : Peas (mz)
0401060 : Rapeseeds/canola seeds (ma, mz)
0402010 : Olives for oil production (mz, pr)
0500010 : Barley (ma, mz)
0500050 : Oat (ma, mz)
0500070 : Rye (ma, mz)
0500090 : Wheat (ma, mz)
0700000 : HOPS (pr)

CA 6.8 Proposed safety intervals

Residue trials have been conducted with applications made at the latest recommended crop growth stage with harvest taking place at the time of crop maturity following good agricultural practice.

Pre-harvest interval
For grape, the PHI is 56 days. The formulation BAS 222 28 F is intended to be used between BBCH growth stages 05 -79, whereby three applications are intended to be made.
For potato, the PHI is 14 days. The formulation BAS 222 28 F is intended to be used between BBCH growth stages 21 and 89, whereby three applications are intended to be made.

Re-entry period for livestock to areas to be grazed
Because metiram is not intended to be used in areas to be grazed, no re-entry period for livestock has to be defined.

Re-entry period for man to treated crops
Re-entry assessments are given for the representative uses in the supplemental product dossiers (M-CP 7.2). Re-entry is possible after the spray deposits on the crops have dried given the worker is wearing adequate work clothing.

Withholding period for animal feed stuffs
Grapes are not considered as animal feeding stuff since grapes and related process fractions are not fed to livestock. Due to the very favourable residue situation in potatoes with residues consistently below LOQ no withholding period needs to be considered for tubers.

Waiting period between application and crop sowing or planting the crop to be protected
No waiting period is necessary since metiram is not intended in a pre-emergence use.

Waiting period between application and handling treated produce
This is not relevant here since a post-harvest treatment is not intended for grapes and potatoes.

Waiting period between last application and sowing or planting succeeding crops
No accumulation of metiram (BAS 222 F) or any of its degradation products was observed in the confined rotational crop study. The metabolites of metiram were extensively incorporated into the carbon pool and into the natural products of plants. In addition, grapes are considered as permanent crop.

CA 6.9 Estimation of the potential and actual exposure through diet and other sources

Introduction

For assessing the dietary risk for the consumer resulting from uses supported in the present dossier, dietary risk assessments according to the proposed definitions of the relevant residue for risk assessment (see section MCA 6.7) were performed. Thus, assessments were done both for the parent compound metiram and for the degradation product ETU. In addition, section MCA 6.10 summarizes relevance assessments for further metiram transformation products (metiram metabolites identified in nature of the residue studies). These assessments were done to investigate a potential relevance for consumer health and thereby provide robustness to the proposed residue definition (see section MCA 6.7).

Metiram: Exposure to the parent compound was assessed considering (1) either the representative uses supported in the present dossier, (2) or the entirety of metiram uses registered in EU, (3) or comprehensively the dithiocarbamate uses in EU. The assessment considering the dithiocarbamates did take into account, besides metiram data, also information available for dithiocarbamates which have the same toxicological target, noteably maneb, mancozeb and propineb. Acute consumer exposure was not performed due to the low acute toxicity of the active substance metiram (ARfD not allocated).

ETU: Exposure to the metabolite ETU was assessed using the VELS model taking processing factors into account. In addition, to provide an indicative assessment of the contribution from uses supported in the present dossier, the EFSA PRIMo model was used with the assumption that the metiram residue would entirely be converted to ETU during processing (a molecular weight conversion factor of 0.36 was applied to express metiram residues as ETU).

Models used for exposure calculation

The following exposure assessment models were used for the assessments provided in this chapter. They are considered sufficiently representative for the consumer in Europe.

EFSA Calculation model for acute and chronic consumer exposure (version 2) as of October 2007, source: Internet: http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620776373.htm

German VELS consumption figures for children (16.15 kg) as published in the Bundesnachrichtenblatt (2005).

Toxicological reference values used

The following toxicological reference values were used for chronic (ADI) and acute assessments (ARfD).

Table 6.9-1: Toxicological reference values: Metiram

End-Point	Value	Study	Safety factor	Reference
Acceptable Daily Intake (ADI)	0.03 mg/kg bw/d	2-year study in rats	100	SANCO/4059/2001-rev 3.3 (03.06.2005)
Acute Reference Dose (ARfD)	not necessary - not allocated	-	-	SANCO/4059/2001-rev 3.3 (03.06.2005)

Table 6.9-2: Toxicological reference values: ETU

Endpoint	Value	Study	Safety factor	Reference
Acceptable Daily Intake (ADI)	0.002 mg/kg bw/d	2-year study in rats	100	SANCO/4059/2001-rev 3.3 (03.06.2005)
Acute Reference Dose (ARfD)	0.05 mg/kg bw/d	-	-	SANCO/4059/2001-rev 3.3 (03.06.2005)

Acceptable Daily Intake (ADI) and Dietary Exposure Calculation

Metiram exposure calculations

Metiram: TMDI

As first tier assessment, MRL values can be used as input values for exposure assessments (TMDI). MRLs in the European Union are not set specifically for Metiram, but for the group of dithiocarbamates including also maneb, mancozeb, propineb, thiram, zineb expressed as CS_2 (see Table 6.9-3).

Representative uses supported in the present dossier: using an ADI for metiram of 0.03 mg/kg bw/d (Table 6.9-1), intake calculations result in ADI utilization of 2.7 to 70.3 % (see Table 6.9-4 in appendix). The ADI utilization of 70.3% was calculated for the diet “FR all population” with the highested contributer table/wine grapes (68.5% ADI).

Entirety of registered uses of metiram: intake calculations result in ADI utilization of 26.0 - 277.7 % (see Table 6.9-5 in appendix). Here, MRLs were considered for the registered uses of metiram in pome fruits, table/wine grapes, potato, celeriac, onion, garlic, tomato, aubergine, cucurbits with edible peel, cucurbits with inedible peel, lettuce and other salad plants, chervil, chives, asparagus, commodities of animal origin as well as import tolerances for banana and passion fruit. The ADI utilization of 277.7% was calculated for the diet “DE child” with the highest contributor pome fruits with 211.9% of ADI.
Such TMDI calculations however represent a massive overestimation of the actual exposure as it is based on several worst case assumptions. Notably,

- Treatment of a crop is assumed to be 100% (Realistically, percentage of crop area treated with metiram is far below 100%).
- Residue levels in a crop are assumed to be at the upper level, the MRL (Realistically, only very few crops contain residues at the maximum residue level).
- Residues in processed commodities are assumed to be at the level of the unprocessed traded commodity (Realistically, washing, peeling and processing generally results in significant reduction of metiram residues).

Therefore, above TMDI calculation results do not indicate a chronic safety concern for the consumer. This is apparent when the calculations are refined to account for more realistic conditions. As an example see the cumulative intake assessment below, which in addition also considers the contributions from other dithiocarbamates.

Metiram: NEDI

Representative uses supported in the present dossier: Considering grape and potato, refinement of intake calculations using STMR or STMRp are not required since the TMDI calculations result in ADI utilizations below 100% (2.7 – 70.3 % of ADI, see TMDI calculations above).

Considering a comprehensive consumer intake assessment: not only the other registered uses of metiram have to be considered but also the contribution of other dithiocarbamates (see the following chapter on cumulative intake assessment).

Metiram: Cumulative Intake Assessment

Cumulative intake estimations in the context of the present dossier have to consider not only the information from metiram but also from other dithiocarbamates having the same toxicological target (maneb, mancozeb, and propineb). To date, no clear guidance is available how to perform a cumulative dietary exposure assessment. Therefore, reference is made to the previous indicative exposure calculation (page 16 of Reasoned Opinion, EFSA Journal 2012; 10(7):2846).

In brief, the ADI for propineb (0.007 mg/kg bw/d) was used in the PRIMo model version 2. Residue data for maneb, mancozeb, metiram were normalized to propineb (recalculated with combined correction factors to account for differences in molecular weight and relative toxicity (see
Table **6.9-6**) and used as input values (see Table 6.9-7). Specifically,
(a) Exclusion of crops
- with MRLs set at the LOQ (assumption no authorized use)
- with uses only for thiram/zineb (different toxicological target, specific MRLs)
(b) Re-calculation of residue data for crops to "propineb equivalents"
- median residue values available:
  multiplication of median residue value with corresponding correction factor
- established MRL (median value not available):
  multiplication of MRL with correction factor of most toxic AI authorized in this crop
(c) No re-calculation:
  available propineb median residue values used.

Uses supported in the present dossier are covered by previous assessments of chronic dietary exposure (*Reasoned Opinion, EFSA Journal 2012; 10(7):2846*). The total calculated intake values did account for at maximum 98.4% of the ADI (DE child). As stated by EFSA, the highest nominal contribution of apples (39.6%) could be further refined considering that for this subpopulation group apples are consumed to 77% as apple juice resulting in a refined exposure of ca. 70% ADI). In conclusion, the calculations indicate that the uses considered are unlikely to pose a chronic public health concern.
To provide detailed information, the present dossier provides a re-calculated assessment using the data of Table 6.9-7 for input into the EFSA PRIMo model (see in Table 6.9-8).

Conclusion on dietary exposure calculations for metiram

Chronic: Taken together, both the exposure assessment for metiram as well as cumulative exposure assessments for dithiocarbamates indicates that the metiram uses supported in the present dossier do not pose a chronic safety concern for the consumer.
The diet with the highest TMDI is "DE child" with 98.4% ADI. For this diet, the highest contributor is pome fruits with 39.6% of ADI. The second diet with the highest TMDI is "WHO Cluster diet B" with 88.7% ADI, where tomatoes are the major contributor with 14.5% of ADI.
The few MRL modifications subsequent to this assessment performed by EFSA in 2012 have insignificant impact on the exposure calculations of dithiocarbamates. Considering the limited availability of residue data from CS_2 sources other than metiram, a re-calculation would not significantly improve precision of intake calculation done by EFSA. Consequently, these calculations can still be considered a valid indicative exposure assessment showing that the uses considered do not pose a public health concern.
(*note*: Assessment of acute exposure to metiram is not provided due to the low acute toxicity of the active substance. Thus, an ARfD is not allocated, see chapter on NESTI calculations below).

ETU exposure calculations

For assessing the chronic dietary risk of the metabolite ETU, an ADI of 0.002 mg/kg bw/d was used. This ADI represents the worst case (for information, an ADI of 0.004 mg/kg bw/day was used in the assessment by JMPR for setting CODEX MRLs). For the acute assessment an ARfD of 0.05 mg/kg bw was used.
ETU is included in the residue definition for processed commodities, but not in the residue definition for raw agricultural commodities (RAC, see section MCA 6.7). This reflects the fact that ETU is generated from dithiocarbamates during processing, in particular when a heating step is involved. In contrast, residue levels of ETU are very low in RAC or processed fractions not involving a heating step. Thus, ETU is not included in the residue definition for MRL establishment. This position was agreed upon in the Peer Review and is also shared by JMPR.
In consequence, a first tier exposure assessment which uses MRLs (TMDI calculation) is not applicable to ETU. Also, a comprehensive exposure assessment based on ETU residue data is currently not feasible as the data base on ETU formation during processing of commodities treated with dithiocarbamates is incomplete (namely for dithiocarbamates other than metiram, see also *Reasoned Opinion, EFSA Journal 2012; 10(7):2846*).
In order to provide an indicative assessment as part of the present dossier, the data available for metiram was used as input value to derive by calculation the ETU exposure according to the VELS model. The German diet used in this model allows to differentiate residue level in raw commodities and residue level in processed commodities.
In addition, to provide an estimate of the contribution resulting from the uses supported in the present dossier (grape, potato), a worst case calculation derived from dithiocarbamate MRLs for these commodities (assuming 100% degradation to ETU) with the PRIMo vers2 model was performed.
As shown in detail below, theses estimations allow the conclusion that the uses supported in the present dossier will not result in a consumer exposure exceeding the toxicological reference values and therefore are unlikely to pose a public health concern.

ETU: VELS Model

The objective is to prove an indicative assessment of chronic consumer exposure to ETU residues. The VELS model based on the German diet allows to differentiate residue levels in raw commodities from residue levels in processed commodities. The diet "German VELS child" is regarded as the most critical and thus informative for this objective. It has a relatively high consumption of mainly fruit and vegetable crops compared to body weight. Other models available are regarded as less informative. Not allowing differention between residue level in raw and processed commodities, these other models are inherently over- or underestimating ETU exposure.

Residue levels of ETU in processed fractions of plant origin are calculated from metiram residue data and the appropriate processing factor (Cf, see below). For the uses supported in the present dossier the median residues of the field trials are used (CS_2, see section MCA 6.3). If such data is not available (noteably for competitor products), the corresponding MRL values were used (see Table 6.9-7) Regarding residue levels in commodities of animal origin, ETU level of “zero” were used as input value for the assessment based on the result of livestock feeding studies (for rationale see section MCA 6.4).

The relevant processing studies are included in the section MCA 6.5 of the present dossier (They have been previously provided in the annex II dossier of metiram, and are also included in the relevant addenda to the draft assessment reports. They were also available during the setting of the EU temporary MRLs for the dithiocarbamate fungicides and have been used previously for the exposure assessment).

The following formula is used for calculating the processing factors of ETU:

$$PF_{ETU} = \frac{\text{Conc. } ETU \ in \ process \ fraction}{\text{Conc. m}etiram \ (as\, CS2)\, in \ raw\, com\, \text{mod}\, ity}$$

This equation considers that ETU is exclusively formed from the parent molecule metiram (or other dithiocarbamate fungicides). In the absence of metiram or other dithiocarbamates, ETU is not formed and thus residue levels are zero.

The
Table **6.9-9** (see in Appendix) provides an overview over the processing factors used in the assessment, considerations specific for certain processing procedures are described in the notes.

Note on processing of grapes
For wine two different processing factors were derived since the wine production for white wine and red wine differs across Europe. Mainly in Germany, thermo-vinification is used for the production of red wine. During this process the mash is heated for few minutes in order to have a better extraction of the "red colour" of out the skins into the grape juice which is fermented in the next step. This process has a significant impact on the levels of ETU finally present in wine. For white wine and in other parts of Europe, this process is not used resulting in low levels of ETU.

Note on processing of potato
For potatoes, no processing study is available since in more than 50 trials, no detectable residue was found in potato tubers.

Note on other dithiocarbamates
The processing factors derived from other dithiocarbamates, i.e. mancozeb or maneb uses are currently not fully available to BASF. Therefore, the factors given in the table were used. They are considered as sufficiently representative for all types of fruit and vegetable cooking / juice production. For vegetable cooking the factor of 0.02 was used. It was derived from tomato processing study (canning). In case of cereals, the assumption is that ETU will not be found in flour or bread since potential ETU residues being present during processing will remain in the waste products (husk). A similar rationale applies to the production of olive oil or sugar from sugar beets where several cleaning steps are involved.

In summary, estimation of the exposure of the consumer to ETU residues based on dithiocarbamate residue levels in combination with appropriate processing factors using the German VELS model does show that the “German child” is not at risk. A large margin of safety is given since several worst case assumptions were used. This applies to the other, less sensitive population groups. The calculation results using the VELS Model are provided in
Table 6.9-10 to Table 6.9-14.
In conclusion, the calculations indicate that the uses considered are unlikely to pose a public health concern.

ETU: EFSA PRIMo 2

The objective is to prove an indicative assessment of the contribution resulting from the uses supported in the present dossier to the consumer exposure to ETU residues.

For assessing the dietary risk, an ADI of 0.002 mg/kg bw/d was used. This ADI represents the worst case (for comparison an ADI of 0.004 mg/kg bw/day was used in the assessment by JMPR for setting CODEX MRLs).
The worst case situation was calculated assuming that all metiram residues in the processed crops would be converted to ETU during processing. The molecular weight conversion factor of 0.36 was applied to express metiram residues as ETU (MW_{ETU} is 102.16 g/mol, $MW_{metiram}$ is 1088.6 g/mol. One molecule of metiram is expected to form 4 molecules of ETU = 0.36, Reasoned Opinion, EFSA Journal 2012; 10(7):2846).
In summary, the calculation shows that chronic exposure to ETU resulting from uses in grape and potato would account for up to 19.0% of the ADI (similarly, the acute exposure using an ARfD of 0.05 mg/kg is up to 11.8%). The results of the intake calculation are presented in Table 6.9-16 and Table 6.9-17. In conclusion, the calculations indicate that the uses considered are unlikely to pose a public health concern.

Conclusion on dietary exposure calculations for ETU

Chronic: Taken together, the indicative exposure assessments for ETU both using the VELS model as well as the EFSA PRIMo model indicate that the metiram uses supported in the present dossier do not pose an acute safety concern for the consumer.

Acute Reference Dose (ARfD) and Dietary Exposure Calculation

NESTI calculations

Note, for metiram an assessment of acute exposure (NESTI) was not performed due to the low acute toxicity of the active substance (ARfD is not allocated).

For the metabolite ETU the acute dietary exposure was assessed using the VELS model taking processing factors into account. In addition, to provide an indicative assessment of the contribution from the uses supported in the present dossier the EFSA PRIMo model was used with the assumption that the metiram residue would entirely be converted to ETU during processing (molecular weight conversion factor of 0.36 was applied to express metiram residues as ETU). An ARfD of 0.05 mg/kg bw was used.
The assessment strategy is described in detail above (in the paragraph "Acceptable Daily Intake (ADI) and Dietary Exposure Calculation"). With both approaches only very low risk cup utilization were calculated considering either only the crops supported in the present dossier (VELS model: max. 1.4% ARfD, PRIMo model: max. 11.8% ARfD) or considering the entirety of EU registered uses including import tolerances (VELS model: max. 8.6% ARfD).

Conclusion on calculations of acute dietary exposure for metiram

Acute: Assessment of acute exposure to metiram is not provided due to the low acute toxicity of the active substance (ARfD is not allocated).

Conclusion on calculations of acute dietary exposure for ETU

Acute: Estimation of acute exposure to ETU allows the conclusion that the uses supported in the present dossier (grape, potato) will not result in a consumer exposure exceeding the toxicological reference values and therefore are unlikely to pose a public health concern.

APPENDIX

Table 6.9-3: EU MRLs set for the uses of metiram (BAS 222 F) / dithiocarbamate fungicides

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
100000	. FRUITS, FRESH or FROZEN; TREE NUTS	
110000	. Citrus fruits	5
110010	. Grapefruits	5
110020	. Oranges	5
110030	. Lemons	5
110040	. Limes	5
110050	. Mandarins	5
110990	. Others (2)	5
120000	. Tree nuts	
120010	. Almonds	0.05*
120020	. Brazil nuts	0.05*
120030	. Cashew nuts	0.05*
120040	. Chestnuts	0.05*
120050	. Coconuts	0.05*
120060	. Hazelnuts/cobnuts	0.05*
120070	. Macadamias	0.05*
120080	. Pecans	0.05*
120090	. Pine nut kernels	0.05*
120100	. Pistachios	0.05*
120110	. Walnuts	0.1
120990	. Others (2)	0.05*
130000	. Pome fruits	5
130010	. Apples	5
130020	. Pears	5
130030	. Quinces	5
130040	. Medlars	5
130050	. Loquats/Japanese medlars	5
130990	. Others (2)	5
140000	. Stone fruits	
140010	. Apricots	2 (ft)
140020	. Cherries (sweet)	2 (ft)
140030	. Peaches	2 (ft)
140040	. Plums	2 (ft)
140990	. Others (2)	0.05*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)			
Code number		**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
150000	.	Berries and small fruits	
151000	.	(a) grapes	5 (ft)
151010	.	Table grapes	5
151020	.	Wine grapes	5
152000	.	(b) strawberries	10 (ft)
153000	.	(c) cane fruits	0.05*
153010	.	Blackberries	0.05*
153020	.	Dewberries	0.05*
153030	.	Raspberries (red and yellow)	0.05*
153990	.	Others (2)	0.05*
154000	.	(d) other small fruits and berries	
154010	.	Blueberries	5
154020	.	Cranberries	5
154030	.	Currants (black, red and white)	5 (ft)
154040	.	Gooseberries (green, red and yellow)	5
154050	.	Rose hips	0.05*
154060	.	Mulberries (black and white)	0.05*
154070	.	Azaroles/Mediterranean medlars	0.05*
154080	.	Elderberries	0.05*
154990	.	Others (2)	5
160000	.	Miscellaneous fruits with	
161000	.	(a) edible peel	
161010	.	Dates	0.05*
161020	.	Figs	0.05*
161030	.	Table olives	5 (ft)
161040	.	Kumquats	0.05*
161050	.	Carambolas	0.05*
161060	.	Kaki/Japanese persimmons	0.05*
161070	.	Jambuls/jambolans	0.05*
161990	.	Others (2)	0.05*
162000	.	(b) inedible peel, small	0.05*
162010	.	Kiwi fruits (green, red, yellow)	0.05*
162020	.	Litchis/lychees	0.05*
162030	.	Passionfruits/maracujas	0.05*
162040	.	Prickly pears/cactus fruits	0.05*
162050	.	Star apples/cainitos	0.05*
162060	.	American persimmons/Virginia kaki	0.05*
162990	.	Others (2)	0.05*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
163000	. (c) inedible peel, large	
163010	. Avocados	0.05*
163020	. Bananas	2 (ft)
163030	. Mangoes	2 (ft)
163040	. Papayas	7 (ft)
163050	. Granate apples/pomegranates	0.05*
163060	. Cherimoyas	0.05*
163070	. Guavas	0.05*
163080	. Pineapples	0.05*
163090	. Breadfruits	0.05*
163100	. Durians	0.05*
163110	. Soursops/guanabanas	0.05*
163990	. Others (2)	0.05*
200000	. VEGETABLES, FRESH or FROZEN	
210000	. Root and tuber vegetables	
211000	. (a) potatoes	0.3 (ft)
212000	. (b) tropical root and tuber vegetables	0.05*
212010	. Cassava roots/manioc	0.05*
212020	. Sweet potatoes	0.05*
212030	. Yams	0.05*
212040	. Arrowroots	0.05*
212990	. Others (2)	0.05*
213000	. (c) other root and tuber vegetables except sugar beets	
213010	. Beetroots	0.5 (ft)
213020	. Carrots	0.2 (ft)
213030	. Celeriacs/turnip rooted celeries	0.3 (ft)
213040	. Horseradishes	0.2 (ft)
213050	. Jerusalem artichokes	0.05*
213060	. Parsnips	0.2 (ft)
213070	. Parsley roots/Hamburg roots parsley	0.2 (ft)
213080	. Radishes	2 (ft)
213090	. Salsifies	0.2 (ft)
213100	. Swedes/rutabagas	0.05*
213110	. Turnips	0.05*
213990	. Others (2)	0.05*
220000	. Bulb vegetables	
220010	. Garlic	0.6 (ft)
220020	. Onions	1 (ft)
220030	. Shallots	1 (ft)
220040	. Spring onions/green onions and Welsh onions	1 (ft)
220990	. Others (2)	0.05*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
230000	. Fruiting vegetables	
231000	. (a) solanacea	
231010	. Tomatoes	3 (ft)
231020	. Sweet peppers/bell peppers	5 (ft)
231030	. Aubergines/eggplants	3 (ft)
231040	. Okra/lady's fingers	0.5 (ft)
231990	. Others (2)	0.05*
232000	. (b) cucurbits with edible peel	2 (ft)
232010	. Cucumbers	2
232020	. Gherkins	2
232030	. Courgettes	2
232990	. Others (2)	2
233000	. (c) cucurbits with inedible peel	1.5 (ft)
233010	. Melons	1.5 (ft)
233020	. Pumpkins	1.5 (ft)
233030	. Watermelons	1.5 (ft)
233990	. Others (2)	1.5 (ft)
234000	. (d) sweet corn	0.05*
239000	. (e) other fruiting vegetables	0.05*
240000	. Brassica vegetables (excluding brassica roots and brassica baby leaf crops)	
241000	. (a) flowering brassica	1 (ft)
241010	. Broccoli	1
241020	. Cauliflowers	1
241990	. Others (2)	1
242000	. (b) head brassica	
242010	. Brussels sprouts	2 (ft)
242020	. Head cabbages	3 (ft)
242990	. Others (2)	0.05*
243000	. (c) leafy brassica	0.5 (ft)
243010	. Chinese cabbages/pe-tsai	0.5
243020	. Kales	0.5
243990	. Others (2)	0.5
244000	. (d) kohlrabies	1 (ft)

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
250000	. Leaf vegetables, herbs and edible flowers	
251000	. (a) lettuces and salad plants	5 (ft)
251010	. Lamb's lettuces/corn salads	5
251020	. Lettuces	5
251030	. Escaroles/broad-leaved endives	5
251040	. Cresses and other sprouts and shoots	5
251050	. Land cresses	5
251060	. Roman rocket/rucola	5
251070	. Red mustards	5
251080	. Baby leaf crops (including brassica species)	5
251990	. Others (2)	5
252000	. (b) spinaches and similar leaves	
252010	. Spinaches	0.05*
252020	. Purslanes	5
252030	. Chards/beet leaves	0.05*
252990	. Others (2)	0.05*
253000	. (c) grape leaves and similar species	0.05*
254000	. (d) watercresses	0.3 (ft)
255000	. (e) witloofs/Belgian endives	0.5 (ft)
256000	. (f) herbs and edible flowers	5 (ft)
256010	. Chervil	5
256020	. Chives	5
256030	. Celery leaves	5
256040	. Parsley	5
256050	. Sage	5
256060	. Rosemary	5
256070	. Thyme	5
256080	. Basil and edible flowers	5
256090	. Laurel/bay leave	5
256100	. Tarragon	5
256990	. Others (2)	5
260000	. Legume vegetables	
260010	. Beans (with pods)	1 (ft)
260020	. Beans (without pods)	0.1 (ft)
260030	. Peas (with pods)	1 (ft)
260040	. Peas (without pods)	0.2 (ft)
260050	. Lentils	0.05*
260990	. Others (2)	0.05*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
270000	. Stem vegetables	
270010	. Asparagus	0.5 (ft)
270020	. Cardoons	0.05*
270030	. Celeries	0.05*
270040	. Florence fennels	0.05*
270050	. Globe artichokes	0.05*
270060	. Leeks	3 (ft)
270070	. Rhubarbs	0.5 (ft)
270080	. Bamboo shoots	0.05*
270090	. Palm hearts	0.05*
270990	. Others (2)	0.05*
280000	. Fungi, mosses and lichens	0.05*
280010	. Cultivated fungi	0.05*
280020	. Wild fungi	0.05*
280990	. Mosses and lichens	0.05*
290000	. Algae and prokaryotes organisms	0.05*
300000	. PULSES	
300010	. Beans	0.1 (ft)
300020	. Lentils	0.05*
300030	. Peas	0.1 (ft)
300040	. Lupins/lupini beans	0.05*
300990	. Others (2)	0.05*
400000	. OILSEEDS AND OIL FRUITS	
401000	. Oilseeds	
401010	. Linseeds	0.1*
401020	. Peanuts/groundnuts	0.1*
401030	. Poppy seeds	0.1*
401040	. Sesame seeds	0.1*
401050	. Sunflower seeds	0.1*
401060	. Rapeseeds/canola seeds	0.5 (ft)
401070	. Soyabeans	0.1*
401080	. Mustard seeds	0.1*
401090	. Cotton seeds	0.1*
401100	. Pumpkin seeds	0.1*
401110	. Safflower seeds	0.1*
401120	. Borage seeds	0.1*
401130	. Gold of pleasure seeds	0.1*
401140	. Hemp seeds	0.1*
401150	. Castor beans	0.1*
401990	. Others (2)	0.1*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
402000	. Oil fruits	
402010	. Olives for oil production	5 (ft)
402020	. Oil palms kernels	0.1*
402030	. Oil palms fruits	0.1*
402040	. Kapok	0.1*
402990	. Others (2)	0.1*
500000	. CEREALS	
500010	. Barley	2 (ft)
500020	. Buckwheat and other pseudo-cereals	0.05*
500030	. Maize/corn	0.05*
500040	. Common millet/proso millet	0.05*
500050	. Oat	2 (ft)
500060	. Rice	0.05*
500070	. Rye	1 (ft)
500080	. Sorghum	0.05*
500090	. Wheat	1 (ft)
500990	. Others (2)	0.05*
600000	. TEAS, COFFEE, HERBAL INFUSIONS, COCOA AND CAROBS	0.1*
610000	. Teas	0.1*
620000	. Coffee beans	0.1*
630000	. Herbal infusions from	0.1*
631000	. (a) flowers	0.1*
631010	. Chamomile	0.1*
631020	. Hibiscus/roselle	0.1*
631030	. Rose	0.1*
631040	. Jasmine	0.1*
631050	. Lime/linden	0.1*
631990	. Others (2)	0.1*
632000	. (b) leaves and herbs	0.1*
632010	. Strawberry	0.1*
632020	. Rooibos	0.1*
632030	. Mate/maté	0.1*
632990	. Others (2)	0.1*
633000	. (c) roots	0.1*
633010	. Valerian	0.1*
633020	. Ginseng	0.1*
633990	. Others (2)	0.1*
639000	. (d) any other parts of the plant	0.1*
640000	. Cocoa beans	0.1*
650000	. Carobs/Saint John's breads	0.1*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
700000	. HOPS	25 (ft)
800000	. SPICES	
810000	. Seed spices	0.1*
810010	. Anise/aniseed	0.1*
810020	. Black caraway/black cumin	0.1*
810030	. Celery	0.1*
810040	. Coriander	0.1*
810050	. Cumin	0.1*
810060	. Dill	0.1*
810070	. Fennel	0.1*
810080	. Fenugreek	0.1*
810090	. Nutmeg	0.1*
810990	. Others (2)	0.1*
820000	. Fruit spices	0.1*
820010	. Allspice/pimento	0.1*
820020	. Sichuan pepper	0.1*
820030	. Caraway	0.1*
820040	. Cardamom	0.1*
820050	. Juniper berry	0.1*
820060	. Peppercorn (black, green and white)	0.1*
820070	. Vanilla	0.1*
820080	. Tamarind	0.1*
820990	. Others (2)	0.1*
830000	. Bark spices	0.1*
830010	. Cinnamon	0.1*
830990	. Others (2)	0.1*
840000	. Root and rhizome spices	0.1*
840010	. Liquorice	0.1*
840020	. Ginger	0.1*
840030	. Turmeric/curcuma	0.1*
840040	. Horseradish	0.1*
840990	. Others (2)	0.1*
850000	. Bud spices	
850010	. Cloves	0.1*
850020	. Capers	25
850990	. Others (2)	0.1*
860000	. Flower pistil spices	0.1*
860010	. Saffron	0.1*
860990	. Others (2)	0.1*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
870000	. Aril spices	0.1*
870010	. Mace	0.1*
870990	. Others (2)	0.1*
900000	. SUGAR PLANTS	
900010	. Sugar beet roots	2
900020	. Sugar canes	0.05*
900030	. Chicory roots	0.05*
900990	. Others (2)	0.05*
1000000	. PRODUCTS OF ANIMAL ORIGIN - TERRESTRIAL ANIMALS	0.05*
1010000	. Tissues from	0.05*
1011000	. (a) swine	0.05*
1011010	. Muscle	0.05*
1011020	. Fat tissue	0.05*
1011030	. Liver	0.05*
1011040	. Kidney	0.05*
1011050	. Edible offals (other than liver and kidney)	0.05*
1011990	. Others (2)	0.05*
1012000	. (b) bovine	0.05*
1012010	. Muscle	0.05*
1012020	. Fat tissue	0.05*
1012030	. Liver	0.05*
1012040	. Kidney	0.05*
1012050	. Edible offals (other than liver and kidney)	0.05*
1012990	. Others (2)	0.05*
1013000	. (c) sheep	0.05*
1013010	. Muscle	0.05*
1013020	. Fat tissue	0.05*
1013030	. Liver	0.05*
1013040	. Kidney	0.05*
1013050	. Edible offals (other than liver and kidney)	0.05*
1013990	. Others (2)	0.05*
1014000	. d) goat	0.05*
1014010	. Muscle	0.05*
1014020	. Fat tissue	0.05*
1014030	. Liver	0.05*
1014040	. Kidney	0.05*
1014050	. Edible offals (other than liver and kidney)	0.05*
1014990	. Others (2)	0.05*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
1015000	. (e) equine	0.05*
1015010	. Muscle	0.05*
1015020	. Fat tissue	0.05*
1015030	. Liver	0.05*
1015040	. Kidney	0.05*
1015050	. Edible offals (other than liver and kidney)	0.05*
1015990	. Others (2)	0.05*
1016000	. (f) poultry	0.05*
1016010	. Muscle	0.05*
1016020	. Fat tissue	0.05*
1016030	. Liver	0.05*
1016040	. Kidney	0.05*
1016050	. Edible offals (other than liver and kidney)	0.05*
1016990	. Others (2)	0.05*
1017000	. (g) other farmed terrestrial animals	0.05*
1017010	. Muscle	0.05*
1017020	. Fat tissue	0.05*
1017030	. Liver	0.05*
1017040	. Kidney	0.05*
1017050	. Edible offals (other than liver and kidney)	0.05*
1017990	. Others (2)	0.05*
1020000	. Milk	0.05*
1020010	. Cattle	0.05*
1020020	. Sheep	0.05*
1020030	. Goat	0.05*
1020040	. Horse	0.05*
1020990	. Others (2)	0.05*
1030000	. Birds eggs	0.05*
1030010	. Chicken	0.05*
1030020	. Duck	0.05*
1030030	. Geese	0.05*
1030040	. Quail	0.05*
1030990	. Others (2)	0.05*
1040000	. Honey and other apiculture products	0.05*
1050000	. Amphibians and Reptiles	0.05*
1060000	. Terrestrial invertebrate animals	0.05*
1070000	. Wild terrestrial vertebrate animals	0.05*

Pesticides - Web Version - EU MRLs (File created on 06/02/2015)		
Code number	**Groups and examples of individual products to which the MRLs apply (a)**	**Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)**
Pesticide residue	**Legislation**	**Entry in to force**
Dithiocarbamates (dithiocarbamates expressed as CS2, including maneb, mancozeb, metiram, propineb, thiram and ziram)		
	Reg. (EU) No 34/2013	26.01.2013

Pesticide residues and maximum residue levels (mg/kg)
*** indicates lower limit of analytical determination**

The MRLs expressed as CS_2 can arise from different dithiocarbamates and therefore they do not reflect a single Good Agricultural Practice (GAP). It is therefore not appropriate to use these MRLs to check compliance with a GAP.
(ft) Footnotes
In brackets the origin of the residue (ma: maneb mz: mancozeb me: metiram pr: propineb t: thiram z: ziram).

0140020 : Cherries (sweet) (mz, me, pr, t, z)
0140030 : Peaches (mz, t)
0140040 : Plums (mz, me, t, z)
0151000 : (a) grapes (ma, mz, me, pr, t)
0152000 : (b) strawberries (t)
0154030 : Currants (black, red and white) (mz)
0161030 : Table olives (mz, pr)
0163020 : Bananas (mz, me, t)
0163030 : Mangoes (mz)
0163040 : Papayas (mz)
0211000 : (a) potatoes (ma, mz, me, pr)
0213010 : Beetroots (mz)
0213020 : Carrots (mz)
0213030 : Celeriacs/turnip rooted celeries (ma, me, pr, t)
0213040 : Horseradishes (mz)
0213060 : Parsnips (mz)
0213070 : Parsley roots/Hamburg roots parsley (mz)
0213080 : Radishes (mz)
0213090 : Salsifies (mz)
0220010 : Garlic (me)
0220020 : Onions (ma, mz)
0220030 : Shallots (ma, mz)
0220040 : Spring onions/green onions and Welsh onions (mz)
0231010 : Tomatoes (ma, mz, me, pr)
0231020 : Sweet peppers/bell peppers (mz, pr)
0231030 : Aubergines/eggplants (mz, me)
0231040 : Okra/lady's fingers (mz)
0232000 : (b) cucurbits with edible peel (mz, pr)
0233000 : (c) cucurbits with inedible peel (me)
0233010 : Melons (me)
0233020 : Pumpkins (me)
0233030 : Watermelons (me)
0233990 : Others (2) (me)
0241000 : (a) flowering brassica (mz)
0242010 : Brussels sprouts (mz)
0242020 : Head cabbages (mz)
0243000 : (c) leafy brassica (mz)
0244000 : (d) kohlrabies (mz)
0251000 : (a) lettuces and salad plants (mz, me, t)
0254000 : (d) watercresses (mz)
0255000 : (e) witloofs/Belgian endives (mz)
0256000 : (f) herbs and edible flowers (mz, me)
0260010 : Beans (with pods) (mz)
0260020 : Beans (without pods) (mz)
0260030 : Peas (with pods) (ma, mz)
0260040 : Peas (without pods) (mz)
0270010 : Asparagus (mz)
0270060 : Leeks (ma, mz)
0270070 : Rhubarbs (mz)
0300010 : Beans (mz)
0300030 : Peas (mz)
0401060 : Rapeseeds/canola seeds (ma, mz)
0402010 : Olives for oil production (mz, pr)
0500010 : Barley (ma, mz)
0500050 : Oat (ma, mz)
0500070 : Rye (ma, mz)
0500090 : Wheat (ma, mz)
0700000 : HOPS (pr)

Table 6.9-4: Metiram: TMDI calculation based on input values of representative commodities (grapes, potatoes and livestock)

Metiram

Status of the active substance:		Code no.	
LOQ (mg/kg bw):	0.056	proposed LOQ:	
Toxicological end points			
ADI (mg/kg bw/day):	**0.03**	ARfD (mg/kg bw):	**n.n.**
Source of ADI:	**Dir 06/72**	Source of ARfD:	**Dir 06/72**
Year of evaluation:		Year of evaluation:	

Explain choice of toxicological reference values.
The risk assessment has been performed on the basis of the MRLs collected from Member States in April 2006. For each pesticide/commodity the highest national MRL was identified (proposed temporary MRL = pTMRL).
The pTMRLs have been submitted to EFSA in September 2006.

Chronic risk assessment

TMDI (range) in % of ADI
minimum - maximum
3 70

No of diets exceeding ADI: ---

Highest calculated TMDI values in % of ADI	MS Diet	Highest contributor to MS diet (in % of ADI)	Commodity / group of commodities	2nd contributor to MS diet (in % of ADI)	Commodity / group of commodities	3rd contributor to MS diet (in % of ADI)	Commodity / group of commodities	pTMRLs at LOQ (in % of ADI)
70.3	FR all population	68.5	Table and wine grapes	1.1	Potatoes	0.4	Milk and cream,	0.7
51.4	PT General population	46.1	Table and wine grapes	5.3	Potatoes		FRUIT (FRESH OR FROZEN)	
39.8	WHO Cluster diet B	35.7	Table and wine grapes	2.7	Potatoes	0.6	Meat, preparations of meat,	1.4
34.9	WHO cluster diet E	29.9	Table and wine grapes	3.8	Potatoes	0.5	Milk and cream,	1.2
27.2	DK adult	24.5	Table and wine grapes	1.5	Potatoes	0.9	Milk and cream,	1.3
26.5	DE child	21.2	Table and wine grapes	2.6	Potatoes	2.4	Milk and cream,	2.8
24.0	NL child	12.7	Table and wine grapes	5.9	Potatoes	4.9	Milk and cream,	5.5
22.7	IE adult	19.4	Table and wine grapes	2.3	Potatoes	0.5	Meat, preparations of meat,	1.0
21.0	UK Adult	18.9	Table and wine grapes	1.4	Potatoes	0.5	Milk and cream,	0.7
18.4	NL general	14.3	Table and wine grapes	2.7	Potatoes	1.1	Milk and cream,	1.4
17.0	WHO Cluster diet F	12.2	Table and wine grapes	3.4	Potatoes	0.7	Milk and cream,	1.4
16.8	UK vegetarian	14.8	Table and wine grapes	1.4	Potatoes	0.5	Milk and cream,	0.6
15.7	FR toddler	6.6	Milk and cream,	5.1	Potatoes	3.5	Table and wine grapes	7.2
14.5	WHO cluster diet D	9.2	Table and wine grapes	4.1	Potatoes	0.8	Milk and cream,	1.2
12.1	WHO regional European diet	6.4	Table and wine grapes	4.0	Potatoes	0.8	Milk and cream,	1.7
12.1	UK Toddler	4.7	Table and wine grapes	3.5	Potatoes	3.4	Milk and cream,	3.9
10.9	UK Infant	6.5	Milk and cream,	3.3	Potatoes	0.7	Table and wine grapes	7.0
10.9	SE general population 90th percentile	4.2	Potatoes	3.8	Table and wine grapes	2.1	Milk and cream,	2.9
10.0	FR infant	4.3	Milk and cream,	4.1	Potatoes	1.3	Table and wine grapes	4.6
9.9	ES adult	7.7	Table and wine grapes	0.9	Potatoes	0.8	Milk and cream,	1.3
8.8	PL general population	5.3	Table and wine grapes	3.4	Potatoes		FRUIT (FRESH OR FROZEN)	
8.4	DK child	3.0	Table and wine grapes	2.4	Potatoes	2.1	Milk and cream,	2.9
7.8	FI adult	5.4	Table and wine grapes	1.2	Potatoes	0.9	Milk and cream,	1.1
5.5	ES child	2.1	Milk and cream,	1.8	Potatoes	0.7	Meat, preparations of meat,	2.9
4.4	LT adult	3.2	Potatoes	0.7	Milk and cream,	0.3	Meat, preparations of meat,	1.0
2.8	IT adult	2.2	Table and wine grapes	0.6	Potatoes		FRUIT (FRESH OR FROZEN)	
2.7	IT kids/toddler	1.8	Table and wine grapes	0.9	Potatoes		FRUIT (FRESH OR FROZEN)	

Conclusion:
The estimated Theoretical Maximum Daily Intakes (TMDI), based on pTMRLs were below the ADI.
A long-term intake of residues of Metiram is unlikely to present a public health concern.

Table 6.9-5: Metiram: TMDI calculation based on input values of registered metiram uses

Metiram

Status of the active substance:		Code no.	
LOQ (mg/kg bw):	0.056	proposed LOQ:	
Toxicological end points			
ADI (mg/kg bw/day):	**0.03**	ARfD (mg/kg bw):	**n.n.**
Source of ADI:	**Dir 06/72**	Source of ARfD:	**Dir 06/72**
Year of evaluation:		Year of evaluation:	

Explain choice of toxicological reference values.
The risk assessment has been performed on the basis of the MRLs collected from Member States in April 2006. For each pesticide/commodity the highest national MRL was identified (proposed temporary MRL = pTMRL).
The pTMRLs have been submitted to EFSA in September 2006.

Chronic risk assessment

TMDI (range) in % of ADI
minimum - maximum
26 278

No of diets exceeding ADI: 3

Highest calculated TMDI values in % of ADI	MS Diet	Highest contributor to MS diet (in % of ADI)	Commodity / group of commodities	2nd contributor to MS diet (in % of ADI)	Commodity / group of commodities	3rd contributor to MS diet (in % of ADI)	Commodity / group of commodities	pTMRLs at LOQ (in % of ADI)
277.7	DE child	211.9	Pome fruit	21.2	Table and wine grapes	20.6	Bananas	2.8
174.3	NL child	112.7	Pome fruit	22.7	Bananas	12.7	Table and wine grapes	5.5
119.4	WHO Cluster diet B	35.7	Table and wine grapes	30.8	Tomatoes	23.4	Pome fruit	1.4
96.6	FR all population	68.5	Table and wine grapes	10.0	Pome fruit	5.3	Lettuce and other salad plants	0.7
94.5	DK child	50.0	Pome fruit	15.2	Bananas	10.9	Cucurbits - edible peel	2.9
93.4	FR toddler	48.0	Pome fruit	17.2	Bananas	7.7	Tomatoes	7.2
91.6	PT General population	46.1	Table and wine grapes	23.6	Pome fruit	9.0	Tomatoes	
79.9	SE general population 90th percentile	24.1	Bananas	23.5	Pome fruit	7.7	Tomatoes	2.9
74.0	IE adult	24.8	Pome fruit	19.4	Table and wine grapes	10.4	Bananas	1.0
73.2	FR infant	47.0	Pome fruit	9.5	Bananas	4.5	Cucurbits - edible peel	4.6
68.9	WHO cluster diet E	29.9	Table and wine grapes	17.1	Pome fruit	5.3	Tomatoes	1.2
65.9	UK Toddler	31.4	Pome fruit	14.3	Bananas	5.9	Tomatoes	3.9
65.8	ES child	27.2	Pome fruit	13.5	Bananas	9.8	Tomatoes	2.9
64.9	UK Infant	30.3	Pome fruit	19.5	Bananas	6.5	Milk and cream,	7.0
60.7	PL general population	38.7	Pome fruit	8.8	Tomatoes	5.3	Table and wine grapes	
58.0	DK adult	24.5	Table and wine grapes	16.5	Pome fruit	5.0	Bananas	1.3
55.2	NL general	22.6	Pome fruit	14.3	Table and wine grapes	4.3	Tomatoes	1.4
55.1	IT kids/toddler	20.6	Pome fruit	14.3	Tomatoes	7.1	Bananas	
54.6	WHO regional European diet	14.3	Pome fruit	11.0	Tomatoes	6.9	Lettuce and other salad plants	1.7
52.8	ES adult	18.5	Pome fruit	8.9	Lettuce and other salad plants	7.8	Tomatoes	1.3
52.0	WHO Cluster diet F	12.8	Pome fruit	12.2	Table and wine grapes	7.5	Bananas	1.4
49.3	LT adult	33.8	Pome fruit	6.2	Tomatoes	3.2	Potatoes	1.0
48.0	WHO cluster diet D	12.6	Pome fruit	10.1	Tomatoes	9.2	Table and wine grapes	1.2
47.6	IT adult	17.1	Pome fruit	11.6	Tomatoes	9.3	Lettuce and other salad plants	
43.9	UK vegetarian	14.8	Table and wine grapes	11.1	Pome fruit	6.2	Tomatoes	0.6
41.0	UK Adult	18.9	Table and wine grapes	7.8	Pome fruit	4.7	Bananas	0.7
26.0	FI adult	7.2	Pome fruit	5.4	Table and wine grapes	4.3	Tomatoes	1.1

Conclusion:
The estimated Theoretical Maximum Daily Intakes based on MS and WHO diets and pTMRLs were in the range of 26 % to 278 % of the ADI.
For 3 diets the ADI is exceeded. Further refinements of the dietary intake estimates have not been performed. A public health risk can not be excluded at the moment.

Table 6.9-6: Overview of the toxicological equivalence factors and molecular weight correction factors

Compound	ADI (mg/kg bw per day)	Toxicological equivalence factor (TEF)	Molecular weight (g/mol)	Molecular weight/carbon disulfide[a]	Combined correction factor (Cf) (=TEF*MW)
Propineb	0.007	1	289.8	1.86	1.86
Metiram	0.03	0.23	1088.7	1.79	0.41
Mancozeb	0.05	0.14	271.3	1.78	0.25
Maneb	0.05	0.14	265.3	1.75	0.25

(a): molecular weight of carbon disulfide (CS_2): 76.1 g/mol

Table 6.9-7: Input values for the consumer dietary exposure assessment (EFSA reasoned opinion, Italy 2012)

Commodity	Chronic exposure assessment	
	Input value (mg/kg)	Comment
The CS_2 values originating from the use of maneb, mancozeb, metiram and propineb, were expressed as propineb by applying the combined correction factor (Cf); the calculated chronic exposure was compared with the ADI of propineb, which has the highest toxicity of these active substances who share the common critical effect of the long-term toxicity.		
Garlic	0.07	Median residue (0.17 onion) (Table 3-2 EFSA reasoned opinion, Italy, 2012) * Cf_{me}
Citrus fruits (except mandarins)	0.11	Median residue $(3.0)_{mz}$ (Italy, 2008b) * Cf_{mz} * PF (0.14) (Italy, 2007)
Mandarins	0.08	Median residue $(2.35)_{mz}$ (Italy, 2008b) * Cf_{mz} * PF (0.14) (Italy, 2007)
Walnuts	0.03	MRL * Cf_{mz}
Pome fruits	0.23	Median residue $(0.56)_{me}$ (Italy, 2008a) * Cf_{me}
Apricots	0.11	Median residue $(0.44)_{mz}$ (Italy, 2008b) * Cf_{mz}
Cherries	0.27	Median residue $(0.65)_{me}$ (Italy, 2008a) * Cf_{me}
Peaches	0.11	Median residue $(0.44)_{mz}$ (Italy, 2008b) * Cf_{mz}

Commodity	Chronic exposure assessment	
	Input value (mg/kg)	Comment
Plums	0.08	Median residue $(0.2)_{me}$ (Italy, 2008a) * Cf_{me}
Table grapes	0.52	Median residue $(2.06)_{mz}$ (Italy, 2008b) * Cf_{mz}
Wine grapes	0.03	Median residue $(2.06)_{mz}$ * Cf_{mz} * yield factor (0.7)[1)] * PF (0.09) (Italy, 2008b)
Blueberries, cranberries, currants (red, black and white), goose berries	1.25	MRL * Cf_{mz}
Table olives	0.46	Median residue $(1.82)_{mz}$ (Italy, 2008b) * Cf_{mz}
Bananas	0.03	Median residue $(0.37)_{me}$ (Italy, 2008a) * CF_{me} * PF $(0.19)_{me}$ (Italy, 2010b)
Mangoes	0.5	MRL * Cf_{mz}
Papaya	1.75	MRL * Cf_{mz}
Potatoes	0.1	Median residue (pr) (Italy, 2010a)
Beetroot	0.13	MRL * Cf_{mz}
Carrots	0.05	MRL * Cf_{mz}
Celeriac	0.19	Median residue $_{pr}$ (Italy, 2010a)
Horseradish	0.05	MRL * Cf_{mz}
Parsnips	0.05	MRL * Cf_{mz}
Parsley root	0.05	MRL * Cf_{mz}
Radishes	0.22	Median residue $(0.89)_{mz}$ * CF_{mz} (EFSA, 2011)
Salsify	0.05	MRL * Cf_{mz}
Onions, shallots, spring onions	0.25	MRL * Cf_{mz}
Tomatoes	0.33	Median residue $_{pr}$ (Italy, 2010a)
Peppers	0.41	Median residue $(1.63)_{mz}$ (Italy, 2008b) * Cf_{mz}
Aubergines	1.23	MRL * CF_{me}
Okra	0.13	MRL * Cf_{mz}

Commodity	Chronic exposure assessment	
	Input value (mg/kg)	Comment
Cucurbits (edible peel)	0.53	Median residue (cucumbers)$_{pr}$ (Italy, 2010a)
Melons, watermelons, pumpkins	0.36[2)]	Median residue (melons)$_{pr}$ (Italy, 2010a)
Flowering brassica	0.25	MRL * Cf_{mz}
Brussels sprouts	0.5	MRL * Cf_{mz}
Head cabbage	0.75	MRL * Cf_{mz}
Leafy brassica	0.13	MRL * Cf_{mz}
Kohlrabi	0.25	MRL * Cf_{mz}
Lettuce and other salad plants	0.35	Median residue (0.86)$_{me}$ (Italy, 2010b) * Cf_{me}
Purslane	1.25	MRL * Cf_{mz}
Watercress	0.08	MRL * Cf_{mz}
Witloof	0.13	MRL * Cf_{mz}
Herbs	2.05	MRL * Cf_{me}
Beans (with pods)	0.25	MRL * Cf_{mz}
Beans (without pods)	0.03	MRL * Cf_{mz}
Peas (with pods)	0.25	MRL * Cf_{mz}
Peas (without pods)	0.01	Median residue (0.056)$_{mz}$ * Cf_{mz} (EFSA, 2010)
Asparagus	0.13	MRL * Cf_{mz}
Leek	0.75	MRL * Cf_{mz}
Rhubarb	0.13	MRL * Cf_{mz}
Beans	0.03	MRL * Cf_{mz}
Peas	0.03	MRL * Cf_{mz}
Rapeseed	0.13	MRL * Cf_{mz}
Olives for oil production	0.46	Median residue (1.86)$_{mz}$ (Italy, 2008b) * Cf_{mz}
Barley	0.32	Median residue (1.28)$_{ma}$ (Italy, 2009) * Cf_{ma}
Oats	0.5	MRL * Cf_{mz}
Rye	0.25	MRL * Cf_{mz}
Wheat	0.06	Median residue (0.23)$_{ma}$ (Italy, 2009) * Cf_{ma}
Hops	16.35	Median residue$_{pr}$ (Italy, 2010a)

Commodity	Chronic exposure assessment	
	Input value (mg/kg)	Comment
Capers	46.5	MRL * Cf_{pr}
Sugar beet Other commodities of plant and animal origin	0[3)] MRL	See table footnote See EU Pesticides database[4)]

1) Consumption figures in the EFSA PRIMo are expressed as wine grapes. Since it is assumed that all wine grapes are consumed as wine, the consumption is recalculated to wine using a yield factor (1 kg of wine grapes is needed to produce 0.7 kg of wine) to perform the refined intake calculation for wine grapes.
2) The new intended use of metiram on cucurbits (edible peel) would result in an input value of 0.04 mg/kg; a more critical value representing the existing use on propineb (Italy, 2010) was selected therefore as an input value. No use on pumpkins was reported by the RMS, but, as the existing EU MRL refers to a group tolerance, it was assumed that existing MRL for pumpkins was derived as an extrapolation from melons.
3) No residues are expected in sugar
4) MRLs are only used for all others (not mentioned in the Table)

Table 6.9-8: Metiram (BAS 222 F): TMDI calculation based on input values listed in Table 6.9-7 (recalculation)

Metiram (calculated as propineb)

Status of the active substance:		Code no.	
LOQ (mg/kg bw):	0.05	proposed LOQ:	
Toxicological end points			
ADI (mg/kg bw/day):	**0.007**	ARfD (mg/kg bw):	**n.n.**
Source of ADI:	**Dir 05/72**	Source of ARfD:	**Dir 05/72**
Year of evaluation:		Year of evaluation:	

Explain choice of toxicological reference values.
The risk assessment has been performed on the basis of the MRLs collected from Member States in April 2006. For each pesticide/commodity the highest national MRL was identified (proposed temporary MRL = pTMRL).
The pTMRLs have been submitted to EFSA in September 2006.

Chronic risk assessment

TMDI (range) in % of ADI
minimum - maximum
16 99

No of diets exceeding ADI: ---

Highest calculated TMDI values in % of ADI	MS Diet	Highest contributor to MS diet (in % of ADI)	Commodity / group of commodities	2nd contributor to MS diet (in % of ADI)	Commodity / group of commodities	3rd contributor to MS diet (in % of ADI)	Commodity / group of commodities	pTMRLs at LOQ (in % of ADI)
98.6	DE child	41.8	Pome fruit	9.4	Table grapes	6.0	Oranges	1.5
88.7	WHO Cluster diet B	14.5	Tomatoes	12.6	Olives for oil production	7.3	Wheat	1.4
74.9	NL child	22.2	Pome fruit	8.4	Potatoes	5.6	Table grapes	1.2
61.8	DK child	15.8	Rye	12.4	Cucurbits - edible peel	9.9	Pome fruit	1.5
56.9	IE adult	5.7	Barley	4.9	Aubergines (egg plants)	4.9	Pome fruit	1.9
50.5	FR toddler	9.5	Pome fruit	7.7	Leek	7.2	Potatoes	2.4
45.6	SE general population 90th percentile	6.7	Head cabbage	6.0	Potatoes	4.9	Cucurbits - edible peel	1.5
43.5	WHO cluster diet E	5.5	Potatoes	3.7	Barley	3.4	Wheat	1.3
43.5	WHO cluster diet D	5.8	Potatoes	5.6	Wheat	4.8	Tomatoes	0.5
41.8	WHO regional European diet	5.7	Potatoes	5.2	Tomatoes	3.9	Head cabbage	0.6
36.7	FR infant	9.3	Pome fruit	5.9	Potatoes	5.1	Cucurbits - edible peel	2.2
35.7	WHO Cluster diet F	4.9	Potatoes	3.2	Tomatoes	3.1	Wheat	0.9
34.9	ES child	5.4	Pome fruit	4.8	Olives for oil production	4.6	Tomatoes	0.7
32.8	NL general	4.5	Pome fruit	3.9	Potatoes	2.3	Oranges	0.6
32.3	PT General population	7.6	Potatoes	4.6	Pome fruit	4.2	Tomatoes	1.9
31.9	UK Toddler	6.2	Pome fruit	5.0	Potatoes	3.4	Wheat	1.2
29.7	IT kids/toddler	6.7	Tomatoes	5.7	Wheat	4.1	Pome fruit	0.4
29.7	PL general population	7.6	Pome fruit	4.9	Potatoes	4.2	Tomatoes	0.4
29.3	LT adult	6.7	Pome fruit	4.5	Potatoes	4.3	Head cabbage	0.2
28.5	ES adult	3.7	Tomatoes	3.7	Pome fruit	2.8	Olives for oil production	0.5
27.0	IT adult	5.5	Tomatoes	3.5	Wheat	3.4	Pome fruit	0.2
27.0	UK Infant	6.0	Pome fruit	4.6	Potatoes	2.2	Wheat	1.9
23.3	FR all population	2.8	Wheat	2.0	Tomatoes	2.0	Pome fruit	2.0
20.9	DK adult	3.3	Pome fruit	2.4	Rye	2.1	Cucurbits - edible peel	1.1
20.7	UK vegetarian	2.9	Tomatoes	2.2	Pome fruit	2.0	Potatoes	0.9
18.1	FI adult	2.4	Rye	2.0	Cucurbits - edible peel	2.0	Tomatoes	0.4
15.8	UK Adult	2.1	Tomatoes	2.0	Potatoes	1.5	Pome fruit	0.9

Conclusion:
The estimated Theoretical Maximum Daily Intakes (TMDI), based on pTMRLs were below the ADI.
A long-term intake of residues of Metiram (calculated as propineb) is unlikely to present a public health concern.

Table 6.9-9: Processing factors: ETU (OECD data point number IIA 6.5 and IIIA 8.5)

Crop/Processed Crop	Number of Studies	Mean Transfer Factor	% Transference
Apples - fresh juice	1	0.07	
Apples - cooked juice	1	0.06	
Apples - wet pomace	1	0.18	
Apples - dry pomace	1	0.85	
Apples - sauce	1	0.06	
Apples - baby food	1	0.06	
Cherries - washed cherries	4	0.03	
Cherries - wash water	4	0.03	
Cherries - juice	4	0.03	
Cherries - canned cherries	4	0.04	
Cherries - fruit syrup	4	0.05	
Plums - wash water	4	0.01	
Plums - washed plums	4	0.02	
Plums - remainder of the staining process	4	0.02	
Plums - condensed water	4	0.01	
Plums - purree	4	0.08	
Plums - dipping water	4	0.01	
Plums - prunes	4	0.01	
Grapes - wine (thermo-vinification)	10	0.22	
Grapes - wine	10	0.06	
Grapes - juice	10	0.20	
Grapes - pomace (thermo-vinification)	10	0.08	
Grapes - pomace	10	0.02	
Grapes - raisins	4	0.05	
Banana - pulp*	12	<0.2	
Banana - peel*	12	<0.2	
Tomatoes - washed tomatoes	4	0.01	
Tomatoes - wash water	4	0.01	
Tomatoes - cooking water	4	0.01	
Tomatoes - peeled tomatoes	4	0.01	
Tomatoes - peel	4	0.05	
Tomatoes - canned tomatoes	4	0.02	
Tomatoes - vegetable stock	4	0.04	
Tomato puree	4	0.24	
Tomato - condensed water	4	0.01	
Tomato - tomato juice	4	0.07	
Tomato - remainder	4	0.03	
Onions - onions peel	4	0.2	
Onions -peeled onions	4	0.0	
Onions - wash water	4	0.0	
Onions - dried onions	4	0.043	
Gherkins - wash water	4	0.04	
Gherkins - washed gherkins	4	0.18	
Gherkins -canned gherkins	4	0.08	
Gherkins -vegetable stock	4	0.04	

* all ETU residues below LOQ of 0.05 mg/kg

Table 6.9-10: ETU - calculation of input values - VELS model

Crop	Crop/Processed Crop - VELS Model	Transfer factor ETU (TF)	Processed Crop Transfer factor	Median (STMR) residue Metiram RAC	Highest residue (HR) Metiram RAC	Median residue (STMR) ETU RAC	Highest residue (HR) ETU RAC	Input values - VELS model chronic		Input values - VELS model acute	
Apples	Apples, processed	0.85	Apples - dry pomace	0.205	0.40	0.018	0.047	0.174	$STMR_{me}$ x TF 0.205 x 0.85	0.34	HR_{me} x TF 0.40 x 0.85
	Apples, portion in juice	0.07	Apples - fresh juice					0.014	$STMR_{me}$ x TF 0.205 x 0.07	0.028	HR_{me} x TF 0.40 x 0.07
Grapes	Grapes, processed	0.08	Grapes - pomace (thermo-vinification)	0.41	0.88	0.01	0.046	0.033	$STMR_{me}$ x TF 0.41 x 0.08	0.07	HR_{me} x TF 0.88 x 0.08
	Grapes, portion in juice	0.20	Grapes - juice					0.082	$STMR_{me}$ x TF 0.41 x 0.2	0.176	HR_{me} x TF 0.88 x 0.2
	Wine	0.22	Grapes - wine (thermo-vinification)					0.09	$STMR_{me}$ x TF 0.41 x 0.22	0.194	HR_{me} x TF 0.88 x 0.22
Passion fruit	Passion fruit, total	-	-	0.415	0.58	0.05	0.05	0.05	$STMR_{ETU}$	0.05	HR_{ETU}
Banana	Banana, total	0.2	Banana - peel & Banana - pulp	0.365	2.8	0.05	0.05	0.073	$STMR_{me}$ x TF 0.365 x 0.2	0.56	HR_{me} x TF 2.8 x 0.2
Potato	Potatoes	-	-	0.056	0.056	0.01	0.038	0.01	$STMR_{ETU}$	0.038	HR_{ETU}
Celeriac	Root celery, total	-	-	0.10	0.20	0.01	0.02	0.01	$STMR_{ETU}$	0.02	HR_{ETU}
Onion	Onion, raw	0.043	Onions - dried onions	0.174	0.24	0.01	0.01	0.007	$STMR_{me}$ x TF 0.174 x 0.043	0.01	HR_{me} x TF 0.24 x 0.043
	Onion, processed	0.2	Onions - onions peel					0.035	$STMR_{me}$ x TF 0.174 x 0.2	0.048	HR_{me} x TF 0.24 x 0.2
Tomato	Tomatoes, processed	0.24	Tomato puree	0.71	0.13	0.01	0.05	0.17	$STMR_{me}$ x TF 0.71 x 0.24	0.031	HR_{me} x TF 0.13 x 0.24
	Tomatoes, portion in juice	0.07	Tomato - tomato juice					0.05	$STMR_{me}$ x TF 0.71 x 0.07	0.009	HR_{me} x TF 0.13 x 0.07
Cucumber /Zucchini	Cucumber, processed	0.08	Gherkins -canned gherkins	0.101	0.25	0.013	0.03	0.008	$STMR_{me}$ x TF 0.101 x 0.08	0.02	HR_{me} x TF 0.25 x 0.08

Crop	Crop/Processed Crop - VELS Model	Transfer factor ETU (TF)	Processed Crop Transfer factor	Median (STMR) residue Metiram RAC	Highest residue (HR) Metiram RAC	Median residue (STMR) ETU RAC	Highest residue (HR) ETU RAC	Input values - VELS model chronic		Input values - VELS model acute	
Melons	Melon, raw	-	-	0.081	0.713	0.012	0.032	0.012	$STMR_{ETU}$	0.032	HR_{ETU}
Lettuce	Lettuce and other salad plants including Brassicacea	-	-	0.06	2.76	0.01	0.06	0.01	$STMR_{ETU}$	0.06	HR_{ETU}
Asparagus	Stem vegetables (fresh)	-	-	0.056	0.056	0.01	0.01	0.01	$STMR_{ETU}$	0.01	HR_{ETU}

Table 6.9-11: ETU - VELS model chronic - representative crops (grapes and potatoes)

	Active substance:	no active substance	Total intake in mg/kg bw:	9.62601E-05	
	ADI (mg/kg bw):	0.002	% ADI	4.813003096	
	Mean bodyweight (kg):	16.15	Calculation method:	TMDI	

Code number	Commodity	chronic			
		Mean consumption (g/d)	MRL (mg/kg)	STMR (mg/kg)	Intake (mg/kg bw)
0151010	Grapes, portion in juice	12	0.082		6.09288E-05
0211000	a) Potatoes	41.4	0.01		2.56347E-05
0151010	Grapes, processed	4.2	0.033		8.58204E-06
0151020	Wine	0.2	0.09		1.11455E-06

Table 6.9-12: ETU - VELS model acute - representative crops (grapes and potatoes)

Active substance:	ETU
ARfD (mg/kg bw):	0.05
Mean bodyweight (kg):	16.15

Code number	Commodity	acute								
		Percentile	Large portion (g)	Unit weight (g)	HR or HR-P (mg/kg)	STMR-P (mg/kg)	Variability-factor	Case	Intake (mg/kg bw)	ARfD (%)
151010	Grapes, processed	97.5	162.9	679	0.07			1	0.000706068	1.4
211000	Potatoes, processed	97.5	218.8	90	0.038			2a/2b	0.000303059	0.6

Table 6.9-13: ETU - VELS model chronic - registered metiram uses

	Active substance:	no active substance	Total intake in mg/kg bw:	0.000543276	
	ADI (mg/kg bw):	0.002	% ADI	27.16377709	
	Mean bodyweight (kg):	16.15	Calculation method:	TMDI	
Code number	**Commodity**	**chronic**			
		Mean consumption (g/d)	MRL (mg/kg)	STMR (mg/kg)	Intake (mg/kg bw)
0130010	Apples, portion in juice	150.2	0.014		0.000130204
0163020	Banana, total	25	0.073		0.000113003
0231010	Tomatoes, processed	10.3	0.17		0.000108421
0130010	Apples, processed	6.8	0.174		7.32632E-05
0151010	Grapes, portion in juice	12	0.082		6.09288E-05
0211000	a) Potatoes	41.4	0.01		2.56347E-05
0162030	Passion fruit, total	3.7	0.05		1.14551E-05
0151010	Grapes, processed	4.2	0.033		8.58204E-06
0220020	Onions, processed	2.7	0.035		5.85139E-06
0151020	Wine	0.2	0.09		1.11455E-06
0270000	vii) Stem vegetables (fresh)	1.8	0.01		1.11455E-06
0231010	Tomatoes, portion in juice	0.3	0.05		9.28793E-07
0251000	a) Lettuce and other salad plants including Brassicacea	1.4	0.01		8.66873E-07
0232010	Cucumber, processed	1.6	0.008		7.9257E-07
0233010	Melons, raw	0.8	0.012		5.94427E-07
0213030	Root celery, total	0.7	0.01		4.33437E-07
0220020	Onions, raw	0.2	0.007		8.66873E-08

Table 6.9-14: ETU - VELS model acute - registered metiram uses

Active substance:	ETU								
ARfD (mg/kg bw):	0.05								
Mean bodyweight (kg):	16.15								

Code number	Commodity	acute							
		Percentile	Large portion (g)	Unit weight (g)	HR or HR-P (mg/kg)	STMR-P (mg/kg)	Variability-factor	Case	Intake (mg/kg bw)	ARfD (%)
163020	Banana, raw	97.5	239.2	115	0.56			2a/2b	0.004306625	8.6
151010	Grapes, processed	97.5	162.9	679	0.07			1	0.000706068	1.4
211000	Potatoes, processed	97.5	218.8	90	0.038			2a/2b	0.000303059	0.6
270010	Asparagus, processed	97.5	155.4	25	0.01			2a/2b	8.0743E-05	0.2
231010	Tomatoes, processed	97.5	111.2	99	0.031			2a/2b	2.3418E-05	0.0
220010	Garlic, raw*	97.5	2.7		0.01			1	1.67183E-06	0.0

Table 6.9-15: ETU - calculation of input values - EFSA PRIMo model version 2

Crop	Transfer factor ETU (TF)	Processed Crop Transfer factor	Median (STMR) residue Metiram RAC	Highest residue (HR) Metiram RAC	Median residue (STMR) ETU RAC	Highest residue (HR) ETU RAC	Input values - PRIMo model vers2 chronic		Input values - PRIMo model vers2 acute	
Grapes	0.08	Grapes - pomace (thermo-vinification)	0.410	0.88	0.01	0.046	0.09	$STMR_{me}$ x highest TF 0.41 x 0.22	0.09	$STMR_{me}$ x highest TF 0.41 x 0.22
	0.20	Grapes - juice								
	0.22	Grapes - wine (thermo-vinification)								
Potato	-	-	0.056	0.056	0.01	0.038	0.01	$STMR_{ETU}$	0.038	HR_{ETU}

Table 6.9-16: ETU - PRIMo model vers2 chronic - representative crops (grapes and potatoes)

ETU

Status of the active substance:		Code no.	
LOQ (mg/kg bw):	0.01	proposed LOQ:	
Toxicological end points			
ADI (mg/kg bw/day):	**0.002**	ARfD (mg/kg bw):	**0.05**
Source of ADI:		Source of ARfD:	
Year of evaluation:		Year of evaluation:	

Explain choice of toxicological reference values.
The risk assessment has been performed on the basis of the MRLs collected from Member States in April 2006. For each pesticide/commodity the highest national MRL was identified (proposed temporary MRL = pTMRL).
The pTMRLs have been submitted to EFSA in September 2006.

Chronic risk assessment

TMDI (range) in % of ADI
minimum - maximum
1 19

No of diets exceeding ADI: ---

Highest calculated TMDI values in % of ADI	MS Diet	Highest contributor to MS diet (in % of ADI)	Commodity / group of commodities	2nd contributor to MS diet (in % of ADI)	Commodity / group of commodities	3rd contributor to MS diet (in % of ADI)	Commodity / group of commodities	pTMRLs at LOQ (in % of ADI)
19.0	FR all population	18.5	Table and wine grapes	0.6	Potatoes		FRUIT (FRESH OR FROZEN)	0.6
15.1	PT General population	12.5	Table and wine grapes	2.7	Potatoes		FRUIT (FRESH OR FROZEN)	2.7
11.0	WHO Cluster diet B	9.6	Table and wine grapes	1.3	Potatoes		FRUIT (FRESH OR FROZEN)	1.3
10.0	WHO cluster diet E	8.1	Table and wine grapes	1.9	Potatoes		FRUIT (FRESH OR FROZEN)	1.9
7.3	DK adult	6.6	Table and wine grapes	0.7	Potatoes		FRUIT (FRESH OR FROZEN)	0.7
7.0	DE child	5.7	Table and wine grapes	1.3	Potatoes		FRUIT (FRESH OR FROZEN)	1.3
6.4	IE adult	5.2	Table and wine grapes	1.1	Potatoes		FRUIT (FRESH OR FROZEN)	1.1
6.4	NL child	3.4	Table and wine grapes	2.9	Potatoes		FRUIT (FRESH OR FROZEN)	2.9
5.8	UK Adult	5.1	Table and wine grapes	0.7	Potatoes		FRUIT (FRESH OR FROZEN)	0.7
5.2	NL general	3.9	Table and wine grapes	1.4	Potatoes		FRUIT (FRESH OR FROZEN)	1.4
5.0	WHO Cluster diet F	3.3	Table and wine grapes	1.7	Potatoes		FRUIT (FRESH OR FROZEN)	1.7
4.7	UK vegetarian	4.0	Table and wine grapes	0.7	Potatoes		FRUIT (FRESH OR FROZEN)	0.7
4.5	WHO cluster diet D	2.5	Table and wine grapes	2.0	Potatoes		FRUIT (FRESH OR FROZEN)	2.0
3.7	WHO regional European diet	2.0	Potatoes	1.7	Table and wine grapes		FRUIT (FRESH OR FROZEN)	2.0
3.5	FR toddler	2.5	Potatoes	0.9	Table and wine grapes		FRUIT (FRESH OR FROZEN)	2.5
3.2	PL general population	1.7	Potatoes	1.4	Table and wine grapes		FRUIT (FRESH OR FROZEN)	1.7
3.1	SE general population 90th percentile	2.1	Potatoes	1.0	Table and wine grapes		FRUIT (FRESH OR FROZEN)	2.1
3.0	UK Toddler	1.7	Potatoes	1.3	Table and wine grapes		FRUIT (FRESH OR FROZEN)	1.7
2.5	ES adult	2.1	Table and wine grapes	0.5	Potatoes		FRUIT (FRESH OR FROZEN)	0.5
2.4	FR infant	2.1	Potatoes	0.4	Table and wine grapes		FRUIT (FRESH OR FROZEN)	2.1
2.1	FI adult	1.5	Table and wine grapes	0.6	Potatoes		FRUIT (FRESH OR FROZEN)	0.6
2.0	DK child	1.2	Potatoes	0.8	Table and wine grapes		FRUIT (FRESH OR FROZEN)	1.2
1.8	UK Infant	1.6	Potatoes	0.2	Table and wine grapes		FRUIT (FRESH OR FROZEN)	1.6
1.6	LT adult	1.6	Potatoes	0.1	Table and wine grapes		FRUIT (FRESH OR FROZEN)	1.6
1.1	ES child	0.9	Potatoes	0.2	Table and wine grapes		FRUIT (FRESH OR FROZEN)	0.9
0.9	IT kids/toddler	0.5	Table and wine grapes	0.4	Potatoes		FRUIT (FRESH OR FROZEN)	0.4
0.9	IT adult	0.6	Table and wine grapes	0.3	Potatoes		FRUIT (FRESH OR FROZEN)	0.3

Conclusion:
The estimated Theoretical Maximum Daily Intakes (TMDI), based on pTMRLs were below the ADI.
A long-term intake of residues of ETU is unlikely to present a public health concern.

Table 6.9-17: ETU - PRIMo model vers2 acute - representative crops (grapes and potatoes)

Acute risk assessment /children						Acute risk assessment / adults / general population					
The acute risk assessment is based on the ARfD.											
For each commodity the calculation is based on the highest reported MS consumption per kg bw and the corresponding unit weight from the MS with the critical consumption. If no data on the unit weight was available from that MS an average European unit weight was used for the IESTI calculation.											
In the **IESTI 1** calculation, the variability factors were 10, 7 or 5 (according to JMPR manual 2002), for lettuce a variability factor of 5 was used. In the **IESTI 2** calculations, the variability factors of 10 and 7 were replaced by 5. For lettuce the calculation was performed with a variabilty factor of 3.											
Threshold MRL is the calculated residue level which would leads to an exposure equivalent to 100 % of the ARfD.											
Unprocessed commodities											
No of commodities for which ARfD/ADI is exceeded (IESTI 1):		---	**No of commodities for which ARfD/ADI is exceeded (IESTI 2):**		---	**No of commodities for which ARfD/ADI is exceeded (IESTI 1):**		---	**No of commodities for which ARfD/ADI is exceeded (IESTI 2):**		---
IESTI 1	*)	**)	**IESTI 2**	*)	**)	**IESTI 1**	*)	**)	**IESTI 2**	*)	**)
Highest % of ARfD/ADI	Commodities	pTMRL/ threshold MRL (mg/kg)	Highest % of ARfD/ADI	Commodities	pTMRL/ threshold MRL (mg/kg)	Highest % of ARfD/ADI	Commodities	pTMRL/ threshold MRL (mg/kg)	Highest % of ARfD/ADI	Commodities	pTMRL/ threshold MRL (mg/kg)
11.8	Table grapes	0.09 / -	11.8	Table grapes	0.09 / -	5.7	Table grapes	0.09 / -	5.7	Table grapes	0.09 / -
11.7	Potatoes	0.038 / -	8.3	Potatoes	0.038 / -	4.3	Wine grapes	0.09 / -	4.3	Wine grapes	0.09 / -
1.4	Wine grapes	0.09 / -	1.4	Wine grapes	0.09 / -	2.3	Potatoes	0.038 / -	1.8	Potatoes	0.038 / -
No of critical MRLs (IESTI 1)			---			**No of critical MRLs (IESTI 2)**			---		

Processed commodities					
No of commodities for which ARfD/ADI is exceeded:		---	**No of commodities for which ARfD/ADI is exceeded:**		---
		***)			***)
Highest % of ARfD/ADI	Processed commodities	pTMRL/ threshold MRL (mg/kg)	Highest % of ARfD/ADI	Processed commodities	pTMRL/ threshold MRL (mg/kg)
5.9	Grape juice	0.09 / -	0.7	Wine	0.09 / -
1.0	Potato puree (flakes)	0.038 / -	0.1	Raisins	0.09 / -
0.1	Fried potatoes	0.038 / -	0.1	Potato uree (flakes)	0.038 / -
0.1	Wine	0.09 / -	0.1	Fried potatoes	0.038 / -
0.1	Grapes (raisins)	0.09 / -			

*) The results of the IESTI calculations are reported for at least 5 commodities. If the ARfD is exceeded for more than 5 commodities, all IESTI values > 90% of ARfD are reported.
**) pTMRL: provisional temporary MRL
***) pTMRL: provisional temporary MRL for unprocessed commodity

Conclusion:

For ETU IESTI 1 and IESTI 2 were calculated for food commodities for which pTMRLs were submitted and for which consumption data are available.
No exceedance of the ARfD/ADI was identified for any unprocessed commodity.

REFERENCES

EFSA (European Food Safety Authority), 2010. Reasoned opinion on the modification of the existing MRL(s) for mancozeb in fresh peas (without pods) prepared by EFSA Pesticide Risk Assessment Peer Review (PRAPeR) Unit. EFSA Journal (2010);8(1):1451.

EFSA (European Food Safety Authority), 2011. Reasoned opinion on the modification of the existing MRL for dithiocarbamates (mancozeb) in radishes. EFSA Journal 2011; 9(3):2108, 28 pp.

EFSA (European Food Safety Authority), 2012 Reasoned opinion on the modification of the existing MRLs for dithiocarbamates (expressed as carbon disulfide) in bulb vegetables, cucurbits and asparagus. EFSA Journal 2012; 10(7):2846

Italy, 2007. Dithiocarbamates. Acute and Chronic Risk Assessment, Residue Trials data and GAPs. Rome, June 2007.

Italy, 2008a. Pesticide Residues Overview File (PROFile) on metiram prepared by the rapporteur Member State Italy. Submitted to EFSA on 1 September 2008.

Italy, 2008b. Pesticide Residues Overview File (PROFile) on mancozeb prepared by the rapporteur Member State Italy. Submitted to EFSA on 12 December 2008.

Italy, 2009. Pesticide Residues Overview File (PROFile) on maneb prepared by the rapporteur Member State Italy. Submitted to EFSA on 26 October 2009.

Italy, 2010a. Pesticide Residues Overview File (PROFile) on propineb prepared by the rapporteur Member State Italy. Submitted to EFSA on 20 April 2010.

Italy, 2010b. Evaluation report on the setting of MRLs for metiram in plant commodities prepared by the evaluating Member State Italy under Article 8 of Regulation (EC) No 396/2005, 15 November 2010, 174 p.

CA 6.10 Other studies

Report: CA 6.10/1
Schopfer C.,Wenzel M., 2015a
Metiram (BAS 222 F): Transformation products: Assessment of dietary exposure including application of TTC concept (threshold of toxicological concern)
2015/1087922
Guidelines: none
GLP: no

1. OBJECTIVE

As part of the metiram re-registration process (Metiram AIR3 Dossier, 2015), the dietary risk assessment for consumers resulting from the use of metiram has to take into account the actual toxicological burden of the components making up the residue. Therefore, the establishment of the residue definition for risk assessment purposes involves a decision on which transformation products of metiram are of toxicological concern. To achieve robustness of such a residue definition, the data base considered should be reasonably broad. Thus, first, any metiram transformation product identified in a *nature of the residue* study is included (i.e. crop metabolism, rotational crop metabolism, livestock metabolism, high temperature hydrolysis). And second, dietary exposure is assessed for two crop scopes, first scope, the representative uses supported in the AIR3 dossier (grape, potato) and second scope, all uses registered in EU including import tolerances.
All nature of the residue studies taken together, a total of eleven potentially relevant transformation products were identified (M222F004=EBIS, M222F003=EU, M222F002=ETU, M222F001, M222F007, M222F008, M222F013, M222F021, M222F022, M222F023, and glycine).
To allow to reproduce the exposure calculations for each of the metiram transformation products, the detailed steps are provided in a separate document: the present report (CA6.10/1) provides a summary of the relevant data base, derivation of estimated residue levels and result of the calculations for dietary exposure.

2. EVALUATION APPROACH

For an initial evaluation of relevance of transformation products, the estimated human exposure can be compared with a safe threshold derived using the assumptions of the TTC concept and information on the transformation product (i.e. molecular structure and genotoxic potential). To this end, a stepwise approach is envisaged (see decision tree in *Scientific Opinion on Evaluation of the Toxicological Relevance of Pesticide Metabolites for Dietary Risk Assessment, EFSA Journal 2012;10(07):2799*). The threshold of toxicological concern (TTC) is based on the concept that exposure levels can be defined (pending the molecular structure) below which human exposure to the chemical results in “no appreciable risk to human health”. In consequence, a transformation product with calculated exposure lower than the corresponding TTC does not need to be considered further. In particular, regarding the exposure threshold of 0.0025 µg/kg bw/day: if the human exposure is estimated to be below this threshold, only absence of certain structural elements (exclusion citeria) has to be confirmed. Alternatively, if the human exposure is estimated to exceed this threshold, any genotoxic concern has to be excluded via (computational) QSAR or (experimental) testing. The transformation products considered in this document were all assessed or tested for genotoxicity. In conclusion, the Cramer class III threshold of 1.5 µg/kg bw/day is considered to represent a safe value covering all toxicological endpoints. In thoses cases, where toxicological information is available for the transformation product, compound specific reference values are derived. The specific derivation can be found in the metiram AIR3 Dossier, section MCA5.8.
A further refined evaluation is to be done for transformation products with calculated exposure exceeding the corresponding TTC or specific reference values. The initial estimations generally result in considerable overestimations of exposure since typically based on various worst case assumptions, in particular in the absence of data from *magnitude of the residue* studies. (In general, all intake for the crop considered is from treated crop, which is an overestimation as market share is well below 100%. For a certain diet all included food items are assumed to have residues at the upper limit which is an overestimation as most crops have residues well below the MRL. Data generation such as crop field trials are conducted to represent the worst case condition as far as residues are concerned.) Therefore, on a case-by-case basis further detailed considerations allow to refine to more realistic exposure scenarios.

3. RESULTS OF EVALUATION

3.1 Initial evaluations

Metiram belongs to the class of dithiocarbamate fungicides. The parent molecule is neither carcinogenic nor genotoxic in vivo. The ADI is 0.03 mg/kg bw/d. An ARfD is not allocated due to low acute toxicity. Based on nature of the residue studies (crop metabolism, rotational crop metabolism, high temperature hydrolysis study, livestock metabolism) the pathway of metiram is well elucidated (metiram AIR3 dossier MCA6.2): desintegration of the parent complex results in dynamic intermediates, their incorporation into the carbon pool of primary metabolism with natural constituents as the terminal residue. All nature of the residue studies taken together, a total of eleven potentially relevant transformation products were identified (M222F004=EBIS, M222F003=EU, M222F002=ETU, M222F001, M222F007, M222F008, M222F013, M222F021, M222F022, M222F023, and glycine). The molecular structure is provided in Table 6.10-5.

For all nine of these transformation products dietary exposure is estimated and expressed in percentage of the corresponding toxicological reference value (see Table 6.10-1 to Table 6.10-4). The exposure calculation model used is EFSA PRIMo vers.2. The residue levels in food commodities is estimated based on available data from *nature and magnitude of the residue studies* (metabolism and residue studies, see section MCA6.2 and MCA6.3). No evaluation of relevance is provided in the context of the present document for two of the listed transformation products: first, glycine is a naturally occurring amino acid. Second, M222F002 (=ETU) is a relevant metabolite and is included in the existing definition of the residue for risk assessment (processed commodities).

For all nine of these transformation products, the indicative exposure calculation is below ot at the corresponding chronic and acute toxicological reference value. Results of the chronic exposure calculations are provided in Table 6.10-1 (scope: representative uses of metiram) and Table 6.10-2 (scope: entirety of metiram uses registered in EU including import tolerances).

Results of the acute exposure calculations are provided in Table 6.10-3 (scope: representative uses of metiram) and Table 6.10-4 (scope: entirety of metiram uses registered in EU including import tolerances).

Table 6.10-1: Results of chronic exposure assessments (potatoes and grapes, AIR3 dossier)

Metabolite	Dietary exposure [% reference value], most critical diet		Commodity with highest contribution [% reference value]	
EBIS	3.9	FR all population	Table and wine grapes	[3.8]
EU*	0.6	FR all population	Table and wine grapes	[0.6]
EU**	0.1	PT General population	Potatoes	[0.1]
EDA/N-AcEDA	0.0	FR all population	Table and wine grapes	[0.0]
Jaffe`s Base	1.9	FR toddler	Milk and cream	[1.8]
TDIT	0.0	not measurable	not applicable	[0.0]
M222F001	2.8	NL child	Potatoes	[2.8]
M222F008	1.5	FR all population	Table and wine grapes	[1.4]
M222F013	0.0	not measurable	not applicable	[0.0]

EU* using residue estimated based on metabolism data
EU** using residue data obtained in crop field trials (grape, potato, see chapter 2.2)

Table 6.10-2: Results of chronic exposure assessments (entirety of EU-registered uses*)

Metabolite	Dietary exposure [% reference value], most critical diet		Commodity with highest contribution [% reference value]	
EBIS	8.6	DE child	Pome fruit	[6.1]
EU	1.4	DE child	Pome fruit	[1.0]
EDA/N-AcEDA	0.1	DE child	Pome fruit	[0.1]
Jaffe`s Base	9.1	FR toddler	Milk and cream	[8.7]
TDIT	0.0	IT adult	Lettuce and other salad	[0.0]
M222F001	5.3	WHO Cluster diet B	Tomatoes	[1.4]
M222F008	3.1	DE child	Pome fruit	[2.3]
M222F013	0.1	DK child	Cucurbits – edible peel	[0.0]

* including import tolerances

Table 6.10-3: Overview: acute exposure assessments (potatoes, grapes, AIR3 dossier)

Metabolite	Commodity with highest contribution [% reference value]	Commodity with 2nd highest contribution [% reference value]	Commodity with 3rd highest contribution [% reference value]
EBIS	17.8 (Table grapes, children)	8.6 (Table grapes, adults)	6.5 (Wine grapes, adults)
EU*	21.4 (Table grapes, children)	10.4 (Table grapes, adults)	7.7 (Wine grapes, adults)
EU**	7.7 (Potatoes, children)	6.7 (Table grapes, children)	3.2 (Table grapes, adults)
EDA/N-AcEDA	0.6 (Table grapes, children)	0.3 (Table grapes, adults)	0.2 (Wine grapes, adults)
Jaffe`s Base	1.9 (Table grapes)	1.7 (Cattle milk and milk products, children)	1.0 (Bovine kidney, children)
TDIT	0.0 (not measurable)	0.0 (not measurable)	0.0 (not measurable)
M222F001	21.5 (Potatoes, children)	4.2 (Potatoes, adults)	0.0 (not measurable)
M222F008	15.2 (Table grapes, children)	7.4 (Table grapes, adults)	5.5 (Wine grapes, adults)
M222F013	0.0 (not measurable)	0.0 (not measurable)	0.0 (not measurable)

EU* using residue estimated based on metabolism data
EU** using residue data obtained in crop field trials (grape, potato, see chapter 2.2)

Table 6.10-4: Overview: acute exposure assessments (uses registered in EU)

Metabolite	Commodity with highest contribution [% reference value]	Commodity with 2nd highest contribution [% reference value]	Commodity with 3rd highest contribution [% reference value]
EBIS	33.7 (Melons, children)	27.1 (Watermelons, children)	17.8 (Table grapes, children)
EU	73.6 (Scarole, children)	57.0 (Melons, children)	45.9 (Watermelons, children)
EDA/N-AcEDA	1.2 (Melons, children)	1.0 (Watermelons, children)	0.6 (Table grapes, children)
Jaffe`s Base	14.3 (Cattle milk and milk products, children)	12.2 (Bovine kidney, children)	5.5 (Bovine kidney, adults)
TDIT	100.9 (Scarole, children)	31.0 (Lettuce, children)	12.7 (Lettuce, adults)
M222F001	80.4 (Scarole, children)	24.8 (Lettuce, children)	21.5 (Potatoes, children)
M222F008	28.7 (Melons, children)	23.2 (Watermelons, children)	15.2 (Table grapes, children)
M222F013	32.6 (Scarole, children)	14.7 (Melons, children)	11.8 (Watermelons, children)

4. CONCLUSION

In order to assess the contribution of eleven metiram transformation products to the actual toxicological burden, indicative exposure calculations are provided including the TTC approach (*Scientific Opinion on Evaluation of the Toxicological Relevance of Pesticide Metabolites for Dietary Risk Assessment, EFSA Journal 2012;10(07):2799*). Dietary exposure is assessed for two crop scopes, first scope, the representative uses supported in the metiram AIR3 dossier (grape, potato) and second scope, all metiram uses registered in EU including import tolerances.

For nine of these transformation products dietary exposure is estimated and expressed as percentage of the corresponding toxicological reference value or the TTC. The exposure calculation model used is EFSA PRIMo vers.2. The residue levels in food commodities is estimated based on available data from *nature and magnitude of the residue studies* (metabolism and residue studies).

For two of the listed transformation products no evaluation of relevance is provided in the context of the present document: first, glycine is a naturally occurring amino acid. Second, M222F002 (=ETU) is a relevant metabolite and is included in the existing definition of the residue for risk assessment (processed commodities). A detailed dietary exposure assessment is provided as part of the metiram AIR3 dossier (section MCA6.9).

For all of these transformation products, the indicative exposure calculation provide results at or below the corresponding toxicological reference value. In summary, the relevance of nine transformation products of metiram has been assessed (M222F004, M222F003, M222F0023, M222F021, M222F022, M222F007, M222F001, M222F008, M222F013). The results show for each transformation product that its contribution to the overall toxicological burden is insignificant. Therefore no further compound has to be included in the definition of the relevant residue for risk assessment.

In conclusion, this relevance assessment confirms the present established definition of the metiram residue for risk assessment (section MCA6.7). This definition of the residue is robust in that it applies not only to the representative uses supported in the metiram AIR3 dossier, but also to the entirety of metiram uses currently registered in EU including established import tolerances.

5. APPENDIX

Table 6.10-5: Overview metabolites: occurrence, molecular structure and molecular mass

Parent			
Name	**Occurrence**	**Structure**	**Molecular mass [g/mol]**
Metiram (BAS 222 F)	not relevant		1088.7

Metabolites			
Name	**Occurrence**	**Structure**	**Molecular mass [g/mol]**
M222F004 (EBIS)	crop (lettuce, apple, potato)* goat** poultry***		176.3
M222F003 (EU)	crop (lettuce, apple, potato)* goat** poultry***		86.1
M222F002 (ETU)	crop (lettuce, apple, potato)* goat** poultry***		102.2
M222F023 (EDA)	crop (apple)* goat** poultry***		60.1
M222F021 (N-AcEDA)	crop (apple)* goat** poultry***		102.1
M222F022 (Jaffe`s Base)	crop (apple)* goat** poultry***		170.2
M222F007 (TDIT)	crop (lettuce)*		212.3
M222F001	rotational crop****		230.2
Glycine	crop (apples, potato)* goat** poultry***		75.1
M222F008 (Hydantoin)	crop (apple)* goat** poultry***		100.1
M222F013	crop (lettuce)*		114.1

* 'Metabolism of 14C-Metiram (14C-BAS 222 F) in lettuce', BASF DocID 2009/1049027
'Metiram: Nature of Residues in Apples', BASF DocID 1990/10669
'Metabolism of 14C-Metiram Complex in Potatoes', BASF DocID 1990/10668

** 'Metabolism of 14C-Metiram Complex in Lactating Goats', BASF DocID 1989/10487

*** 'Metabolism of 14C-Metiram Complex in Laying Hens', BASF DocID 1990/5080

**** 'Confined rotational crop study with 14C-BAS 222 F', BASF DocID 2009/1017248

Table 6.10-6: Transformation products of metiram: molecular weight correction factors

Metabolite	Units[1)]	Total molecular weight[2)]	Molecular weight correction factor[3)]
M222F004 (EBIS)	4	705.2	0.65
M222F003 (EU)	4	344.4	0.32
M222F002 (ETU)	n.a.[4)]	n.a.	n.a.
M222F023 (EDA)	4	240.4	0.22
M222F021 (N-AcEDA)	4	408.4	0.38
M222F022 (Jaffe`s Base)	2	340.4	0.31
M222F007 (TDIT)	2	426.6	0.39
M222F001	2	460.4	0.42
Glycine	n.a.	n.a.	n.a.
M222F008	4	400.4	0.37
M222F013	4	456.4	0.42

1) number of molecules that may potentially be formed from one molecule metiram (= n)
2) n x molecular weight [g/mol]
3) calculated as (n x molecular weight)/1088.7 g/mol)
4) n.a. not applicable Rationale: the transformation product M222F002=ETU is a relevant metabolite and since included in the existing definition of the residue for risk assessment is subject to a dietary risk assessment (see Metiram AIR3 dossier, in preparation). The transformation product glycine is a naturally occurring amino acid. Therefore, in the context of the present document, both for ETU and for glycine, no further evaluation of relevance for the metiram residue definitions is performed.

Table 6.10-7: Toxicological reference values used for chronic dietary exposure calculations

Metabolite	Reference value [mg/kg bw/d]	Source
EBIS	0.02	AIR3 dossier, MCA 06.09
EU	0.06	AIR3 dossier, MCA 06.09
EDA/N-AcEDA	0.2	AIR3 dossier, MCA 06.09
Jaffe`s Base	0.0015	TTC Cramer class III*
TDIT	0.0015	TTC Cramer class III*
M222F001	0.0015	TTC Cramer class III*
M222F008	0.0015	TTC Cramer class III*
M222F013	0.0015	TTC Cramer class III*

The following reference values were used for the assessment of acute exposure to metiram metabolites:

Table 6.10-8: Toxicological reference values used for acute dietary exposure calculations

Metabolite	Reference value [mg/kg bw]	Source
EBIS	0.15	AIR3 dossier, MCA 06.09
EU	0.06	AIR3 dossier, MCA 06.09
EDA/N-AcEDA	0.5	AIR3 dossier, MCA 06.09
Jaffe`s Base	0.005	recommended by EFSA PPR*
TDIT	0.005	recommended by EFSA PPR*
M222F001	0.005	recommended by EFSA PPR*
M222F008	0.005	recommended by EFSA PPR*
M222F013	0.005	recommended by EFSA PPR*

* Scientific Opinion on Evaluation of the Toxicological Relevance of Pesticide Metabolites for Dietary Risk Assessment (EFSA Journal 2012;10(07):2799), page 32

CA 6.10.1 Effect on the residue level in pollen and bee products

The objective of these studies would be to determine the residue in pollen and bee products for human consumption resulting from residues taken up by honeybees from crops at blossom.

So far, for metiram no study for pollen and bee products was conducted as official published guideline for such a study is as-yet not available. Moreover, the representative uses supported in the present dossier, grape and potato are not considered as important for honey production. Metiram is a non-systemic fungicide non-soluble in water. In summary, the dietary risk resulting from the supported uses of metiram is considered negligible.

Tier 1 Summaries of the Supervised Field Residue Trials for the Representative Crops

Grapes

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)
b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Wine grapes / Berries and small fruits	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	CS_2, BAS 222 F (by CS_2), BAS 222 F (by EBDC)
Region	**Central and Northern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I** CS_2[1]	**II** metiram (by CS_2)	**III** metiram (by EBDC)		
2012/1272626 Landauer Str. 10 76831 Eschbach Germany L120532	FB 1236 Riesling	1. 1959 2. 15.-25.06.2012 3. 13.10.2012	Knap-sack sprayer air assisted - SOLO	0.18	800	1.4	3 01.08.2012	77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	2.50 0.43 0.38 0.30	4.48 0.76 0.68 0.53	6.61 1.52 1.12 1.12	0 50 56 62	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2012/1272626 Mainzer Str. 21 55437 Ockenheim Germany L120533	FB 1236 Johanniter	1. 2008 2. 05.-15.06.2012 3. 28.09.2012	Knap-sack sprayer air assisted - SOLO	0.18	800	1.4	3 26.07.2012	81	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	4.57 0.97 0.57 0.88	8.19 1.74 1.01 1.57	11.10 2.82 1.87 3.08	0 49 56 63	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2012/1272626 B4215 Gnew ent GL 18 1LS Gloucestershire United Kingdom L120535	FB 1236 Sauvignon Blanc	1. 1997 2. 04.06.-07.07.2012 3. 21.10.2012	Atomizer with lance - Stihl	0.18	800	1.4	3 19.08.2012	75	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	1.94 0.11 0.26 0.09	3.47 0.20 0.46 0.15	5.74 0.33 0.85 0.29	0 49 57 63	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Wine grapes / Berries and small fruits	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	CS_2, BAS 222 F (by CS_2), BAS 222 F (by EBDC)
Region	**Central and Northern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I** CS_2[1]	**II** metiram (by CS_2)	**III** metiram (by EBDC)		
2014/1161880 Upton Estate Stratford Road Banbury, OX15 6 EP United Kingdom L130015	FB 1236 Sauvignon Blanc	1. 1997 2. 01.-06.07.2013 3. 01.-06.11.2013	Atomizer	0.18	800	1.4	3 03.09.2013	73	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.96 < 0.056 < 0.056 < 0.056	1.71 < 0.1 < 0.1 < 0.1	3.12 0.10 0.16 0.084	0 49 56 62	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2014/1000221 55437 Ockenheim Germany L130086	FB 0269 Johanniter	1. 2008 2. 20.06.-28.06.2013 3. 02.10.2013	Broadcast foliar appl.	0.18	800	1.4	3 31.07.2013	79	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	2.21 0.36 0.40 0.44	3.95 0.64 0.72 0.78	1.97 2.12 1.70 2.05	0 50 56 62	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2014/1000221 67150 Niederkirchen Germany L130087	FB 0269 Bacchus	1. 1985 2. 20.06.-24.06.2013 3. 26.09.2013	Broadcast foliar appl.	0.18	800	1.4	3 23.07.2013	75	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	2.79 0.37 0.48 0.49	5.00 0.67 0.86 0.88	7.50 1.24 1.93 1.49	0 49 57 65	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2014/1000221 45160 Saint-Hilaire-Saint-Mesmin France (N) L130088	FB 0269 Pino Noir	1. 15.09.1980 2. 15.05-30.05.2013 3. 09.10.2013	Broadcast foliar appl.	0.18	800	1.4	3 07.08.2013	77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.64* 0.16* 0.11* 0.09*	1.14* 0.28* 0.21* 0.18*	6.59 0.63 0.31 0.34	0 49 56 63	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Wine grapes / Berries and small fruits	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	CS_2, BAS 222 F (by CS_2), BAS 222 F (by EBDC)
Region	**Central and Northern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I** CS_2[1]	**II** metiram (by CS_2)	**III** metiram (by EBDC)		
2014/1000221 45420 Bonny sur Loire France (N) L130089	FB 0269 Sauvignon	1. 09.12.2013 2. 10.06.-22.06.2012 3. 08.10.2013	Broadcast foliar appl.	0.18	800	1.4	3 06.08.2012	77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.30* 0.11* < 0.056* < 0.056*	0.54* 0.20* < 0.1* < 0.1*	0.46* 0.18* 0.067* 0.088*	0 49 56 63	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2015/1000322 55437 Ockenheim Germany L140355	FB 0269 Regent	1. 1995 2. 02.-11.06.2014 3. 11.09.2014	SOLO knapsack sprayer	0.18	800	1.4	3 16.07.2014	79	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	3.1 0.36 0.31 0.41	5.5 0.65 0.55 0.74	11 2.6 1.9 2.4	0 49 56 62	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2015/1000322 76831 Eschbach Germany L140356	FB 0269 Weißburg-under	1. 2005 2. 09.-23.06.2014 3. 10.10.2014	SOLO knapsack sprayer	0.18	800	1.4	3 12.08.2014	78	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	1.7 0.52 0.52 0.20	3.1 0.93 0.94 0.35	6.3 1.9 1.8 0.63	0 49 56 63	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2015/1000322 GL 18 1LS Newent Gloucestershire United Kingdom L140357	FB 0269 Sauvignon Blanc	1. 1997 2. 25.06.-02.07.2014 3. 10.-25.10.2014	Pulvexper Lance	0.18 0.17 0.18	227 229 233	0.40 0.40 0.41	3 22.08.2014	73	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.31 < 0.056 < 0.056 < 0.056	0.56 < 0.1 < 0.1 < 0.1	0.89 0.11 0.050 0.053	0 48 57 62	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Wine grapes / Berries and small fruits	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	CS_2, BAS 222 F (by CS_2), BAS 222 F (by EBDC)
Region	**Central and Northern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
										I	**II**	**III**		
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**CS_2[1]**	**metiram (by CS_2)**	**metiram (by EBDC)**		
2015/1000322 4400 Nýregyháza Hungary L140358	FB 0269 Cserszegi Füszeres	1. 2002 2. 11.-20.06.2014 3. 06.-10.10.2014	Airblast Stihl	0.18	800	1.4	3 04.08.2014	77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	1.7 0.63 0.28 0.45	3.1 1.1 0.51 0.81	6.4 1.9 1.2 1.3	0 49 56 63	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01

1) conversion factor from metiram to CS_2 is 1.79

n.a. not applicable

* mean value of two individual extractions

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Wine grapes / Berries and small fruits	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	ETU, EU, EBIS
Region	**Central and Northern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I ETU**	**II EU**	**III EBIS**		
2012/1272626 Landauer Str. 10 76831 Eschbach Germany L120532	FB 1236 Riesling	1. 1959 2. 15.-25.06.2012 3. 13.10.2012	Knap-sack sprayer air assisted - SOLO	0.18	800	1.4	3 01.08.2012	77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.053 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 0.011 < 0.01	0.19 0.022 0.015 < 0.01	0 50 56 62	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01
2012/1272626 Mainzer Str. 21 55437 Ockenheim Germany L120533	FB 1236 Johanniter	1. 2008 2. 05.-15.06.2012 3. 28.09.2012	Knap-sack sprayer air assisted - SOLO	0.18	800	1.4	3 26.07.2012	81	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.074 0.013 0.010 0.025	0.015 0.013 0.012 0.017	0.27 0.036 0.025 < 0.01	0 49 56 63	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01
2012/1272626 B4215 Gnew ent GL 18 1LS Gloucestershire United Kingdom L120535	FB 1236 Sauvignon Blanc	1. 1997 2. 04.06.-07.07.2012 3. 21.10.2012	Atomizer with lance - Stihl	0.18	800	1.4	3 19.08.2012	75	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.046 < 0.01 < 0.01 < 0.01	0.012 < 0.01 < 0.01 < 0.01	0.17 < 0.01 0.014 < 0.01	0 49 57 63	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01
2014/1161880 Upton Estate Stratford Road Banbury, OX15 6 EP United Kingdom L130015	FB 1236 Sauvignon Blanc	1. 1997 2. 01.-06.07.2013 3. 01.-06.11.2013	Atomizer	0.18	800	1.4	3 03.09.2013	73	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.015 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0.039 < 0.01 < 0.01 < 0.01	0 49 56 62	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Wine grapes / Berries and small fruits	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	ETU, EU, EBIS
Region	**Central and Northern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
										I	**II**	**III**		
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**ETU**	**EU**	**EBIS**		
2014/1000221 55437 Ockenheim Germany L130086	FB 0269 Johanniter	1. 2008 2. 20.-28.06.2013 3. 02.10.2013	broadcast foliar application	0.175	800	1.4	3 31.07.2013	79	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.02 0.01 < 0.01 0.01	< 0.01 0.01 0.01 0.01	0.17 0.02 0.03 0.02	0 50 56 62	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2014/1000221 67150 Niederkirchen Germany L130087	FB 0269 Bacchus	1. 1985 2. 20.-24.06.2013 3. 26.09.2013	broadcast foliar application	0.175	800	1.4	3 23.07.2013	75	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.05 < 0.01 < 0.01 0.01	< 0.01 0.01 < 0.01 0.01	0.17 0.02 0.02 0.04	0 49 57 65	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2014/1000221 45160 Saint-Hilaire-Saint-Mesmin, France (N) L130088	FB 0269 Pinot Noir	1. 15.09.1980 2. 15.-30.05.2013 3. 09.10.2013	broadcast foliar application	0.175	800	1.4	3 07.08.2013	77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.02 < 0.01 < 0.01 < 0.01	0.01 < 0.01 < 0.01 < 0.01	0.06 < 0.01 < 0.01 < 0.01	0 49 56 63	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2014/1000221 45420 Bonny sur Loire France (N) L130089	FB 0269 Sauvignon	1. 2004 2. 10.-22.06.2013 3. 06.10.2013	broadcast foliar application	0.175	800	1.4	3 06.08.2013	77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0.01 < 0.01 < 0.01 < 0.01	0 49 56 63	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Wine grapes / Berries and small fruits	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	ETU, EU, EBIS
Region	**Central and Northern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
										I	**II**	**III**		
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**ETU**	**EU**	**EBIS**		
2015/1000322 55437 Ockenheim Germany L140355	FB 0269 Regent	1. 1995 2. 02.-11.06.2014 3. 11.09.2014	SOLO knapsack sprayer	0.18	800	1.4	3 16.07.2014	79	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.065 0.027 0.027 0.025	0.023 0.019 0.018 0.021	0.31 0.19 0.017 0.017	0 49 56 62	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2015/1000322 76831 Eschbach Germany L140356	FB 0269 Weißburg-under	1. 2005 2. 09.-23.06.2014 3. 10.10.2014	SOLO knapsack sprayer	0.18	800	1.4	3 12.08.2014	78	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.065 0.031 0.035 0.019	0.025 0.026 0.034 0.023	0.17 0.029 0.019 <0.01	0 49 56 63	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2015/1000322 GL 18 1LS Newent Gloucestershire United Kingdom L140357	FB 0269 Sauvignon Blanc	1. 1997 2. 25.06.-02.07.2014 3. 10.-25.10.2014	Pulvexper Lance	0.18 0.17 0.18	227 229 233	0.40 0.40 0.41	3 22.08.2014	73	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0.019 < 0.01 < 0.01 < 0.01	0 48 57 62	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2015/1000322 4400 Nýregyháza Hungary L140358	FB 0269 Cserszegi Füszeres	1. 2002 2. 11.-20.06.2014 3. 06.-10.10.2014	Airblast Stihl	0.18	800	1.4	3 04.08.2014	77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.065 0.027 0.016 0.014	0.050 0.063 0.061 0.054	0.080 0.013 < 0.01 < 0.01	0 49 56 63	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Wine grapes / Berries and small fruits	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	CS_2, BAS 222 F (by CS_2), BAS 222 F (by EBDC)
Region	**Southern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
										I	**II**	**III**		
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**CS_2[1]**	**metiram (by CS_2)**	**metiram (by EBDC)**		
2012/1272626 Herreros N°11 4°3° 29700 Velez Malaga Spain L120536	FB 1236 Moscatel	1. 1995 2. 19.05.-08.06.2012 3. 02.09.2012	Atomizer with lance - Stihl SR 420	0.18	800	1.4	3 09.07.2012	77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	2.91 0.50 0.51 0.61	5.20 0.90 0.91 1.09	8.07 1.76 1.85 2.29	0 49 55 62	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2012/1272626 Polymilos Kozani West Macedonia GR 50100 Greece L120537	FB 1236 Muscat	1. 02.1972 2. 05.-20.06.2012 3. 19.-30.09.2012	Knapsack with lance - AZO	0.18	800	1.4	3 26.07.2012	75-77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	1.39 <0.056 0.059 0.062	2.48 <0.1 0.11 0.11	3.94 0.21 0.21 0.23	0 49 56 63	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2012/1272626 C/Forfe No. 6 29718 Almanchar Spain L120539	FB 1236 Moscatel de Alejandria	1. 01.1997 2. 14.05.-12.06.2012 3. 03.09.2012	Atomizer with lance - Stihl SR 420	0.18	800	1.4	3 10.07.2012	80	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.68 0.12 0.10 0.082	1.22 0.21 0.18 0.15	2.33 0.47 0.34 0.28	0 49 55 62	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2014/1161880 Corso Verbone 175 18036 Soldano Italy L130016	FB 1236 Rossese	1. 2007 2. 03.-16.06.2013 3. 19.-26.09.2013	Lance sprayer	0.18	800	1.4	3 25.07.2013	81	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	2.32 0.38 0.24 0.29	4.16 0.69 0.42 0.52	8.91 1.89 1.26 1.41	0 49 56 63	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Wine grapes / Berries and small fruits	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	CS_2, BAS 222 F (by CS_2), BAS 222 F (by EBDC)
Region	**Southern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I** CS_2[1]	**II** metiram (by CS_2)	**III** metiram (by EBDC)		
2014/1000221 82700 Bourret France (S) L130090	FB 0269 Cabernet Sauvignon	1. April 1998 2. n.a. 3. 22.10.2013	Broadcast foliar appl.	0.18	800	1.4	3 20.08.2013	79-81	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	1.39 0.37 0.462 0.58	2.49 0.67 0.82 1.03	5.06 1.32 2.03 1.52	0 49 56 63	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2014/1000221 50100 Polymilos Greece L130091	FB 0269 Muscat	1. Feb 1972 2. 05.06.-20.06.2013 3. 15.09.-30.09.2013	Broadcast foliar appl.	0.18	800	1.4	3 26.07.2013	75 / 77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	3.56 0.22 0.32 0.29	6.38 0.40 0.58 0.53	9.86 0.46 0.61 0.81	0 48 55 63	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2014/1000221 40050 Bagnarola Italy L130092	FB 0269 Trebbiano	1. 2002 2. May-June 2013 3. 18.09.2013	Broadcast foliar appl.	0.18	800	1.4	3 19.07.2013	80	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	1.67 0.11 0.13 0.095	2.99 0.20 0.24 0.17	5.61 0.34 0.52 0.31	0 48 55 61	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2014/1000221 11540 Sanlúcar de Barrameda Spain L130093	FB 0269 Palomino Fino	1. 15.02.1975 2. 06.05.-13.05.2013 3. 28.08.2013	Broadcast foliar appl.	0.18	800	1.4	3 26.06.2013	77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	1.60 0.33 0.28 0.35	2.87 0.59 0.51 0.62	6.15 1.30 1.41 1.40	0 49 56 63	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2015/1000322 33640 Isle St Georges France (S) L140359	FB 0269 Cabernet	1. 11.1996 2. 10.-15.06.2014 3. 02.10.2014	Airblast	0.18	800	1.4	3 31.07.2014	79	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.53 0.14 0.10 < 0.056	1.0 0.26 0.18 < 0.1	1.5 0.41 0.32 0.24	0 49 57 63	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Wine grapes / Berries and small fruits	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	CS_2, BAS 222 F (by CS_2), BAS 222 F (by EBDC)
Region	**Southern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I** CS_2[1]	**II** metiram (by CS_2)	**III** metiram (by EBDC)		
2015/1000322 50100 Polymyos Greece L140360	FB 0269 Muscat	1. 1972 2. 01.-20.06.2014 3. 01.-25.09.2014	Knapsack sprayer with lance	0.18	800	1.4	3 16.07.2014	75	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	1.1 0.73 0.29 0.27	2.0 1.3 0.52 0.49	3.1 3.0 1.2 0.96	0 48 57 61	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2015/1000322 18036 Soldano Italy L140361	FB 0269 Rossese	1. 1992 2. 25.05.-05.06.2014 3. 02.-09.10.2014	Lance GLP SPR07	0.18	800	1.4	3 08.08.2014	79	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.59 0.24 0.23 0.54	1.1 0.43 0.42 0.97	2.2 1.0 0.84 2.7	0 48 56 62	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2015/1000322 29700 Velez Malaga Spain L140362	FB 0269 Moscatel	1. 01.1995 2. 02.-16.05.2014 3. 21.-28.08.2014	Airblast	0.18	800	1.4	3 26.06.2014	77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	3.1 0.88 0.65 < 0.056	5.6 1.6 1.2 < 0.1	9.0 4.3 2.7 1.0	0 48 56 63	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Wine grapes / Berries and small fruits	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	ETU, EU, EBIS
Region	**Southern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I ETU**	**II EU**	**III EBIS**		
2012/1272626 Herreros N°11 4°3° 29700 Velez Malaga Spain L120536	FB 1236 Moscatel	1. 1995 2. 19.05.-08.06.2012 3. 02.09.2012	Atomizer with lance - Stihl SR 420	0.18	800	1.4	3 09.07.2012	77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.044 0.019 0.026 0.022	< 0.01 0.012 0.012 0.016	0.19 0.024 0.025 < 0.01	0 49 55 62	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01
2012/1272626 Polymilos Kozani West Macedonia GR 50100 Greece L120537	FB 1236 Muscat	1. 02.1972 2. 05.-20.06.2012 3. 19.-30.09.2012	Knapsack with lance - AZO	0.18	800	1.4	3 26.07.2012	75-77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.020 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0.10 < 0.01 < 0.01 < 0.01	0 49 56 63	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01
2012/1272626 C/Forfe No. 6 29718 Almanchar Spain L120539	FB 1236 Moscatel de Alejandria	1. 01.1997 2. 14.05.-12.06.2012 3. 03.09.2012	Atomizer with lance - Stihl SR 420	0.18	800	1.4	3 10.07.2012	80	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.013 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0.063 < 0.01 < 0.01 < 0.01	0 49 55 62	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01
2014/1161880 Corso Verbone 175 18036 Soldano Italy L130016	FB 1236 Rossese	1. 2007 2. 03.-16.06.2013 3. 19.-26.09.2013	Lance sprayer	0.18	800	1.4	3 25.07.2013	81	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.039 0.015 0.013 < 0.01	0.013 0.013 0.016 0.018	0.20 0.034 0.017 0.020	0 49 56 63	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Wine grapes / Berries and small fruits	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	ETU, EU, EBIS
Region	**Southern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I ETU**	**II EU**	**III EBIS**		
2014/1000221 82700 Bourett France (S) L130090	FB 0269 Cabernet Sauvignon	1. April 1998 2. n.r. 3. 22.10.2013	broadcast foliar application	0.175	782 - 914	1.4	3 20.08.2013	79-81	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.02 < 0.01 < 0.01 < 0.01	0.01 0.02 0.02 0.02	0.08 < 0.01 0.01 0.03	0 49 56 63	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2014/1000221 50100 Polymilos Greece L130091	FB 0269 Muscat	1. Feb 1972 2. 05.-20.06.2013 3. 15.-30.09.2013	broadcast foliar application	0.175	800	1.4	3 26.07.2013	75/77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.05 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0.21 < 0.01 0.01 0.01	0 48 55 63	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2014/1000221 40050 Bagnarola Italy L130092	FB 0269 Trebbino	1. 2002 2. May - Jun 2013 3. 18.09.2013	broadcast foliar application	0.175	800	1.4	3 19.07.2013	80	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.03 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0.12 < 0.01 0.01 0.01	0 48 55 61	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2014/1000221 11540 Sanlúcar de Barrameda Spain L130093	FB 0269 Palomino Fino	1. 15.02.1975 2. 06.-13.05.2013 3. 28.08.2013	broadcast foliar application	0.175	800	1.4	3 26.06.2013	77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.06 0.03 < 0.01 0.03	< 0.01 0.02 0.02 0.02	0.14 0.04 0.05 0.05	0 49 56 63	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Wine grapes / Berries and small fruits	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	ETU, EU, EBIS
Region	**Southern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I ETU**	**II EU**	**III EBIS**		
2015/1000322 33640 Isle St Georges France (S) L140359	FB 0269 Cabernet	1. 11.1996 2. 10.-15.06.2014 3. 02.10.2014	Airblast	0.18	800	1.4	3 31.07.2014	79	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.018 0.014 < 0.01 < 0.01	0.011 0.021 0.017 0.013	0.032 0.011 < 0.01 < 0.01	0 49 57 63	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2015/1000322 50100 Polymyos Greece L140360	FB 0269 Muscat	1. 1972 2. 01.-20.06.2014 3. 01.-25.09.2014	Knapsack sprayer with lance	0.18	800	1.4	3 16.07.2014	75	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.027 0.018 0.011 < 0.01	0.012 0.019 0.015 0.014	0.088 0.028 < 0.01 < 0.01	0 48 57 61	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2015/1000322 18036 Soldano Italy L140361	FB 0269 Rossese	1. 1992 2. 25.05.-05.06.2014 3. 02.-09.10.2014	Lance GLP SPR07	0.18	800	1.4	3 08.08.2014	79	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.046 0.039 0.017 0.038	< 0.01 0.016 0.012 0.029	0.097 0.024 0.030 0.035	0 48 56 62	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2015/1000322 29700 Velez Malaga Spain L140362	FB 0269 Moscatel	1. 01.1995 2. 02.-16.05.2014 3. 21.-28.08.2014	Airblast	0.18	800	1.4	3 26.06.2014	77	grapes (fruit) grapes (fruit) grapes (fruit) grapes (fruit)	0.16 0.059 0.046 0.022	0.014 0.040 0.027 0.024	0.26 0.037 0.026 < 0.01	0 48 56 63	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg

n.r. not reported

Potato

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Potato / Root and Tuber Vegetables	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	CS_2, BAS 222 F (by CS_2), BAS 222 F (by EBDC)
Region	**Central and Northern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I CS_2[1]**	**II metiram (by CS_2)**	**III metiram (by EBDC)**		
2012/1272625 Ormersheimerhof 4 67227 Frankenthal Germany L120524	VR0589 Marabell	1. 27.03.2012 2. n.a. 3. 21.09.2012	plot-sprayer with boom	0.7	200	1.4	3 01.08.2012	47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 7 14 22	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2012/1272625 Oggersheimer Str. 60 67227 Studernheim Germany L120525	VR0589 Quarta	1. 17.04.2012 2. 15.06.-11.07.2012 3. 24.08.-21.09.2012	plot-sprayer with boom	0.7	200	1.4	3 31.07.2012	48	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 8 14 21	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2012/1272625 Kings New ham, Rugby Warwickshire CV 23 0JT United Kingdom L120526	VR0589 Markies	1. 21.04.2012 2. 25.07.-20.08.2012 3. 12.10.2012	Pulv-exper boom sprayer	0.56	250	1.4	3 20.09.2012	45	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 7 13 20	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2012/1272625 Stradford Road, Banbury Oxfordshire OX 15 6EP United Kingdom L120527	VR0589 Markies	1. 06.05.2012 2. 01.-09.08.2012 3. 19.10.2012	Pulv-exper boom sprayer	0.56	250	1.4	3 04.10.2012	47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 8 15 22	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Potato / Root and Tuber Vegetables	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	CS_2, BAS 222 F (by CS_2), BAS 222 F (by EBDC)
Region	**Central and Northern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I** **CS_2[1]**	**II** **metiram (by CS_2)**	**III** **metiram (by EBDC)**		
2014/1000222 Ormsheimerhof 4 67227 Frankenthal Germany L130118	VR0589 Marabell	1. 11.03.2013 2. n.a. 3. 14.08.2013	mono-cycle plot-sprayer with boom	0.7	200	1.4	3 16.07.2013	47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 8 14 21	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2014/1000222 Oggersheimer Str. 60 67227 Studernheim Germany L130119	VR0589 Alians	1. 18.04.2013 2. 06.06.-11.07.2013 3. 30.08.-12.09.2013	plot-sprayer with boom	0.7	200	1.4	3 08.08.2013	48	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 7 14 21	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2014/1000222 La Bonde, 49850 Albnes France (N) L130120	VR0589 Spunta	1. 22.04.2013 2. 20.06.-15.07.2013 3. 15.-22.08.2013	Pulv-exper boom sprayer	0.7	200	1.4	3 29.07.2013	45	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 7 14 22	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2014/1000222 The Meadows Alkerton Oaks Business Park Stratford Road Banbury, OX156EP United Kingdom L130121	VR0589 Harmony	1. 17.04.2013 2. 01.-29.07.2013 3. 20.-30.08.2013	Pulv-exper boom sprayer	0.7	200	1.4	3 09.08.2013	47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 6 13 20	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2015/1000321 Omersheimerhof 4 67227 Frankenthal Germany L140382	VR0589 Musica	1. 21.03.2014 2. n.a. 3. 21.08.2014	Boom sprayer BASF Gloria	0.7	200	1.4	3 17.06.2014	46	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 8 14 22	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Potato / Root and Tuber Vegetables	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	CS_2, BAS 222 F (by CS_2), BAS 222 F (by EBDC)
Region	**Central and Northern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
										I	**II**	**III**		
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**CS_2[1]**	**metiram (by CS_2)**	**metiram (by EBDC)**		
2015/1000321 Oggersheimer Str. 60 67227 Studernheim Germany L140383	VR0589 Toscana	1. 11.04.2014 2. 23.06.-16.07.2014 3. 21.08.-30.09.2014	Boom sprayer BASF Gloria	0.7	200	1.4	3 31.07.2014	47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 6 13 20	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2015/1000321 Akerton Oaks Buisness Park Upton Estate, Stradford Road Banbury, OX 15 6EP United Kingdom L140384	VR0589 Home guard	1. 26.03.2014 2. 19.05.-13.06.2014 3. 07.08.2014	Pulv-exper boom sprayer	0.7	200	1.4	3 16.07.2014	45	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 8 13 22	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2015/1000321 De Streek 13 9414 VL Hooghalen The Netherlands L140385	VR0589 Lady Claire	1. 28.04.2014 2. 10.-17.07.2014 3. 28.08.-03.09.2014	Boom sprayer	0.7	200	1.4	2* 14.08.2014	47	potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05	7 14 20	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01

1) conversion factor from metiram to CS_2 is 1.79

n.a. not applicable

* due to an infection with phytophtora infestans only two applications could be carried out and not all sampling were possible (no 0 DALA sampling)

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Potato / Root and Tuber Vegetables	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	ETU, EU, EBIS
Region	**Central and Northern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I ETU**	**II EU**	**III EBIS**		
2012/1272625 Ormersheimerhof 4 67227 Frankenthal Germany L120524	VR0589 Marabell	1. 27.03.2012 2. n.a. 3. 21.09.2012	plot-sprayer with boom	0.7	200	1.4	3 01.08.2012	47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	0.019 0.022 0.026 0.021	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 7 14 22	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01
2012/1272625 Oggersheimer Str. 60 67227 Studernheim Germany L120525	VR0589 Quarta	1. 17.04.2012 2. 15.06.-11.07.2012 3. 24.08.-21.09.2012	plot-sprayer with boom	0.7	200	1.4	3 31.07.2012	48	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 8 14 21	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01
2012/1272625 Kings New ham, Rugby Warwickshire CV 23 0JT United Kingdom L120526	VR0589 Markies	1. 21.04.2012 2. 25.07.-20.08.2012 3. 12.10.2012	Pulv-exper boom sprayer	0.56	250	1.4	3 20.09.2012	45	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 7 13 20	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01
2012/1272625 Stradford Road, Banbury Oxfordshire OX 15 6EP United Kingdom L120527	VR0589 Markies	1. 06.05.2012 2. 01.-09.08.2012 3. 19.10.2012	Pulv-exper boom sprayer	0.56	250	1.4	3 04.10.2012	47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 8 15 22	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Potato / Root and Tuber Vegetables	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	ETU, EU, EBIS
Region	**Central and Northern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I ETU**	**II EU**	**III EBIS**		
2014/1000222 Ormsheimerhof 4 67227 Frankenthal Germany L130118	VR0589 Marabell	1. 11.03.2013 2. n.a. 3. 14.08.2013	mono-cycle plot-sprayer with boom	0.7	200	1.4	3 16.07.2013	47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	0.037 0.028 0.038 0.031	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 8 14 21	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2014/1000222 Oggersheimer Str. 60 67227 Studernheim Germany L130119	VR0589 Alians	1. 18.04.2013 2. 06.06.-11.07.2013 3. 30.08.-12.09.2013	plot-sprayer with boom	0.7	200	1.4	3 08.08.2013	48	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	0.011 0.020 0.017 0.013	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 7 14 21	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2014/1000222 La Bonde, 49850 Albnes France (N) L130120	VR0589 Spunta	1. 22.04.2013 2. 20.06.-15.07.2013 3. 15.-22.08.2013	Pulv-exper boom sprayer	0.7	200	1.4	3 29.07.2013	45	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 7 14 22	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2014/1000222 The Meadows Alkerton Oaks Business Park Stratford Road Banbury, OX156EP United Kingdom L130121	VR0589 Harmony	1. 17.04.2013 2. 01.-29.07.2013 3. 20.-30.08.2013	Pulv-exper boom sprayer	0.7	200	1.4	3 09.08.2013	47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 6 13 20	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Potato / Root and Tuber Vegetables	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	ETU, EU, EBIS
Region	**Central and Northern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I ETU**	**II EU**	**III EBIS**		
2015/1000321 Omersheimerhof 4 67227 Frankenthal Germany L140382	VR0589 Musica	1. 21.03.2014 2. n.a. 3. 21.08.2014	Boom sprayer BASF Gloria	0.7	200	1.4	3 17.06.2014	46	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 8 14 22	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2015/1000321 Oggersheimer Str. 60 67227 Studernheim Germany L140383	VR0589 Toscana	1. 11.04.2014 2. 23.06.-16.07.2014 3. 21.08.-30.09.2014	Boom sprayer BASF Gloria	0.7	200	1.4	3 31.07.2014	47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 6 13 20	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2015/1000321 Akerton Oaks Buisness Park Upton Estate, Stradford Road Banbury, OX 15 6EP United Kingdom L140384	VR0589 Home guard	1. 26.03.2014 2. 19.05.-13.06.2014 3. 07.08.2014	Pulv-exper boom sprayer	0.7	200	1.4	3 16.07.2014	45	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 8 13 22	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2015/1000321 De Streek 13 9414 VL Hooghalen The Netherlands L140385	VR0589 Lady Claire	1. 28.04.2014 2. 10.-17.07.2014 3. 28.08.-03.09.2014	Boom sprayer	0.7	200	1.4	2* 14.08.2014	47	potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01	7 14 20	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg

* due to an infection with phytophtora infestans only two applications could be carried out and not all sampling were possible (no 0 DALA sampling)

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Potato / Root and Tuber Vegetables	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	CS_2, BAS 222 F (by CS_2), BAS 222 F (by EBDC)
Region	**Southern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I** CS_2[1]	**II** metiram (by CS_2)	**III** metiram (by EBDC)		
2012/1272625 Platahos, Imathia, Central Macedonia GR-59032 Greece L120528	VR0589 Agria	1. 04.04.2012 2. 05.-30.05.2012 3. 10.-30.07.2012	boom sprayer AZO	0.47	300	1.4	3 02.07.2012	45-47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 7 14 22	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2012/1272625 Polymilos, Kozani, West Macedonia GR-50100 Greece L120529	VR0589 Agria	1. 05.05.2012 2. 01.07.-05.08.2012 3. 20.08.-15.09.2012	boom sprayer AZO	0.35	400	1.4	3 03.08.2012	45-47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 7 14 20	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2012/1272625 Entrada de Velez No. 7 Zafarraya Spain L120530	VR0589 Kennebec	1. 24.05.2012 2. n.a. 3. 19.09.2012	boom sprayer Agrar-test	0.56	250	1.4	3 05.09.2012	46	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 6 14 20	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2012/1272625 Hermanos Pinzon No. 22 29700 Velez-Malaga Spain L120531	VR0589 Kennebec	1. 24.05.2012 2. n.a. 3. 26.09.2012	boom sprayer Agrar-test	0.56	250	1.4	3 13.09.2012	46	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 8 13 21	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Potato / Root and Tuber Vegetables	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	CS_2, BAS 222 F (by CS_2), BAS 222 F (by EBDC)
Region	**Southern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
										I	**II**	**III**		
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**CS_2[1]**	**metiram (by CS_2)**	**metiram (by EBDC)**		
2014/1000222 84480 Bonnieux France (S) L130122	VR0589 Jaerla	1. 20.06.2013 2. 15.08.-01.09.2013 3. 24.09.2013	plot-sprayer with boom	0.7	200	1.4	3 10.09.2013	44	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 8 14 21	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2014/1000222 Nea Magnisia, Thessaloniki Central Macedonia, GR-57008 Greece L130123	VR0589 Spunta	1. 18.04.2013 2. 01.-20.06.2013 3. 05.-20.07.2013	AZO boom-sprayer	0.7	200	1.4	3 21.06.2013	42-45	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 7 14 21	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2014/1000222 Via Giugni 3 23010 Albosaggia Italy L130124	VR0589 Primura	1. 28.05.2013 2. 25.06.-04.07.2013 3. 16.-23.08.2013	backpack sprayer with boom	0.7	200	1.4	3 02.08.2013	47-48	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	0.07 < 0.05 < 0.05 < 0.05	0 7 14 21	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2014/1000222 Cale Olivio No. 50 14400 Pozoblanco Spain L130125	VR0589 Condor	1. 02.03.2013 2. 22.05.-03.06.2013 3. 03.06.2013	knapsack boom-sprayer	0.7	200	1.4	3 20.06.2013	45	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 6 14 21	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2015/1000321 C/San Francisco No 6 14412 Pedrpche Cordoba Spain L140386	VR0589 Carlita	1. 10.02.2014 2. n.a. 3. 02.-09.06.2014	Boom sprayer	0.7	200	1.4	3 19.06.2014	47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 7 14 21	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Potato / Root and Tuber Vegetables	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	CS_2, BAS 222 F (by CS_2), BAS 222 F (by EBDC)
Region	**Southern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
										I	**II**	**III**		
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**CS_2[1]**	**metiram (by CS_2)**	**metiram (by EBDC)**		
2015/1000321 57008 Nea Magnisis Greece L140387	VR0589 Jaerla	1. 19.03.2014 2. 05.-25.05.2014 3. 05.-25.06.2014	AZO Boom sprayer	0.7	200	1.4	3 26.05.2014	45/47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 6 15 21	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2015/1000321 23036 Teglio Italy L140388	VR0589 Kennebeck	1. 24.04.2014 2. 07.-23.07.2014. 3. 11.09.2014	Boom sprayer	0.7	200	1.4	3 28.08.2014	48	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	0.064 < 0.05 < 0.05 < 0.05	0 7 14 21	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01
2015/1000321 C/Union Del Iano No 3 18128 Zafarraya Spain L140389	VR0589 Kennebeck	1. 14.05.2014 2. 21.-28.07.2014 3. 18.-25.08.2014	Boom sprayer	0.7	200	1.4	3 04.08.2014	47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.056 < 0.056 < 0.056 < 0.056	< 0.1 < 0.1 < 0.1 < 0.1	< 0.05 < 0.05 < 0.05 < 0.05	0 7 14 21	metiram (CS_2): BASF method No. L0234/01 metiram (EBDC): BASF method No. L0089/01

1) conversion factor from metiram to CS_2 is 1.79

n.a. not applicable

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Potato / Root and Tuber Vegetables	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	ETU, EU, EBIS
Region	**Southern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
										I	**II**	**III**		
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**ETU**	**EU**	**EBIS**		
2012/1272625 Platahos, Imathia, Central Macedonia GR-59032 Greece L120528	VR0589 Agria	1. 04.04.2012 2. 05.-30.05.2012 3. 10.-30.07.2012	boom sprayer AZO	0.47	300	1.4	3 02.07.2012	45-47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 7 14 22	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01
2012/1272625 Polymilos, Kozani, West Macedonia GR-50100 Greece L120529	VR0589 Agria	1. 05.05.2012 2. 01.07.-05.08.2012 3. 20.08.-15.09.2012	boom sprayer AZO	0.35	400	1.4	3 03.08.2012	45-47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 7 14 20	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01
2012/1272625 Entrada de Velez No. 7 Zafarraya Spain L120530	VR0589 Kennebec	1. 24.05.2012 2. n.a. 3. 19.09.2012	boom sprayer Agrar-test	0.56	250	1.4	3 05.09.2012	46	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 6 14 20	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01
2012/1272625 Hermanos Pinzon No. 22 29700 Velez-Malaga Spain L120531	VR0589 Kennebec	1. 24.05.2012 2. n.a. 3. 26.09.2012	boom sprayer Agrar-test	0.56	250	1.4	3 13.09.2012	46	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 8 13 21	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Potato / Root and Tuber Vegetables	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	ETU, EU, EBIS
Region	**Southern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
										I	**II**	**III**		
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**ETU**	**EU**	**EBIS**		
2014/1000222 84480 Bonnieux France (S) L130122	VR0589 Jaerla	1. 20.06.2013 2. 15.08.-01.09.2013 3. 24.09.2013	plot-sprayer with boom	0.7	200	1.4	3 10.09.2013	44	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 0.011 0.013	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 8 14 21	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2014/1000222 Nea Magnisia, Thessaloniki Central Macedonia, GR-57008 Greece L130123	VR0589 Spunta	1. 18.04.2013 2. 01.-20.06.2013 3. 05.-20.07.2013	AZO boom-sprayer	0.7	200	1.4	3 21.06.2013	42-45	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 7 14 21	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2014/1000222 Via Giugni 3 23010 Albosaggia Italy L130124	VR0589 Primura	1. 28.05.2013 2. 25.06.-04.07.2013 3. 16.-23.08.2013	backpack sprayer with boom	0.7	200	1.4	3 02.08.2013	47-48	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 7 14 21	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Potato / Root and Tuber Vegetables	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	ETU, EU, EBIS
Region	**Southern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**I ETU**	**II EU**	**III EBIS**		
2014/1000222 Cale Olivio No. 50 14400 Pozoblanco Spain L130125	VR0589 Condor	1. 02.03.2013 2. 22.05.-03.06.2013 3. 03.06.2013	knapsack boom-sprayer	0.7	200	1.4	3 20.06.2013	45	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 0.018 0.023 0.023	< 0.01 < 0.01 0.010 0.010	< 0.01 < 0.01 < 0.01 < 0.01	0 6 14 21	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2015/1000321 C/San Francisco No 6 14412 Pedrpche Cordoba Spain L140386	VR0589 Carlita	1. 10.02.2014 2. n.a. 3. 02.-09.06.2014	Boom sprayer	0.7	200	1.4	3 19.06.2014	47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 7 14 21	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2015/1000321 57008 Nea Magnisis Greece L140387	VR0589 Jaerla	1. 19.03.2014 2. 05.-25.05.2014 3. 05.-25.06.2014	AZO Boom sprayer	0.7	200	1.4	3 26.05.2014	45/47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 6 15 21	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg

RESIDUE DATA SUMMARY FROM SUPERVISED TRIALS (SUMMARY)

b

Active substance (common name)	Metiram	**Commercial Product (name)**	Polyram DF, Polyram WG
Crop/crop group:	Potato / Root and Tuber Vegetables	**Producer of commercial product**	BASF SE, Ludwigshafen, Germany
Responsible body for reporting (name, address)	BASF SE, 67056 Ludwigshafen	**Indoor/Glasshouse/Outdoor**	outdoor
Country	Germany	**Other active substance in the formulation (common name and content)**	none
Content of active substance (g/kg or g/L)	700 g/kg (metiram)		
Formulation (e.g. WP)	BAS 222 28 F (WG)	**Residues calculated as:**	ETU, EU, EBIS
Region	**Southern Zone**		

1	2	3	4	5			6	7	8	9			10	11
Report-No. Location (trial no.)	**Commo-dity/ Variety**	**Date of 1. Sowing / planting 2. Flowering 3. Harvest**	**Method of treat-ment**	**Application rate per treatment**			**No of treat-ment(s) and last date**	**Growth stage at last treat. or date**	**Portion analysed**	**Residues (mg/kg parent equiv.)**			**PHI (days)**	**Remarks**
										I	**II**	**III**		
				kg a.s./hL	**Water L/ha**	**kg a.s./ha**				**ETU**	**EU**	**EBIS**		
2015/1000321 23036 Teglio Italy L140388	VR0589 Kennebeck	1. 24.04.2014 2. 07.-23.07.2014 3. 11.09.2014	Boom sprayer	0.7	200	1.4	3 28.08.2014	48	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 7 14 21	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg
2015/1000321 C/Union Del Iano No 3 18128 Zafarraya Spain L140389	VR0589 Kennebeck	1. 14.05.2014 2. 21.-28.07.2014 3. 18.-25.08.2014	Boom sprayer	0.7	200	1.4	3 04.08.2014	47	potato (tuber) potato (tuber) potato (tuber) potato (tuber)	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01 < 0.01	0 7 14 21	ETU: BASF method No. L0176/01 EU and EBIS: BASF method No. L0233/01 LOQ: 0.01 mg/kg

Metiram

Document M-CA, Section 7

FATE AND BEHAVIOUR IN THE ENVIRONMENT

Compiled by:

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number
30/Jun/2015		BASF DocID 2015/1029567
31/Aug/2017	CA 7.1.3.1.2/2 Insertion of Report Amendment No. 1 to report Harder (2014b), BASF DocID 2014/1111056: Harder U., 2016 BASF DocID 2016/1322724 An additional evaluation (see report below) considered additional experimental study raw data. The original report of Harder (2014b) was amended accordingly. CA 7.1.3.1.2/3 Insertion of an evaluation of the ETU sorption study of Harder (2014b)), BASF DocID 2014/1111056: Platz K., 2016 BASF DocID 2016/1297349 The ETU sorption study of Harder (2014b) was evaluated under consideration of a draft checklist for soil sorption experiments developed by MS experts in order to investigate the reliability of the calculated sorption values. The checklist was circulated by the EFSA PRAS Secretariat on 29/10/2015.	BASF DocID 2017/1161178

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 7 FATE AND BEHAVIOUR IN THE ENVIRONMENT

Metiram (BAS 222 F), a fungicide for use in grapes, potatoes and other crops, is registered in Europe since many years.
Metiram was included in Annex I of Directive 91/414/EEC on 1 July 2006 (entry into force) under Inclusion Directive 2005/72/EC. The Review Report (Metiram - SANCO/4059/2001-rev.3.3) is dated 03 June 2005 and provides endpoints agreed during first inclusion evaluation (Appendix II to the Review Report).

For the current registration renewal under Regulation 1107/2009, a data gap analysis according to new guidelines, new guidance documents and new procedures in kinetic evaluations and exposure assessments was performed and new studies or kinetic evaluations were initiated where considered necessary. All new data are provided in this section.

Furthermore, a literature search was performed and scientific publications were evaluated for their endpoint relevance and quality. Although title and abstract of some publications indicated a potential connection to respective environmental fate chapters of this dossier, the detailed evaluation of these publications showed no endpoint which could be used for the required risk assessments. Consequently, for environmental fate no summaries of public literature data are provided in this section. Further information on the literature assessment and respective justifications can be found in M-CA 9.

An overview of metabolites discussed in this section is given below. The table includes the different code numbers that are available for each metabolite. In the most recent studies the new systematic metabolite codes (M222Fxxx) were used, but in many old studies (and their summaries) the other code names or trivial names were used. In the following chapters and study summaries synonym metabolite codes or trivial names are partly given in brackets.

Substance/ Metabolite Code	Reference code (Reg.No.)	Synonyms	Structure
Metiram BAS 222 F	250284	M222F000 Metiram TK 85 BAS 222 29 F	H N S S Zn(NH$_3$) S N H S 3 H N S S S N H S x
222F002	146099	BF222-ETU ETU	S HN NH
M222F003	27270	BF222-EU EU	O HN NH

Substance/ Metabolite Code	Reference code (Reg.No.)	Synonyms	Structure
M222F004	243959	BF 222-EBIS EBIS ETM	
M222F005	251072	BF222-Carbimid Carbimid ETT	
M222F007	4670450	TDIT M212	
M222F008	132345	Hydantoin	
M222F009	not assigned		
M222F010	not assigned		
M222F011	70964		
M222F012	not assigned		
M222F013	6014473		
M222F014	not assigned		

Substance/ Metabolite Code	Reference code (Reg.No.)	Synonyms	Structure
M222F015	6012392		
M222F016	6014472		
M222F017	283749		
M222F018	not assigned		or or
M222F019	not assigned		
M222F020	not assigned		

Metiram is an ethylene-bisdithiocarbamate complex, i.e. its structure is built from a rather simple monomer. It contains no diverse structural elements like different ring systems. With regard to this structure it is sufficient to use one single radiolabel in the monomer for the investigation of the environmental fate of metiram. All environmental fate studies involving radiolabeled test item were performed with ethylene-^{14}C-labeled metiram.

Metiram is a compound with very unusual characteristics:
It is an organic Zn-containing polymeric complex that forms as a precipitate during synthesis. Once formed this polymeric precipitate is no longer soluble in water or any organic solvent. If dissolution is observed, this occurs only under decomposition of the intact metiram complex.

These characteristics have a serious impact on the conduct of environmental fate studies:
- no homogeneous solutions can be prepared for application or analysis
- applications have to be performed using a suspension which is inherently less homogeneous than a solution
- with radiolabeled test item the issue of inhomogeneity is even more serious: Radiosynthesis can only performed on a very small scale where specific techniques used in the large scale production process (e.g. drying and milling) are not applicable
- common analytical techniques like HPLC cannot be used for the active substance since these require that the compound is dissolved; therefore the active substance can only be measured by indirect methods, e.g. determination of liberated CS_2
- the determination of the active substance and its metabolites requires two completely different methods which bears the risk of inconsistencies

Due to these very special characteristics, procedures used in environmental fate studies with metiram frequently need to deviate from established procedures that are applicable to most other compounds. Also an inherently lower homogeneity and repeatability of individual treatments, samples and analyses is due to these characteristics. This should be taken into account when evaluating studies with metiram.

The previous data set for environmental fate of metiram included rather old studies. In many of the old studies quite high and varying amounts of metabolites were detected. A thorough evaluation revealed that many of the old studies where subject to experimental artefacts that were the reason for the elevated amount of metabolites. The main sources for artefacts were:

(1) The active substance was "dissolved" in dimethyl-sulfoxide (DMSO) prior to application. This leads to an immediate decomposition of the intact metiram complex, i.e. the dissolved material is no longer intact active substance but consists of various degradates. That means that in those studies not the active substance but degradates thereof have been applied. Even if those degradates were identical to environmental metabolites, this is misleading since high amounts of these metabolites instead of the active substance are applied.

(2) Treated soil was extracted with inappropriate solvents. Metiram is subject to substantial solvolysis, depending on the nature of the solvent. Metiram and its solvolysis products are relative stable in pure organic solvents like acetonitrile or methanol. However, if water is additionally present (e.g. in mixtures of solvent with water) this leads to enhanced solvolysis and the formation of the respective solvolysis products which were reported as metabolites. In some cases aqueous EDTA solutions were used as first/main extractant. EDTA is complexing the Zn atom of the metiram complex leading to a fast decomposition of the active substance and the formation of solvolysis products.
Although solvolysis cannot be completely avoided, it can be minimized by the use of pure organic solvents.

(3) Analysis by TLC (normal phase) can lead to further decomposition on the TLC plate resulting in degradate patterns that are difficult to interpret and may not represent the actual constituents of the extract.

(4) In a number of studies the active substance and its decrease was not appropriately quantified. This was mainly in those cases where degradates (upon dissolution in DMSO) were applied instead of the active substance.

Apart from that there were some cases where more specific deficiencies were observed with certain old studies. These are discussed in the context of the respective studies.

It is proposed to invalidate the old studies that are affected by one or more of the above deficiencies. New environmental fate studies have been conducted that tried to avoid as far as possible the reasons for the artefacts. The new state-of-the-art studies are used to replace the old invalid studies. In fact this leads to a more consistent and obviously more realistic picture of the environmental fate of Metiram and its metabolites.

Upon recommendation of the RMS not only the new information has been summarized in this dossier but also information on valid older (already peer reviewed) studies is included. Summaries of the peer reviewed studies were taken from the monograph or its addenda. Changes of these summaries were kept at a minimum. However, some of them were partly outdated or required a revision for other reasons.

CA 7.1 Fate and behaviour in soil

CA 7.1.1 Route of degradation in soil

Metiram is degraded rapidly in soil with half-lives <1 day, presumably by abiotic mechanisms. Upon application of metiram to soil, primary metabolites (EBIS (=M222F004), TDIT (=M222F007)) are formed. The initial processes are very fast and these metabolites reach their maximum immediately after metiram is getting into contact with the soil. The primary metabolites are very short-lived and are further degraded with half-lives of mostly <1 day. Secondary metabolites, ethylenethiourea (ETU (=M222F002)) and ethylene urea (EU (=M222F003)) are formed which are also very transient with average half-lives of <2 days.

In older studies the formation of metabolites was largely overestimated. In these studies inappropriate procedures were used that led to artificial decomposition of metiram. More recent studies show that only EBIS (max. 25.7% TAR at day 0), TDIT (max. 13.5% TAR at day 0), ETU (max. 11.2% TAR at day 2) and EU (max. 6.9% TAR at day 1) exceed 10% of the applied radioactivity or 5% of applied in two successive sampling events. Carbimid is only observed in very low amounts and Hydantoin not at all. Other metabolites are formed only in minor percentages.
Further degradation results in a high percentage of CO_2 (up to 51.6% TAR) and the formation of bound residues up to 54.9% TAR.

Photolytic degradation on soil leads to the same degradation products (EBIS, ETU and EU) as in the aerobic metabolism studies. No special photoproducts are detected in significant amounts. Thus no differentiation is made between biological and photolytical degradation.

Metiram and all of its degradation products are degraded rapidly in soil under aerobic conditions. Anaerobic conditions are not expected to prevail for extended time periods after application of metiram. Therefore, data on the route of degradation of metiram under anaerobic are considered only as supplementary information.
The metabolic profile of metiram under anaerobic conditions is similar to that found under aerobic conditions. Thus metabolites EBIS, ETU and EU are also found under anaerobic conditions. However, the degradation processes are partly slower than under aerobic conditions leading to longer half-lives and higher amounts of the metabolites ETU and EU. No other metabolites exceed 10% of the applied radioactivity or 5% of applied in two successive sampling events. Final degradation under anaerobic conditions results in CO_2 (up to 13.4% TAR) and bound residues (up to 54.0% TAR).

CA 7.1.1.1 Aerobic degradation

Studies presented in the first Annex I inclusion process:

Five aerobic soil metabolism studies were presented in the first Annex I inclusion process.
An overview is given in Table 7.1.1.1-1. Two studies of Staudenmaier H. [*BASF DocID 2002/1011913 and BASF DocID 2002/1012954*] and one study of Keller E. [*BASF DocID 1993/10578*] are considered still valid and are summarized in CA 7.1.1.1/1, CA 7.1.1.1/2 and CA 7.1.1.1/5.

The studies of Keller E., Huber R. and Klein W., Koerdel W. et al. [*Keller E. and Huber R, 1985, BASF DocID 1985/10059; Klein W., Koerdel W. et al., 1986, BASF DocID 1986/10134*] are considered invalid for the following reasons:
In both studies the quantification of metabolites relied solely on radio-TLC analysis which was later on shown to be not a suitable method for the investigation of degradation products of metiram [*see CA 7.1.1.1/5, BASF DocID 1993/10578*]. Although HPLC is mentioned in the report of Klein W., Koerdel W. et al., 1986, this was not used for quantification.
In the study of Klein W., Koerdel W. et al., 1986, additionally inappropriate extraction solutions (solvent mixtures with water) were used. In this study also no data on the degradation of the active substance are provided.
Note that in the study of Keller E., Huber R. [*Keller E. and Huber R, 1985, BASF DocID 1985/10059*] also soil column leaching experiments were performed. These are considered still valid and are reported under CA 7.1.4.1.1/4.

Table 7.1.1.1-1: Studies on aerobic soil degradation of metiram

Reference	BASF DocID	Soil type	Application rate [mg kg^{-1}]	Incubation temperature [°C]	Incubation period [days]	Remark
Keller E., Huber R., 1985	1985/10059	Loamy sand Loam	10	22	365	Invalid
Klein W., Koerdel W. et al., 1986	1986/10134	Loamy sand	10	25	21	Invalid
Keller E., 1993	1993/10578	-- [a]				Relevant
Staudenmaier H., 2002a	2002/1011913	Loamy sand	7.5	20°	365	Relevant
Staudenmaier H., 2002b	2002/1012954	Loamy sand	3.9	20	133	Relevant

[a] Investigation of solvolytic decomposition of metiram in different solvents.

Submission of not yet peer-reviewed studies in this AIR3-Dossier:

The old soil metabolism studies of Keller E. et al. 1985 and Klein, W. et al. 1986 are considered as no longer valid. The same applies for the very old soil degradation study of Anonymous 1974 [BASF DocID 1974/10028] (justification see 7.1.2.1.1). For this reason not enough valid DT_{50} values for the parent metiram were available any more. New soil degradation experiments were performed for the aerobic degradation of metiram which are summarized in CA 7.1.1.1/3 and CA 7.1.1.1/4.

Peer reviewed studies (Summary taken from the Monograph or its Addenda):

Report:	*CA 7.1.1.1/1* *Staudenmaier H., 2002a* *Aerobic metabolism of BAS 222 F (Metiram) in Cashmere soil* *2002/1011913*
Guidelines:	*SETAC, BBA IV 4-1, EPA 162-1*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Report:	*CA 7.1.1.1/2* *Staudenmaier H., 2002b* *Aerobic metabolism of BAS 222 F (Metiram) in soil* *2002/1012954*
Guidelines:	*SETAC, BBA IV 4-1, EPA 162-1*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Both studies were run in parallel in the same laboratory and are summarized together:

Materials and methods:

[Ethylene-^{14}C]-BAS 222 F (metiram) with a specific radioactivity of 4.68 MBq mg^{-1} and a purity of > 98% was applied on two soils, a German silty sand soil (Li 35 b) and a loamy sand soil from the US (Cashmere). The soil characteristics are summarized in Table 7.1.1.1-2.
The equivalent field application rates for the German and the US soils were respectively 2.7 kg and 5.4 kg active substance/ha, assuming an equal distribution in the top 5 cm soil layer and a soil density of 1.5 g cm^{-3}.
The incubation conditions were: aerobic, in the dark, 20°C, 40% maximum water holding capacity for the German soil and 75% of field capacity at 1/3 bar for the US soil. A system with continuous aeration and trapping of volatiles was used.
Metiram is extractable only under decomposition and was therefore quantified in soil by the CS_2 method without extraction. Degradates were quantified by extraction with methanol and subsequent analysis by radio-HPLC.

Table 7.1.1.1-2: Soils used to investigate the degradation and metabolism of metiram

Soil designation	Cashmere	Li 35 b
Origin	Payette, Idaho, USA	Limburgerhof, Germany
Textural class (German scheme)	loamy sand	silty sand
Textural class (USDA scheme)	loamy sand	loamy sand
Particle size distribution [%] (German scheme): 0.063 - 2 mm 0.002 - 0.063 mm < 0.002 mm (USDA scheme): 0.05 - 2 mm 0.002 - 0.05 mm < 0.002 mm	 77 16 7 77 16 7	 67.7 26.8 5.5 74.9 21.9 3.1
Organic carbon [%]	0.8*	1.2
Organic matter [%]	1.4	2.1*
Microbial biomass [mg C/100g dry soil]	7.95	18.2
CEC [mVal/100 g]	11.6	8.1
pH [$CaCl_2$]	6.6	6.9
pH [H_2O]	7.1	7.3
MWC [g H_2O/100 g dry soil]	29.6	34.2
FC [g H_2O/100 g dry soil], 0.33 bar	12.8	n.a.

CEC cation exchange capacity
MWC maximum water holding capacity
FC field capacity
n.a. not available
* recalculated from organic matter by a factor of 1.72 or vice versa

Findings:

For the American Cashmere soil a rapid decrease of metiram was observed soon after application (Table 7.1.1.1-3). When analyzing as soon as possible after application (with the Cashmere soil), 90.4 % of the applied metiram was detectable as CS_2. This percentage decreased to 65.4% after 3 hours, 37.0% after one day and 5.1% after 7 days. From 62 DAT on, less than 1% of the applied metiram could be detected. The degradation in the German soil was even faster.

Table 7.1.1.1-3: Degradation of Metiram as determined by the CS_2 method

DAT	mg kg^{-1}	% of applied
Soil: Cashmere		
0 h	6.509	90.4
3 h	4.708	65.4
0 (3 h)	5.133	68.2
1	2.784	37.0
2	1.643	21.8
3	1.085	14.4
7	0.383	5.1
14	0.187	2.5
30	0.131	1.7
62	0.070	0.9
90	0.067	0.9
121	0.037	0.5
240	0.029	0.4
365	0.036	0.5
Soil: Li 35 b		
0	2.675	67.9
1	0.647	16.4
2	0.308	7.8
3	0.238	6.0
7	0.133	3.4
14	0.103	2.6
35	0.033	0.8
62	0.032	0.8
90	0.043	1.1
133	0.042	1.1

The distribution of radioactivity at different sampling dates is shown in Table 7.1.1.1-4. Significant mineralization was detected already after two days and increased continuously to 40.8% of the Total Applied Radioactivity (TAR) at 365 Days After Treatment (DAT) or 28.2%TAR at 133 DAT, for the American and the German soil respectively. Bound residues were formed immediately after application in amounts of 22.0 – 30.5%TAR. They increased to a maximum of 48.1 or 50.3%TAR at 14 DAT and later on decreased slowly down to 32.0%TAR at 365 DAT or 35.9%TAR at 133 DAT, respectively.

The metabolites EU, ETU, Carbimid and EBIS could be identified in the methanol extracts of the Cashmere soil / Li 35 b soil: The concentrations were generally low with a maximum of 11.2% of TAR for ETU in the American soil. After one week none of these metabolites were found in concentrations higher than 0.5% of TAR.

A further peak was detected at a maximum of 13.5%TAR / 4.6%TAR at 0 DAT. A molecular mass of M = 212 was determined and a tentative structure was proposed from LC-MS data for this peak which received the designation TDIT. This compound was formed immediately after application of metiram to soil and then was degraded rapidly without further detectable formation. No peak matched the retention time of hydantoin confirming the absence of this metabolite.

Table 7.1.1.1-4: Recovery of radioactivity in %TAR (Total applied radioactivity) and distribution of metabolites after application of [14C]-Metiram to soil and incubation under aerobic conditions

DAT	$^{14}CO_2$	EU	ETU	Carb-imid	EBIS	M 212 (TDIT)	Others* Sum	EDTA** Extract	Bound resi-dues	Material balance TRR*** + CO_2
Soil: Cashmere										
0 (3h)	n.d.	0.6	1.4	0.6	1.6	13.5	7.0	44.5	22.0	102.2
1	0.2		7.6	0.3	1.7	11.1	11.6	28.9	29.7	98.0
2	1.7		11.2	0.3	1.5	6.9	8.4	27.3	36.1	104.3
3	4.6	3.0	3.1	0.2	1.7	6.0	7.5	26.1	36.7	106.0
7	14.3	0.1	0.2	0.1	0.4	2.0	5.6	19.6	43.8	99.4
14	21.3	0.0	0.0	0.1	0.3	0.8	3.1	15.3	48.1	97.3
30	26.9	0.0		0.1	0.1	0.2	1.8	15.8	46.1	96.1
62	31.2				0.1	0.1	1.5	11.5	43.6	90.5
90	33.8				0.1	0.1	1.3	9.8	38.2	89.7
121	36.1				0.0	0.1	1.0	8.0	39.2	89.1
240	38.9				0.0	0.1	0.8	6.8	35.3	85.2
365	40.8							6.3	32.0	85.1
Mean										95.3
Soil: Li 35 b										
0	n.d.		1.3	0.1	11.4	4.6	5.8	43.5	30.5	99.3
1	0.3		8.3		1.1	1.1	15.7	32.9	33.8	100.4
2	1.2		9.2	0.1	0.5	0.7	12.2	29.3	39.5	98.2
3	2.9		10.1		0.5	0.4	9.2	27.4	41.1	97.8
7	6.6	0.1	0.1	0.1	0.5	0.1	4.8	23.5	44.1	87.5
14	11.3			0.1	0.2	0.0	3.3	18.1	50.3	88.2
35	18.4		0.0	0.0	0.0	0.3	2.3	15.0	44.9	86.3
62	22.6						1.7	13.0	37.8	85.0
90	25.4			0.0			1.6	12.0	38.3	80.2
133	28.2						1.2	10.8	35.9	79.9
Mean										90.3

n.d. = not determined
* numerous peaks, < 3 %TAR each
** Metiram which is insoluble in all solvents was destroyed by 1 % aqueous EDTA after extraction of metabolites in order to distinguish it principally from bound residues; due to the chelating activity of EDTA a certain amount of bound residues was also extracted
*** TRR = total radioactive residues in soil prior to extraction

After NaOH extraction roughly half of the bound radioactivity remained non extractable and was assigned to the humin fraction. The minor portion of radioactivity was precipitated at pH 1.5 and was attributed to the humic acids fraction. The major portion remained in solution and was attributed to the fulvic acids fraction. However, only a very minor portion of the fulvic acids was extractable into ethyl acetate.

Conclusion:

As a conclusion a metabolic pathway for the degradation of metiram in soil based on the results of the latter two studies is proposed. The degradation pathway is slightly deviating from the previously proposed by including the possible metabolite M212 (TDIT) in an early step but excluding the presence of hydantoin as a metabolite following EU. The new studies are considered to be superior compared to the older studies done under non-GLP conditions in the eighties both with respect to study design as with respect to the analytical methods applied.
The slow decrease with time in the material balance from about 100% of TAR down to 85% of TAR (American soil) and 80% of TAR (German soil) could not be explained by reversible adsorption to vessels or Teflon tubing nor to formation of volatiles escaping the ethylene glycol trap.
The overall quality of the studies is acceptable and the studies followed recommended guidelines.

[*pathway deleted and replaced by modified pathway in Figure 7.1.1.3-1*]

Report:	CA 7.1.1.1/3 Voelkel W., 2015a 14C-Metiram - Degradation and metabolism in two soils incubated under aerobic conditions 2013/1095967
Guidelines:	OECD 307 (2002), EPA 835.4100
GLP:	yes (certified by Swiss Federal Office of Public Health, Berne, Switzerland)

Executive Summary

The aerobic soil metabolism of ^{14}C-labeled metiram was investigated in two soils (Speyer 2.2 and Am Fischteich), a loamy sand and a silt loam (USDA). The nominal application rate was 5.6 mg kg^{-1} dry soil (corresponding to 1.4 kg test item ha^{-1}). Soil aliquots of 100 g (dry weight basis) were weighed into test vessels and incubated at 20.7 ± 0.2ºC and water capacity of pF 2.0 in the dark for 122 days. A closed incubation system with continuous aeration was used with an attached trapping system for the determination of volatile compounds.

Soil samples were taken at 2.5 hours after treatment (Day 0) and after 1, 2, 3, 6, 14, 29, 58, 99/92 and 122 days of incubation. Each replicate was divided into two sub-samples of about 50 g dry weight equivalents each. One sub-sample was used for metiram determination. The other sub-sample was used for extraction of radioactivity in order to determine the material balance and formation and decline of metabolites. Metiram had to be quantified by an indirect CS_2 method, during which metiram was decomposed by stannous hydrochloric acid and the formation of CS_2 was quantified. Two replicates per soil were extracted separately for up to four times using methanol, followed by extractions using up to five times 1% EDTA in ultra-pure water. The individual extracts were combined according to the extraction solution used and analyzed for the degradation products by HPLC.

A fast degradation of metiram was observed in both soils. 2.5 hours after treatment, the amount of metiram in Speyer 2.2 soil accounted for 76.4% of the applied amount, 9.6% of the applied test item were detected after 6 days of incubation, and after 122 days of incubation amounts were below the limit of quantification. In Am Fischteich soil, the amount of metiram decreased from 69.7% (day 0 = 2.5 hours) to 9.3% of applied test item after 6 days of incubation, and accounted for 1.4% after 122 days. The reported values of ^{14}C-metiram (in % of applied) were subjected to a kinetic evaluation and resulted in DT_{50} values of 0.3 and 0.5 days. The corresponding DT_{90} values were 7.3 and 4.2 days.

The amount of radioactivity recovered from Speyer 2.2 soil by methanol extraction decreased from 22.7% AR (day 0, 2.5 hours after treatment) to 1.6% AR after 14 days and accounted for 0.4% AR after 122 days of incubation. The corresponding values for Am Fischteich soil were 36.6% AR (day 0), 1.8% AR after 14 days and 0.3% AR, respectively.

The amount of radioactivity recovered by extraction with 1% EDTA on day 0 accounted for 34.1% AR (Speyer 2.2) and 31.7% AR (Am Fischteich), respectively. The amount of radioactivity in the 1% EDTA extracts slowly decreased over time, and accounted for 12.3% AR (Speyer 2.2) and 7.9% AR (Am Fischteich) after 122 days of incubation.

For Speyer 2.2 soil, the amount of bound residues increased from 36.9% AR (day 0) to a maximum of 53.0% AR after 29 days, followed by a decrease to 39.9% AR after 122 days. Bound residues for Am Fischteich soil showed a similar pattern, with the non-extractable radioactivity increasing from 43.0% AR (day 0) to a maximum of 54.9% AR after 29 days, and accounting for 39.2% AR on day 122.

The residual radioactivity remaining in selected soil samples following extraction after 0, 2, 6, 29, 58 and 122 days of incubation) was subjected to fractionation of the soil organic matter into fulvic acids, humic acids and humin fractions. For Speyer 2.2 soil, fulvic acids accounted for 25.0 to 36.1% of applied radioactivity. The amount of humic acids ranged from 7.8 to 12.3% of applied radioactivity. The remaining humin fraction accounted for 3.9 to 5.4% of applied radioactivity.
Similar results were observed for Am Fischteich soil. The amount of fulvic acids was between 25.6 and 36.0% of applied radioactivity, while the amount of humic acids ranged between 7.6 and 11.5% of applied radioactivity. The humin fraction accounted for 4.8 to 7.5% of applied radioactivity.

A significant mineralization of ^{14}C-metiram was observed in both soils tested. By the end of the incubation after 122 days, $^{14}CO_2$ reached maximum levels of 46.5% and 51.6% AR for Speyer 2.2 and Am Fischteich soils, respectively. Other radioactive volatile products did not exceed 0.2% AR at any sampling interval. Amounts of CS_2 trapped varied in the individual traps but never exceeded 3.8% of the theoretical amount applied.

^{14}C-metiram degraded into 20 mostly minor radioactive fractions. M222F004 (EBIS) was the only major radioactive fraction exceeding 10% of the amount applied in the methanol extracts of both soils. In the methanol extracts of Speyer 2.2 soil, M222F004 (EBIS) accounted for 15.0% of AR after 2.5 hours, followed by a fast decrease to 2.4% of AR after 3 days of incubation. The corresponding values for Am Fischteich soil were 25.7% of AR after 2.5 hours and 3.1% of AR after 3 days. M222F004 (EBIS) was not detectable anymore after 14 days of incubation in both soils. The calculated DT_{50} values were 0.4 and 0.3 days and the corresponding DT_{90} values 1.3 and 1.0 days. As minor fractions M222F003 (ethylene urea, EU) and M222F002 (ethylene thiourea, ETU) were detected.

The polymeric metiram is degraded into small fragments with M222F004 (EBIS) as main product, which is then further degraded to M222F003 (EU) and M222F002 (ETU). The most important degradation end product was the formation of bound residues and the complete mineralization to $^{14}CO_2$.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Common name: metiram (BAS 222 F)
Reg. No.: 250284
CAS-No.: 9006-42-2
Chemical name (IUPAC): zinc ammoniate ethylenebis(dithiocarbamate)-poly-(ethylenethiuramdisulphide)
Molecular formular: $(C_4H_9N_3S_4Zn)_3$; $(C_4H_6N_2S_4)_x$
Molar mass: 1088.7 g mol^{-1}

Labeled test item
Batch No.: 153-6001
Position of radiolabel: ^{14}C-ethylene
Specific radioactivity of a.s.: 7.64 MBq mg^{-1}
Releasable CS_2: 89.0%

Non-labeled test item
Batch No.: CP052744
Purity: 93.6%

2. Soil

Two different soils originating from Germany were used in the study: one soil ("Speyer 2.2") was sampled from a meadow in Hanhofen (Rhineland-Palatinate, Germany), the other one ("Am Fischteich") from an agricultural area in Mannichswalde (Crimmitschau, Germany). The soils were sampled from 0-20 cm depth. The soil was passed through a 2 mm sieve and stored moist at 4°C for less than one month. The soil characteristics are summarized in Table 7.1.1.1-5.

Table 7.1.1.1-5: Characteristics of soils used for soil metabolism study with ^{14}C-metiram

Soil designation	Speyer 2.2	Am Fischteich
Origin	Hanhofen Germany	Crimmitschau Germany
USDA Particle size distribution [%]		
Sand 0.050 – 2 mm	78.9	13.7
Silt 0.002 – 0.050 mm	13.8	67.6
Clay < 0.002 mm	7.3	18.7
Textural class	Loamy sand	Silt loam
Total organic carbon [%]	1.77	1.60
Organic matter [%] [a)]	3.05	2.76
pH (H_2O)	n.a.	6.5
pH ($CaCl_2$)	5.5	5.9
Cation exchange capacity [$cmol^+ kg^{-1}$]	10.1	16.7
Microbial biomass start of incubation [mg C per 100g dry soil]	76.9	50.8
Microbial biomass at end of incubation [mg C per 100g dry soil]	45.6	29.0
Water retention characteristics (pF 2) [g per 100g dry soil]	14.7	26.8

n.a. = not analyzed
[a)] % Organic matter = 1.724 × % organic carbon

B. STUDY DESIGN

1. Experimental conditions

The test substance was applied at a nominal concentration of 5.6 mg a.s. kg^{-1} dry soil which corresponds to a field application rate of 1.4 kg a.s. ha^{-1} (calculated on the basis of an equal distribution in the top 2.5 cm soil layer and a soil density of 1.0 g cm^{-3}). Portions of 100 g soil (dry weight basis) were then filled into test vessels.

To determine the actual applied radioactivity and the homogeneous distribution of test item, small aliquots of soil (0.03-0.05 g) were combusted and the evolved $^{14}CO_2$ was absorbed in OxySolve and the radioactivity measured by LSC.
The results showed a high variability, at least for the loamy soil Am Fischteich, indicating an inhomogeneous distribution of the radioactivity in soil at a small scale. The variability is due to the batch application of a suspended insoluble test substance. The overall recovery of radioactivity in the sub-samples however showed a much lower variability, indicating that a homogeneous distribution of the test item was achieved at a larger scale (50 g dry weight equivalents). Therefore, the overall recoveries of the sub-samples were normalized to 100% applied radioactivity and used to calculate the treatment rate.

The degradation experiment was performed in an open gas-flow system (all-glass flasks with a volume of approximately 1 L) and all samples were ventilated with moistened air. The exiting air was passed through a trapping system consisting of flasks of sodium hydroxide, potassium hydroxide/methanol and ethylene glycol in series. For sampling on day 0, no adsorption traps were set up. The treated soils were incubated at pF 2.0 water holding capacity and 20.7 ± 0.2°C in the dark.

Control samples used for the determination of the microbial biomass were incubated under the same conditions as the treated samples. The exiting air of the untreated control samples was passed through a flask containing water.

2. Sampling

Two replicates (A and B) of treated soil samples were taken for work-up and analyzed 2.5 hours after treatment (day 0). Additional replicates were taken after 1, 2, 3, 6, 14, 29, 58, 99 and 122 days of incubation for soil 1, and after 1, 2, 3, 6, 14, 29, 58, 92 and 122 days of incubation for soil 2, respectively.

Trapping solutions of samples were taken on the corresponding sampling days. Trapping solutions were in addition exchanged at regular intervals, depending on the formation of CS_2 or $^{14}CO_2$.

3. Description of analytical procedures

In order to determine the degradation rate of metiram and the rates of formation and decline of transformation products, different analytical methods were used. At each sampling interval, the samples were divided into two sub-samples of about 50 g dry weight equivalents each. One sub-sample was used for metiram determination. The other sub-sample was used for extraction of radioactivity in order to determine the material balance and formation and decline of metabolites.

Metiram determination

Metiram is insoluble in all solvents and can therefore not be quantified by direct analysis. Hence, metiram had to be quantified by an indirect CS_2 method, during which metiram is decomposed by stannous hydrochloric acid and the formation of CS_2 is quantified. The procedure was based on an existing method, which was adapted to meet the requirements of this study.
Soil sub-samples (50 g dry weight equivalents) were transferred into an Erlenmeyer flask and connected to a decomposition and distillation apparatus ventilated with nitrogen. The outlet gas passed through a condenser and a trapping system consisting of flasks of zinc acetate, sulfuric acid and methanolic solution of potassium hydroxide in series. 150 mL of a decomposition solution (8 g tin (II) chloride dehydrate dissolved in hydrochloric acid/water (4:21, v/v)) was added drop-wise to the soil sample and the suspension was heated until a slight boil was observed, once the whole decomposition solution was added. As soon as the soil-solution-mixture started to boil, the nitrogen flow was stopped until no further gas formation was observed. The suspension was then boiled for additional 45 minutes.

The CS_2 formed by decomposition of metiram under reductive conditions was trapped in the methanolic solution of potassium chloride and quantified by UV detection

Extraction and isolation of radioactivity from soil

Sub-samples (50 g dry weight equivalents) of two replicates per soil were exhaustively extracted separately using methanol (up to four times) followed by extraction using 1% EDTA in ultra pure water adjusted to pH 8 by addition of 10M NaOH (five or six times).

The amount of solvent used for each extraction step was about 2 mL g^{-1} soil. Each extraction was performed in a shaker at approximately 150 rpm for 15 minutes. The individual extracts were filtered and quantified by LSC.

The extracts containing more than 2% of the radioactivity applied were combined separately according to the extraction solution used. If needed, pooled extracts were in addition concentrated in a rotary evaporator at 30°C or 35°C. The pooled extracts were measured by LSC for recovery and submitted to HPLC analysis.

The residual radioactivity remaining in soil after the extraction procedure was quantified by LSC after combusting aliquots of the air-dried and homogenized soil.

For sampling interval 0, 2, 6, 29, 59 and 122 days, the exhaustively extracted soil was submitted to an organic matter fractionation, in order to determine the amount of radioactivity in the fulvic acids, humic acids and humin fractions. Aliquots of the extracted soil samples were extracted five times using 0.5M NaOH. The liquid phase was separated from the solid by centrifugation and the non-extractable residues (containing the humin fraction) were quantified by LSC after combusting aliquots of the air-dried and homogenized soil.
The radioactivity in the NaOH extracts (containing the humic and fulvic acids) was quantified by LSC. The extracts were pooled, adjusted to pH 1 by adding HCl, and left standing over night at about 4°C. Thereafter, the combined extracts were centrifuged. The precipitate (containing the humic acids) was dissolved in 0.5M NaOH and submitted to LSC. The radioactivity in the supernatant (containing the fulvic acids) was quantified by LSC. In addition, an aliquot of the supernatant was adjusted to pH 2-3 by adding NaOH and submitted to HPLC analysis.

Structure elucidation

Degradates of metiram were subjected to structure elucidation using HPLC-MS/MS. For this purpose, an aliquot of Speyer 2.2 soil (57.7 g wet weight) was treated with an exaggerated amount of metiram (393 mg unlabeled metiram and about 0.4 mg ^{14}C-metiram). After 24 hours of incubation, the sample was extracted twice with methanol and submitted to HPLC-MS/MS analysis.

Trapping solutions

The volumes of the trapping solutions were recorded. Thereafter, radioactivity present in the trapping solutions was determined by LSC.
In order to confirm the presence of $^{14}CO_2$, the radioactivity contained in the sodium hydroxide traps was precipitated with barium hydroxide on a pooled sample. The absence of radioactivity in the supernatants after precipitation was taken as proof that only $^{14}CO_2$ was present in the sodium hydroxide solutions.

Microbial biomass

The microbial biomass was determined with untreated soil before and at the end of incubation. Prior to use, the soils were sieved through a 2 mm mesh and adjusted to the same soil moisture (pF 2.0) as used for the treated soils.

The biomass was determined using about 20 g soil samples (dry weight equivalent). The soil samples were fumigated for 24 hours with $CHCl_3$ and the released carbon extracted with 0.5M K_2SO_4 solution. Non-fumigated control soil samples were also extracted with 0.5M K_2SO_4 solution. The carbon content (TOC) in the extracts was determined with a photometric method.

4. Calculation of the degradation rate

Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints) and for modeling purposes (modeling endpoints). Kinetic analysis and calculation of $DegT_{50}$ and $DegT_{90}$ values was performed following the recommendations of the FOCUS Kinetics workgroup [*FOCUS (2006) "Guidance Document on Estimating Persistence and Degradation Kinetics from Environmental Fate Studies on Pesticides in EU Registration" Report of the FOCUS Work Group on Degradation Kinetics, EC Document Reference Sanco/10058/2005 version 2.0, 434pp.*].

The detailed kinetic evaluation of the degradation behavior of metiram and its metabolites is presented in a separate report [*see CA 7.1.2.1.1/5, BASF DocID 2014/1311117*].

II. RESULTS AND DISCUSSION

A. MASS BALANCE

The detailed results on the extractability of radioactive residues are presented in Table 7.1.1.1-6 and Table 7.1.1.1-7. The mass balance ranged from 89.4% to 106.1% for Speyer 2.2 soil and from 81.6% to 116.1% for Am Fischteich soil. The variability is due to the batch application of a suspended insoluble test substance.

B. EXTRACTABLE AND BOUND RESIDUES

The extraction procedure of soil treated with metiram must take into account the very special characteristics of this active substance. Intact polymeric metiram itself will not be extracted since it is insoluble in all solvents. However, during the course of the extraction the influence of the extractant will lead to a certain decomposition of the active substance accompanied by the formation of artificial amounts of degradates. It is known that this decomposition is relatively limited with pure solvents like methanol but is more significant with mixtures of organic solvents with water. The solvent extraction scheme has to be understood as a compromise between obtaining highest extractability and avoiding formation of high amounts of artificial degradates.

The intention of the final EDTA extraction was to destroy metiram remaining in the soil after the solvent extraction. Since metiram is insoluble in methanol it would remain unextracted which would lead to false (i.e. artificially increased) amounts of bound residues.

The distribution of radioactivity in the soil is shown in Table 7.1.1.1-6 and Table 7.1.1.1-7. The amount of radioactivity recovered from Speyer 2.2 soil by methanol extraction decreased from 22.7% AR (day 0, 2.5 hours after treatment) to 1.6% AR after 14 days and accounted for 0.4% AR after 122 days of incubation. The corresponding values for Am Fischteich soil were 36.6% AR (day 0), 1.8% AR (14 days) and 0.3% AR (122 days).

The amount of radioactivity recovered by extraction with 1% EDTA on day 0 accounted for 34.1% AR (Speyer 2.2) and 31.7% AR (Am Fischteich), respectively and slowly decreased over time to 12.3% AR (Speyer 2.2) and 7.9% AR (Am Fischteich) after 122 days of incubation. As a consequence, the total amount of extractable radioactivity slowly decreased over time ranging between 56.8% and 68.3% AR (day 0) to 8.2% - 12.6% AR after 122 days.

Table 7.1.1.1-6: Material balance and distribution of radioactivity after application of ^{14}C-metiram to Speyer 2.2 soil [% AR]

Incubation Time (days)	Replicate	Methanol Extracts *	EDTA Extracts **	Total extractable residues	Non-extractable residues	$^{14}CO_2$	Other volatiles in EG	Other volatiles in KOH/ Methanol	TOTAL	MEAN ± SD
0 (2.5h)	A	22.6	34.5	57.1	36.6	n.p.	n.p.	n.p.	93.7	**100.0 ± 4.9**
	B	22.8	33.7	56.5	37.3	n.p.	n.p.	n.p.	93.7	
	Mean	**22.7**	**34.1**	**56.8**	**36.9**	**n.p.**	**n.p.**	**n.p.**	**93.7**	
1	A	21.7	30.0	51.7	46.3	4.7	<0.1	<0.1	102.7	
	B	21.8	30.1	51.9	49.4	4.3	<0.1	<0.1	105.6	
	Mean	**21.7**	**30.1**	**51.8**	**47.9**	**4.5**	**<0.1**	**<0.1**	**104.2**	
2	A	16.1	28.2	44.3	48.5	8.9	<0.1	<0.1	101.7	
	B	15.9	28.0	43.8	49.0	9.4	<0.1	<0.1	102.2	
	Mean	**16.0**	**28.1**	**44.0**	**48.7**	**9.2**	**<0.1**	**<0.1**	**101.9**	
3	A	12.0	25.9	38.0	50.5	10.6	<0.1	<0.1	99.1	
	B	12.7	25.2	37.8	50.3	12.4	<0.1	0.1	100.6	
	Mean	**12.4**	**25.6**	**37.9**	**50.4**	**11.5**	**<0.1**	**0.1**	**99.9**	
6	A	5.6	21.1	26.7	45.4	20.8	<0.1	0.1	93.0	
	B	5.7	18.9	24.6	43.7	21.1	<0.1	<0.1	89.4	
	Mean	**5.6**	**20.0**	**25.7**	**44.6**	**20.9**	**<0.1**	**<0.1**	**91.2**	
14	A	1.6	17.0	18.6	51.5	27.6	<0.1	0.3	98.1	
	B	1.6	17.5	19.1	54.1	28.5	<0.1	<0.1	101.8	
	Mean	**1.6**	**17.3**	**18.9**	**52.8**	**28.1**	**<0.1**	**0.2**	**99.9**	
29	A	0.9	14.4	15.3	52.8	35.3	<0.1	<0.1	103.3	
	B	0.9	14.4	15.3	53.3	35.8	<0.1	0.1	104.6	
	Mean	**0.9**	**14.4**	**15.3**	**53.0**	**35.5**	**<0.1**	**<0.1**	**103.9**	
58	A	0.5	11.7	12.2	51.4	41.8	<0.1	0.1	105.4	
	B	0.4	12.0	12.5	51.3	42.4	<0.1	<0.1	106.1	
	Mean	**0.5**	**11.8**	**12.3**	**51.3**	**42.1**	**<0.1**	**<0.1**	**105.8**	
99	A	0.3	11.9	12.3	46.1	46.9	<0.1	<0.1	105.3	
	B	0.3	11.7	12.0	36.6	45.9	<0.1	0.1	94.7	
	Mean	**0.3**	**11.8**	**12.1**	**41.3**	**46.4**	**<0.1**	**0.1**	**100.0**	
122	A	0.4	12.2	12.6	39.1	46.7	<0.1	<0.1	98.4	
	B	0.4	12.4	12.7	40.7	46.2	<0.1	0.1	99.6	
	Mean	**0.4**	**12.3**	**12.6**	**39.9**	**46.5**	**<0.1**	**<0.1**	**99.0**	

n.p. = not performed
SD = standard deviation
* Extracted with methanol up to four times
** Extraction with 1% EDTA (adjusted to pH 8 with 10 M NaOH), five times

Table 7.1.1.1-7: Material balance and distribution of radioactivity after application of ^{14}C-metiram to Am Fischteich soil [% AR]

Incubation Time (days)	Replicate	Methanol Extracts *	EDTA Extracts **	Total extractable residues	Non-extractable residues	$^{14}CO_2$	Other volatiles in EG	Other volatiles in KOH/ Methanol	TOTAL	MEAN ± SD
0 (2.5h)	A	38.3	32.4	70.7	45.4	n.p.	n.p.	n.p.	116.1	
	B	34.8	31.1	65.9	40.5	n.p.	n.p.	n.p.	106.5	
	Mean	**36.6**	**31.7**	**68.3**	**43.0**	**n.p.**	**n.p.**	**n.p.**	**111.3**	
1	A	26.8	23.9	50.6	44.2	2.1	<0.1	<0.1	96.9	
	B	32.7	27.1	59.9	47.3	2.1	<0.1	<0.1	109.2	
	Mean	**29.8**	**25.5**	**55.3**	**45.7**	**2.1**	**<0.1**	**<0.1**	**103.1**	
2	A	23.2	27.7	50.9	51.7	7.2	<0.1	0.1	109.9	
	B	22.5	26.6	49.0	49.7	4.7	<0.1	<0.1	103.5	
	Mean	**22.8**	**27.2**	**50.0**	**50.7**	**6.0**	**<0.1**	**0.1**	**106.7**	
3	A	15.3	24.5	39.8	55.8	11.2	<0.1	<0.1	106.8	
	B	13.8	22.1	35.9	53.0	11.3	<0.1	<0.1	100.1	
	Mean	**14.6**	**23.3**	**37.8**	**54.4**	**11.2**	**<0.1**	**<0.1**	**103.4**	
6	A	6.2	19.0	25.3	40.7	19.4	<0.1	<0.1	85.4	
	B	6.5	19.7	26.1	37.8	17.7	<0.1	<0.1	81.6	
	Mean	**6.4**	**19.4**	**25.7**	**39.2**	**18.6**	**<0.1**	**<0.1**	**83.5**	**100.0 ± 8.5**
14	A	1.8	12.6	14.4	45.4	30.6	<0.1	<0.1	90.4	
	B	1.8	13.7	15.5	46.1	30.4	<0.1	<0.1	92.0	
	Mean	**1.8**	**13.1**	**15.0**	**45.7**	**30.5**	**<0.1**	**<0.1**	**91.2**	
29	A	0.7	10.6	11.3	55.5	37.2	<0.1	<0.1	104.0	
	B	0.7	10.8	11.4	54.4	37.2	<0.1	<0.1	103.0	
	Mean	**0.7**	**10.7**	**11.3**	**54.9**	**37.2**	**<0.1**	**<0.1**	**103.5**	
58	A	0.5	8.7	9.2	51.0	44.4	<0.1	<0.1	104.6	
	B	0.5	8.0	8.5	51.9	42.7	<0.1	<0.1	103.1	
	Mean	**0.5**	**8.3**	**8.8**	**51.4**	**43.5**	**<0.1**	**<0.1**	**103.8**	
92	A	0.3	7.3	7.7	42.1	45.4	<0.1	0.1	95.1	
	B	0.3	6.8	7.2	41.5	44.8	<0.1	0.1	93.6	
	Mean	**0.3**	**7.1**	**7.4**	**41.8**	**45.1**	**<0.1**	**0.1**	**94.4**	
122	A	0.3	8.1	8.4	36.0	53.4	<0.1	<0.1	97.7	
	B	0.3	7.7	8.0	42.5	49.9	<0.1	<0.1	100.4	
	Mean	**0.3**	**7.9**	**8.2**	**39.2**	**51.6**	**<0.1**	**<0.1**	**99.1**	

n.p. = not performed
SD = standard deviation
* Extracted with methanol up to four times
** Extraction with 1% EDTA (adjusted to pH 8 with 10M NaOH), five times

C. VOLATILIZATION

A significant mineralization of ^{14}C-metiram was observed in both soils tested (Table 7.1.1.1-6 and Table 7.1.1.1-7). By the end of the incubation after 122 days, $^{14}CO_2$ reached maximum levels of 46.5% and 51.6% AR for Speyer 2.2 and Am Fischteich soils, respectively. The identity of $^{14}CO_2$ was confirmed by precipitation with barium hydroxide in selected samples of both soils.

Other radioactive volatile products than $^{14}CO_2$ did not exceed 0.2% AR at any sampling interval. CS_2 was determined in methanolic potassium hydroxide traps (Table 7.1.1.1-8). The amount of CS_2 detected varied in the individual traps but never exceeded 3.8% of the theoretical amount applied (Speyer 2.2 soil, day 2).

Table 7.1.1.1-8: Volatiles in potassium hydroxide solutions of Speyer 2.2 soil and Am Fischteich soil as determined by the CS_2 method

Speyer 2.2 soil				Am Fischteich soil			
Incubation Time (days)	**Replicate**	**CS_2 [mg]**	**[% AR]**	**Incubation Time (days)**	**Replicate**	**CS_2 [mg]**	**[% AR]**
0 (2.5h)	A	n.p.		**0 (2.5h)**	A	n.p.	
	B	n.p.			B	n.p.	
	Mean	**n.p.**	**n.p.**		**Mean**	**n.p.**	**n.p.**
1	A	0.007		**1**	A	0.009	
	B	0.007			B	0.005	
	Mean	**0.007**	**2.5**		**Mean**	**0.007**	**2.1**
2	A	0.009		**2**	A	0.014	
	B	0.013			B	0.005	
	Mean	**0.011**	**3.8**		**Mean**	**0.010**	**2.9**
3	A	0.009		**3**	A	0.013	
	B	0.009			B	0.007	
	Mean	**0.009**	**3.1**		**Mean**	**0.010**	**3.1**
6	A	0.005		**6**	A	n.p.	
	B	0.007			B	n.p.	
	Mean	**0.006**	**2.1**		**Mean**	**n.p.**	**n.p.**
14	A	0.002		**14**	A	0.003	
	B	0.001			B	0.003	
	Mean	**0.002**	**0.6**		**Mean**	**0.003**	**0.8**
29	A	0.001		**29**	A	<LOQ	
	B	0.001			B	<LOQ	
	Mean	**0.001**	**0.5**		**Mean**	**<LOQ**	**<LOQ**
58	A	<LOQ		**58**	A	<LOQ	
	B	0.002			B	<LOQ	
	Mean	**0.001**	**0.3**		**Mean**	**<LOQ**	**<LOQ**
99	A	0.002		**92**	A	0.002	
	B	<LOQ			B	0.004	
	Mean	**0.001**	**0.4**		**Mean**	**0.003**	**1.0**
122	A	<LOQ		**122**	A	0.004	
	B	<LOQ			B	0.003	
	Mean	**<LOQ**	**<LOQ**		**Mean**	**0.003**	**1.0**

n.p. = not performed
LOQ (Speyer 2.2 soil) = 0.26% AR (0.0008 mg CS_2)
LOQ (Am Fischteich soil) = 0.23% AR (0.0008 mg CS_2)

D. TRANSFORMATION OF PARENT COMPOUND

Degradation of metiram

The degradation of metiram, as determined by the CS_2 method, in Speyer 2.2 and Am Fischteich soils are summarized in Table 7.1.1.1-9.

A fast degradation of metiram was observed in both soils. 2.5 hours after treatment, the amount of metiram in Speyer 2.2 soil accounted for 76.4% of the applied amount. After 6 days of incubation, 9.6% of applied test item could be attributed to metiram, and the amount of metiram detected after 122 days of incubation was below the limit of quantification. In Am Fischteich soil, the amount of metiram decreased from 69.7% (day 0 = 2.5 hours) to 9.3% of applied test item after 6 days of incubation, and accounted for 1.4% after 122 days.

Table 7.1.1.1-9: Degradation of metiram in Speyer 2.2 soil as determined by the CS_2 method

Speyer 2.2 soil				Am Fischteich soil			
Incubation Time (days)	**Replicate**	**^{14}C-metiram [mg kg^{-1}]**	**[% AR]**	**Incubation Time (days)**	**Replicate**	**^{14}C-metiram [mg kg^{-1}]**	**[% AR]**
0 (2.5h)	A	3.807		**0 (2.5h)**	A	3.978	
	B	4.047			B	4.161	
	Mean	**3.927**	**76.4**		**Mean**	**4.070**	**69.7**
1	A	1.128		**1**	A	2.384	
	B	1.402			B	2.198	
	Mean	**1.265**	**24.6**		**Mean**	**2.291**	**39.2**
2	A	1.064		**2**	A	1.224	
	B	1.546			B	1.409	
	Mean	**1.305**	**25.4**		**Mean**	**1.316**	**22.5**
3	A	0.983		**3**	A	0.931	
	B	0.894			B	1.106	
	Mean	**0.938**	**18.3**		**Mean**	**1.018**	**17.4**
6	A	0.568		**6**	A	0.554	
	B	0.417			B	0.528	
	Mean	0.493	9.6		**Mean**	**0.541**	**9.3**
14	A	0.274		**14**	A	0.322	
	B	0.222			B	0.340	
	Mean	**0.248**	**4.8**		**Mean**	**0.331**	**5.7**
29	A	0.212		**29**	A	0.073	
	B	0.229			B	0.080	
	Mean	**0.220**	**4.3**		**Mean**	**0.076**	**1.3**
58	A	0.055		**58**	A	0.112	
	B	0.123			B	0.123	
	Mean	**0.089**	**1.7**		**Mean**	**0.117**	**2.0**
99	A	0.122		**92**	A	0.154	
	B	0.093			B	0.142	
	Mean	**0.107**	**2.1**		**Mean**	**0.148**	**2.5**
122	A	<LOQ		**122**	A	0.039	
	B	<LOQ			B	0.128	
	Mean	**<LOQ**	**<LOQ**		**Mean**	**0.083**	**1.4**

LOQ = 0.045 mg kg^{-1} metiram (0.8% of applied)

Pattern of metabolite formation

The results for the methanol extracts are summarized in Table 7.1.1.1-10 and Table 7.1.1.1-11.

The metabolite pattern was also investigated in the EDTA extracts. As mentioned above, the intention of the EDTA extraction was to destroy metiram remaining in the soil after the methanol extraction in order not to generate artificially high amounts of bound residues. However, when comparing the amount of radioactivity in the EDTA extracts with the amounts of metiram as determined by the CS_2 method it became obvious that the radioactivity concentration in the EDTA solution was higher than was expected from the presence of metiram. It is concluded that extraction with EDTA solution not only leads to a release of metiram "monomers" by complexation of the Zn atom but also to a beginning disaggregation of bound radioactivity in the high molecular weight humic substances potentially due to the same metal complexing mechanism.

However, the use of complexing agents like EDTA is very artificial and the radioactive compounds released by EDTA are considered to be of no environmental relevance. Therefore, the results of the EDTA extracts were not used for further evaluation.

^{14}C-metiram degraded into 20 radioactive fractions. Most of them were minor fractions.

M222F004 (EBIS) was the only major radioactive fraction exceeding 10% of the amount applied in the methanol extracts of both soils. In the methanol extracts of Speyer 2.2 soil, M222F004 (EBIS) accounted 15.0% of AR after 2.5 hours, followed by a fast decrease to 2.4% of AR after 3 days. The corresponding values for Am Fischteich soil were 25.7% of AR after 2.5 hours and 3.1% of AR after 3 days. M222F004 (EBIS) was not detectable anymore after 14 days of incubation in both soils.

In Am Fischteich soil, the minor radioactive fraction M222F003 (EU) exceeded 5% AR at two consecutive sampling intervals. M222F003 (EU) was detected as two distinct peaks, but LC/MS analyses showed that both corresponded to the same compound. M222F003 (EU) was present at 2.7% of AR after 2.5 hours and increased to a maximum of 6.9% of AR after 1 day, followed by a decrease to 0.8% after 14 days.

Metabolites M222F004 (EBIS), M222F002 (ETU), M222F003 (EU), M222F007 (TDIT) and M222F010 were identified and confirmed by structure elucidation using HPLC-MS/MS.

None of the other radioactive fractions reached a concentration of 10% AR or 5% AR at two consecutive sampling intervals, respectively. Therefore, the remaining radioactive fractions were not identified.

Table 7.1.1.1-10: Radio HPLC analysis of methanol extracts of Speyer 2.2 soil treated with ^{14}C-metiram [% AR]

Inc. Time (days)	Replicate	Metabolic Fraction								s. o. [1]	n. a.[2]
		EU		EU total	ETU	M222F010	EBIS	UK1	UK4		
		Retention Time (min)									
		2.90-3.80	3.80-4.80	2.90-4.80	4.80-6.20	28.60-30.00	30.50-30.70	34.40-35.90	40.00-40.70		
0 (2.5h)	A	1.9	*	1.9	*	*	14.6	6.1	*	*	
	B	2.2	*	2.2	*	*	15.4	5.1	*	*	
	Mean	**2.1**	*	**2.1**	*	*	**15.0**	**5.6**	*	*	
1	A	3.3	*	3.3	*	3.3	11.2	2.9	0.9	*	
	B	2.7	*	2.7	*	4.6	10.5	3.2	0.8	*	
	Mean	**3.0**	*	**3.0**	*	**4.0**	**10.8**	**3.1**	**0.9**	*	
2	A	2.3	*	2.3	*	2.8	7.4	2.4	1.2	*	
	B	2.6	*	2.6	*	3.8	6.3	2.2	0.9	*	
	Mean	**2.4**	*	**2.4**	*	**3.3**	**6.9**	**2.3**	**1.1**	*	
3	A	0.6	0.3	0.9	1.1	2.7	2.1	1.0	0.6	3.6	
	B	0.6	1.2	1.8	0.4	2.7	2.6	1.4	0.6	3.2	
	Mean	**0.6**	**0.7**	**1.4**	**0.7**	**2.7**	**2.4**	**1.2**	**0.6**	**3.4**	
6	A	1.0	0.2	1.3	0.2	1.6	1.3	0.5	0.3	0.4	
	B	1.0	0.2	1.2	0.1	1.3	1.0	0.4	0.3	1.2	
	Mean	**1.0**	**0.2**	**1.3**	**0.2**	**1.5**	**1.2**	**0.5**	**0.3**	**0.8**	
14	A	0.6	0.2	0.7	*	0.4	0.1	0.1	0.1	0.1	
	B	0.6	0.2	0.8	*	0.5	0.1	*	0.1	*	
	Mean	**0.6**	**0.2**	**0.8**	*	**0.5**	**0.1**	**0.1**	**0.1**	*	
29	A	0.7	*	0.7	*	0.2	*	*	*	*	
	B	0.6	*	0.6	0.1	0.2	*	*	*	0.1	
	Mean	**0.6**	*	**0.6**	*	**0.2**	*	*	*	**0.1**	
58	A	*	*	*	*	*	*	*	*	*	0.5
	B	*	*	*	*	*	*	*	*	*	0.4
	Mean	*	*	*	*	*	*	*	*	*	**0.5**
99	A	*	*	*	*	*	*	*	*	*	0.3
	B	*	*	*	*	*	*	*	*	*	0.3
	Mean	*	*	*	*	*	*	*	*	*	**0.3**
122	A	*	*	*	*	*	*	*	*	*	0.4
	B	*	*	*	*	*	*	*	*	*	0.4
	Mean	*	*	*	*	*	*	*	*	*	**0.4**

Inc. Time = Incubation time, S.o. = Sum others, N.a. = Not analyzed
* Not detected. (LOD: 0.0235 % AR)
[1] sum others, each metabolite < 1% of applied radioactivity
[2] not analyzed

Table 7.1.1.1-11: Radio HPLC analysis of methanol extracts of Am Fischteich soil treated with ^{14}C-metiram [% AR]

Inc. Time (days)	Repl.	Metabolic Fraction											s. o. [1]	n. a.[2]
		EU		EU total	ETU	UK18	UK7	UK8	M222F010	EBIS	UK15	UK1		
		Retention Time (min)												
		2.90-3.80	3.80-4.80	2.90.-4.80	4.80-6.20	6.40-7.00	7.20-8.20	9.90-12.20	28.60-30.00	30.50-30.70	31.80-33.70	34.40-35.90		
0 (2.5h)	A	1.7	1.1	2.8	*	*	*	*	*	26.3	*	8.5	0.7	
	B	2.6	*	2.6	*	*	*	*	*	25.1	*	7.2	*	
	Mean	**2.1**	**0.5**	**2.7**	*	*	*	*	*	**25.7**	*	**7.9**	**0.3**	
1	A	5.3	1.0	6.3	0.6	0.8	1.1	1.3	1.8	9.3	1.0	3.3	1.3	
	B	4.6	3.0	7.6	1.4	*	1.2	1.9	1.0	13.4	*	4.1	2.2	
	Mean	**4.9**	**2.0**	**6.9**	**1.0**	**0.4**	**1.2**	**1.6**	**1.4**	**11.3**	**0.5**	**3.7**	**1.7**	
2	A	4.1	2.3	6.5	0.6	1.2	1.0	0.7	1.9	7.3	1.5	2.6	*	
	B	5.4	1.9	7.2	1.3	1.1	0.7	1.2	1.3	7.2	0.8	1.8	*	
	Mean	**4.7**	**2.1**	**6.8**	**0.9**	**1.1**	**0.8**	**0.9**	**1.6**	**7.2**	**1.1**	**2.2**	*	
3	A	3.4	0.7	4.1	0.6	0.5	0.7	1.1	1.3	3.0	0.6	1.3	3.1	
	B	2.6	0.6	3.2	0.8	0.4	0.3	1.1	1.5	3.2	0.4	1.0	2.1	
	Mean	**3.0**	**0.7**	**3.6**	**0.7**	**0.4**	**0.5**	**1.1**	**1.4**	**3.1**	**0.5**	**1.1**	**2.6**	
6	A	0.6	0.9	1.5	0.7	0.3	0.2	0.7	0.9	0.7	*	0.3	1.0	
	B	0.5	1.0	1.6	0.4	0.3	0.3	0.6	1.0	0.9	*	0.3	1.1	
	Mean	**0.6**	**1.0**	**1.5**	**0.5**	**0.3**	**0.2**	**0.7**	**0.9**	**0.8**	*	**0.3**	**1.0**	
14	A	0.5	0.3	0.8	0.1	*	0.1	0.1	0.3	*	*	*	0.3	
	B	0.4	0.3	0.7	0.1	0.1	0.1	0.1	0.3	0.1	0.1	*	0.2	
	Mean	**0.4**	**0.3**	**0.8**	**0.1**	**0.1**	**0.1**	**0.1**	**0.3**	**0.1**	*	*	**0.2**	
29	A	*	*	*	*	*	*	*	*	*	*	*	*	0.7
	B	*	*	*	*	*	*	*	*	*	*	*	*	0.7
	Mean	*	*	*	*	*	*	*	*	*	*	*	*	**0.7**
58	A	*	*	*	*	*	*	*	*	*	*	*	*	0.5
	B	*	*	*	*	*	*	*	*	*	*	*	*	0.5
	Mean	*	*	*	*	*	*	*	*	*	*	*	*	**0.5**
92	A	*	*	*	*	*	*	*	*	*	*	*	*	0.3
	B	*	*	*	*	*	*	*	*	*	*	*	*	0.3
	Mean	*	*	*	*	*	*	*	*	*	*	*	*	**0.3**
122	A	*	*	*	*	*	*	*	*	*	*	*	*	0.3
	B	*	*	*	*	*	*	*	*	*	*	*	*	0.3
	Mean	*	*	*	*	*	*	*	*	*	*	*	*	**0.3**

Inc. Time = Incubation time, S.o. = Sum others, N.a. = Not analyzed

* Not detected (LOD: 0.1749 % AR)

[1] sum others, each metabolite < 1% of applied radioactivity

[2] not analyzed

E. CHARACTERIZATION OF NON-EXTRACTABLE RESIDUES (NER)

The residual radioactivity remaining in selected soil samples after extraction (after 0, 2, 6, 29, 58 and 122 days of incubation) was subjected to a fractionation of the soil organic matter into humic acids, fulvic acids and humin fractions (Table 7.1.1.1-12 and Table 7.1.1.1-13).

For Speyer 2.2 soil, the proportions of fulvic acids, humic acids and humin fractions in percent of non-extractable radioactivity remained stable over time. Fulvic acids accounted for 25.0 to 36.1% of applied radioactivity. The amount of humic acids ranged from 7.8 to 12.3% of applied radioactivity. The remaining humin fraction accounted for 3.9 to 5.4% of applied radioactivity.
Similar results were observed for Am Fischteich soil. The amount of fulvic acids was between 25.6 and 36.0% of applied radioactivity, while the amount of humic acids ranged between 7.6 and 11.5% of applied radioactivity. Humin fraction accounted for 4.8 to 7.5% of applied radioactivity.

Table 7.1.1.1-12: Organic matter fractionations of Speyer 2.2 soil at various interval days [% AR]

Interval day	Replicate	NER	Fulvic acids [a)]	Humic acids [b)]	Humin [c)]
0 (2.5h)	A	36.6	24.7	7.6	4.2
	B	37.3	25.3	8.0	4.0
	Mean	**36.9**	**25.0**	**7.8**	**4.1**
2	A	48.5	33.6	10.6	4.3
	B	49.0	34.1	10.5	4.4
	Mean	**48.7**	**33.8**	**10.6**	**4.3**
6	A	45.4	30.6	9.8	5.0
	B	43.7	31.8	9.2	2.7
	Mean	**44.6**	**31.2**	**9.5**	**3.9**
29	A	52.8	36.9	12.7	3.2
	B	53.3	35.2	12.0	6.1
	Mean	**53.0**	**36.1**	**12.3**	**4.7**
58	A	51.4	34.2	12.8	4.4
	B	51.3	33.4	11.4	6.5
	Mean	**51.3**	**33.8**	**12.1**	**5.4**
122	A	39.1	25.5	9.1	4.5
	B	40.7	27.2	8.9	4.6
	Mean	**39.9**	**26.4**	**9.0**	**4.5**

NER Non-extractable residues
% AR Percent of applied radioactivity.
a) soluble at low pH
b) soluble at high pH
c) insoluble fraction

Table 7.1.1.1-13: Organic matter fractionations of Am Fischteich soil at various interval days [% AR]

Interval day	Replicate	NER	Fulvic acids [a)]	Humic acids [b)]	Humin [c)]
0 (2.5h)	A	45.4	30.3	8.9	6.1
	B	40.5	27.4	8.0	5.1
	Mean	**43.0**	**28.9**	**8.5**	**5.6**
2	A	51.7	35.5	10.3	5.9
	B	49.7	35.0	9.7	5.0
	Mean	**50.7**	**35.2**	**10.0**	**5.5**
6	A	40.7	28.1	7.9	4.7
	B	37.8	25.7	7.2	4.9
	Mean	**39.2**	**26.9**	**7.6**	**4.8**
29	A	55.5	36.6	11.9	7.0
	B	54.4	35.3	11.2	7.9
	Mean	**54.9**	**36.0**	**11.5**	**7.5**
58	A	51.0	35.4	10.5	5.1
	B	51.9	33.7	10.6	7.6
	Mean	**51.4**	**34.5**	**10.5**	**6.4**
122	A	36.0	23.7	7.5	4.8
	B	42.5	27.5	9.1	5.8
	Mean	**39.2**	**25.6**	**8.3**	**5.3**

NER Non-extractable residues
% AR Percent of applied radioactivity.
a) soluble at low pH
b) soluble at high pH
c) insoluble fraction

F. KINETIC MODELING RESULTS

The reported values of ^{14}C-metiram and its metabolites M222F004 (EBIS), M222F003 (EU) and M222F002 (ETU) [in % of applied radioactivity] were subjected to degradation kinetics evaluations and described in a separate report [*see CA 7.1.2.1.1/5, BASF DocID 2014/1311117*] to obtain trigger and modeling endpoints.

The calculated DT_{50} (half-life) and DT_{90} values (trigger endpoints) for ^{14}C-metiram as well as its metabolites in soil are presented in Table 7.1.1.1-14.

Table 7.1.1.1-14: Trigger endpoints for metiram, M222F004 (EBIS), M222F003 (EU), M222F002 (EU)

Soil	Model	χ^2 error	DT_{50} [d]	DT_{90} [d]
Metiram				
Speyer 2.2	FOMC	8.3	0.3	7.3
Am Fischteich	DFOP	9.6	0.5	4.2
M222F004 (EBIS)				
Speyer 2.2	SFO	19.2	0.4	1.3
Am Fischteich	SFO	6.7	0.3	1.0
M222F003 (EU)				
Speyer 2.2	SFO	No reliable endpoints derived		
Am Fischteich	SFO	20.2	0.7	2.5
M222F002 (ETU)				
Speyer 2.2	SFO	No reliable endpoints derived		
Am Fischteich	SFO	15.9	1.7	5.7

III. CONCLUSION

A fast degradation of metiram was observed in both soils, DT_{50} values of 0.3 and 0.5 days were determined.
M222F004 (EBIS) was the only major radioactive fraction exceeding 10% of the amount applied in both soils. M222F003 (EU) exceeded 5% TAR at two successive sampling events in one soil. Their DT_{50} values were calculated to be 0.4 and 0.3 for EBIS and 0.7 days for EU. Additionally minor metabolites including M222F002 (ETU) were formed. The degradation finally resulted in substantial formation of carbon dioxide up to 47/52 %TAR and the formation of bound residues.

Report: CA 7.1.1.1/4
Staudenmaier H.,Kuhnke G., 2011b
Formation of CS2 from Metiram in soil
2010/1090462

Guidelines: OECD 307 (2002), EPA 835.4100, BBA IV 4-1, EPA 162-1, SETAC Procedures for assessing the environmental fate and ecotoxicity for pesticides (March 1995)

GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

The objective of the study was to determine the formation of carbon disulfide (CS_2) from metiram (BAS 222 F) in soil under aerobic conditions. Furthermore, the degradation of metiram in soil was followed. The investigations were performed in one soil originating from Germany.

The soil was treated with metiram at a nominal rate of 4.85 mg per kg dry soil, which corresponds to a field application rate of 1820 g metiram per hectare, calculated on the basis of an equal distribution in the top 2.5 cm soil layer and a soil density of 1.5 g cm^{-3}. The incubation was carried out in the dark in the laboratory under aerobic conditions at soil moisture of 40% of the maximum water holding capacity and a temperature of 20°C. A closed incubation system with continuous aeration was used with an attached trapping system for the determination of CS_2. Samples were taken at 0, 1, 2, 3, 4, 7, 10 and 15 days after treatment (DAT).

At all sampling times the absorber flask for CS_2 was changed and analyzed. Soil samples were analyzed in duplicate except for 0 DAT, where 4 soil aliquots were worked up and analyzed. The trapping solution (methanolic KOH) was analyzed for CS_2 by UV detection of the xanthogenate formed.
The amount of metiram in the soil samples was determined by means of CS_2 release after chemical decomposition with stannous hydrochloric acid. CS_2 is absorbed in a methanolic solution of potassium hydroxide and quantification again is performed by UV detection of the xanthogenate.

The amount of metiram decreased rapidly from 95.8% of applied at day 0 to 5.2% of applied after 15 days. CS_2 was formed only in small amounts of maximum 1.2% of the applied amount per day. After 15 days the cumulative amount accounted for 2.1% of applied, where "% of applied" for CS_2 relates to theoretical amount of CS_2 in the applied metiram. The quantitative release of CS_2 from soil and its trapping was verified with a control experiment.

Degradation times of metiram were estimated using the software package KinGUI version 1.1. According to FOMC kinetics DT_{50} and DT_{90} values were 0.72 days and 6.6 days, respectively.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

BAS code:	BAS 222 F
Common name:	metiram
Reg. No.:	250284
Chemical name (IUPAC):	zinc ammoniate ethylenebis(dithiocarbamate)-poly(ethylenethiuramdisulfide)
Molecular formula:	3 $C_4H_9N_3S_4Zn$ * $C_4H_6N_2S_4$
Molecular weight:	1088.7 g mol^{-1} (theoretical value for monomeric compound)
Batch No.:	CP052744
Chemical Purity:	93.6%

2. Soils

One soil collected from an area near Limburgerhof (Germany) was used for treatment and incubation. The soil was sieved through a 2 mm sieve before use, remoistened to approximately 8-12% soil moisture and stored at about 4°C in the dark. The soil characteristics are summarized in Table 7.1.1.1-15.

Table 7.1.1.1-15: Soil characteristics

Soil designation	Li 10 (10/1680/01)
Origin	Limburgerhof, RP, Germany
DIN Particle size distribution [%] Sand 0.063 – 2 mm Silt 0.002 – 0.063 mm Clay < 0.002 mm Textural class	 81.2 13.5 5.3 loamy sand
USDA Particle size distribution [%] Sand 0.050 – 2 mm Silt 0.002 – 0.050 mm Clay < 0.002 mm Textural class	 82.0 12.7 5.3 loamy sand
Total organic carbon [%]	0.93
pH (H_2O) pH ($CaCl_2$)	7.0 6.3
Cation exchange capacity [$cmol^+ kg^{-1}$]	4.7
Maximum water holding capacity [g per 100g dry soil]	27.1
Microbial biomass (before start of study) [mg C per 100g dry soil]	24.8
Microbial biomass (after 20 DAT) [mg C per 100g dry soil]	14.1
Water retention characteristics (pF 2) [g soil moisture per g dry soil]	0.111 (11.1%)

DAT = days of treatment

B. STUDY DESIGN

1. Experimental conditions

The soil was adjusted to 30% of the maximum water holding capacity (MWHC) and was pre-incubated for four days in an incubation chamber at 20°C in the dark until use. The soil was treated at a nominal concentration of 4.85 mg metiram per kg dry soil, which corresponds to a field application rate of 1850 g a.s. ha^{-1}, assuming an equal distribution in the top 2.5 cm soil layer and a soil density of 1.5 g cm^{-3}.

Metiram is insoluble in water and organic solvents and was applied as a suspension water. By addition of the aqueous suspension, the soil was adjusted to 40% of the MWHC.

Soil aliquots of about 110 g (dry soil equivalents) were filled into 10 test vessels. The test vessels (except day 0 samples) were connected in series via gas inlet and outlet tubes. The vessels were incubated in the dark for up to 15 days at 20°C. Throughout the incubation period, the samples were continuously aerated with a slight stream of moistened synthetic air. The outgoing air was led through a series of gas-washing bottles containing trapping solutions for potential volatiles (0.5 N methanolic KOH for CS_2 trapping, ethylene glycol). The water content of the soils was monitored throughout the incubation period by weighing the sampled vessels. Water content was re-adjusted if necessary.

The day 0 samples were not incubated, but worked up immediately.

2. Sampling

Sampling times were 0 (no sampling of volatile), 1, 2, 3, 4, 7, 10 and 15 DAT.

The soil samples at day 0 were sampled and worked up as soon as possible after application. At each following sampling time, one vessel with soil was removed from the incubator and two soil aliquots from the vessel were analyzed for metiram. Moreover, at each sampling time besides day 0, the traps for volatiles were sampled and replaced by new flasks with fresh solutions.

3. Description of the analytical procedures

At 0 DAT four soil aliquots of one vessel (4 x 25 g) were worked up to determine the amount of metiram by use of CS_2 method. At the other sampling time points only two aliquots (2 x 25 g) of one test vessel were analyzed.

The principle of the determination of metiram in soil was based on an existing residue analytical method. The sample (soil or suspension of the test substance) was filled into an Erlenmeyer flask and connected to a decomposition apparatus with attached traps (methanolic KOH). The active substance in the sample was hydrolyzed under reductive conditions by boiling with stannous hydrochloric acid. The assay was kept boiling for 45 minutes while a slight stream of nitrogen was led through the apparatus. After passage through washing bottles filled with zinc acetate and sulfuric acid, CS_2 was absorbed in a methanolic solution of potassium hydroxide. The methanolic KOH of the trap was made up to volume with methanolic KOH and the formed xanthogenate was measured using a HPLC system equipped with a UV detector at 302 nm.

At each sampling date (apart from day 0) the CS_2 trap containing methanolic KOH was exchanged. The absorber vial and the absorber connection tube were rinsed with 3 mL of methanolic KOH, which was combined with the trapping solution. An aliquot of the solution was used for analysis of CS_2 by quantifying the formed xanthogenate by using HPLC and UV detection at 302 nm.

A control experiment was set up in order to show that CS_2 potentially formed in the soil will be quantitatively released. Soil (110.8 g, moist) was dosed with a stock solution of CS_2 in methanol in an Erlenmeyer flask.
A slight stream of air was led through the flask containing the treated soil that was connected to a trap containing methanolic KOH. The trap was exchanged after 1.5, 3, 6 and 24 hours against a freshly filled one. CS_2 trapped in the methanolic KOH was determined as described above and summed up.

4. Calculation of the degradation rate

Kinetic analysis and calculations of DT_{50} and DT_{90} values were performed following the recommendations of the FOCUS Kinetics workgroup [*FOCUS (2006)*] in order to derive appropriate endpoints for use as triggers for additional work. The analysis was done by non-linear regression methods using the software package KinGUI version 1.1. According to FOCUS Kinetics the observed residues were fitted with SFO and FOMC kinetics. The appropriate kinetic was selected under consideration of the “visual fit assessment” and the χ^2 test. If the better fit was derived by the FOMC kinetic it was checked if other bi-phasic models could further improve the fit quality. The observed metiram soil residues were considered for the fit approach as recommended by FOCUS. Because of the fast decline of the compound, the day 0 sample was corrected for the time period needed for the analytical work-up of the samples of about 1.5 hours (0.0625 d).

The $DegT_{50}$ value of metiram was normalized to reference moisture conditions (pF 2) in a separate kinetic evaluation [*see CA 7.1.2.1.1/7, BASF DocID 2014/1314053*].

II. RESULTS AND DISCUSSION

Degradation of metiram and formation of CS_2

The amount of metiram decreased rapidly from 95.8% of the nominal applied amount at day 0 to 5.2% of the applied amount after 15 days. CS_2 was formed only in small amounts during the course of the study. The highest amount of CS_2 was detected at the first day after treatment with only 1.2% of the theoretical amount in the applied metiram (= "% of applied CS_2"). On the following days, the amount of released CS_2 decreased to very low values. From 7 DAT, the signals were very low and uncharacteristic for CS_2 and the concentration of CS_2 was set to zero.

At the end of the incubation after 15 days, only a low percentage of metiram (including its CS_2 forming metabolites) was still present and formation of CS_2 was no longer detectable. The overall formation of CS_2 from metiram in this study accounted for 2.1% of the theoretical amount in the applied metiram. The time course of the degradation of metiram and the formation of CS_2 in treated soil is shown in Table 7.1.1.1-16.

Table 7.1.1.1-16: Time course of metiram concentration in aerobic soil

DAT	metiram [mg kg^{-1}]	metiram [% AD] [a]	CS_2 (cumulated) [mg kg^{-1}]	CS_2 (cumulated) [% AD] [a]
0	6.042	129.1		
0	6.439	137.6		
0	2.845	60.8		
0	2.600	55.6		
0 mean	4.482	95.8 [b]	n.d.	n.d.
1	2.078	44.4		
1	2.152	46.0		
1 mean	2.115	45.2	0.030	1.2
2	1.120	23.9		
2	1.236	26.4		
2 mean	1.178	25.2	0.042	1.6
3	0.854	18.2		
3	1.081	23.1		
3 mean	0.967	20.7	0.051	2.0
4	0.749	16.0		
4	0.777	16.6		
4 mean	0.763	16.3	0.056	2.1
7	0.436	9.3		
7	0.358	7.7		
7 mean	0.397	8.5	0.056	2.1
10	0.417	8.9		
10	0.476	10.2		
10 mean	0.447	9.5	0.056	2.1
15	0.271	5.8		
15	0.214	4.6		
15 mean	0.242	5.2	0.056	2.1

n.d. = not determined

[a] % AD = % applied dose ("% of applied CS_2" relates to the theoretical amount of CS_2 in the applied metiram)

[b] The 0 DAT sampling was done about 1.5 hours after application. Values showed rather high variation although mean value was acceptable with 95.8%

The quantitative release of CS_2 from soil and its trapping was verified with a control experiment. CS_2 was quantitatively found in the traps (114.1%) with the major part (77.5%) already being trapped after 1.5 hours.

Kinetic modeling results

The soil residues could be best described by the FOMC kinetic fit approach (Table 7.1.1.1-17). DT_{50} and DT_{90} values of metiram according to FOMC kinetics were 0.72 days and 6.6 days, respectively.

Table 7.1.1.1-17: Estimated parameters and DT_{50} and DT_{90} values of metiram

Data Set	Kinetic Model	χ^2 error [%]	$DegT_{50}$ [d]	$DegT_{90}$ [d]
Li 10	FOMC	4.3	0.72	6.6

III. CONCLUSION

The amount of metiram decreased rapidly in aerobic soil. Only small amounts of CS_2 were formed. The cumulative amount of CS_2 formed accounted for 2.1% of the theoretical amount in the applied metiram.

DT_{50} and DT_{90} values of 0.72 and 6.6 days, respectively, were calculated for metiram according to FOMC kinetics.

Special study

In old environmental metabolism studies with metiram (roughly before 1990) difficulties were experienced with the analysis of metabolites and partly high and varying amounts of metabolites were detected. For this reason the following investigation on solvolysis of metiram was performed which is considered an important study in the understanding of environmental fate studies with metiram.

Peer reviewed study (Summary taken from the Monograph or its Addenda):

Report:	*CA 7.1.1.1/5* *Keller E., 1993a* *Identification of Metiram solvolytic degradation products* *1993/10578*
Guidelines:	*<none>*
GLP:	*yes* *(certified by Ministerium fuer Arbeit, Soziales, Familie und Gesundheit, Postfach 3180, 6500 Mainz)*

This study was initiated because it was very difficult to interpret finally the analytical results (mainly TLC data) of the previous studies with metiram. This was mainly due to the following reasons:
- By its chemical nature metiram is insoluble and therefore, upon extraction of the matrices (e.g. soil), the parent material is decomposed.
- Matrix effects (e.g. natural soil constituents) impaired the chromatographic analyses and identification of the degradation products.
- Almost all components were already found in the 0 day samples, often, in addition, with the highest residues compared with the other samplings.

It may be concluded from this that very fast formation of degradation products is rather a solvolytic effect caused by the treatment of metiram with solvents than a biotic or abiotic (in photolysis) process.
To prove this hypothesis the following experiments were carried out.

Materials and Methods:

0.3 - ca. 1 mg [^{14}C]-metiram was added to 1 - 1.6 mL of MeOH, acetonitrile (ACN) or aqueous EDTA solution (10%, pH 8 - 9). The suspension was sonified and samples were taken for analysis from 0 up to 24 hours. The samples were analyzed by radio-HPLC; some of them were also analyzed by radio-TLC in order to have a comparison with the metabolism study.
Final identification of the solvolysis products was done by a series of MS investigations using different application, ionization and record modes.
Application modes: flow injection-API, HPLC-API, flow injection-particle beam, HPLC-particle beam, GC
Ionization modes: positive ions, negative ions, EI, ion spray, CAD-Ar (MS/MS)
Record modes: mass spectrum, MS/MS daughter scan

Findings:

As metiram decomposition products were identified: ethylene urea (EU) (only in few cases and small amounts), ethylene thiourea (ETU), ethylene bis(isothiocyanate) sulfide (EBIS), ethylene thiourea-N-thiocarboxamide (carbimide), and ethylene bis isothiocyanate (EBIC) which proved to be only transient; EBIC could be detected only in the first few hours of incubation.
Using this data a solvolytic decomposition pathway of metiram can be set up which is shown in Figure 7.1.1.1-1.

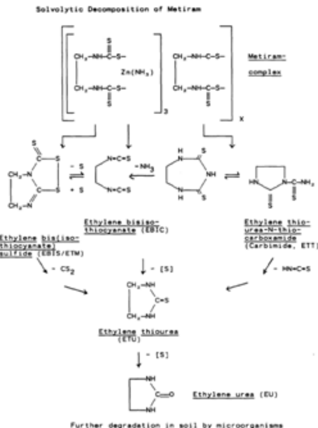


Figure 7.1.1.1-1: Solvolytic decomposition of metiram

Conclusions were that TLC characterizations are not the best means for elucidation of metiram degradation products because solvolysis products decomposed themselves on TLC plates producing complex TLC patterns difficult to be interpreted and that once the two metabolites ETU and EU are formed in soil metabolism studies (aerobic and anaerobic) no further metabolites should be found in substantial amounts because these metabolites are very close to endogenous microbial compounds.

CA 7.1.1.2 Anaerobic degradation

Metiram and all of its degradation products are degraded rapidly in soil under aerobic conditions. Anaerobic conditions are not expected to prevail for extended time periods after application of metiram. Therefore, data on the route of degradation of metiram under anaerobic conditions are not relevant and are considered only as supplementary information.

Studies presented in the first Annex I inclusion process:

Two anaerobic soil metabolism studies were presented in the first Annex I inclusion process [*Keller E. and Huber R., 1985, DocID 1985/10059; Ruedel H., 1990, DocID 1990/0014*] and an overview is given in Table 7.1.1.2-1. Both studies are considered invalid for the following reasons: In the old study of Keller E. and Huber R., 1985 the quantification of metabolites relied solely on radio-TLC analysis which was later on shown to be not a suitable method for the investigation of degradation products of metiram [*see CA 7.1.1.1/5, BASF DocID 1993/10578*].
In the study of Ruedel H. (1990), the soil samples were extracted with 10% EDTA solution in the first extraction step. EDTA leads to immediate destruction of metiram leading to artificially high amounts of degradates. Furthermore also here the analyses for metabolites were mostly done by radio-TLC analysis. The degradation of the active substance was not appropriately followed since the degradation after day 1 was derived from the mineralization rate.

Table 7.1.1.2-1: Studies on anaerobic soil degradation of metiram

Reference	BASF DocID	Soil type	Application rate [mg kg^{-1}]	Incubation temperature [°C]	Incubation period [days]	Remark
Keller E., Huber R., 1985	1985/10059	Loamy sand Loam	10	22	365	Invalid
Ruedel H., 1990	1990/0014	Loamy sand	10	20	60	Invalid

Submission of not yet peer-reviewed study in this AIR3-Dossier:

Since the old anaerobic soil metabolism study is considered invalid, a new study was performed with metiram, which is summarized in CA 7.1.1.2/1.

Report:	CA 7.1.1.2/1 Voelkel W., 2015b 14C-Metiram - Degradation and metabolism in one soil incubated under anaerobic conditions 2013/1095969
Guidelines:	OECD 307 (2002), EPA 835.4100
GLP:	yes (certified by Swiss Federal Office of Public Health, Berne, Switzerland)

Executive Summary

The anaerobic soil metabolism of ^{14}C-labeled metiram was investigated in one soil (Am Fischteich), a silt loam, following treatment with the test item at a nominal application rate of 1.4 kg a.s. ha^{-1} and incubation at 20.9 ± 0.2°C one day under aerobic conditions and after flooding 120 days under anaerobic conditions.

The samples were incubated in a dark, air-conditioned room. Treated samples of 50 g dry weight soil were initially incubated under aerobic conditions for 24 hours, water-logged, and incubated for further 120 days under anaerobic conditions. The incubation system was aerated with moist air for 24 hours during the aerobic phase and thereafter the incubation system was flushed with nitrogen at regular intervals to maintain anaerobic conditions. The exiting air was passed through a trapping system consisting of flasks of sodium hydroxide, potassium hydroxide/methanol, and ethylene glycol in series. Prior to treatment and at the end of the incubation period, the microbial biomass was determined, showing that the soil was viable during the study.

Ten sampling intervals were set for the study: 0, 1, 2, 4, 7, 14, 28, 60, 90 and 120 days after flooding.

Metiram had to be quantified by an indirect CS_2 method, during which metiram was decomposed by stannous hydrochloric acid and the formation of CS_2 was quantified. Two replicates per soil were extracted separately for up to four times using methanol, followed by extractions using up to five times 1% EDTA in ultra-pure water. The individual extracts were combined and analyzed for the degradation products by HPLC.

A fast degradation of metiram was observed. At aerobic conditions, 2.5 hours after treatment, the amount of metiram accounted for 69.7% of the applied amount. The degradation of metiram under anaerobic conditions continued very fast and detected amounts represented 37.1% of the total applied radioactivity (TAR) shortly after flooding (0 days after induced anaerobicity = DAIA) and 21.9% TAR at 1 DAIA. By the end of the incubation period (120 DAIA), only 2.0% TAR could be attributed to metiram. The kinetic evaluation resulted in $DegT_{50}$ and $DegT_{90}$ values of 1.5 and 77.5 days.

The overall mean recovery amounted to 99.6 ± 5.6% TAR for the anaerobic incubation. Total individual recovery of radioactivity ranged from 93.7% to 103.7% TAR.
The amount of radioactivity recovered from soil by methanol extraction decreased slowly from 36.6% TAR at time 0 of the aerobic incubation and 35.7% TAR on day 0 of anaerobic incubation to 24.8% TAR by the end of the 120-day anaerobic incubation period.
The amount of radioactivity recovered by extraction with 1% EDTA on day 0 of the aerobic incubation accounted for 31.7% TAR. The amount of radioactivity in the 1% EDTA extracts under anaerobic conditions slowly decreased over time from 22.2% TAR (day 0 of anaerobic incubation) to a minimum of 10.0% TAR at 90 DAIA and still represented 13.4% TAR at 120 DAIA.

Bound residues (non-extractable) at time 0 of the aerobic incubation amounted to 43.0% TAR. Under anaerobic conditions non-extractable radioactivity increased from 39.5% TAR (0 DAIA) to a maximum of 54.0% TAR at 90 DAIA, followed by a slight decrease to 46.9% TAR at 120 DAIA.

The residual radioactivity remaining in selected soil samples after extractions (after 0, 60 and 120 DAIA) was subjected to a fractionation of the soil organic matter into humic acids, fulvic acids, and humin fractions. Their proportions in percent of non-extractable radioactivity remained stable over time. Fulvic acids accounted for 20.6 to 26.0% TAR. The amount of humic acids ranged from 11.1 to 12.3% TAR. The remaining humin fraction accounted for 7.9 to 13.6% TAR.

The amount of radioactive carbon dioxide increased from 2.6% TAR shortly after flooding (0 DAIA) to 13.4% TAR at 120 DAIA. Other radioactive volatile products did not exceed 0.1% TAR at any sampling interval. Amounts of CS_2 trapped varied in the individual traps but never exceeded 6% of the theoretical amount applied.

^{14}C-metiram degraded into 20 mostly minor radioactive fractions. M222F004 (EBIS) was one of the two major radioactive fractions exceeding 10% of the total amount applied in the methanol extracts. In the methanol extracts, M22F004 (EBIS) accounted for 25.7% TAR at time 0 of the aerobic incubation, followed by a fast decrease to 6.2% TAR at time 0 of anaerobic incubation. Thereafter, M222F004 (EBIS) further degraded and was not detectable anymore after 120 days of anaerobic incubation. The second major radioactive fraction M222F003 (EU) amounte to 2.7% TAR at time 0 of the aerobic incubation, followed by an increase to 8.2% TAR at time 0 of anaerobic incubation. M222F003 (EU) increased to 13.3% TAR after 7 days of anaerobic incubation and remained at that level until the end of anaerobic incubation after 120 days, representing 11.8% TAR. M222F002 (ETU) was not detected at time 0 of aerobic incubation, but amounted to 1.8% TAR on day 0 of anaerobic incubation. Thereafter, it slowly increased to a maximum of 9.3% TAR on day 14 of anaerobic incubation and decreased to 6.3% TAR after 120 days. None of the other radioactive fractions reached a concentration of 10% TAR or 5% TAR at two consecutive sampling intervals, respectively.

The reported values of ^{14}C-metiram (in % TAR) were subjected to kinetic analysis resulting in $DegT_{50}$ and $DegT_{90}$ values of 1.5 and 77.5 days. The calculated $DegT_{50}$ value of the metabolites EBIS, ETU, and EU were 1.4, 188 and greater 1000 days, respectively, and the corresponding $DegT_{90}$ values were 4.6, 624, and greater 1000 days, respectively.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

BAS-Code:	BAS 222 F (metiram)
Registry No.:	250284
Chemical name:	Zinc ammoniate ethylenebis(dithiocarbamate)-poly-(ethylenethiuramdisulfide)
Molecular formula:	$(C_4H_9N_3S_4Zn)_3$; $(C_4H_6N_2S_4)x$
Molar mass:	1088.7 g mol^{-1} (unlabeled)

Labeled test item

Label:	^{14}C-ethylene
Batch No.:	153-6001
Specific activity of a.s.:	7.64 MBq mg^{-1}
Releasable CS_2:	89.0%

Non-labeled test item

Batch No.:	CP052744
Purity:	93.6%

2. Soil

The study was conducted with one soil (Am Fischteich) classified as silt loam (USDA) and was collected from the top layer (0-20 cm depth) of a field near Crimmitschau, Germany. The soil was passed through a 2 mm sieve and stored moist at 4°C for a period of less than one month. An overview of the soil parameters is listed in Table 7.1.1.2-2.

Table 7.1.1.2-2: Characteristics of Am Fischteich soil used for anaerobic soil metabolism study with ^{14}C-metiram

Soil designation	**Am Fischteich** **11/12** Crimmitschau, Germany
USDA Particle size distribution [%] sand 0.050 – 2 mm silt 0.002 – 0.050 mm clay < 0.002 mm textural class	 11.9 67.2 20.9 silt loam
Organic C [%]	1.62
Organic matter [%] [a)]	2.79
pH ($CaCl_2$) pH (H_2O)	6.3 6.7
Cation exchange capacity [mmol 100 g^{-1} soil]	15.8
Water holding capacity (at pF 2) [g 100g^{-1} dry weight]	33.0
Biomass Start of anaerobic incubation [mg C/kg soil] End of anaerobic incubation [mg C/kg soil]	 442.0 309.7

[a)] % organic matter = 1.724 x % organic carbon

B. STUDY DESIGN

1. Experimental conditions

The soil was treated with the ^{14}C-test item by batch application. The application suspension was applied drop-wise to the soil surface (2700 g dry weight). The soil was mixed thoroughly along the way using a hand mixer. The test item was applied with a rate of 5.6 mg active ingredient (a.s.) per kg dry soil, which corresponds to an application rate of 1.4 kg a.s. ha^{-1}.

Control samples used for microbial biomass determination were not treated.

50 g soil aliquots were incubated in an air-conditioned room in the dark at a temperature of 20.9 ± 0.23°C. The experiment was performed in an open gas-flow system. The soil moisture content was approximately 22.3% during the initial aerobic incubation period.

The incubation system was aerated with moist air for 24 hours during the initial aerobic phase. After flooding, the samples were ventilated with nitrogen at regular intervals to maintain anaerobic conditions. Each interval lasted about 10 minutes at a minimum flow rate to pass through the trapping system consisting of flasks of sodium hydroxide, potassium hydroxide/methanol, and ethylene glycol in series. For the aerobic sampling on day 0, no absorption traps were set up.

At each sampling interval during the anaerobic incubation period, the following parameters were measured in the treated flasks to be sampled and in two untreated control samples: pH, oxygen concentration in water, and redox potential of the aqueous layer.

2. Sampling

Samples were taken at 0, 1, 2, 4, 7, 14, 28, 60, 90 and 120 days after flooding. Trapping solutions of samples were taken on the corresponding sampling day. Trapping solutions were in addition exchanged at regular intervals, depending on the formation of CS_2 or $^{14}CO_2$.

Untreated samples were taken for the determination of the microbial biomass on day 0 of aerobic incubation (start) and on day 120 of anaerobic incubation (end).

3. Description of analytical procedures

Metiram determination

Metiram was quantified by an indirect CS_2 method, during which metiram is decomposed by stannous hydrochloric acid. The formed CS_2 was trapped in the methanolic solution of potassium chloride and quantified by UV detection.

The limit of detection (LOD) and limit of quantification (LOQ) for the UV-spectrophotometrical analysis of CS_2 were determined to be 0.37% and 0.74% TAR, respectively.

Extraction and isolation of radioactivity from soil

Duplicate samples were extracted separately using methanol (up to four times) followed by extractions with 1% EDTA in ultra-pure water adjusted to pH 8 with 10 M NaOH (five times). The extracts containing more than 2% TAR were combined separately according to the extraction solution used. If needed, pooled extracts were in addition concentrated in a rotary evaporator at 30°C or 35°C. The pooled extracts were measured by LSC for recovery and submitted to HPLC analysis. Degradates of metiram were subjected to structure elucidation using HPLC-MS/MS.

The residual radioactivity remaining in soil after the extraction procedure was quantified by LSC after combusting aliquots of the air-dried and homogenized soil.

For sampling intervals 0, 60 and 120 days of anaerobic incubation, the extracted soil was submitted to an organic matter fractionation in order to determine the amount of radioactivity in the fulvic acids, humic acids, and humin fractions. Aliquots of the extracted soil samples were extracted four times using 0.5 M NaOH, centrifuged and the volume of the supernatant was recorded. The extraction with 0.5 M NaOH was repeated once more. The liquid phase was again separated from the solid by centrifugation and the non-extractable residues (containing the humin fraction) were quantified by LSC after combusting aliquots of the air-dried and homogenized soil.

The radioactivity in the NaOH extracts (containing the humic and fulvic acids) was quantified by LSC. The extracts were then pooled and adjusted to pH 1 by adding HCl. After centrifugation, the precipitate (containing the humic acids) was dissolved in 0.5 M NaOH and submitted to LSC. The radioactivity in the supernatant (containing the fulvic acids) was quantified by LSC. In addition, an aliquot of the supernatant was adjusted to pH 2-3 by adding NaOH and was submitted to HPLC analysis.

For HPLC, the LOQ was calculated to be 0.370% (0.020 mg kg^{-1}) and 0.340% TAR (0.019 mg kg^{-1}) for methanol and EDTA, respectively. The LOD was determined to be half of these amounts, i.e. 0.180% (0.010 mg kg^{-1}) and 0.170% TAR (0.009 mg kg^{-1}) for methanol and EDTA, respectively.

Trapping solutions

The volumes of the trapping solutions were recorded and radioactivity was determined by LSC.

In order to confirm the presence of $^{14}CO_2$, the radioactivity contained in the sodium hydroxide traps was precipitated with barium hydroxide on a pooled sample. The absence of radioactivity in the supernatants after precipitation was taken as proof that only $^{14}CO_2$ was present in the sodium hydroxide solutions.

The 24-hour aerobic incubation phase was analyzed in a separate study and the results at time 0 were taken from this study [*see CA 7.1.1.1/3, BASF DocID 2013/1095967*].

4. Calculation of the degradation rate

Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints). Kinetic analysis and calculation of $DegT_{50}$ and $DegT_{90}$ values was performed following the recommendations of the FOCUS Kinetics workgroup [*FOCUS (2006)*]. The software package KinGUII (version 2.2014.224.1704) was used for parameter fitting.

For the parent substance metiram and the metabolites EBIS, ETU, and EU replicate measurements from analysis were used for the parameter estimation. The endpoints of the parent compound metiram and its metabolites EBIS, ETU, and EU were derived from a compartment modeling approach.

The appropriateness of the distinct kinetic model to describe soil degradation was tested by visual assessment, estimation of the error percentage at which the χ^2 test was passed, and the t-test to evaluate whether estimated degradation parameters differ from zero.
The trigger endpoints were derived from the kinetic models that provided the best-fit to the measured data, generally indicated by the lowest χ^2 – error and a visual assessment of the fit.

II. RESULTS AND DISCUSSION

Following flooding of the flasks containing the treated samples, dissolved oxygen concentrations decreased rapidly in the samples to levels below 1 mg/L, indicating anaerobic conditions. The redox potential was measured on 0, 60, 90 and 120 DAIA; between 0 and 60 DAIA no measurement was possible due to an instrument malfunction. The positive redox potential immediately following flooding was followed by negative redox potential on 60 DAIA, then strongly negative values on 90 and 120 DAIA also indicated anaerobic conditions, similar to the trend observed with the oxygen concentration.

A. MASS BALANCE

The two samples from the aerobic incubation time 0 interval amounted to a mean recovery of 111.3% TAR. The overall mean recovery of total applied radioactivity for the samples under anaerobic conditions amounted to 99.6 ± 5.6% TAR. Total individual recovery of radioactivity during the 120-day anaerobic incubation period ranged from 93.7% to 103.7% TAR (Table 7.1.1.2-3).

Table 7.1.1.2-3: Material balance and distribution of radioactivity after application of ^{14}C-metiram to soil Am Fischteich under aerobic and anaerobic conditions [% TAR]

Incubation Time (days)	Replicate	Methanol Extracts	EDTA Extracts	Total extractable residues	Non-extractable residues	$^{14}CO_2$	Other volatiles in EG [b]	Other volatiles in KOH/Methanol	TOTAL	MEAN ± SD
0(2.5h) [a] (aerobic)	A	38.3	32.4	70.7	45.4	n.p.	n.p.	n.p.	116.1	
	B	34.8	31.1	65.9	40.5	n.p.	n.p.	n.p.	106.5	
	Mean	**36.6**	**31.7**	**68.3**	**43.0**	**n.p.**	**n.p.**	**n.p.**	**111.3**	
0 (anaerobic)	A	32.8	20.9	53.7	37.9	2.2	<0.1	<0.1	93.7	99.6 ± 5.6
	B	38.6	23.6	62.2	41.1	3.1	<0.1	<0.1	106.5	
	Mean	**35.7**	**22.2**	**58.0**	**39.5**	**2.6**	**<0.1**	**<0.1**	**100.1**	
1	A	32.0	19.3	51.4	42.8	2.6	<0.1	<0.1	96.8	
	B	31.8	19.9	51.7	45.9	3.0	<0.1	<0.1	100.6	
	Mean	**31.9**	**19.6**	**51.5**	**44.3**	**2.8**	**<0.1**	**<0.1**	**98.7**	
2	A	26.6	18.0	44.6	41.8	3.8	<0.1	<0.1	90.2	
	B	31.8	19.9	51.8	43.0	2.4	<0.1	<0.1	97.2	
	Mean	**29.2**	**19.0**	**48.2**	**42.4**	**3.1**	**<0.1**	**<0.1**	**93.7**	
4	A	33.0	19.5	52.5	43.3	3.3	<0.1	<0.1	99.1	
	B	28.5	17.5	45.9	41.6	2.9	<0.1	<0.1	90.5	
	Mean	**30.7**	**18.5**	**49.2**	**42.4**	**3.1**	**<0.1**	**<0.1**	**94.8**	
7	A	33.0	20.3	53.3	46.4	4.1	<0.1	0.1	103.9	
	B	31.6	19.6	51.2	47.2	4.2	<0.1	<0.1	102.6	
	Mean	**32.3**	**20.0**	**52.3**	**46.8**	**4.1**	**<0.1**	**0.1**	**103.2**	
14	A	30.6	18.4	49.0	49.1	6.0	<0.1	<0.1	104.1	
	B	30.7	16.3	46.9	46.7	3.8	<0.1	<0.1	97.4	
	Mean	**30.6**	**17.3**	**48.0**	**47.9**	**4.9**	**<0.1**	**<0.1**	**100.7**	
28	A	34.1	16.8	50.9	49.1	5.8	<0.1	<0.1	105.8	
	B	30.2	16.9	47.1	41.8	7.3	<0.1	<0.1	96.3	
	Mean	**32.1**	**16.9**	**49.0**	**45.5**	**6.5**	**<0.1**	**<0.1**	**101.0**	
60	A	26.9	14.4	41.3	52.2	12.6	<0.1	<0.1	106.1	
	B	27.5	13.1	40.6	51.6	9.1	<0.1	0.1	101.3	
	Mean	**27.2**	**13.8**	**40.9**	**51.9**	**10.8**	**<0.1**	**<0.1**	**103.7**	
90	A	25.0	7.9	32.9	52.5	10.8	<0.1	<0.1	96.2	
	B	27.6	12.0	39.6	55.5	12.2	<0.1	<0.1	107.3	
	Mean	**26.3**	**10.0**	**36.3**	**54.0**	**11.5**	**<0.1**	**<0.1**	**101.7**	
120	A	20.5	11.2	31.8	46.0	13.7	<0.1	<0.1	91.4	
	B	29.0	15.6	44.6	47.8	13.2	<0.1	<0.1	105.7	
	Mean	**24.8**	**13.4**	**38.2**	**46.9**	**13.4**	**<0.1**	**<0.1**	**98.5**	

TAR = Total applied radioactivity
SD = Standard deviation
n.p.= Not performe
[a] Aerobic incubation lasted 24 hours before flooding
[b] ethylene glycol

B. EXTRACTABLE AND BOUND RESIDUES

The extraction procedure of soil treated with metiram must take into account the very special characteristics of this active substance. Intact polymeric Metiram itself will not be extracted since it is insoluble in all solvents. However, during the course of the extraction the influence of the extractant will lead to a certain decomposition of the a.s. accompanied by the formation of artificial amounts of degradates. The solvent extraction scheme has to be understood as a compromise between obtaining highest extractability and avoiding formation of high amounts of artificial degradates.

The intention of the final EDTA extraction was to destroy metiram remaining in the soil after the solvent extraction. Since metiram is insoluble in methanol (as in other solvents) it would remain unextracted which would lead to false (i.e. artificially increased) amounts of bound residues.

The amount of radioactivity recovered from soil by methanol extraction decreased slowly from 36.6% TAR at time 0 of the aerobic incubation and 35.7% TAR on day 0 of anaerobic incubation to 24.8% TAR by the end of the 120-day anaerobic incubation period.

The amount of radioactivity recovered by extraction with 1% EDTA on day 0 of the aerobic incubation accounted for 31.7% TAR. The amount of radioactivity in the 1% EDTA extracts under anaerobic conditions slowly decreased over time from 22.2% TAR (day 0 of anaerobic incubation) to a minimum of 10.0% TAR 90 DAIA and still represented 13.4% TAR 120 DAIA.

C. VOLATILIZATION

The amount of radioactive carbon dioxide increased from 2.6% TAR shortly after flooding (0 DAIA) and accounted for 13.4% TAR at 120 DAIA.

Radioactive volatile products other than $^{14}CO_2$ did not exceed 0.1% TAR at any sampling interval. CS_2 was determined in methanolic potassium hydroxide traps. The amount of CS_2 detected varied in the individual traps but never exceeded 6% (day 0) of the theoretical amount applied.

D. TRANSFORMATION OF PARENT COMPOUND

Degradation of metiram

The degradation of metiram, as determined by the CS_2 method, is summarized in Table 7.1.1.2-4.

A fast degradation of metiram was observed. 2.5 hours after treatment, the amount of metiram accounted for 69.7% TAR. The degradation of metiram under anaerobic conditions continued very fast resulting in an average of 37.1% TAR shortly after flooding (0 days anaerobic conditions) and reaching 21.9% TAR one day after flooding. Metiram continued to degrade rapidly, representing only 2.0% TAR by the end of the incubation period (120 DAIA).

Table 7.1.1.2-4: Degradation of metiram in Am Fischteich soil as determined by the CS_2 method

Incubation Time (days)	Replicate	^{14}C-metiram [mg kg^{-1}]	[% AR]
Aerobic Incubation			
0 (2.5h) [a]	A	3.978	
	B	4.161	
	Mean	**4.070**	**69.7**
Anaerobic incubation			
0	A	1.969	
	B	2.187	
	Mean	**2.078**	**37.1**
1	A	1.110	
	B	1.338	
	Mean	**1.224**	**21.9**
2	A	0.885	
	B	0.914	
	Mean	**0.899**	**16.1**
4	A	0.589	
	B	0.495	
	Mean	**0.542**	**9.7**
7	A	0.477	
	B	0.349	
	Mean	**0.413**	**7.4**
14	A	0.783	
	B	0.234	
	Mean	**0.508**	**9.1**
28	A	0.358	
	B	0.212	
	Mean	**0.285**	**5.1**
60	A	0.346	
	B	0.266	
	Mean	**0.306**	**5.5**
90	A	0.208	
	B	0.192	
	Mean	**0.200**	**3.6**
120	A	0.111	
	B	0.111	
	Mean	**0.111**	**2.0**

AR = Applied Residue
[a] Aerobic incubation lasted 24 hours before flooding

Pattern of metabolite formation

The pattern of anaerobic degradation and formation of metabolites in methanol extracts of Am Fischteich soil treated with ^{14}C-metiram is summarized in Table 7.1.1.2-5.

^{14}C-Metiram degraded into 20 radioactive fractions. Most of them were minor fractions.

M222F004 (EBIS) was one of the two major radioactive fractions exceeding 10% of the total amount applied in the methanol extracts. In the methanol extracts, EBIS accounted for 25.7% TAR at time 0 of the aerobic incubation, followed by a fast decrease to 6.2% TAR at time 0 of anaerobic incubation. Thereafter, EBIS further degraded and was not detectable anymore after 120 days of anaerobic incubation.

The second major radioactive fraction M222F003 (EU) accounted for 2.7% of TAR at time 0 of the aerobic incubation, followed by an increase to 8.2% of TAR at time 0 of anaerobic incubation. M222F003 increased to 13.3% TAR after 7 days of anaerobic incubation and remained at that level until the end of anaerobic incubation after 120 days representing 11.8% TAR. M222F003 was detected as two distinct peaks, but LC/MS analyses showed that both corresponded to the same compound.

M222F002 (ETU) was not detected at time 0 of aerobic incubation, but amounted to 1.8% TAR on day 0 of anaerobic incubation. It slowly increased to 9.3% TAR on day 14 of anaerobic incubation and decreased to 6.3% TAR after 120 days.

The minor radioactive fraction UK1, exceeding 5% TAR only once, decreased from 7.9% TAR on day 0 of aerobic incubation to 2.9% TAR on day 0 of the anaerobic incubation and was not any longer detected after 2 days.

The minor radioactive fraction M222F010 exceeded 5% TAR only once with 8.5% TAR at day 0 of the anaerobic incubation. Further on it decreased to lower, however slightly vaying amounts.

Metabolites M222F004 (EBIS), M222F002 (ETU), M222F003 (EU) and M222F010 were identified and confirmed by structure elucidation using HPLC-MS/MS.

None of the other radioactive fractions reached amounts of 10% or 5% TAR at two consecutive sampling intervals, respectively.

Table 7.1.1.2-5: Radio-HPLC analysis of methanol extracts of Am Fischteich soil with ^{14}C-metiram [% TAR]

		Metabolic Fraction															
		EU		EU total	ETU	UK18	UK7	UK17	UK8	UK16	UK9	UK11	UK14	M222F010	EBIS	UK15	UK1
Time (days)	Repli-cate	Retention Time (min)															
		2.90-3.80	3.80-4.80	2.90-4.80	4.80-6.20	6.40-7.00	7.20-8.20	8.40-9.70	9.90-12.20	12.20-13.10	13.30-14.80	17.10-18.70	22.40-25.20	28.60-30.00	30.50-30.70	31.80-33.70	34.40-35.90
0 (2.5h)[a] aerobic	A	1.7	1.1	2.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.7	n.d.	n.d.	n.d.	26.3	n.d.	8.5
	B	2.6	n.d.	2.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	25.1	n.d.	7.2
	Mean	**2.1**	**0.5**	**2.7**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**0.3**	**n.d.**	**n.d.**	**n.d.**	**25.7**	**n.d.**	**7.9**
0 anaerobic	A	5.4	2.5	7.9	1.8	1.2	n.d.	n.d.	2.7	n.d.	1.4	n.d.	1.0	8.2	6.1	n.d.	2.7
	B	6.1	2.4	8.5	1.8	1.7	1.6	1.6	2.5	n.d.	1.4	n.d.	1.2	8.9	6.4	n.d.	3.2
	Mean	**5.8**	**2.4**	**8.2**	**1.8**	**1.5**	**0.8**	**0.8**	**2.6**	**n.d.**	**1.4**	**n.d.**	**1.1**	**8.5**	**6.2**	**n.d.**	**2.9**
1	A	1.3	7.9	9.2	4.0	3.7	1.7	n.d.	2.0	n.d.	n.d.	1.0	n.d.	1.7	4.8	2.3	1.5
	B	1.4	8.5	9.9	6.6	2.7	1.3	n.d.	1.8	n.d.	n.d.	n.d.	n.d.	1.8	6.4	1.3	n.d.
	Mean	**1.4**	**8.2**	**9.6**	**5.3**	**3.2**	**1.5**	**n.d.**	**1.9**	**n.d.**	**n.d.**	**0.5**	**n.d.**	**1.8**	**5.6**	**1.8**	**0.7**
2	A	1.0	9.5	10.5	2.4	1.8	1.7	n.d.	2.2	n.d.	n.d.	1.3	n.d.	1.9	2.7	2.0	n.d.
	B	1.0	8.3	9.3	5.6	3.1	2.3	n.d.	1.9	1.3	n.d.	1.6	n.d.	1.9	3.3	1.5	n.d.
	Mean	**1.0**	**8.9**	**9.9**	**4.0**	**2.5**	**2.0**	**n.d.**	**2.0**	**0.7**	**n.d.**	**1.4**	**n.d.**	**1.9**	**3.0**	**1.8**	**n.d.**
4	A	3.8	7.8	11.6	6.2	2.3	2.5	n.d.	1.2	0.5	n.d.	1.5	n.d.	3.2	1.7	1.8	n.d.
	B	3.0	7.6	10.6	3.6	4.7	2.6	n.d.	1.2	n.d.	n.d.	1.0	n.d.	2.7	1.4	0.8	n.d.
	Mean	**3.4**	**7.7**	**11.1**	**4.9**	**3.5**	**2.5**	**n.d.**	**1.2**	**0.2**	**n.d.**	**1.2**	**n.d.**	**3.0**	**1.6**	**1.3**	**n.d.**
7	A	1.2	13.3	14.5	5.5	2.1	2.8	n.d.	2.0	n.d.	n.d.	0.7	n.d.	4.0	0.7	0.7	n.d.
	B	0.9	11.2	12.1	8.1	n.d.	2.2	2.0	0.7	0.7	0.9	n.d.	n.d.	3.4	1.5	n.d.	n.d.
	Mean	**1.0**	**12.3**	**13.3**	**6.8**	**1.0**	**2.5**	**1.0**	**1.4**	**0.4**	**0.4**	**0.4**	**n.d.**	**3.7**	**1.1**	**0.4**	**n.d.**
14	A	1.2	n.d.	1.2	11.8	6.2	3.7	1.2	n.d.	2.2	n.d.	n.d.	n.d.	4.3	n.d.	n.d.	n.d.
	B	1.2	9.0	10.2	6.9	1.7	2.0	1.8	2.5	0.7	n.d.	n.d.	n.d.	3.6	1.3	n.d.	n.d.
	Mean	**1.2**	**4.5**	**5.7**	**9.3**	**3.9**	**2.8**	**1.5**	**1.2**	**1.5**	**n.d.**	**n.d.**	**n.d.**	**4.0**	**0.7**	**n.d.**	**n.d.**
28	A	1.0	15.0	15.9	5.7	3.2	2.5	n.d.	1.5	1.3	n.d.	n.d.	n.d.	3.2	0.8	n.d.	n.d.
	B	0.7	9.9	10.6	9.3	1.6	2.6	n.d.	0.8	1.3	n.d.	n.d.	n.d.	2.0	2.0	n.d.	n.d.
	Mean	**0.8**	**12.4**	**13.3**	**7.5**	**2.4**	**2.5**	**n.d.**	**1.2**	**1.3**	**n.d.**	**n.d.**	**n.d.**	**2.6**	**1.4**	**n.d.**	**n.d.**
60	A	1.4	11.7	13.1	7.5	n.d.	2.5	1.1	1.1	n.d.	n.d.	n.d.	n.d.	1.5	n.d.	n.d.	n.d.
	B	1.9	11.1	13.0	5.5	3.7	2.6	0.8	0.5	n.d.	n.d.	n.d.	n.d.	1.3	n.d.	n.d.	n.d.
	Mean	**1.7**	**11.4**	**13.1**	**6.5**	**1.8**	**2.6**	**0.9**	**0.8**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**1.4**	**n.d.**	**n.d.**	**n.d.**
90	A	1.2	11.6	12.8	2.2	4.9	1.6	1.1	0.6	0.4	n.d.	n.d.	n.d.	0.9	0.5	n.d.	n.d.
	B	1.9	13.2	15.1	6.2	2.3	1.6	0.3	0.7	n.d.	n.d.	n.d.	n.d.	0.7	0.6	n.d.	n.d.
	Mean	**1.6**	**12.4**	**14.0**	**4.2**	**3.6**	**1.6**	**0.7**	**0.6**	**0.2**	**n.d.**	**n.d.**	**n.d.**	**0.8**	**0.5**	**n.d.**	**n.d.**
120	A	1.4	8.9	10.3	3.7	2.4	1.1	1.3	0.5	0.3	n.d.	n.d.	n.d.	1.1	n.d.	n.d.	n.d.
	B	2.0	11.3	13.3	8.8	2.7	2.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.8	n.d.	n.d.	n.d.
	Mean	**1.7**	**10.1**	**11.8**	**6.3**	**2.5**	**1.7**	**0.6**	**0.3**	**0.1**	**n.d.**	**n.d.**	**n.d.**	**1.4**	**n.d.**	**n.d.**	**n.d.**

TAR = Total applied radioactivity
n.d. = Not detected (LOD: 0.18 % TAR)
[a] Aerobic incubation lasted 24 hours before flooding

The EDTA extracts were also analyzed. Due to higher amounts of radioactivity than expected, it was concluded that extraction with aqueous EDTA not only leads to a release of metiram "monomers" by complexation of the Zn atom but also to a beginning disaggregation of bound radioactivity in the high molecular weight humic substances potentially due to the same metal complexing mechanism.

The use of complexing agents like EDTA is very artificial and the radioactive compounds released by EDTA are considered to be of no environmental relevance. Therefore, the results of the EDTA extracts were not used for further evaluation.

E. CHARACTERIZATION OF NON-EXTRACTABLE RESIDUES (NER)

Bound residues (non-extractable) at time 0 of the aerobic incubation amounted to 43.0% TAR. Under anaerobic conditions non-extractable radioactivity increased from 39.5% TAR (0 DAIA) to a maximum of 54.0% TAR at 90 DAIA, followed by a slight decrease to 46.9% TAR at 120 DAIA.

The residual radioactivity remaining in selected soil samples after extraction (after 0, 60 and 120 DAIA) was subjected to a fractionation of the soil organic matter into humic acids, fulvic acids, and humin fractions.

The proportions of fulvic acids, humic acids, and humin fractions in percent of non-extractable radioactivity remained stable over time (Table 7.1.1.2-6). Fulvic acids accounted for 20.6 to 26.0% TAR. The amount of humic acids ranged from 11.1 to 12.3% TAR. The remaining humin fraction accounted for 7.9 to 13.6% TAR.

Table 7.1.1.2-6: Organic matter fractionations of Am Fischteich soil at various interval days [% TAR]

Anaerobic incubation Interval day	Replicate	NER	Fulvic acids	Humic acids	Humin
0	A	37.9	20.0	10.4	7.5
	B	41.1	21.2	11.7	8.3
	Mean	**39.5**	**20.6**	**11.1**	**7.9**
60	A	52.2	26.0	12.4	13.9
	B	51.6	26.0	12.1	13.4
	Mean	**51.9**	**26.0**	**12.3**	**13.6**
120	A	46.0	21.6	11.3	13.1
	B	47.8	22.4	11.9	13.5
	Mean	**46.9**	**22.0**	**11.6**	**13.3**

NER = Non-extractable radioactive residues
% TAR = Percent of total applied radioactivity

F. KINETIC MODELING RESULTS

The reported values of ^{14}C-metiram (in % TAR) and its metabolites M222F004 (EBIS), M222F003 (EU), and M222F002 (ETU) were subjected to kinetic analysis.

The visual assessment indicated plausible fits for the parent compound and the metabolites. For the metabolites χ^2-error values above 15 % were obtained which can be attributed to the low level of measured residues as well as to the rapid transformation processes that are symptomatic for the degradation of Metiram in soil. As the residuals were randomly scattered around the zero line, bi-phasic degradation behavior of the metabolites were not indicated.

Their calculated $DegT_{50}$ (half-live) and $DegT_{90}$ values for use as triggers for additional work are presented in Table 7.1.1.2-7.

Table 7.1.1.2-7: Rates of degradation of ^{14}C-metiram and its metabolites EBIS, ETU, and EU under anaerobic conditions

Analyte	Model	χ^2-error [%]	$DegT_{50}$ [d]	$DegT_{90}$ [d]
^{14}C-Metiram	DFOP	5.9	1.5	77.5
M222F004 (EBIS)	SFO	24.6	1.4	4.6
M222F002 (ETU)	SFO	16.8	187.7	623.5
M222F003 (EU)	SFO	15.9	>1000*	>1000*

*default value as the degradation rate constant was not estimated significantly different from zero

III. CONCLUSION

The anaerobic degradation of ^{14}C-metiram was studied in one soil (Am Fischteich) by incubating at about 20°C for one day under aerobic conditions and after flooding for 120 days under anaerobic conditions.

A fast initial degradation of metiram was observed and a $DegT_{50}$ value of 1.5 days was determined using the CS_2 method. However, the $DegT_{90}$ was longer with 77.5 days.

Under anaerobic conditions, the same metabolites were observed as under aerobic conditions, however at slightly different concentrations and with different degradation rates. Major metabolites >10% TAR were M222F004 (EBIS) and M222F003 (EU). M222F002 (ETU) exceeded 5% TAR at successive sampling events. Their $DegT_{50}$ values were calculated to be 1.4 d, >1000 d and 188 d, respectively. Additionally, a range of minor metabolites was observed. Finally, moderate amounts of CO_2 and bound residues were formed.

CA 7.1.1.3 Soil photolysis

Studies presented in the first Annex I inclusion process:

Two soil photolysis studies were presented, one with metiram and the other one with its metabolite ETU. An overview is given in Table 7.1.1.3-1.
The study with ETU [*BASF DocID 1987/10155*] is considered still valid and is summarized in CA 7.1.1.3/2.
The study with metiram [*Klein W., 1986, DocID 1986/10171*] is considered invalid, for the following reasons:
The quantification of metabolites relied solely on radio-TLC analysis which was later on shown to be not a suitable method for the investigation of degradation products of metiram [*see CA 7.1.1.1/5, BASF DocID 1993/10578*]. Although HPLC is mentioned in the report this was not used for quantification. As the second and third extractant inappropriate extraction solutions (solvent mixtures with water or water alone) were used which lead to enhanced destruction of metiram and the formation of artificially high concentration of degradates. The degradation of the active substance was not appropriately followed since only the extractable radioactivity was taken as a measure for the degradation of the active substance.

Table 7.1.1.3-1: Studies on soil photolysis performed with metiram and ETU

Reference	BASF DocID	Soil type	Application rate [mg kg^{-1}]	Wavelength [nm]	Irradiation period [days]	Remark
Klein W., 1986	1986/10171	Loamy sand	8.5 (metiram)	≥ 290	30	Invalid
Carpenter M., 1987	1987/10155	Silty loam	9.6 (ETU)	> 290	8	Relevant

Submission of not yet peer-reviewed study in this AIR3-Dossier:

Since the old soil photolysis study with metiram is considered invalid, a new study was performed, which is summarized in CA 7.1.1.3/1.

Report:	CA 7.1.1.3/1 Adam D., 2015a 14C-Metiram: Photolysis on soil surface 2013/1095968
Guidelines:	EPA 835.2410, Draft OECD Guideline: Phototransformation of Chemicals in Soil Surfaces (Jan. 2002)
GLP:	yes (certified by Swiss Federal Office of Public Health)

Executive Summary

A soil photolysis was conducted with ^{14}C-labeled-metiram (BAS 222 F) to investigate its behavior on soil surface at 20 ± 2°C under the influence of light. For this purpose, ^{14}C-metiram was applied to a moist soil layer (Am Fischteich; silt loam). Each sample (irradiated and dark) was uniformly treated with 148.4 µg of test item, corresponding to 1.187 kg ha^{-1}.

The total irradiation time was 15 days of continuous irradiation, equivalent to approximately 27.5 days of natural summer sunlight at latitudes 30°N to 50°N. Dark control samples were also incubated for 15 days under the same conditions.

Two samples of about 3 g dry weight equivalents each were used for metiram determination. Two further samples were used for extraction of radioactivity in order to determine the material balance and formation and decline of metabolites. Metiram had to be quantified by an indirect CS_2 method, during which metiram was decomposed by stannous hydrochloric acid and the formation of CS_2 was quantified. Two replicates per soil were extracted separately for up to four times using methanol, followed by extractions using up to five times 1% EDTA in ultra-pure water. The individual extracts were combined and analyzed for the degradation products by HPLC.

Duplicate samples were taken and analyzed 1.5 hours after treatment (time 0), and after 1, 2, 4, 7 and 15 days of continuous irradiation. Dark control samples were taken at the same sampling intervals. The individual extracts were combined and analyzed for the degradation products by HPLC. At all sampling intervals (except time 0), duplicate NaOH, methanolic KOH, ethylene glycol and H_2SO_4 volatile trapping solutions were removed for analysis.

A fast degradation of metiram was observed under irradiation and in the dark. The first sample analyzed was obtained after 1.5 hours of contact between metiram and the soil, due to the work-up procedure. 1.5 hours after treatment, the amount of metiram in the irradiated samples accounted for 42.6% of the applied amount. After 15 days of incubation, 2.1% of applied test item could be attributed to metiram. In the dark control samples, the amount of metiram decreased from 42.6% (day 0 = 1.5 hours) to 4.1% of applied test item after 15 days of incubation.

On day 0 (1.5 hours after treatment), the overall extractable radioactivity (sum of methanol and EDTA extracts) was 71.3% TAR. In the irradiated samples, the overall extractable radioactivity continuously decreased to 30.9% TAR on day 15. In the dark samples, the overall extractable radioactivity continually decreased to a minimum of 25.8% TAR on day 15.

On day 0 (1.5 hours after treatment), the amount of radioactivity represented by non-extractable (bound) residues was 28.7% TAR. In the irradiated samples, bound residues increased consistently to a maximum of 62.3% TAR after 15 days of irradiation. In the dark control samples, bound residues also increased consistently to a maximum of 64.5% TAR by the end of the 15-day study period.

Moderate mineralization of ^{14}C-metiram was observed in both the irradiated and dark control samples. On day 15 $^{14}CO_2$ reached maximum levels of 6.8% TAR and 9.6% TAR in the irradiated and dark samples, respectively.

In both the irradiated and dark control samples, ^{14}C-metiram degraded into 20 radioactive fractions. Most of them were minor fractions. In both the irradiated and dark samples, M222F004 (EBIS) was the only major radioactive fraction exceeding 10% of the amount applied in the methanol extracts. EBIS accounted for 27.0% of TAR shortly after application (time 0) and decreased continuously to a minimum of 0.3% TAR by the end of the 15-day irradiation period. After time 0 in the dark samples, the amount of EBIS decreased continuously to a minimum of 2.7% TAR by the end of the 15-day study period.

In both the irradiated samples and the dark control samples, M222F003 (EU) and M222F002 (ETU) were detected as minor radioactive fractions.

^{14}C-Metiram photodegraded with a half-life (DT_{50}) of <0.1 day of exposure to artificial sunlight. Corresponding degradation of ^{14}C-metiram in the dark control samples also resulted in a DT_{50} of <0.1 day.

Degradation in both the irradiated and dark control samples proceeded through the formation of the major metabolite M222F004 (EBIS), with subsequent formation of the minor metabolites M222F003 (EU) and M222F002 (ETU). In the irradiated samples, EBIS degraded with a DT_{50} of 0.6 days, while EU and ETU degraded with DT_{50} values of 14.0 and 6.4 days, respectively. In the dark control samples, EBIS degraded with a DT_{50} values of 1.2 days, while EU and ETU degraded with DT_{50} of 2.7 and 2.5 days, respectively. Several other minor metabolites were observed in both the irradiated and dark control samples, but were neither identified nor further characterized. The degradation finally resulted in the formation of radioactive carbon dioxide and bound residues.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

BAS-Code: BAS 222 F (metiram)
Registry No.: 250284
Chemical name: Zinc ammoniate ethylenebis(dithiocarbamate)-poly-(ethylenethiuramdisulfide)
Molecular formula: $(C_4H_9N_3S_4Zn)_3$; $(C_4H_6N_2S_4)x$
Molar mass: 1088.7 g mol^{-1} (unlabeled)

<u>Labeled test item</u>
Label: ^{14}C-ethylene
Batch No.: 153-6001
Specific activity of a.s.: 7.64 MBq mg^{-1}
Releasable CS_2: 89.0%

<u>Non-labeled test item</u>
Batch No.: CP052744
Purity: 93.6%

2. Soil

The study was conducted with one soil (Am Fischteich) classified as silt loam (USDA) and was collected from the top layer (0-20cm depth) of a field near Crimmitschau, Germany. The soil was passed through a 2 mm sieve and stored moist at 4°C for a period of less than one month. An overview of the soil parameters is listed in Table 7.1.1.3-2.

Table 7.1.1.3-2: Characteristics of Am Fischteich soil used for the soil photolysis study with ^{14}C-metiram

Soil designation	Am Fischteich 11/12 Crimmitschau, Germany
USDA Particle size distribution [%] sand 0.050 – 2 mm silt 0.002 – 0.050 mm clay < 0.002 mm textural class	 11.9 67.2 20.9 silt loam
Organic C [%]	1.62
Organic matter [%] [a]	2.79
pH [H_2O] pH [$CaCl_2$]	6.7 6.3
Cation exchange capacity [mmol 100 g^{-1} soil]	15.8
Max. water holding capacity (at pF 2) [g $100g^{-1}$ dry weight]	33.0

[a] % organic matter = 1.724 x % organic carbon

B. STUDY DESIGN

1. Experimental conditions

Soil thin-layers of 12.5 cm^2 each were prepared by applying an aqueous soil slurry to 48 separate glass plates with a layer thickness of approximately 2 mm. Thereafter, the moist soil layers were allowed to dry, then adjusted to a soil moisture content of pF 2.0. Approximately 3 g soil was applied to each plate.

The soil test plates were irradiated in a "Suntest" accelerated exposure table unit, fitted to a Xenon arc light source and filters to cut-off light of less than 290 nm wavelength. In order to control the temperature of the irradiated samples and to trap any volatile degradation products formed, the glass plates containing the soil were placed in a stainless steel cooling tank. The tank was sealed with a quartz lid and placed beneath the xenon burner. All samples were continuously irradiated for up to 15 days at a temperature of 20 ± 2°C in the exposure tank. Control samples were incubated the same way except that they were kept in the dark.

^{14}C-metiram was applied to the soil thin-layers at a nominal amount of 175 µg per sample, corresponding to a nominal field application rate of 1.4 kg a.s. ha^{-1}.

For trapping volatile degradation products, moistened air, controlled by a pump, was passed through the system. The outlet air was passed through a series of four absorption traps, each containing specific trapping solutions (2 M NaOH, methanolic KOH, 0.5 M H_2SO_4, ethylene glycol).

2. Sampling

For both irradiated and dark samples, two replicates of treated soil samples were taken for work-up and analyzed 1.5 hours after treatment (day 0). Additional replicates were taken and analyzed after 1, 2, 4, 7 and 15 days of continuous irradiation. Dark control samples were taken at the same sampling intervals. At all sampling intervals (except time 0), volatile trapping solutions were removed for analysis.

3. Description of analytical procedures

Metiram is insoluble in all solvents and was therefore quantified by an indirect CS_2 method, during which metiram is decomposed by stannous hydrochloric acid. The formed CS_2 was trapped in the methanolic solution of potassium chloride and quantified by UV detection.

On the day of sampling, soil samples were extracted using methanol (up to four times) followed by extractions with 1% EDTA in ultra-pure water adjusted to pH 8 with 10 M NaOH (five times). Each extraction was performed in a shaker and the individual extracts were filtered and analyzed by liquid scintillation counting (LSC).

The extracts were combined according to the extraction solutions used. The pooled extracts were measured by LSC for recovery and submitted to HPLC analysis. Degradates of metiram were subjected to structure elucidation using HPLC-MS/MS.

The residual radioactivity remaining in soil after sequential extractions was quantified by LSC after combusting aliquots of the air-dried and homogenized soil. Radioactivity present in the trapping solutions was determined by LSC.

4. Calculation of the degradation rate

Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints). Kinetic analysis and calculation of $DegT_{50}$ and $DegT_{90}$ values was performed following the recommendations of the FOCUS Kinetics workgroup [*FOCUS (2006)*]. The software package KinGUII (version 2.2014.224.1704) was used for parameter fitting.

The endpoints of the parent compound Metiram and its metabolites EBIS, ETU and EU were derived from a compartment modeling approach.

For each data set, the kinetic models proposed by the FOCUS Kinetics guidance document were tested to identify the best-fit model. The recommended kinetic models, i.e. the single first order kinetics (SFO), the Gustafson-Holden model (FOMC), and bi-exponential kinetics DFOP), are already implemented in KinGUI. The Goodness-of-fit was evaluated by visual assessment, χ^2 error value, and type-I-error rate (t-test).

II. RESULTS AND DISCUSSION

A. MASS BALANCE

Total recoveries of radioactivity extracted from soil are summarized in Table 7.1.1.3-3 (irradiated) and Table 7.1.1.3-4 (dark control). The recoveries of the individual replicates were normalized to 100%.

Table 7.1.1.3-3: Recovery and distribution of radioactivity in soil Am Fischteich after treatment with ^{14}C-labeled metiram and incubation under irradiated conditions [% TAR]

Incubation time (days) Suntest	Repl.	Extractable residues			NER	Volatiles				Mass balance
		Methanol[a)]	EDTA[b)]	Total		$^{14}CO_2$	Ethylene glycol	KOH/ methanol	H_2SO_4	
0 (1.5h)	A	47.9	25.6	73.4	26.6	n.p.	n.p.	n.p.	n.p.	100.0
	B	43.6	25.7	69.3	30.7	n.p.	n.p.	n.p.	n.p.	100.0
	Mean	**45.7**	**25.6**	**71.3**	**28.7**	**n.p.**	**n.p.**	**n.p.**	**n.p.**	**100.0**
1	A	27.7	27.1	54.8	44.9	0.3	<0.1	<0.1	<0.1	100.0
	B	30.8	26.4	57.2	42.5	0.2	<0.1	<0.1	<0.1	100.0
	Mean	**29.3**	**26.7**	**56.0**	**43.7**	**0.3**	**<0.1**	**<0.1**	**<0.1**	**100.0**
2	A	22.0	26.6	48.5	51.1	0.4	<0.1	<0.1	<0.1	100.0
	B	26.8	27.3	54.1	45.5	0.4	<0.1	<0.1	<0.1	100.0
	Mean	**24.4**	**27.0**	**51.3**	**48.3**	**0.4**	**<0.1**	**<0.1**	**<0.1**	**100.0**
4	A	19.5	26.2	45.7	52.9	1.4	<0.1	<0.1	<0.1	100.0
	B	22.5	25.3	47.7	50.9	1.3	<0.1	<0.1	<0.1	100.0
	Mean	**21.0**	**25.8**	**46.7**	**51.9**	**1.4**	**<0.1**	**<0.1**	**<0.1**	**100.0**
7	A	13.9	25.4	39.3	57.7	3.1	<0.1	<0.1	<0.1	100.0
	B	19.0	25.9	44.9	52.2	2.9	<0.1	<0.1	<0.1	100.0
	Mean	**16.5**	**25.6**	**42.1**	**54.9**	**3.0**	**<0.1**	**<0.1**	**<0.1**	**100.0**
15	A	7.1	25.1	32.1	61.1	6.8	<0.1	<0.1	<0.1	100.0
	B	7.8	21.8	29.6	63.5	6.8	<0.1	<0.1	<0.1	100.0
	Mean	**7.4**	**23.4**	**30.9**	**62.3**	**6.8**	**<0.1**	**<0.1**	**<0.1**	**100.0**

NER = Non-extractable radioactive residues
n.p. = Not performed
a) Extracted with methanol (four times)
b) Extraction with 1% EDTA, adjusted to pH 8 with 10M NaOH (five times)

Table 7.1.1.3-4: Recovery and distribution of radioactivity in soil Am Fischteich after treatment with ^{14}C-labeled metiram and incubation under dark conditions [% TAR]

Incubation time (days)	Replicate	Extractable residues			NER	Volatiles				Mass balance
Suntest		Methanol[a)]	EDTA[b)]	Total		$^{14}CO_2$	Ethylene glycol	KOH/ methanol	H_2SO_4	
0 (1.5h)	A	47.9	25.6	73.4	26.6	n.p.	n.p.	n.p.	n.p.	100.0
	B	43.6	25.7	69.3	30.7	n.p.	n.p.	n.p.	n.p.	100.0
	Mean	**45.7**	**25.6**	**71.3**	**28.7**	**n.p.**	**n.p.**	**n.p.**	**n.p.**	**100.0**
1	A	34.2	21.5	55.8	44.1	<0.1	<0.1	<0.1	<0.1	100.0
	B	36.3	21.4	57.7	42.2	<0.1	<0.1	<0.1	<0.1	100.0
	Mean	**35.3**	**21.5**	**56.7**	**43.2**	**<0.1**	**<0.1**	**<0.1**	**<0.1**	**100.0**
2	A	28.9	24.1	53.0	46.4	0.6	<0.1	<0.1	<0.1	100.0
	B	30.7	19.6	50.3	49.1	0.5	<0.1	<0.1	<0.1	100.0
	Mean	**29.8**	**21.9**	**51.7**	**47.8**	**0.6**	**<0.1**	**<0.1**	**<0.1**	**100.0**
4	A	19.3	21.8	41.0	56.4	2.5	<0.1	<0.1	<0.1	100.0
	B	17.2	19.1	36.3	61.4	2.2	<0.1	<0.1	<0.1	100.0
	Mean	**18.2**	**20.4**	**38.7**	**58.9**	**2.4**	**<0.1**	**<0.1**	**<0.1**	**100.0**
7	A	14.4	19.6	34.0	61.5	4.5	<0.1	<0.1	<0.1	100.0
	B	11.7	22.5	34.1	59.0	6.8	<0.1	<0.1	<0.1	100.0
	Mean	**13.0**	**21.0**	**34.1**	**60.3**	**5.7**	**<0.1**	**<0.1**	**<0.1**	**100.0**
15	A	7.5	18.1	25.6	64.8	9.6	<0.1	<0.1	<0.1	100.0
	B	7.4	18.7	26.1	64.3	9.6	<0.1	<0.1	<0.1	100.0
	Mean	**7.4**	**18.4**	**25.8**	**64.5**	**9.6**	**<0.1**	**<0.1**	**<0.1**	**100.0**

NER = Non-extractable radioactive residues
n.p. = Not performed
a) Extracted with methanol (four times)
b) Extraction with 1% EDTA, adjusted to pH 8 with 10M NaOH (five times)

B. EXTRACTABLE AND BOUND RESIDUES

In the irradiated samples, the amount of radioactivity recovered by methanol extraction decreased on day 0 (1.5 hours after treatment) from 45.7% TAR to 29.3% TAR on day 1 and continued to decrease slowly to 7.4% TAR on day 15. In the dark samples, the amount of radioactivity recovered by methanol extraction decreased on day 0 (1.5 hours after treatment) from 45.7% TAR to 35.3% TAR on day 1 and continued to decrease slowly to 7.4% TAR on day 15.

On day 0 (1.5 hours after treatment), the amount of radioactivity recovered by EDTA extraction was 25.6% TAR. In the irradiated samples, the amount of radioactivity recovered by EDTA extraction remained stable throughout the 15-day irradiation period, ranging from a minimum of 23.4% to a maximum of 27.0% TAR. Similarly in the dark control samples, the amount of radioactivity recovered by EDTA extraction remained relatively stable, still representing 18.4% TAR on day 15.

On day 0 (1.5 hours after treatment), the amount of radioactivity represented by non-extractable (bound) residues was 28.7% TAR. In the irradiated samples, bound residues increased to 43.7% TAR on day 1 and increased consistently over time to a maximum of 62.3% TAR on day 15. In the dark control samples, bound residues increased to 43.2% TAR on day 1 and increased consistently over time to a maximum of 64.5% TAR on day 15.

C. VOLATILIZATION

Moderate mineralization of ^{14}C-metiram was observed in the irradiated and dark control samples. By the end of 15 day, $^{14}CO_2$ reached a maximum level of 6.8% TAR and 9.6% TAR in the irradiated and dark samples, respectively.

The concentration of other radioactive volatile products, extracted in each of ethylene glycol, potassium hydroxide (KOH) in methanol, and sulfuric acid (H_2SO_4) remained < 0.1% TAR at all sampling intervals for both the irradiated and dark control samples.

D. TRANSFORMATION OF PARENT COMPOUND

Degradation of metiram

The degradation of metiram, as determined by the CS_2 method, is summarized in Table 7.1.1.3-5. A fast degradation of metiram was observed in the Am Fischteich soil in both the irradiated and dark control samples. About 1.5 hours after treatment, the amount of metiram accounted for 42.6% of the applied amount. In the irradiated samples, the amount of radioactivity that could be attributed to metiram had already decreased to 18.5% TAR one day after application. By the end of the 15-day irradiation period, radioactivity attributed to metiram accounted for only 2.1% TAR. In the dark control samples, radioactivity attributed to metiram had decreased significantly after one day, accounting for only 25.8% TAR. By the end of the 15-day incubation period, radioactivity attributed to metiram accounted for only 4.1% TAR.

Table 7.1.1.3-5: Degradation of metiram in Am Fischteich soil as determined by the CS_2 method

Am Fischteich		^{14}C-metiram [mg kg^{-1}]		[% TAR]	
Incubation time (days)	Replicate	Irradiated	Dark	Irradiated	Dark
0 (1.5h)	A	30.9	30.9	60.1	60.1
	B	18.2	18.2	25.2	25.2
	Mean	**24.5**	**24.5**	**42.6**	**42.6**
1	A	10.2	12.5	24.9	25.8
	B	7.0	<LOQ	12.2	<LOQ
	Mean	**8.6**	**12.5**	**18.5**	**25.8**
2	A	13.5	16.7	28.2	31.7
	B	3.5	10.8	6.3	18.9
	Mean	**8.5**	**13.7**	**17.2**	**25.3**
4	A	3.7	5.9	7.4	12.5
	B	0.6	7.6	1.1	14.2
	Mean	**2.1**	**6.8**	**4.2**	**13.3**
7	A	1.8	5.5	3.6	8.6
	B	2.0	<LOQ	3.7	<LOQ
	Mean	**1.9**	**5.5**	**3.7**	**8.6**
15	A	0.6	2.1	1.2	5.1
	B	1.5	1.3	3.0	3.2
	Mean	**1.1**	**1.7**	**2.1**	**4.1**

TAR = Total applied radioactivity
LOQ = 0.52 mg kg^{-1} (1.0% TAR)

Pattern of metabolite formation

Several degradation products were detected. The results of radio-HPLC of the methanol extracts are summarized in Table 7.1.1.3-6 (irradiated) and Table 7.1.1.3-7 (dark control).

In both the irradiated and dark samples, M222F004 (EBIS) was the only major radioactive fraction exceeding 10% of the amount applied or 5% of applied in two consecutive sampling inervals in the methanol extracts. M222F004 accounted for 27.0% TAR 1.5 hours after treatment (time 0). After time 0 in the irradiated samples, the amount of M222F004 decreased continuously to a minimum of 0.3% TAR by the end of the 15-day irradiation period. After time 0 in the dark samples, the amount of M222F004 decreased continuously to a minimum of 2.7% TAR by the end of the 15-day study period.

In the irradiated samples, the minor radioactive fractions M222F003 (EU) and M222F002 (ETU) reached maximum levels on day 4 of 6.2% TAR and 2.1% TAR, respectively. In the dark control samples, M222F003 reached a maximum of 3.4% TAR on day 1, while M222F002 reached a maximum of 1.9% TAR on day 2. M222F003 was detected as two distinct peaks, but LC/MS analyses showed that both corresponded to the same compound.

Metabolites M222F004 (EBIS), M222F002 (ETU), and M222F003 (EU) were identified and confirmed by structure elucidation using HPLC-MS/MS.

None of the other radioactive fractions reached a concentration of 10% TAR or 5% TAR at two consecutive sampling intervals, respectively. Therefore, the remaining radioactive fractions were not identified or further characterized.

Table 7.1.1.3-6: Radio-HPLC analysis of methanol extracts: after treatment of Am Fischteich soil with ^{14}C-metiram under irradiated conditions [% TAR]

		Metabolic fraction													
		EU		EU total	ETU	UK18	UK7	UK17	UK10	UK5	EBIS	UK15	UK1	UK4	Sum others a)
Incubation time (days)	Replicate	Retention time [min]													
Suntest		2.9- 3.8	3.8- 4.8	2.9- 4.8	4.8- 6.2	6.4- 7.0	7.2- 8.2	8.4- 9.7	15 1- 16.8	28.6- 30.0	30.5- 30.7	31.8- 33.7	34.4- 35.9	40.0- 40.7	
0 (1.5h)	A	n.d.	n.d.	n.d.	1.4	0.6	1.0	0.5	n.d.	n.d.	28.0	6.9	6.9	2.6	n.d.
	B	n.d.	n.d.	n.d.	1.2	0.8	0.5	0.2	n.d.	n.d.	26.1	5.4	7.6	1.8	n.d.
	Mean	**n.d.**	**n.d.**	**n.d.**	**1.3**	**0.7**	**0.7**	**0.3**	**n.d.**	**n.d.**	**27.0**	**6.1**	**7.3**	**2.2**	**n.d.**
1	A	0.3	0.4	0.7	1.4	1.7	1.2	1.5	1.9	n.d.	10.4	1.9	2.8	1.4	2.9
	B	0.2	0.4	0.5	2.3	0.9	0.9	0.8	1.6	0.4	11.7	3.2	4.1	1.5	3.4
	Mean	**0.3**	**0.4**	**0.6**	**1.8**	**1.3**	**1.0**	**1.1**	**1.7**	**0.2**	**11.1**	**2.6**	**3.5**	**1.4**	**3.1**
2	A	1.3	3.1	4.4	1.5	0.6	0.6	0.6	0.7	0.7	5.7	2.5	1.1	0.8	3.3
	B	0.2	0.3	0.5	2.3	1.9	1.3	1.5	0.8	0.2	9.6	3.3	1.8	0.9	2.9
	Mean	**0.8**	**1.7**	**2.5**	**1.9**	**1.3**	**1.0**	**1.0**	**0.8**	**0.4**	**7.7**	**2.9**	**1.4**	**0.9**	**3.1**
4	A	0.9	4.8	5.8	1.7	1.4	2.8	0.8	0.3	0.6	2.6	1.5	0.4	0.6	1.5
	B	0.9	5.8	6.7	2.6	2.4	1.3	1.5	0.3	0.6	2.3	1.4	0.6	0.4	2.9
	Mean	**0.9**	**5.3**	**6.2**	**2.1**	**1.9**	**2.1**	**1.2**	**0.3**	**0.6**	**2.4**	**1.5**	**0.5**	**0.5**	**2.2**
7	A	1.8	1.3	3.0	1.2	1.2	1.0	0.6	0.2	0.7	2.5	0.5	0.5	0.5	2.6
	B	2.7	2.8	5.5	2.0	1.6	1.5	1.6	0.2	0.9	1.9	0.9	0.3	0.4	3.1
	Mean	**2.2**	**2.0**	**4.3**	**1.6**	**1.4**	**1.3**	**1.1**	**0.2**	**0.8**	**2.2**	**0.7**	**0.4**	**0.5**	**2.9**
15	A	1.5	0.8	2.3	0.8	0.6	0.3	0.2	0.1	0.1	0.4	0.3	0.2	0.1	1.7
	B	1.0	1.1	2.1	0.6	0.4	0.4	0.2	n.d.	0.3	0.2	0.1	0.2	0.1	1.2
	Mean	**1.3**	**1.0**	**2.2**	**0.7**	**0.5**	**0.3**	**0.2**	**0.1**	**0.2**	**0.3**	**0.2**	**0.2**	**0.1**	**1.5**

n.d. = Not detected (LOD = 0.085% TAR)
a) Each individual metabolite < 1% of applied radioactivity

Table 7.1.1.3-7: Radio-HPLC analysis of methanol extracts: after treatment of Am Fischteich soil with ^{14}C-metiram and incubation in the dark [% TAR]

		Metabolic fraction													Sum others a)
		EU		EU total	ETU	UK18	UK7	UK16	UK10	UK5	EBIS	UK15	UK1	UK4	
Incubation time (days)	Replicate	Retention time [min]													
Suntest		2.9-3.8	3.8-4.8	2.9-4.8	4.8-6.2	6.4-7.0	7.2-8.2	12.2-13.1	15.1-16.8	28.6-30.0	30.5-30.7	31.8-33.7	34.4-35.9	40.0-40.7	
0 (1.5h)	A	n.d.	n.d.	n.d.	1.4	0.6	1.0	n.d.	n.d.	n.d.	28.0	6.9	6.9	2.6	0.5
	B	n.d.	n.d.	n.d.	1 2	0.8	0.5	n.d.	n.d.	n.d.	26 1	5.4	7.6	1.8	0.2
	Mean	**n.d.**	**n.d.**	**n.d.**	**1.3**	**0.7**	**0.7**	**n.d.**	**n.d.**	**n.d.**	**27.0**	**6.1**	**7.3**	**2.2**	**0.3**
1	A	n.d.	3.7	3.7	1.3	n.d.	1.3	1.3	1.2	0.3	15.1	3.5	3.0	1.4	2.2
	B	0.2	2.9	3.1	2 1	n.d.	0.9	1.3	0.9	0.4	15.0	4.3	3.3	1.6	3.2
	Mean	**0.1**	**3.3**	**3.4**	**1.7**	**n.d.**	**1.1**	**1.3**	**1.1**	**0.4**	**15.1**	**3.9**	**3.1**	**1.5**	**2.7**
2	A	0.4	2.2	2.6	1.6	1.2	1.7	0.9	0.5	0.5	14.7	n.d.	1.9	1.3	2.0
	B	0.2	3.2	3.4	2 2	1.4	1.2	0.9	0.6	0.6	10 9	4.1	1.6	1.1	2.6
	Mean	**0.3**	**2.7**	**3.0**	**1.9**	**1.3**	**1.5**	**0.9**	**0.6**	**0.6**	**12.8**	**2.0**	**1.8**	**1.2**	**2.3**
4	A	0.4	2.8	3.3	1.7	1.1	1.1	0.1	0.2	0.8	5 3	2.4	0.8	0.6	2.0
	B	0.9	2.2	3.1	1.0	0.8	0.5	0.3	0.3	0.6	5.2	2.3	0.9	0.7	1.5
	Mean	**0.7**	**2.5**	**3.2**	**1.4**	**0.9**	**0.8**	**0.2**	**0.2**	**0.7**	**5.3**	**2.3**	**0.9**	**0.6**	**1.8**
7	A	0.4	2.3	2.7	1.1	0.9	0.6	0.3	0.2	0.4	4.0	0.9	0.7	0.5	2.0
	B	0.9	1.1	2.0	0.7	0.2	0.6	0.3	0.1	1.0	2.8	1.0	0.6	0.7	1.8
	Mean	**0.6**	**1.7**	**2.4**	**0 9**	**0.6**	**0.6**	**0.3**	**0.1**	**0.7**	**3.4**	**1.0**	**0.6**	**0.6**	**1.9**
15	A	0.8	0.7	1.5	0.3	n.d.	n.d.	n.d.	n.d.	1.0	2.8	1.4	0.5	n.d.	n.d.
	B	1.5	n.d.	1.5	0.7	n.d.	n.d.	n.d.	n.d.	1.1	2.6	0.7	0.7	n.d.	n.d.
	Mean	**1.2**	**0.3**	**1.5**	**0.5**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**1.1**	**2.7**	**1.0**	**0.6**	**n.d.**	**n.d.**

n.d. = Not detected (LOD = 0.085% TAR)
a) Each individual metabolite < 1% of applied radioactivity

The EDTA extracts were also analyzed. Due to higher amounts of radioactivity than expected, it was concluded that extraction with aqueous EDTA not only leads to a release of metiram "monomers" by complexation of the Zn atom but also to a beginning disaggregation of bound radioactivity in the high molecular weight humic substances potentially due to the same metal complexing mechanism.

The use of complexing agents like EDTA is very artificial and the radioactive compounds released by EDTA are considered to be of no environmental relevance. Therefore, the results of the EDTA extracts were not used for further evaluation.

E. KINETIC MODELING RESULTS

The calculated $DegT_{50}$ and $DegT_{90}$ values of metiram and its metabolites EBIS, ETU, and EU used as triggers for additional work are given in Table 7.1.1.3-8.

Table 7.1.1.3-8: Trigger endpoints for metiram and its metabolites EBIS, ETU, and EU

Test system	Analytes	Best-fit model	χ^2 error	Trigger endpoints	
				$DegT_{50}$ [d]	$DegT_{90}$ [d]
Irradiated	^{14}C-metiram	DFOP	12.2	<0.1	3.4
	EBIS	SFO	9.9	0.6	2.0
	ETU	SFO	4.7	6.4	21.4
	EU	SFO	38.1	14.0	46.6
Dark control	^{14}C-metiram	DFOP	22.7	<0.1	8.0
	EBIS	SFO	10.8	1.2	4.1
	ETU	SFO	9.2	2.5	8.2
	EU	SFO	20.1	2.7	8.9

III. CONCLUSION

Metiram photodegraded with a half-life ($DegT_{50}$) of <0.1 day of exposure to artificial sunlight. Corresponding degradation of ^{14}C-metiram in the dark control samples also resulted in a $DegT_{50}$ of <0.1 day.

Degradation in the irradiated and dark control samples was similar and proceeded through the formation of the major metabolite M222F004 (EBIS). Metabolites M222F002 (ETU) and M222F003 (EU) were also observed, however only in minor amounts. Several other minor metabolites were observed in both, the irradiated and dark control samples. Final degradation resulted in the formation of carbon dioxide and bound residues. Overall the same degradation products were observed as in the aerobic and anaerobic degradation, i.e. the influence of light has no significant influence on the degradation of metiram.

In the irradiated samples, EBIS degraded with a DT_{50} of 0.6 days, while EU and ETU degraded with DT_{50} of 14.0 and 6.4 days, respectively. In the dark control samples, EBIS degraded with a DT_{50} of 1.2 days, while EU and ETU degraded with DT_{50} of 2.7 and 2.5 days, respectively. The slightly longer DT50 values of of EU and ETU under irradiated conditions are considered to be due to different experimental conditions (e.g. drying of the soil under light) that cannot be completely avoided in this study type.

Peer reviewed study (Summary taken from the Monograph or its Addenda):

Report: *CA 7.1.1.3/2*
Carpenter M., 1987a
Determination of the photolysis rate of Ethylenethiourea (ETU) on the surface of soil
1987/10155
Guidelines: *EPA 161-3*
GLP: *yes*
(certified by <none>)

Materials and Methods:

A silty loam soil (9.6% sand, 64% silt, 26.4% clay, about 1% organic carbon) was spiked with [^{14}C]-ETU at a rate of 9.59 mg ETU/kg soil and irradiated with a Xenon arc lamp at 25°C. The Xenon light was filtered for elimination of radiation below 290 nm. The samples were positioned in such a distance from the soil samples that the photon flux density was approximately one half that of sunlight, i.e. the permanent exposure of the samples was approximately equivalent to a daily sunlight irradiation of 12 h.
Soil samples were extracted with MeOH/H_2O (70/30), measured by LSC and analyzed by radio-TLC. Traps for volatiles were adopted.

Findings:

The distribution of radioactivity in the light exposed and dark control samples is shown in Table 7.1.1.3-9. Good recoveries were obtained (93.4 and 97.1% respectively for exposed and dark samples).

Table 7.1.1.3-9: Balance of radioactivity in soil photolysis of ETU. Data in % IRR.

DAT[1]	ERR		RRR		Traps[2]		Others[3]		Balance	
	Exposed	Dark	Exposed	Dark	Exposed	Dark	Exposed	Dark	Exposed	Dark
0	91.2	91.2	6.0	6.0	-	-	-	-	97.3	97.3
1	65.3	74.5	29.0	22.1	-	-	0.8	0.2	95.1	96.7
2	57.4	66.2	35.0	27.7	0.1	-	0.8	0.2	93.3	94.1
3	57.6	67.3	36.7	33.7	0.1	-	0.8	0.2	95.2	101.1
4	50.6	62.1	42.1	36.4	0.1	-	0.8	0.2	93.6	98.7
5	46.0	56.2	44.6	40.1	0.1	-	0.8	0.2	91.5	96.5
6	44.4	53.7	45.8	42.8	0.1	-	0.8	0.2	91.1	96.6
7	44.8	47.4	44.6	47.8	0.1	0.2	0.8	0.2	90.4	95.6
8	45.6	53.9	45.7	44.3	0.1	0.2	0.8	0.2	92.2	98.6

1: Sampling dates rounded; exact data: 0, 0.91, 1.98, 2.94, 3.88, 5.06, 6.06, 6.92, 7.81
2: Trapping solutions were: ethylene glycol, 1 N H_2SO_4 and 1 N KOH
3: "Others" comprise: Photo chamber rinse and Sep Pak Cartridge rinse (Sep Pak was at the final air outlet after NaOH trap)

The result of TLC analysis of ERR is given in Table 7.1.1.3-10.

Table 7.1.1.3-10: Radio-TLC analysis of ERR upon ETU soil photolysis. Data in % radioactivity in the specific sample.

DAT	ETU		EU		IMID[2]		u k. 1[3]		u.k. 2		Origin[3]	
	E[1]	D[1]	E	D	E	D	E	D	E	D	E	D
0	48.4	48.4	7.1	7.1	-[4]	-	-	-	-	-	6.2	6.2
1	17.4	31.4	18.2	13.5	3.6	-	4.0	4.4	-	-	6.4	7.5
2	10.8	19.0	20.4	21.1	-	-	3.6	3.0	-	-	8.3	7.3
3	7.0	22.7	19.5	12.8	-	-	9.3	9.0	-	-	7.0	8.1
4	5.2	18.6	23.5	21.4	-	-	5.5	3.9	4.2	-	-	6.5
5	-	8.5	15.0	17.8	5.0	-	-	5.6	3.7	-	5.7	8.8
6	-	8.8	18.4	20.8	1.9	1.4	6.7	3.2	-	-	3.0	5.6
7	-	-	19.8	25.2	1.8	-	5.7	2.9	-	-	3.4	5.7
8	-	7.8	13.6	15.9	2.7	1.7	4.7	7.4	7.3	-	3.0	5.5

1: E = exposed, D = dark sample
2: IMID = 2-imidazoline
3: u.k. = unknown, origin = unknown remaining at the start dot on TLC
4: - = not detected

Radioactivity missing for the balance (cp. ERR data Table 7.1.1.3-9 and Σ components on TLC) may be allocated to "diffuse radioactivity" on TLC not accounted for by a distinct peak.
The similarity of the degradate profiles in both samples is indicative of a non-photolytic process but of an oxidation process analogous to that observed in the metabolism study. […] No volatiles were detected during the study.
The evaluator considers therefore that the contribution of photolysis to the soil metabolism of ETU can be considered low.

Summary: Route of degradation of metiram in soil

Metiram is degraded rapidly in soil, presumably by abiotic mechanisms. Upon application of metiram to soil, primary metabolites (EBIS (=M222F004), TDIT (=M222F007)) are formed. The initial processes are very fast and these metabolites reach their maximum immediately after metiram is getting into contact with the soil. The primary metabolites are very short-lived and are further degraded forming secondary metabolites, ethylenethiourea (ETU (=M222F002) and ethylene urea (EU (=M222F003)) which are also very transient. Other metabolites are formed only in minor percentages. Further degradation results in a high percentage of CO_2 and the formation of bound residues.

Photolytic degradation in soil leads to the same degradation products (EBIS, ETU and EU) as in the aerobic metabolism studies. No special photoproducts are detected in significant amounts. Thus no differentiation is made between biological and photolytical degradation.
Also under anaerobic conditions, the metabolic profile of metiram is similar to that found in aerobic soil.

In older studies the formation of metabolites was largely overestimated. In these studies inappropriate procedures were used that led to artificial decomposition of metiram. More recent studies show that only EBIS, TDIT, ETU and EU exceed trigger values whereas the Carbimid is only observed in very low amounts and Hydantoin not at all. Other metabolites are formed only in minor percentages.

Metiram (BAS 222 F)

EBIS (M222F004)

TDIT (M222F007)

Carbimid (M222F005)

ETU (M222F002)

EU (M222F003)

Hydantoin (M222F008)

CO_2 + Bound Residues

Figure 7.1.1.3-1: Proposed route of degradation of metiram in soil

CA 7.1.2 Rate of degradation in soil

CA 7.1.2.1 Laboratory studies

CA 7.1.2.1.1 Aerobic degradation of the active substance

Studies presented in the first Annex I inclusion process:

Several studies on the aerobic degradation rate of metiram were presented in the first Annex I inclusion process. An overview is given in Table 7.1.2.1.1-1.
Two studies of Staudenmaier H. (2002) are considered still valid and are summarized in CA 7.1.1.1/1 and CA 7.1.1.1/2.

The old studies of Keller E., Huber R. and Klein W., Koerdel W. et al. [*Keller E., Huber H., 1985, BASF DocID 1985/10059; Klein W., Koerdel W. et al., 1986, BASF DocID 1986/10134*] are considered invalid as discussed in CA 7.1.1.1.
The very old study of Anonymous [*Anonymous, 1974, BASF DocID 1974/10021*] is considered invalid as it was performed with non-viable soils which resulted in an unrealistic degradation rate for metiram. This was already stated in the list of endpoints of the 3rd Addendum to the Monograph (May 2003).
The kinetic evaluation of Goetz N. von [*Goetz N. von, 2000, BASF DocID 2000/1004064*] is outdated as it is not according to the current FOCUS guidance and it relies on the old, invalid studies of Anonymous, Keller E., Huber R. and Klein W., Koerdel W. et al.

Table 7.1.2.1.1-1: Studies on aerobic degradation rates in soil of metiram

Reference	BASF DocID	Soil type	Application rate [mg kg^{-1}]	Incubation temperature [°C]	Incubation period [days]	Remark
Anonymous, 1974	1974/10021	Soil 1 [a] Soil 2 [a]	10	22	56	Invalid
Keller E., Huber R., 1985	1985/10059	Loamy sand Loam	10	22	365	Invalid
Klein W., Koerdel W. et al., 1986	1986/10134	Loamy sand	10	25	21	Invalid
Goetz N. von, 2000 (kinetic evaluation)	2000/1004064	-- [b]				Invalid (outdated)
Staudenmaier H., 2002b	2002/1011913	Loamy sand	7.5	20	365	Relevant
Staudenmaier H., 2002c	2002/1012954	Loamy sand	3.9	20	133	Relevant

[a] Soil types not reported
[b] Kinetic evaluation

Submission of not yet peer-reviewed studies in this AIR3-Dossier:

As some of the old soil metabolism/degradation studies are considered invalid, not enough valid DT_{50} values for the parent metiram were available any more. For this reason new aerobic soil degradation studies were performed for metiram which are summarized in CA 7.1.1.1/3 and CA 7.1.1.1/4. The corresponding kinetic evaluations are reported in separate studies and summarized in CA 7.1.2.1.1/5 and CA 7.1.2.1.1/7. For the studies of Staudenmaier H. [see CA 7.1.1.1/1 and CA 7.1.1.1/2] a new kinetic evaluation was performed following the recommendations of the FOCUS Kinetics workgroup [FOCUS (2006)], considering the newest guidelines and model versions and is summarized in CA 7.1.2.1.1/3.

Report:	CA 7.1.2.1.1/1 Staudenmaier H., 2002a Aerobic metabolism of BAS 222 F (Metiram) in Cashmere soil 2002/1011913
Guidelines:	SETAC, BBA IV 4-1, EPA 162-1
GLP:	yes (certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)

Report:	CA 7.1.2.1.1/2 Staudenmaier H., 2002b Aerobic metabolism of BAS 222 F (Metiram) in soil 2002/1012954
Guidelines:	SETAC, BBA IV 4-1, EPA 162-1
GLP:	yes (certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)

The experimental studies [*BASF DocID 2002/1011913 and 2002/1012954*] are already described in detail in CA 7.1.1.1/1 and CA 7.1.1.1/2. The kinetic evaluation is presented below.

Report:	CA 7.1.2.1.1/3 Pape L., 2015a Kinetic evaluation of a laboratory soil degradation study with BAS 222 F - Metiram according to FOCUS Degradation Kinetics 2014/1228382
Guidelines:	FOCUS Kinetics (2006) SANCO/10058/2005 version 1.1 of Dec. 2014
GLP:	no (certified by <none>)

Executive Summary

The aerobic degradation of metiram has been investigated in the laboratory in two soils in two studies using ethylene-^{14}C-labeled metiram. The purpose of this evaluation was to analyze the degradation kinetics of metiram and its metabolites observed in these studies, taking into account the current guidance of the FOCUS workgroup on degradation kinetics.

Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints) and degradation parameters for environmental fate models (modeling endpoints).

For metiram, best-fit $DegT_{50}$ values (trigger endpoints) were 0.2 and 0.4 days, and $DegT_{90}$ values were 1.7 and 3.9 days. Normalized modeling $DegT_{50}$ (20°C, pF 2) were 0.5 and 1.0 days.

For metabolite TDIT, trigger $DegT_{50}$ values were 0.2 and 0.6 days, and $DegT_{90}$ values were 0.5 and 2.1 days. Normalized modeling $DegT_{50}$ (20°C, pF 2) were 0.2 and 0.4 days with corresponding formation fractions from metiram of 0.187 and 0.416.

For metabolite EBIS, trigger $DegT_{50}$ values were 0.1 and 1.7 days, and $DegT_{90}$ values were 0.2 and 5.7 days. Normalized modeling $DegT_{50}$ (20°C, pF 2) were 0.1 and 1.1 days with corresponding formation fractions from metiram of 0.713 and 0.041.

For metabolite ETU, trigger $DegT_{50}$ values were 0.6 and 2.4 days, and $DegT_{90}$ values were 1.9 and 8.0 days. Normalized modeling $DegT_{50}$ (20°C, pF 2) were 0.4 and 2.4 days with corresponding formation fractions from TDIT of 1 and 0.806.

I. MATERIAL AND METHODS

The aerobic degradation of metiram has been investigated in the laboratory in two soils in two studies [*see CA 7.1.1.1/1, BASF DocID 2002/1011913 and CA 7.1.1.1/2, BASF DocID 2002/1012954*]. In both studies, ethylene-^{14}C-labeled metiram was used. The resulting data were analyzed taking into account the current guidance of the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*].

Kinetic modeling strategy

Kinetic evaluation was performed for metiram and its metabolites TDIT, EBIS and ETU considering the procedures and kinetic models proposed by the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*]. According to FOCUS, degradation endpoints were derived for use as triggers for future work and for use as modeling inputs.

The metabolites EU and carbimide were not included in the evaluation as there was either only a limited number of data points available (which would lead to an insufficient number of degrees of freedom) or the residues were too low for a reliable parameter estimation.

The kinetic models SFO, FOMC and DFOP were employed for the evaluation of the parent. For the metabolites, only SFO kinetics were used.

Trigger endpoints were derived from the kinetic models that provided the best fit to the measured data, generally indicated by the lowest χ^2 – error. Modeling endpoints were derived preferably from SFO model. If the SFO model was not appropriate, pragmatic procedures were used to derive conservative pseudo-SFO degradation rates from the appropriate biphasic model.

The appropriateness of the distinct kinetic model to describe soil degradation was tested by visual assessment, estimation of the error percentage at which the χ^2 test was passed and the t-test to evaluate whether estimated degradation parameters differ from zero.

A kinetic model was considered appropriate for deriving trigger or modeling endpoints if the χ^2 -error value was low (ideally below 15%, but may be larger if the overall pattern of decline in pesticide concentrations was represented adequately by the model and the distribution of the residuals was random, i.e. not a cut-off criterion) and the t-test for the degradation parameters was passed at 5% error level.

Where applicable, the endpoints of the parent compound metiram and its metabolites TDIT, EBIS and ETU were derived from a combined fit (compartment modeling approach).

According to FOCUS, the most appropriate conceptual model to describe the degradation pathway with simultaneous formation of TDIT and EBIS from the parent as well as formation of ETU from TDIT and EBIS was assumed as initial pathway which was further adapted during the evaluation.

Because of significant correlations between the ETU formation pathways TDIT->ETU and EBIS->ETU the compartment model was simplified by excluding the (relatively low) formation pathway EBIS->ETU. This approach has no influence on the subsequent risk assessment as both possible ETU precursors degrades very fast and have a similar sorption behaviour that ETU would be formed in the same soil horizon, in any case.

The final degradation pathway is shown in Figure 7.1.2.1.1-1.

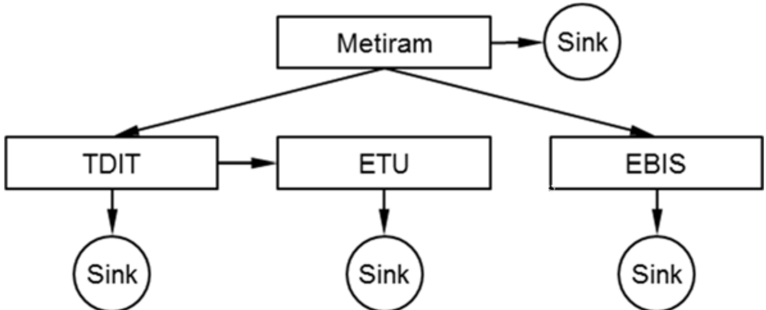


Figure 7.1.2.1.1-1: Final degradation pathway of metiram

Data handling

The software package KinGUI version 2.2012.320.1629 was used for parameter fitting. The time points reported in days were converted into hours in order to increase the calculation accuracy of the optimization approach.

For the parent substance metiram replicate measurements from analysis of two aliquots of the individual soil samples were used for the parameter estimation while for the metabolites single measurements were available.

In the study on Soil Cashmere [*see CA 7.1.1.1/1, BASF DocID 2002/1011913*], additional data for metiram analyzed immediately after application and after 3 hours storage at room temperature were available. The additional data were included in the data set for kinetic evaluation as 0 and 3 h (0.125 DAT) sampling time and the data from the original first sampling were assigned to 3 h (0.125 DAT) sampling time. As the material balance at early sampling times was close to 100% TAR, the initial concentration was set to 100% TAR for metiram and to 0% TAR for the metabolites.

In the study on Soil Li 35b [*see CA 7.1.1.1/2, BASF DocID 2002/1012954*], the results from the first sampling corresponded very well to the results of the first sampling of the study on Soil Cashmere, which were assigned to 3 h sampling time. As it is likely that the time between application and first sampling was the same for both experiments, the results of the first sampling were also assigned to 3 h sampling time. For the kinetic evaluation, an additional data point was added to the data set of Soil Li 35b for 0 h sampling time with 100% TAR for metiram and 0% TAR for the metabolites.

Values below the limit of quantification (LOQ) were treated as recommended by FOCUS [*FOCUS (2006)*].

Experimental data used for kinetic analysis

In the study BASF DocID 2002/1011913 [*see CA 7.1.1.1/1*], the test soil Cashmere was treated with ethylene-^{14}C-labeled metiram at a nominal rate of 7.2 mg a.s. kg^{-1} dry soil which corresponds to a field application rate of 5.4 kg a.s. ha^{-1}. The actual application rate, determined from analysis of the total radioactivity in six aliquots of treated soil immediately after application, was 7.53 mg a.s. kg^{-1}. The treated soil was incubated under dark aerobic conditions at 20°C and 75% of soil moisture at 1/3 bar for 365 days.

In the study BASF DocID 2002/1012954 [*see CA 7.1.1.1/2*], the test soil Li 35b was treated with ethylene-^{14}C-labeled metiram at a nominal rate of 3.6 mg a.s. kg^{-1} dry soil which corresponds to a field application rate of 2.7 kg a.s. ha^{-1}. The actual application rate, determined from analysis of the total radioactivity in eight aliquots of treated soil immediately after application, was 3.94 mg a.s. kg^{-1}. The treated soil was incubated under dark aerobic conditions at 20°C and 40% of soil maximum water holding capacity (MWHC) for 133 days.

The measured data as well as resulting datasets submitted to kinetic analysis are summarized in Table 7.1.2.1.1-2 and Table 7.1.2.1.1-3.

Table 7.1.2.1.1-2: Data for Soil Cashmere for kinetic evaluation [*BASF DocID 2002/1011913*]

Time [d]	Residue data					Input data according to FOCUS				
	Metiram [a] [mg kg^{-1}]	Metiram [b] [% TAR]	TDIT [% TAR]	EBIS [% TAR]	ETU [% TAR]	Time [h]	Metiram [% TAR]	TDIT [% TAR]	EBIS [% TAR]	ETU [% TAR]
0 [c]	6.492	90.2	n.a.	n.a.	n.a.	0	100.0 [d]	0.0 [e]	0.0 [e]	0.0 [e]
0 [c]	6.527	90.6								
0.125 [c]	4.777	66.4				3	66.4	-	-	-
0.125 [c]	4.638	64.4				3	64.4	-	-	-
0.125	5.054	67.1	13.5	1.6	1.4	3	67.1	13.5	1.6	1.4
	5.212	69.2				3	69.2	-	-	-
1	2.736	36.3	11.1	1.7	7.6	24	36.3	11.1	1.7	7.6
	2.832	37.6				24	37.6	-	-	-
2	1.583	21.0	6.9	1.5	11.2	48	21.0	6.9	1.5	11.2
	1.702	22.6				48	22.6	-	-	-
3	1.082	14.4	6.0	1.7	3.1	72	14.4	6.0	1.7	3.1
	1.089	14.5				72	14.5	-	-	-
7	0.400	5.3	2.0	0.4	0.2	168	5.3	2.0	0.4	0.2
	0.366	4.9				168	4.9	-	-	-
14	0.185	2.5	0.8	0.3	<0.05 [g]	336	2.5	0.8	0.3	0.025 [f]
	0.190	2.5				336	2.5	-	-	-
30	0.135	1.8	0.2	0.1	n.d.	720	1.8	0.2	0.1	-
	0.128	1.7				720	1.7	-	-	-
62	0.084	1.1	0.1	0.1	n.d.	1488	1.1	0.1	0.1	-
	0.056	0.7				1488	0.7	-	-	-
90	0.066	0.9	0.1	0.1	n.d.	2160	0.9	0.1	0.1	-
	0.068	0.9				2160	0.9	-	-	-
121	0.043	0.6	0.1	<0.05 [g]	n.d.	2904	0.6	0.1	0.025 [f]	-
	0.032	0.4				2904	0.4	-	-	-
240	0.030	0.4	0.1	<0.05 [g]	n.d.	5760	0.4	0.1	0.025 [f]	-
	0.028	0.4				5760	0.4	-	-	-
365	0.035	0.5	n.d.	n.d.	n.d.	8760	0.5	0.025 [f]	-	-
	0.037	0.5				8760	0.5	-	-	-

n.a. not analyzed
n.d. not detected
[a] Reported values
[b] Calculated, 100% TAR corresponds to 7.53 mg kg^{-1}
[c] Separate application without analysis of metabolites
[d] Set to 100%
[e] Set to zero
[g] Detectable amounts <0.05% TAR rounded down
[f] Set to ½ LOQ (LOQ = 0.05% TAR)

Table 7.1.2.1.1-3: Data for Soil Li 35b for kinetic evaluation [*BASF DocID 2002/1012954*]

Time [d]	Residue data					Input data according to FOCUS					
	Metiram [a] [mg kg^{-1}]	Metiram [b] [% TAR]	TDIT [% TAR]	EBIS [% TAR]	ETU [% TAR]	Time [h]	Metiram [% TAR]	TDIT [% TAR]	EBIS [% TAR]	ETU [% TAR]
0 [c]						0	100.0 [d]	0.0 [e]	0.0 [e]	0.0 [e]
0.125 [f]	2.690	68.3	4.6	11.4	1.3	3	68.3	4.6	11.4	1.3
	2.660	67.5				3	67.5	-	-	-
1	0.647	16.4	1.1	1.1	8.3	24	16.4	1.1	1.1	8.3
	0.647	16.4				24	16.4	-	-	-
2	0.308	7.8	0.7	0.5	9.2	48	7.8	0.7	0.5	9.2
	0.308	7.8				48	7.8	-	-	-
3	0.238	6.0	0.4	0.5	10.1	72	6.0	0.4	0.5	10.1
	0.238	6.0				72	6.0	-	-	-
7	0.144	3.7	0.1	0.5	0.1	168	3.7	0.1	0.5	0.1
	0.122	3.1				168	3.1	-	-	-
14	0.111	2.8	<0.05 [g]	0.2	n.d.	336	2.8	0.025 [h]	0.2	0.025 [h]
	0.094	2.4				336	2.4	-	-	-
35	0.035	0.9	0.3	<0.05 [g]	<0.05 [g]	840	0.9	0.3	0.025 [h]	-
	0.031	0.8				840	0.8	-	-	-
62	0.032	0.8	n.d.	n.d.	n.d.	1488	0.8	0.025 [h]	-	-
	0.032	0.8				1488	0.8	-	-	-
90	0.043	1.1	n.d.	n.d.	n.d.	2160	1.1	-	-	-
	0.042	1.1				2160	1.1	-	-	-
133	0.039	1.0	n.d.	n.d.	n.d.	3192	1.0	-	-	-
	0.045	1.1				3192	1.1	-	-	-

n.d. not detected
[a] Reported values
[b] Calculated, 100% TAR corresponds to 3.94 mg kg^{-1}
[c] Inserted for kinetic analysis; no measurement available
[d] Set to 100%
[e] Set to zero
[f] Estimated analysis time
[g] Detectable amounts <0.05% TAR rounded down
[h] Set to ½ LOQ (LOQ = 0.05% TAR)

Normalization to reference conditions

The $DegT_{50}$ values suitable for modeling were normalized to reference conditions (20°C, pF 2). As the studies were performed at 20°C, temperature correction was not necessary. The moisture normalization was performed using the moisture dependency equations by Walker, with a Walker coefficient of 0.7 according to FOCUS [*FOCUS (2012): Generic Guidance for Tier 1 FOCUS Ground Water Assessments, version 2.1, 64 pp*].

II. RESULTS AND DISCUSSION

The kinetic evaluation showed that the degradation of metiram in soil is best described with biphasic kinetic models (FOMC, DFOP). For the metabolites the evaluation showed no indication of a biphasic degradation pattern as the SFO model was appropriate for the two experiments. For the evaluated soils, the visual assessment indicated plausible fit. For the combined fits of parent and metabolites, χ^2 error values above 15% were obtained for the metabolite fits. They were attributed to the low level of measured residues as well as to the rapid transformation processes that are symptomatic for the degradation of metiram and its metabolites in soil. As the observations were generally well described by the fitted curves the high χ^2 error values were considered acceptable. The t-test was passed for the respective model parameters. Therefore, the resulting $DegT_{50}$ values were considered reliable.

Trigger endpoints

The trigger endpoints for metiram and its metabolites TDIT, EBIS and ETU calculated for two soils are given in Table 7.1.2.1.1-4 to Table 7.1.2.1.1-7.

Table 7.1.2.1.1-4: Trigger endpoints for metiram

DocID	Soil	Best-fit model	χ^2 error [%]	Trigger endpoints	
				$DegT_{50}$ [d]	$DegT_{90}$ [d]
2002/1011913	Cashmere	DFOP	5.8	0.4	3.9
2002/1012954	Li 35b	FOMC	4.4	0.2	1.7

Table 7.1.2.1.1-5: Trigger endpoints for TDIT

DocID	Soil	Best-fit model	χ^2 error [%]	Trigger endpoints	
				$DegT_{50}$ [d]	$DegT_{90}$ [d]
2002/1011913	Cashmere	SFO [a]	15.7	0.6	2.1
2002/1012954	Li 35b	SFO [b]	19.6	0.2	0.5

[a] DFOP kinetics for parent
[b] FOMC kinetics for parent

Table 7.1.2.1.1-6: Trigger endpoints for EBIS

DocID	Soil	Best-fit model	χ^2 error [%]	Trigger endpoints	
				$DegT_{50}$ [d]	$DegT_{90}$ [d]
2002/1011913	Cashmere	SFO [a]	20.2	1.7	5.7
2002/1012954	Li 35b	SFO [b]	9.9	0.1	0.2

[a] DFOP kinetics for parent
[b] FOMC kinetics for parent

Table 7.1.2.1.1-7: Trigger endpoints for ETU

DocID	Soil	Best-fit model	χ^2 error [%]	Trigger endpoints	
				$DegT_{50}$ [d]	$DegT_{90}$ [d]
2002/1011913	Cashmere	SFO [a]	36.0	0.6	1.9
2002/1012954	Li 35b	SFO [b]	27.1	2.4	8.0

[a] DFOP kinetics for parent
[b] FOMC kinetics for parent

Modeling endpoints

The $DegT_{50}$ values suitable for modeling obtained under different incubation conditions were normalized to reference conditions (20°C, pF 2). Parameters included in the normalization procedure are shown in Table 7.1.2.1.1-8. Modeling endpoints for metiram and its metabolites are summarized Table 7.1.2.1.1-9 to Table 7.1.2.1.1-12.

Table 7.1.2.1.1-8: Normalization of $DegT_{50}$ values for metiram and its metabolites TDIT, EBIS and ETU to reference conditions

Soil (soil type)	Compound	Kinetic model	θ_{act} [g 100 g^{-1}]	θ_{ref} [g 100 g^{-1}]	f_{moist} [-]	$DegT_{50act}$ [d]	$DegT_{50ref}$ [d]
Cashmere (Loamy sand)	Metiram	DFOP	8.3	15.1 [a]	0.659	1.5 [c]	1.0
	TDIT	SFO				0.6	0.4
	EBIS	SFO				1.7	1.1
	ETU	SFO				0.6	0.4
Li 35b (Loamy sand)	Metiram	FOMC	13.7	14.0 [b]	0.984	0.5 [d]	0.5
	TDIT	SFO				0.2	0.2
	EBIS	SFO				0.1	0.1
	ETU	SFO				2.4	2.4

θ_{act} Actual soil moisture [g 100 g^{-1} dry soil]
θ_{ref} Reference soil moisture at field capacity (pF 2) according to Ref. 8 or study report [g 100 g^{-1} dry soil]
f_{moist} Moisture correction factor [-]
$DegT_{50act}$ $DegT_{50}$ at study conditions [d]
$DegT_{50ref}$ $DegT_{50}$ at reference conditions [d]
[a] From study report
[b] According to FOCUS [*FOCUS (2012)*]
[c] Calculated as $DegT_{50} = \ln(2)/k2$
[d] Calculated as $DegT_{50} = DT_{90}/3.32$

Table 7.1.2.1.1-9: Modeling endpoints for metiram

DocID	Soil	Kinetic model	χ^2 error [%]	Modeling endpoints	
				$DegT_{50}$ [d]	Normalized $DegT_{50}$ [d] [a]
2002/1011913	Cashmere	DFOP	5.8	1.5 [b]	1.0
2002/1012954	Li 35b	FOMC	4.4	0.5 [c]	0.5

[a] Reference conditions: 20°C, pF 2
[b] Calculated as $DegT_{50} = \ln(2)/k2$
[c] Calculated as $DegT_{50} = DT_{90}/3.32$

Table 7.1.2.1.1-10: Modeling endpoints for TDIT

DocID	Soil	Kinetic model	χ^2 error [%]	Modeling endpoints		
				$DegT_{50}$ [d]	Normalized $DegT_{50}$ [d] [a]	Formation fraction
2002/1011913	Cashmere	SFO [b]	15.7	0.6	0.4	0.416 [d]
2002/1012954	Li 35b	SFO [c]	19.6	0.2	0.2	0.187 [d]

[a] Reference conditions: 20°C, pF 2
[b] DFOP kinetics for parent
[c] FOMC kinetics for parent
[d] From metiram

Table 7.1.2.1.1-11: Modeling endpoints for EBIS

DocID	Soil	Kinetic model	χ^2 error [%]	Modeling endpoints		
				$DegT_{50}$ [d]	Normalized $DegT_{50}$ [d] [a]	Formation fraction
2002/1011913	Cashmere	SFO [b]	20.2	1.7	1.1	0.041 [d]
2002/1012954	Li 35b	SFO [c]	9.9	0.1	0.1	0.713 [d]

[a] Reference conditions: 20°C, pF 2
[b] DFOP kinetics for parent
[c] FOMC kinetics for parent
[d] From metiram

Table 7.1.2.1.1-12: Modeling endpoints for ETU

DocID	Soil	Kinetic model	χ^2 error [%]	Modeling endpoints		
				$DegT_{50}$ [d]	Normalized $DegT_{50}$ [d] [a]	Formation fraction
2002/1011913	Cashmere	SFO [b]	36.0	0.6	0.4	1 [d]
2002/1012954	Li 35b	SFO [c]	27.1	2.4	2.4	0.806 [d]

[a] Reference conditions: 20°C, pF 2
[b] DFOP kinetics for parent
[c] FOMC kinetics for parent
[d] From TDIT

III. CONCLUSION

Trigger and modeling endpoints were calculated for metiram and its metabolites TDIT, EBIS and ETU in two laboratory degradation studies with two different soils. The kinetic evaluation showed that the degradation of metiram in soil was best described with biphasic kinetic models (FOMC, DFOP). For the metabolites the evaluation showed no indication of a biphasic degradation pattern as the SFO model was appropriate for the two experiments.

For metiram, best-fit $DegT_{50}$ values (trigger endpoints) were 0.2 and 0.4 days, and $DegT_{90}$ values were 1.7 and 3.9 days. Normalized modeling $DegT_{50}$ (20°C, pF 2) were 0.5 and 1.0 days.

For metabolite TDIT, trigger $DegT_{50}$ values were 0.2 and 0.6 days, and $DegT_{90}$ values were 0.5 and 2.1 days. Normalized modeling $DegT_{50}$ (20°C, pF 2) were 0.2 and 0.4 days with corresponding formation fractions from metiram of 0.187 and 0.416.

For metabolite EBIS, trigger $DegT_{50}$ values were 0.1 and 1.7 days, and $DegT_{90}$ values were 0.2 and 5.7 days. Normalized modeling $DegT_{50}$ (20°C, pF 2) were 0.1 and 1.1 days with corresponding formation fractions from metiram of 0.713 and 0.041.

For metabolite ETU, trigger $DegT_{50}$ values were 0.6 and 2.4 days, and $DegT_{90}$ values were 1.9 and 8.0 days. Normalized modeling $DegT_{50}$ (20°C, pF 2) were 0.4 and 2.4 days with corresponding formation fractions from TDIT of 1 and 0.806.

Report: CA 7.1.2.1.1/4
Voelkel W., 2015a
14C-Metiram - Degradation and metabolism in two soils incubated under aerobic conditions
2013/1095967
Guidelines: OECD 307 (2002), EPA 835.4100
GLP: yes
(certified by Swiss Federal Office of Public Health, Berne, Switzerland)

This experimental study [*BASF DocID 2013/1095967*] is already described in detail in CA 7.1.1.1/3. The kinetic evaluation is presented below.

Report: CA 7.1.2.1.1/5
Maassen K., 2015a
Kinetic evaluation of a laboratory soil degradation study with BAS 222 F - Metiram and its metabolites according to Focus Degradation Kinetics
2014/1311117
Guidelines: FOCUS Degradation Kinetics (2006) SANCO/10058/2005 version 2.0 (December 2014)
GLP: no
(certified by <none>)

Executive Summary

The aerobic degradation of metiram has been investigated in the laboratory in two soils in one study using ethylene-^{14}C-labeled metiram. The purpose of this evaluation was to analyze the degradation kinetics of metiram and its metabolites observed in this study, taking into account the current guidance of the FOCUS workgroup on degradation kinetics.

Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints) and degradation parameters for environmental fate models (modeling endpoints).

Normalization of endpoints suitable for modeling was not necessary as the study was conducted at reference conditions.

For metiram, best-fit $DegT_{50}$ values (trigger endpoints) were 0.5 and 0.3 days, and $DegT_{90}$ values were 4.2 and 7.3 days. Modeling $DegT_{50}$ (20°C, pF 2) were 1.6 and 2.2 days.

For metabolite EBIS, trigger $DegT_{50}$ values were 0.3 and 0.4 days, and $DegT_{90}$ values were 1.0 and 1.3 days. Modeling $DegT_{50}$ (20°C, pF 2) were 0.3 and 0.4 days and formation fractions from metiram were 0.952 and 0.608.

For metabolite ETU, endpoints could only be derived from soil Am Fischteich. Trigger endpoints were a $DegT_{50}$ of 1.7 days and a $DegT_{90}$ of 5.7 days. Modeling endpoints were a $DegT_{50}$ (20°C, pF 2) of 1.7 and a formation fraction from EBIS of 0.024.

For metabolite EU, endpoints could only be derived from soil Am Fischteich. Trigger endpoints were a $DegT_{50}$ of 0.7 days and $DegT_{90}$ of 2.5 days. Modeling endpoints were a $DegT_{50}$ (20°C, pF 2) of 0.7 and a formation fraction from EBIS of 0.268.

I. MATERIAL AND METHODS

The aerobic degradation of metiram has been investigated in the laboratory in two soils in one study [*see CA 7.1.1.1/3, BASF DocID 2013/1095967*]. In the study, ethylene-^{14}C-labeled metiram was used. The resulting data were analyzed taking into account the current guidance of the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*].

Kinetic modeling strategy

Kinetic evaluation was performed for metiram and its metabolites EBIS, ETU and EU considering the procedures and kinetic models proposed by the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*]. According to FOCUS, degradation endpoints were derived for use as triggers for future work and for use as modeling inputs.

The kinetic models SFO, FOMC and DFOP were employed for the evaluation of the parent. For the metabolites, only SFO kinetics were used.

Trigger endpoints were derived from the kinetic models that provided the best fit to the measured data, generally indicated by the lowest χ^2 – error. Modeling endpoints were derived preferably from SFO model. If the SFO model was not appropriate, pragmatic procedures were used to derive conservative pseudo-SFO degradation rates from the appropriate bi-phasic model.

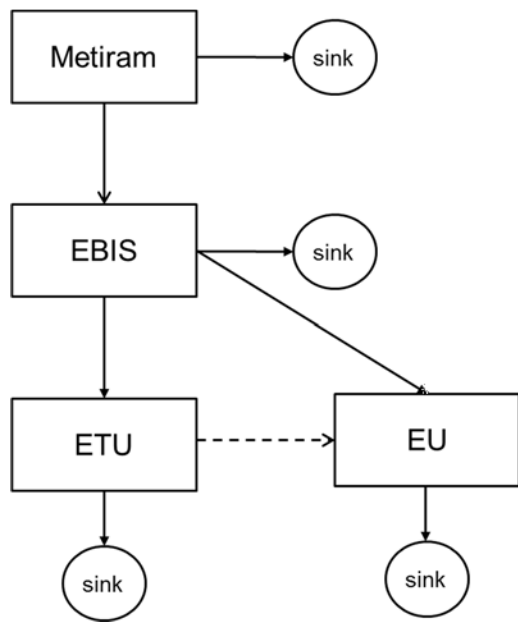
The appropriateness of the distinct kinetic model to describe soil degradation was tested by visual assessment, estimation of the error percentage at which the χ^2 test was passed and the t-test to evaluate whether estimated degradation parameters differ from zero.

A kinetic model was considered appropriate for deriving trigger or modeling endpoints if the χ^2 - error value was low (ideally below 15%, but may be larger if the overall pattern of decline in pesticide concentrations was represented adequately by the model and the distribution of the residuals was random, i.e. not a cut-off criterion) and the t-test for the degradation parameters was passed at 5% error level.

Where applicable, the endpoints of the parent compound metiram and its metabolites EBIS, ETU and EU were derived from a combined fit (compartment modeling approach) in a stepwise approach.

According to FOCUS, the most appropriate conceptual model to describe the degradation pathway, i.e. metiram → EBIS → ETU → EU was used for the first fitting step. Based on the visual and statistical results of the individual fitting steps the compartment model was modified.

The final degradation pathway is shown in Figure 7.1.2.1.1-2.



Dashed line: Pathway only considered in soil Speyer 2.2

Figure 7.1.2.1.1-2: Final degradation pathway of metiram

Data handling

The software package KinGUI version 2.2012.320.1629 [*SCHÄFER et al. (2007); SCHMITT et al. (2011)*] was used for parameter fitting. The time points reported in days were converted into hours in order to increase the calculation accuracy of the optimization approach.

The experimental data were derived from the study reports and adjusted according to FOCUS [FOCUS (2006)].

For all compounds, duplicate measurements from analysis of two individual soil samples were available and used for the parameter estimation.

The first sample was taken and analyzed 2.5 hours (0.104 DAT) following application. Due to the rapid degradation of metiram less than 80% TAR was recovered as parent and more than 20% TAR was recovered as degradation products in the soil extracts of the first sampling. The overall material balance corresponds to the initially applied amount of the test substance. In order to use realistic initial concentrations, a new data point at 0 DAT was added to the data set where the residues of metiram were set to 100% TAR (i.e. the mean of the recovery of the applied radioactivity for each of the two soils) and the residues for the metabolites were set to 0% TAR.

Experimental data used for kinetic analysis

In the study BASF DocID 2013/1095967 [*see CA 7.1.1.1/3*], the test soils were treated with ethylene-^{14}C-labeled metiram at a concentration of 5.2 to 5.8 mg a.s. kg^{-1} dry soil, which corresponds to a field application rate of 1.4 kg a.s. ha^{-1} when assuming an even distribution of the test item in the top 2.5 cm soil layer and a soil bulk density of 1 g cm^3.

The treated soils were incubated under dark aerobic conditions at a temperature of 20°C and a soil moisture adjusted to the water holding capacity at pF 2 for 122 days.

The measured data as well as resulting datasets submitted to kinetic analysis are summarized in Table 7.1.2.1.1-13 and Table 7.1.2.1.1-14.

Table 7.1.2.1.1-13: Data for soil Am Fischteich for kinetic evaluation

Time [d]	Residue data					Input data according to FOCUS				
	Metiram [a] [mg kg^{-1}]	Metiram [b] [% TAR]	EBIS [% TAR]	ETU [% TAR]	EU [% TAR]	Time [h]	Metiram [% TAR]	EBIS [% TAR]	ETU [% TAR]	EU [% TAR]
0	Not sampled					0	100[c]	0[d]	0[d]	0[d]
						0	100[c]	0[d]	0[d]	0[d]
0.104	3.978	68.1	26.3	<LOD	2.8	2.5	68.1	26.3	0.01[e]	2.8
	4.161	71.3	25.1	<LOD	2.6	2.5	71.3	25.1	0.01[e]	2.6
1	2.384	40.8	9.3	0.6	6.3	24	40.8	9.3	0.6	6.3
	2.198	37.6	13.4	1.4	7.6	24	37.6	13.4	1.4	7.6
2	1.224	20.9	7.3	0.6	6.5	48	20.9	7.3	0.6	6.5
	1.409	24.1	7.2	1.3	7.2	48	24.1	7.2	1.3	7.2
3	0.931	15.9	3.0	0.6	4.1	72	15.9	3.0	0.6	4.1
	1.106	18.9	3.2	0.8	3.2	72	18.9	3.2	0.8	3.2
6	0.554	9.5	0.7	0.7	1.5	144	9.5	0.7	0.7	1.5
	0.528	9.1	0.9	0.4	1.6	144	9.1	0.9	0.4	1.6
14	0.322	5.5	<LOD	0.1	0.8	336	5.5	0.01[e]	0.1	0.8
	0.340	5.9	0.1	0.1	0.7	336	5.9	0.1	0.1	0.7
29	0.073	1.2	<LOD	<LOD	<LOD	696	1.2	NaN	0.01[e]	0.01[e]
	0.080	1.4	<LOD	<LOD	<LOD	696	1.4	0.01[e]	0.01[e]	0.01[e]
58	0.112	1.9	<LOD	<LOD	<LOD	1392	1.9	NaN	NaN	NaN
	0.123	2.1	<LOD	<LOD	<LOD	1392	2.1	NaN	NaN	NaN
92	0.154	2.6	<LOD	<LOD	<LOD	2208	2.6	NaN	NaN	NaN
	0.142	2.4	<LOD	<LOD	<LOD	2208	2.4	NaN	NaN	NaN
122	0.039	0.7	<LOD	<LOD	<LOD	2928	0.7	NaN	NaN	NaN
	0.128	2.2	<LOD	<LOD	<LOD	2928	2.2	NaN	NaN	NaN

a Reported
b Calculated
c Set to mean of recovery of radioactivity
d Set to zero following FOCUS
e Set to ½ LOD (LOD for metabolites = 0.02% TAR) following FOCUS

Table 7.1.2.1.1-14: Data for soil Speyer 2.2 for kinetic evaluation

Time [d]	Residue data					Input data according to FOCUS				
	Metiram [a] [mg kg^{-1}]	Metiram [b] [% TAR]	EBIS [% TAR]	ETU [% TAR]	EU [% TAR]	Time [h]	Metiram [% TAR]	EBIS [% TAR]	ETU [% TAR]	EU [% TAR]
0	Not sampled					0	100[c]	0[d]	0[d]	0[d]
						0	100[c]	0[d]	0[d]	0[d]
0.104	3.807	74.1	14.6	<LOD	1.9	2.5	74.1	14.6	NaN	1.9
	4.047	78.7	15.4	<LOD	2.2	2.5	78.7	15.4	NaN	2.2
1	1.128	21.9	11.2	<LOD	3.3	24	21.9	11.2	NaN	3.3
	1.402	27.3	10.5	<LOD	2.7	24	27.3	10.5	NaN	2.7
2	1.064	20.7	7.4	<LOD	2.3	48	20.7	7.4	0.01	2.3
	1.546	30.1	6.3	<LOD	2.6	48	30.1	6.3	0.01	2.6
3	0.983	19.2	2.1	1.1	0.9	72	19.2	2.1	1.1	0.9
	0.894	17.4	2.6	0.4	1.8	72	17.4	2.6	0.4	1.8
6	0.568	11.1	1.3	0.2	1.3	144	11.1	1.3	0.2	1.3
	0.417	8.1	1.0	0.1	1.2	144	8.1	1.0	0.1	1.2
14	0.274	5.3	0.1	<LOD	0.7	336	5.3	0.1	0.01	0.7
	0.222	4.3	0.1	<LOD	0.8	336	4.3	0.1	0.01	0.8
29	0.212	4.1	<LOD	<LOD	0.7	696	4.1	0.01[e]	NaN	0.7
	0.229	4.5	<LOD	0.1	0.6	696	4.5	0.01[e]	0.1	0.6
58	0.055	1.1	<LOD	<LOD	<LOD	1392	1.1	NaN	NaN	0.01[e]
	0.123	2.3	<LOD	<LOD	<LOD	1392	2.3	NaN	0.01[e]	0.01[e]
99	0.122	2.4	<LOD	<LOD	<LOD	2376	2.4	NaN	NaN	NaN
	0.093	1.8	<LOD	<LOD	<LOD	2376	1.8	NaN	NaN	NaN
122	<LOQ	<LOQ	<LOD	<LOD	<LOD	2928	0.63[f]	NaN	NaN	NaN
	<LOQ	<LOQ	<LOD	<LOD	<LOD	2928	0.63[f]	NaN	NaN	NaN

[a] Reported
[b] Calculated
[c] Set to mean of recovery of radioactivity
[d] Set to zero according to FOCUS
[e] Set to ½ LOD, LOD for metabolites = 0.02% TAR, following FOCUS
[f] Set to ½ (LOD + LOQ), LOD for metiram = 0.42% TAR, LOQ for metiram = 0.83% TAR, following FOCUS

Normalization to reference conditions

Normalization of endpoints suitable for modeling was not necessary as the study was conducted at reference conditions (20°C, pF 2).

II. RESULTS AND DISCUSSION

The kinetic evaluation showed that the degradation of metiram in soil is best described with biphasic kinetic models (FOMC, DFOP). For the metabolites, the evaluation showed no indication of a biphasic degradation pattern as the SFO model was appropriate for both soils.

For the combined fits of parent and metabolites, χ^2 error values above 15% were obtained for the metabolite fits. They can be attributed to the low level of measured residues as well as to the rapid transformation processes that are symptomatic for the degradation of metiram and its metabolites in soil. As the observations were generally well described by the fitted curves (the residuals are randomly scattered around zero, hence there is no indication for bi-phasic degradation behavior of the metabolites) the high χ^2 error values are acceptable.

The derived trigger and modeling endpoints for metiram and its metabolites EBIS, ETU and EU calculated from two soils are given in Table 7.1.2.1.1-15 to Table 7.1.2.1.1-18.

Table 7.1.2.1.1-15: Trigger and modeling endpoints for metiram

Soil	Kinetic model	χ^2 error	Trigger endpoints		Modeling $DegT_{50}$ [a] [d]
			$DegT_{50}$ [d]	$DegT_{90}$ [d]	
Am Fischteich	DFOP	9.6	0.5	4.2	1.6 [b]
Speyer 2.2	FOMC	8.3	0.3	7.3	2.2 [c]

[a] At 20°C and pF2
[b] Calculated as $DegT_{50} = \ln(2)/k2$
[c] Calculated as $DegT_{50} = DegT_{90}/3.32$

Table 7.1.2.1.1-16: Trigger and modeling endpoints for EBIS

Soil	Kinetic model	χ^2 error	Trigger endpoints		Modeling endpoints	
			$DegT_{50}$ [d]	$DegT_{90}$ [d]	$DegT_{50}$ [a] [d]	Formation fraction
Am Fischteich	SFO	6.7	0.3	1.0	0.3	0.952 [b]
Speyer 2.2	SFO	19.2	0.4	1.3	0.4	0.608 [b]

[a] At 20°C and pF2
[b] From metiram

Table 7.1.2.1.1-17: Trigger and modeling endpoints for ETU

Soil	Kinetic model	χ^2 error	Trigger endpoints		Modeling endpoints	
			$DegT_{50}$ [d]	$DegT_{90}$ [d]	$DegT_{50}$ [a] [d]	Formation fraction
Am Fischteich	SFO	15.9	1.7	5.7	1.7	0.024 [b]
Speyer 2.2	SFO	No reliable endpoints derived [c]				

[a] At 20°C and pF2
[b] From EBIS
[c] No appropriate fit derived

Table 7.1.2.1.1-18: Trigger and modeling endpoints for EU

Soil	Kinetic model	χ^2 error	Trigger endpoints		Modeling endpoints	
			$DegT_{50}$ [d]	$DegT_{90}$ [d]	$DegT_{50}$ [a] [d]	Formation fraction
Am Fischteich	SFO	20.2	0.7	2.5	0.7	0.268 [b]
Speyer 2.2	SFO	No reliable endpoints derived [c]				

[a] At 20°C and pF2
[b] From EBIS
[c] No appropriate fit derived

III. CONCLUSION

Trigger and modeling endpoints were derived for metiram and its metabolites EBIS, ETU and EU in one laboratory degradation study with two different soils. The kinetic evaluation showed that the degradation of metiram in soil is best described with biphasic kinetic models (FOMC, DFOP). For the metabolites the evaluation showed no indication of a biphasic degradation pattern as the SFO model was appropriate for both soils.

For metiram, best-fit $DegT_{50}$ values (trigger endpoints) were 0.5 and 0.3 days, and $DegT_{90}$ values were 4.2 and 7.3 days. Modeling $DegT_{50}$ (20°C, pF 2) were 1.6 and 2.2 days.

For metabolite EBIS, trigger $DegT_{50}$ values were 0.3 and 0.4 days, and $DegT_{90}$ values were 1.0 and 1.3 days. Modeling $DegT_{50}$ (20°C, pF 2) were 0.3 and 0.4 days and formation fractions from metiram were 0.952 and 0.608.

For metabolite ETU, endpoints could only be derived from soil Am Fischteich. Trigger endpoints were a $DegT_{50}$ of 1.7 days and a $DegT_{90}$ of 5.7 days. Modeling endpoints were a $DegT_{50}$ (20°C, pF 2) of 1.7 and a formation fraction from EBIS of 0.024.

For metabolite EU, endpoints could only be derived from soil Am Fischteich. Trigger endpoints were a $DegT_{50}$ of 0.7 days and $DegT_{90}$ of 2.5 days. Modeling endpoints were a $DegT_{50}$ (20°C, pF 2) of 0.7 and a formation fraction from EBIS of 0.268.

Report: CA 7.1.2.1.1/6
Staudenmaier H.,Kuhnke G., 2011b
Formation of CS2 from Metiram in soil
2010/1090462
Guidelines: OECD 307 (2002), EPA 835.4100, BBA IV 4-1, EPA 162-1, SETAC Procedures for assessing the environmental fate and ecotoxicity for pesticides (March 1995)
GLP: yes
(certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

This experimental study [*BASF DocID 2010/1090462*] is already described in detail in CA 7.1.1.1/4. The normalization is presented below.

Report: CA 7.1.2.1.1/7
Maassen K., 2015b
Derivation of a modeling degradation half-life of Metiram in soil at reference temperature and moisture conditions
2014/1314053
Guidelines: FOCUS Groundwater (2014) Generic Guidance for Tier 1 FOCUS Ground Water Assessments v 2.2.
GLP: no
(certified by <none>)

Executive Summary

A $DegT_{50}$ value of metiram derived from a laboratory study under aerobic soil conditions [*see CA 7.1.1.1/4, BASF DocID 2010/1090462*] was normalized to reference moisture conditions at pF = 2 following FOCUS (2014) in order to derive a modeling endpoint suitable for the use in environmental fate models.

The normalization was performed using the moisture dependency equations recommended by FOCUS (2014). The actual soil moisture (40% at MWHC) and the reference moisture at field capacity at pF 2 were taken from the study report.

No temperature correction was performed as the study was already conducted at reference conditions (20°C).

The normalized $DegT_{50}$ value ($DegT_{50,ref}$) was 2.0 days.

I. MATERIAL AND METHODS

The modeling endpoint reported in BASF DocID 2014/1090462 [*see CA 7.1.1.1/4,*] of metiram in one soil was normalized following the recommendations of FOCUS [*FOCUS (2014): Generic Guidance for Tier 1 FOCUS Ground Water Assessments, version 2.2 (May, 2014)*] as soil $DegT_{50}$ values for environmental fate modeling are required at reference conditions (temperature of 20°C and soil moisture at field capacity at pF 2).

A temperature correction of the data was not necessary as the laboratory study was performed at 20°C. The soil moisture normalization was conducted using a Walker coefficient of 0.7. The actual soil moisture and the water content at pF 2 are shown in Table 7.1.2.1.1-19.

Table 7.1.2.1.1-19: Soil properties needed for the normalization process

Soil	Soil type USDA	T_{act}	T_{ref}	MWHC [g 100g^{-1}]	θ_{act} [% MWHC]	θ_{act} [g 100g^{-1}]	θ_{ref} [g 100g^{-1}]	f_{moist}
Li 10	loamy sand	20	20	27.1	40	10.8	11.1	0.98

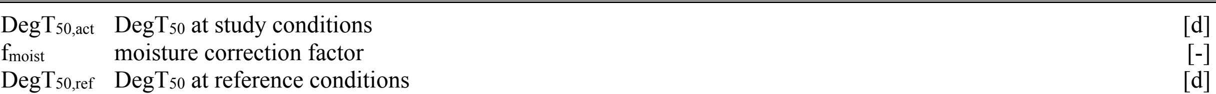
T_{act} actual temperature [°C]
T_{ref} reference temperature [°C]
θ_{act} actual soil moisture [g 100 g^{-1} dry soil]
θ_{ref} reference soil moisture at field capacity (pF 2) as stated in study report [g 100 g^{-1} dry soil]
f_{moist} moisture correction factor [-]

II. RESULTS AND DISCUSSION

The actual laboratory degradation rate and the normalized laboratory degradation rate for metiram are presented in Table 7.1.2.1.1-20.

Table 7.1.2.1.1-20: Normalization of metiram $DegT_{50}$ value to reference conditions

Soil	Soil type USDA	Kinetic model	$DegT_{50,act}$ [d]	f_{moist}	$DegT_{50,ref}$ [d]
Li10	loamy sand	FOMC	2.0[a]	0.98	2.0


$DegT_{50,act}$ $DegT_{50}$ at study conditions [d]
f_{moist} moisture correction factor [-]
$DegT_{50,ref}$ $DegT_{50}$ at reference conditions [d]

[a] Pseudo SFO $DegT_{50,act}$ = FOMC $DegT_{90,act}$ /3.32

III. CONCLUSION

The soil degradation rate at study conditions estimated under aerobic conditions in the laboratory was normalized to reference soil moisture (pF 2) in order to derive a modeling endpoint for environmental fate models.

The normalized $DegT_{50}$ value ($DegT_{50,ref}$) was 2.0 days.

Summary of degradation endpoints for metiram in different soils under aerobic conditions

In the following tables an overview on the results of the laboratory soil degradation studies with metiram is provided.

Table 7.1.2.1.1-21: Summary table of trigger endpoints of metiram obtained in laboratory soil studies

BASF DocID	Soil / Soil type	pH ($CaCl_2$)	Org. C [%]	Temp. [°C]	Moisture [% MWHC]	Trigger values $DegT_{50}$ / $DegT_{90}$ [d]	Method of calculation
2002/1011913 2014/1228382	Cashmere / loamy sand	6.6	0.8	20	75% FC at 1/3 bar	0.4 / 3.9	DFOP
2002/1012954 2014/1228382	Li 35b / loamy sand	6.9	1.2	20	40	0.2 / 1.7	FOMC
2013/1095967 2014/1311117	Speyer 2.2 / loamy sand	5.5	1.8	20[a]	at pF2	0.3 / 7.3	FOMC
	Am Fischteich / silt loam	5.9	1.6	20[a]	at pF2	0.5 / 4.2	DFOP
2010/1090462	Li 10 / loamy sand	6.3	0.9	20	40	0.7 / 6.6	FOMC

MWHC = Maximum water holding capacity
[a] Temperature = 20.7 ± 0.2°C

Table 7.1.2.1.1-22: Summary table on modeling endpoints of metiram obtained in laboratory soil studies (20°C, pF2)

BASF DocID	Soil / Soil type	pH ($CaCl_2$)	Org. C [%]	Temp. [°C]	Moisture [% MWHC]	$DegT_{50}$ at study conditions [d]	Method of calculation	$DegT_{50}$ normalized to 20°C, pF2 [d]
2002/1011913 2014/1228382	Cashmere / loamy sand	6.6	0.8	20	75% FC at 1/3 bar	1.5[a]	DFOP	1.0
2002/1012954 2014/1228382	Li 35b / loamy sand	6.9	1.2	20	40	0.5[b]	FOMC	0.5
2013/1095967 2014/1311117	Speyer 2.2 / loamy sand	5.5	1.8	20[c]	at pF2	2.2[b]	FOMC	2.2
	Am Fischteich / silt loam	5.9	1.6	20[c]	at pF2	1.6[a]	DFOP	1.6
2010/1090462	Li 10 / loamy sand	6.3	0.9	20	40	2.0[b]	FOMC	2.0

MWHC = Maximum water holding capacity
FC = Field capacity
[a] Calculated from slow phase of DFOP model ($DegT_{50} = ln(2)/k2$)
[b] Calculated as $DegT_{50} = DegT_{90}/3.32$
[c] Temperature = 20.7 ± 0.2°C

$DegT_{50}$ values at a temperature of 10°C were calculated by multiplying the normalized $DegT_{50}$ values at 20°C and pF 2 with a default Q_{10} value of 2.58 [*EFSA (2007): "Opinion on a request from EFSA related to the default Q_{10} value used to describe the temperature effect on transformation rates of pesticides in soil. Scientific Opinion of the Panel on Plant Protection Products and their Residues (PPR-Panel). Question No EFSA-Q-2007-048. The EFSA Journal (2007) 622, 1-32*]. A summary of the calculated half-lives of metiram at 10°C is given in the table below.

Table 7.1.2.1.1-23: Summary table on modeling endpoints of metiram obtained in laboratory soil studies normalized to 10°C and pF2

BASF DocID	Soil / Soil type	pH ($CaCl_2$)	Org. C [%]	Temp. [°C]	Moisture [% MWHC]	$DegT_{50}$ at study conditions [d]	Method of calculation	$DegT_{50}$ normalized to 10°C, pF2 [d]
2002/1011913 2014/1228382	Cashmere / loamy sand	6.6	0.8	20	75% FC at 1/3 bar	1.5[a]	DFOP	2.6
2002/1012954 2014/1228382	Li 35b / loamy sand	6.9	1.2	20	40	0.5[b]	FOMC	1.3
2013/1095967 2014/1311117	Speyer 2.2 / loamy sand	5.5	1.8	20[c]	at pF2	2.2[b]	FOMC	5.7
	Am Fischteich / silt loam	5.9	1.6	20[c]	at pF2	1.6[a]	DFOP	4.1
2010/1090462	Li 10 / loamy sand	6.3	0.9	20	40	2.0[b]	FOMC	5.2

MWHC = Maximum water holding capacity
FC = Field capacity
[a] Calculated from slow phase of DFOP model ($DegT_{50}$ = ln(2)/k2)
[b] Calculated as $DegT_{50} = DegT_{90}/3.32$
[c] Temperature = 20.7 ± 0.2°C

CA 7.1.2.1.2 Aerobic degradation of metabolites, breakdown and reaction products

DT_{50} values of metabolites were either derived from studies with the parent compound or from studies where metabolites were applied as test items.

Studies presented in the first Annex I inclusion process:

An overview of the studies on aerobic degradation rates of the metabolites of metiram presented in the first Annex I inclusion process is given in Table 7.1.2.1.2-1.
Two old studies are considered invalid [*Keller E. and Huber R., 1985, BASF DocID 1985/10059 and Klein W., Koerdel W. et al., 1986, BASF DocID 1986/10134*] as discussed in CA 7.1.1.1.
The very old study of Anonymous [*Anonymous, 1974, BASF DocID 1974/10028*] is considered invalid as it was performed at the same time with the same soils as BASF DocID 1974/10021 cited under CA 7.1.2.1.1. For the latter study it was stated in the list of endpoints of the 3rd Addendum to the Monograph (May 2003) that these were non-viable soils.

The report of Dressel J., 2001 [*BASF DocID 2001/1010672*] is no experimental study but a compilation and evaluation of endpoints. This compilation is outdated since many new endpoints have been generated since this time.
A new compilation of current endpoints for EBDC common metabolites has been developed for the calculation of PEC values. This document [*Platz K. and Hardy I., 2015, BASF DocID 2015/1093409*] is referred to in CP 9.1 and CP 9.2.

Table 7.1.2.1.2-1: Studies on aerobic degradation rate in soil of metabolites of metiram

Reference	BASF DocID	Soil type	Application rate [mg kg^{-1}]	Incubation temperature [°C]	Incubation period [days]	Remark
Anonymous, 1974	1974/10028	Soil 1 [a] Soil 2 [a]	3.8 (ETU)	20	6	Invalid
Keller E., Huber R., 1985	1985/10059	Loamy sand Loam	10 (metiram)	22	365	Invalid
Klein W., Koerdel W. et al., 1986	1986/10134	Loamy sand	10 (metiram)	25	21	Invalid
Cornelese A.A. et al. 1995	1995/10772	Sand Humic sand	1 (ETU)	20	7	Relevant
Wright M.C., 2000	2001/5001155	Silt loam Sand	5 (ETU)	25	14	Relevant
Staudenmaier H., 2002b	2002/1011913	Loamy sand	7.5 (metiram)	20	365	Relevant
Staudenmaier H., 2002c	2002/1012954	Loamy sand	3.9 (metiram)	20	133	Relevant
Vette H.Q.M. de, 2002a	2002/1000223, 2002/1004290 (Amendment I)	Sandy loam Loamy sand Loam	2 (carbimide)	20	1	Relevant
Vette H.Q.M. de, Cremers R.K.H., 2002c	2002/1005335	Sandy loam Sand Loam	2 (EBIS)	20	1	Relevant

[a] Soil types not reported
[b] Compilation of aerobic soil half-lives and soil adsorption data of ETU, EU, EBIS, Carbimide and EDA

Submission of not yet peer-reviewed studies in this AIR3-Dossier:

New aerobic degradation studies were performed with the metabolites ETU [see CA 7.1.2.1.2/14, DocID 2014/1189253] and TDIT [see CA 7.1.2.1.2/21, DocID 2014/1189254] in soil and the kinetic evaluations were performed and reported in separate studies [see CA 7.1.2.1.2/15, BASF DocID 2014/1311118; CA 7.1.2.1.2/22, DocID 2014/1311119]. All summaries are provided below.
Additionally, new kinetic re-evaluations were performed for older studies [BASF DocID 2009/1093835; BASF DocID 2010/1056131; BASF DocID 1995/10772; BASF DocID 2001/5001155; BASF DocID 2002/1005335; BASF DocID 2002/1000223] following the recommendations of FOCUS [FOCUS (2006)] and the kinetic re-evaluations are summarized below.

The kinetic re-evaluation of the studies of Staudenmaier H. [BASF DocID 2002/1011913; BASF DocID 2002/1012954] is summarized in CA 7.1.2.1.1/3.

Peer reviewed study:

Report:	*CA 7.1.2.1.2/1* *Staudenmaier H., 2002a* *Aerobic metabolism of BAS 222 F (Metiram) in Cashmere soil* *2002/1011913*
Guidelines:	*SETAC, BBA IV 4-1, EPA 162-1*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz* Rheinland-Pfalz, Mainz)

Peer reviewed study:

Report:	*CA 7.1.2.1.2/2* *Staudenmaier H., 2002b* *Aerobic metabolism of BAS 222 F (Metiram) in soil* *2002/1012954*
Guidelines:	*SETAC, BBA IV 4-1, EPA 162-1*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz* *Rheinland-Pfalz, Mainz)*

Report:	CA 7.1.2.1.2/3 Pape L., 2015a Kinetic evaluation of a laboratory soil degradation study with BAS 222 F - Metiram according to FOCUS Degradation Kinetics 2014/1228382
Guidelines:	FOCUS Kinetics (2006) SANCO/10058/2005 version 1.1 of Dec. 2014
GLP:	no (certified by <none>)

The experimental studies of Staudenmaier, H. [*BASF DocID 2002/1011913 and 2002/1012954*] are already described in detail in CA 7.1.1.1/1 and CA 7.1.1.1/2.

The corresponding new kinetic evaluation of Pape L. [*BASF DocID 2014/1228382*] is already described in detail in CA 7.1.2.1.1/3.

Report:	CA 7.1.2.1.2/4 Voelkel W., 2015a 14C-Metiram - Degradation and metabolism in two soils incubated under aerobic conditions 2013/1095967
Guidelines:	OECD 307 (2002), EPA 835.4100
GLP:	yes (certified by Swiss Federal Office of Public Health, Berne, Switzerland)

Report:	CA 7.1.2.1.2/5 Maassen K., 2015a Kinetic evaluation of a laboratory soil degradation study with BAS 222 F - Metiram and its metabolites according to Focus Degradation Kinetics 2014/1311117
Guidelines:	FOCUS Degradation Kinetics (2006) SANCO/10058/2005 version 2.0 (December 2014)
GLP:	no (certified by <none>)

The experimental study of Voelkel W. [*BASF DocID 2013/1095967*] is already described in detail in CA 7.1.1.1/3.

The corresponding kinetic evaluation of Maasssen K. [*BASF DocID 2014/1311117*] is already described in detail in CA 7.1.2.1.1/5.

Peer reviewed study (Summary taken from the Monograph or its Addenda):

Report:	*CA 7.1.2.1.2/6* *Cornelese A.A. et al., 1995a* *Behaviour of ETU in soil from two Dutch fields - I: Laboratory experiments - II: Field experiment* *1995/10772*
Guidelines:	*none*
GLP:	*yes*

Materials and Methods:
ETU (purity 98%), [^{14}C]-labeled in the ethylene moiety. The soil characteristics are given in Table 7.1.2.1.2-2.

Table 7.1.2.1.2-2: Characteristics of the soils used in the soil degradation study (data for depth 0-20 cm St. Maartensbrug, 0-30 cm Schijndel)

Textural Class	Sand	Humic sand
Origin	**St. Maartensbrug/Netherlands**	**Schijndel/Netherlands**
Particle size distribution < 0.002 mm (%)	2.5	2.6
Organic matter (%)	1.6	3.3
Moisture at pF-2 (field capacity) (%)	12.6	15.2
$CaCO_3$ (%)	0.2	0.1
pH (KCl)	7.1	5.0

ETU was applied at the rate of ca. 1 mg kg^{-1} soil (unlabeled spiked with labeled) to the two soils, a sand and a humic-sand. Soil samples were incubated at 20°C and moisture content equivalent to pF=2 in the dark in a centrifuge vessel with CO_2-traps to collect volatiles. Incubation was up to 168 hours.

Findings:

The half-lives and DT_{90} values are reported in Table 7.1.2.1.2-3.

Table 7.1.2.1.2-3: First order half-lives and DT_{90} values for ETU in the upper soil layer derived from the soil degradation study (based on ^{14}C-results, cited as worst case)

Origin	DT_{50} (h)	DT_{90} (h)
St. Maartensbrug, NL (0-20 cm)	7	24.5
Schijndel, NL (0-30 cm)	24	80*

* not stated in the study, calculated from DT_{50}

Conclusion:

ETU has been shown to be very fast degraded ($DT_{50} \leq 1$ day) in soil.

The following study was submitted as part of the mancozeb data package. Since it was presented in the 1st Addendum to the DAR of mancozeb, this study is considered as already peer reviewed. The summary was taken from the 1st Addendum to the Monograph of mancozeb.

Peer reviewed study:

Report:	*CA 7.1.2.1.2/7* *Wright M.C., 2000a* *Aerobic soil metabolism degradation rate determination for Ethylenethiourea (ETU) on soil* *2001/5001155*
Guidelines:	*EPA 162-1, EPA 40 CFR 158*
GLP:	*yes* *(certified by United States Environmental Protection Agency)*

Materials and methods:

The rate of degradation of ETU was evaluated under aerobic conditions at approximately 25°C at 70% Normal Moisture Holding Capacity (NMHC) and 40% NMHC for a silt loam soil and at 70% NMHC for a sandy soil.

The soil characteristics are reported in Table 7.1.2.1.2-4. The test substance, unlabeled ETU, was applied at each soil sample at a dose rate of 5 µg g^{-1}, based on 50 g d.w.b. soil weight. Each dose soil was immediately mixed thoroughly with a bioprobe to incorporate the ETU dose into soil. The soil moisture was then adjusted to 70% or 40% NMHC. After dosing and moisture adjustment, all test systems were placed in an environmental chamber set at approximately 25°C and set up for aerobic incubation in the dark. Each vessel was connected to an air supply that was scrubbed of CO_2 and then re-humidified by passing the air stream through a water scrubber prior to entering the test chamber.

Table 7.1.2.1.2-4: Soil characteristics

Characteristics	New York Silt Loam	New York Sand
Texture class (USDA) % sand % silt % clay	Silt loam 29 61 10	Sand 90 8 2
% organic matter	3.6	2.1
Bulk density	1.09	1.24
CEC mEq 100 g^{-1}	13.3	5.5
pH	6.1	6.8

The sampling intervals for the silt loam soil at 40% NMHC were at 0 time, 0.25, 0.5, 1, 2, 4, 7 and 14 days post dosing. At each of these time intervals, duplicate samples of the 5-ppm-dosed soil were harvested. The same time intervals were considered for the 70% NMHC soil. The sand soil test system was removed from the soils by solvent extraction. Residue of ETU and EU were removed by solvent extraction and analyzed using HPLC/MS.

Results:

Under the conditions of 70% NMHC, ETU degraded rapidly on New York silt loam soil; reducing the New York silt loam soil moisture to 40% NMHC the time for ETU degradation doubled.
ETU degrades rapidly in soil with a maximum half-life of 3.2 days (lowest soil moisture conditions). The first, second and third half-life of ETU in the silt loam soil at 70% NMCH are 1.6 (r^2=0.9898), 3.2 and 4.8 days, respectively, with DT_{90} of 5.3 days; at 40% NMCH half-lives are 3.2 (r^2=0.9877), 6.3 and 9.5 days with DT_{90} of 10.5 days. The first, second and third half-life of ETU in the sand soil at 70% NMCH are 1.4 (r^2=0.9782), 2.7 and 4.1 days, respectively, with DT_{90} of 4.5 days. There was no correlation between degradation rate and organic matter content and only slight correlation between degradation and soil type. The strongest correlation between ETU degradation rate and soil characteristics was observed with the percent moisture content of the soils.
The half-life of EU in the silt loam soil at 70% NMCH is 1.27 hours (0.0533 days) while at 40% is 1.85 hours (0.077 days). The half-life of EU in the sand soil at 70% NMCH is 1.0 hour (0.042 days).

Conclusions:

Under all soil moisture and soil type aerobic conditions described in this study, both ETU and EU are degraded fast. ETU degrades rapidly in soil with a first-order half-life in the silt loam soil at 70% NMCH of 1.6 days with DT_{90} of 5.3 days. At 40% NMCH ETU half-life is 3.2 days with DT_{90} of 10.5 days while in the sand soil at 70% NMCH half-life is 1.4 days with DT_{90} of 4.5 days. The half-life of EU in the silt loam soil at 70% NMCH is 1.27 hours while at 40% is 1.85. The half-life of EU in the sand soil at 70% NMCH is 1.0 hour.

Report: CA 7.1.2.1.2/8
Budde E., 2015f
Kinetic evaluation of two laboratory soil degradation studies with ETU according to FOCUS Degradation Kinetics
2014/1227796
Guidelines: FOCUS Degradation Kinetics (2006) SANCO/10058/2005 version 1.1 of December 2014
GLP: no

Executive Summary

The degradation of ETU (metabolite of metiram) has been investigated in the laboratory in three soils in two studies. In one study the formation and degradation of the ETU metabolite EU was additionally investigated. The purpose of this evaluation was to analyze the degradation kinetics observed in these studies, taking into account the current guidance of the FOCUS workgroup on degradation kinetics.

Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints) and degradation parameters for environmental fate models (modeling endpoints).

Best-fit $DegT_{50}$ values (trigger endpoints) for ETU were between 0.4 and 3.1 days, and $DegT_{90}$ values were between 1.3 and 10.2 days. Normalized modeling $DegT_{50}$ (20°C, pF 2) ranged between 0.4 and 2.8 days.

Best-fit $DegT_{50}$ values (trigger endpoints) for metabolite EU could only be derived from one soil incubated at two different soil moisture regimes, with a $DegT_{50}$ of 2 times 0.1 days and $DegT_{90}$ of 0.3 and 0.4 days. A normalized modeling endpoint (20°C, pF 2) of 0.1 days could be derived from one only experiment.

I. MATERIAL AND METHODS

The degradation of ETU (metabolite of metiram) has been investigated in the laboratory in three soils in two studies. The first study [*see Ca 7.1.2.1.2/7, BASF DocID 2001/5001155*] used non-labeled ETU and the formation and degradation of the ETU metabolite EU was additionally investigated. In the second study [*see CA 7.1.2.1.2/6, BASF DocID 1995/10772*] ^{14}C-labeled ETU was used. The resulting data were analyzed taking into account the current guidance of the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*].

Kinetic modeling strategy

The endpoints of the parent compound ETU and its metabolite EU were derived from a compartment modeling approach.

Kinetic evaluation was performed considering the procedures and kinetic models proposed by the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*]. According to FOCUS, degradation endpoints were derived for use as triggers for future work and for use as modeling inputs.

Trigger endpoints were derived from the kinetic models that provided the best fit to the measured data, generally indicated by the lowest χ^2 – error.

Modeling endpoints were derived preferably from SFO model. If the SFO model was not appropriate, pragmatic procedures were used to derive conservative pseudo-SFO degradation rates from the appropriate bi-phasic model.

The appropriateness of the distinct kinetic model to describe soil degradation was tested by visual assessment. Furthermore, a kinetic model was considered appropriate for deriving trigger or modeling endpoints of χ^2 – error value was low (ideally below 15%) and the t-test for the degradation parameters passed at 5% error level.

Data handling and software for kinetic evaluation

The experimental data were derived from the study reports and adjusted according to FOCUS [*FOUCS (2006)*].

For the study BASF DocID 2001/5001155 [*see CA 7.1.2.1.2/7*] the individual residue values reported in µg per sample were converted into µg g^{-1} dry soil (ppm) by division with the nominal soil weight (50 g). For the metabolite EU, measured values were additionally converted to parent equivalents on a molar basis. Values below the limit of quantification (LOQ) were treated as recommended by the FOCUS workgroup. The reported LOQ of 0.1 µg mL^{-1} was converted to 0.019 ppm parent equivalents (rounded value) considering the sample volume (8 mL), the nominal soil weight (50 g) and the molar mass correction. A limit of detection (LOD) cannot be extracted from the study report and was therefore defined as LOQ. According to FOCUS, values below LOD were set to 0.5 × LOD = 0.009 ppm parent equivalents (rounded value).

The total extractable residue data of soil St. Maartensbrug [*see CA 7.1.2.1.2/6, BASF DocID 1995/10772*] were used unmodified as model data.

The software package KinGUI version 2.2012.320.1629 was used for parameter fitting.

Experimental data used for kinetic analysis

In the study BASF DocID 2001/5001155 [*see CA 7.1.2.1.2/7*], the test soils were treated with non-labeled ETU at a rate of 5 µg per g, based on 50 g soil dry weight. After dosing, separate sets of soil samples were prepared for one soil sample (silt loam). For one set, the soil moisture was adjusted to 70% of NMHC (normal moisture holding capacity = moisture at 1/3 bar) and to 40% of NMHC for the other set. For the other soil sample (sand soil), only one set of test systems were prepared and soil moisture was adjusted to 70% of NMHC. Test systems were incubated under aerobic conditions at 25 ± 1°C in the dark for up to 14 days.

The test soil used in study BASF DocID 1995/10772 [*see CA 7.1.2.1.2/6*] was treated with ^{14}C-labeled ETU at a rate of 1.06 mg kg^{-1}. Samples were incubated in the dark at 20°C and a soil moisture content equivalent to pF 2 for 96 hours.

The measured data as well as resulting datasets submitted to kinetic analysis are summarized in Table 7.1.2.1.2-5 to Table 7.1.2.1.2-8.

Table 7.1.2.1.2-5: Data for New York silt loam (40% NMHC) for kinetic evaluation [*BASF DocID 2001/5001155*]

Time [d]	Residue data				Input data according to FOCUS	
	ETU		EU		ETU	EU
	[µg] [a]	[µg g^{-1}] [b]	[µg] [a]	[µg g^{-1} p.e.] [b]	[µg g^{-1}]	[µg g^{-1} p.e.]
0	238.08	4.76	2.80	0.066	4.83 [c]	0 [d]
0	212.16	4.24	4.08	0.097	4.34 [c]	0 [d]
0.25	206.46	4.13	6.48	0.154	4.13	0.154
0.25	241.68	4.83	6.16	0.146	4.83	0.146
0.5	189.38	3.79	4.25	0.101	3.79	0.101
0.5	205.20	4.10	4.64	0.110	4.10	0.110
1	168.48	3.37	3.84	0.091	3.37	0.091
1	153.50	3.07	4.62	0.110	3.07	0.110
2	118.57	2.37	3.36	0.080	2.37	0.080
2	122.40	2.45	3.28	0.078	2.45	0.078
4	120.32	2.41	4.56	0.108	2.41	0.108
4	102.58	2.05	3.36	0.080	2.05	0.080
7	47.68	0.95	1.36	0.032	0.95	0.032
7	53.46	1.07	1.31	0.031	1.07	0.031
14	5.67	0.11	<LOQ	<LOQ	0.11	0.009 [e]
14	15.48	0.31	<LOQ	<LOQ	0.31	0.009 [e]

a Reported values
b Parent equivalents; calculated considering nominal soil weight (50 g) and molar mass correction for EU
c Sum of ETU and EU residues at 0 day
d Set to zero
e Set to ½ LOQ (LOQ = 0.019 µg g^{-1} p.e., rounded value)

Table 7.1.2.1.2-6: Data for New York silt loam (70% NMHC) for kinetic evaluation [*BASF DocID 2001/5001155*]

Time [d]	Residue data				Input data according to FOCUS	
	ETU		EU		ETU	EU
	[µg] [a]	[µg g^{-1}] [b]	[µg] [a]	[µg g^{-1} p.e.] [b]	[µg g^{-1}]	[µg g^{-1} p.e.]
0	261.68	5.23	3.44	0.082	5.32 [c]	0 [d]
0	280.54	5.61	3.57	0.085	5.70 [c]	0 [d]
0.25	239.77	4.80	7.79	0.185	4.80	0.185
0.25	235.04	4.70	7.52	0.178	4.70	0.178
0.5	192.16	3.84	6.08	0.144	3.84	0.144
0.5	187.21	3.74	6.40	0.152	3.74	0.152
1	126.24	2.52	4.96	0.118	2.52	0.118
1	134.32	2.69	5.52	0.131	2.69	0.131
2	76.67	1.53	2.54	0.060	1.53	0.060
2	101.44	2.03	3.04	0.072	2.03	0.072
4	48.22	0.96	3.90	0.093	0.96	0.093
4	27.68	0.55	2.00	0.047	0.55	0.047
7	13.84	0.28	1.28	0.030	0.28	0.030
7	8.69	0.17	<LOQ	<LOQ	0.17	0.009 [e]

[a] Reported values
[b] Parent equivalents; calculated considering nominal soil weight (50 g) and molar mass correction for EU
[c] Sum of ETU and EU residues at 0 day.
[d] Set to zero.
[e] Set to ½ LOQ (LOQ = 0.019 µg g^{-1} p.e., rounded value)

Table 7.1.2.1.2-7: Data for New York sand (70% NMHC) for kinetic evaluation [*BASF DocID 2001/5001155*]

Time [d]	Residue data				Input data according to FOCUS	
	ETU		EU		ETU	EU
	[µg] [a]	[µg g^{-1}] [b]	[µg] [a]	[µg g^{-1} p.e.] [b]	[µg g^{-1}]	[µg g^{-1} p.e.]
0	229.20	4.58	0.88	0.021	4.60 [c]	0 [d]
0	240.73	4.81	1.05	0.025	4.84 [c]	0 [d]
0.5	202.66	4.05	6.16	0.146	4.05	0.146
0.5	206.08	4.12	5.20	0.123	4.12	0.123
1	176.00	3.52	5.92	0.141	3.52	0.141
1	183.44	3.67	5.44	0.129	3.67	0.129
2	88.00	1.76	8.64	0.205	1.76	0.205
2	140.56	2.81	8.40	0.199	2.81	0.199
4	55.44	1.11	<LOQ	<LOQ	1.11	0.009 [e]
4	36.52	0.73	<LOQ	<LOQ	0.73	0.009 [e]
7	4.76	0.10	<LOQ	<LOQ	0.10	
7	6.89	0.14	<LOQ	<LOQ	0.14	

[a] Reported values
[b] Parent equivalents; calculated considering nominal soil weight (50 g) and molar mass correction for EU
[c] Sum of ETU and EU residues at 0 day.
[d] Set to zero.
[e] Set to ½ LOQ (LOQ = 0.019 µg g^{-1} p.e., rounded value)

Table 7.1.2.1.2-8: Data for soil St. Maartensbrug for kinetic evaluation [*BASF DocID 1995/10772*]

Time [h]	Residue data [%TAR] 0-20 cm	Input data according to FOCUS [%TAR] 0-20 cm
	ETU	ETU
0	94.0	94.0
1	87.0	87.0
2	80.0	80.0
3	73.0	73.0
4	74.0	74.0
8	56.0	56.0
16	32.0	32.0
24	8.0	8.0
96	4.0	4.0

TAR Total applied radioactivity

Normalization to reference conditions

The $DegT_{50}$ values suitable for modeling were normalized to reference conditions (temperature of 20°C and soil moisture at field capacity, i.e. pF 2). The normalization was performed using the moisture dependency equations by Walker, with a Walker coefficient of 0.7. The temperature normalization was performed using the temperature correction factor f_{temp} and a default Q_{10} value of 2.58 was considered. The actual temperature and soil moisture was used as specified in the study reports. The corresponding water content at field capacity was either taken from the study reports or estimated according to FOCUS [*FOCUS (2012)*].

II. RESULTS AND DISCUSSION

The derived trigger endpoints for ETU and its metabolite EU calculated from four soil experiments are given in Table 7.1.2.1.2-9 and Table 7.1.2.1.2-10. The kinetic evaluation showed that the SFO model is appropriate to derive trigger endpoints in three soil experiments for ETU, while the biphasic kinetic model (DFOP) was selected as best-fit model in one soil experiment (New York silt loam, 70% NMHC).

For the evaluated soils, the visual assessment indicated plausible fit. For some fit approaches the χ^2 error value were above 15% but as the observations were generally well described by the fitted curves the χ^2 error values were considered acceptable. The t-test was passed for the respective model parameters. Therefore, the resulting $DegT_{50}$ values were considered reliable.

Table 7.1.2.1.2-9: Trigger endpoints for ETU

DocID	Soil	Best-fit model	χ^2 error [%]	Trigger endpoints	
				$DegT_{50}$ [d]	$DegT_{90}$ [d]
2000/5001155	New York silt loam (40% NMHC)	SFO	7.7	3.1	10.2
	New York silt loam (70% NMHC)	DFOP	3.8	1.0	4.7
	New York sand (70% NMHC)	SFO	5.0	1.8	6.0
1995/10772	St. Maartensbrug	SFO	5.3	0.4	1.3

Table 7.1.2.1.2-10: Trigger endpoints for the metabolite EU

DocID	Soil	Best-fit model ETU/EU	χ^2 error [%]	Trigger endpoints	
				$DegT_{50}$ [d]	$DegT_{90}$ [d]
2000/5001155	New York silt loam (40% NMHC)	SFO/SFO	21.6	0.1	0.3
	New York silt loam (70% NMHC)	DFOP/SFO	19.5	0.1	0.4
	New York sand (70% NMHC)	no adequate fit derived			

Modeling endpoints for ETU were obtained using SFO kinetics in the four soil experiments. The $DegT_{50}$ values suitable for modeling obtained under different incubation conditions were normalized to a reference moisture of pF 2 and a temperature of 20°C. Parameters including in the normalization procedure are shown in Table 7.1.2.1.2-11, while derived modeling endpoints for ETU and EU are summarized Table 7.1.2.1.2-12 and Table 7.1.2.1.2-13.

Table 7.1.2.1.2-11: Normalization of $DegT_{50}$ values for ETU and its metabolite EU to reference conditions

Soil, soil type, (exp. moisture)	Kinetic model	T_{act} [°C]	T_{ref} [°C]	θ_{act} [g 100g^{-1}]	θ_{ref} [g 100g^{-1}]	f_{temp} [-]	f_{moist} [-]	$DegT_{50act}$ [d]	$DegT_{50ref}$ [d]
ETU									
New York, silt loam (40% NMHC at 1/3 bar)	SFO	25	20	11.4	26 [a]	1.6	0.563	3.1	2.8
New York, silt loam (70% NMHC at 1/3 bar)	SFO	25	20	20.0	26 [a]	1.6	0.833	1.1	1.5
New York, sand (70% NMHC at 1/3 bar)	SFO	25	20	6.0	12 [a]	1.6	0.612	1.8	1.8
St. Maartensbrug, sand (at pF2)	SFO	20	20	12.6	12.6 [b]	1.0	1.000	0.4	0.4
EU									
New York, silt loam (40% NMHC at 1/3 bar)	SFO	25	20	11.4	26 [a]	1.6	0.563	0.1	0.1

T_{act} Actual temperature during incubation [°C]
T_{ref} Reference temperature (20°C) [°C]
θ_{act} Actual soil moisture [g 100 g^{-1} dry soil]
θ_{ref} Reference soil moisture at field capacity (pF 2) [g 100 g^{-1} dry soil]
f_{temp} Temperature correction factor [-]
f_{moist} Moisture correction factor [-]
$DegT_{50act}$ $DegT_{50}$ at study conditions [d]
$DegT_{50ref}$ $DegT_{50}$ at reference conditions [d]
[a] According to FOCUS [*FOCUS (2012)*]
[b] According to study report [*DocID 1995/10772*]

Table 7.1.2.1.2-12: Modeling endpoints for ETU

DocID	Soil	Kinetic model	χ^2 error [%]	Modeling endpoints	
				$DegT_{50}$ [d]	Normalized $DegT_{50}$ [d] [a]
2000/5001155	New York silt loam (40% NMHC)	SFO	7.7	3.1	2.8
	New York silt loam (70% NMHC)	SFO	6.0	1.1	1.5
	New York sand (70% NMHC)	SFO	5.0	1.8	1.8
1995/10772	St. Maartensbrug	SFO	5.3	0.4	0.4

[a] Reference conditions: 20°C, pF 2

Table 7.1.2.1.2-13: Modeling endpoints for the metabolite EU

DocID	Soil	Kinetic model ETU/EU	χ^2 error [%]	Modeling endpoints	
				$DegT_{50}$ [d]	Normalized $DegT_{50}$ [d] [a]
2000/5001155	New York silt loam (40% NMHC)	SFO/SFO	21.6	0.1	0.1
	New York silt loam (70% NMHC)	No reliable endpoints derived			
	New York sand (70% NMHC)	No adequate fit derived			

[a] Reference conditions: 20°C, pF 2

III. CONCLUSION

Trigger and modeling endpoints were derived for ETU and its metabolite EU in two laboratory degradation studies with three different soils. The kinetic evaluation showed that with one exception (New York silt loam, 70% NMHC, DFOP) the SFO model provided the best fit to the measured data for ETU. Modeling endpoints for ETU as well as trigger and modeling endpoints for the metabolite EU were always derived from SFO kinetics.

Best-fit $DegT_{50}$ values (trigger endpoints) for ETU were between 0.4 and 3.1 days, and $DegT_{90}$ values were between 1.3 and 10.2 days. Normalized modeling $DegT_{50}$ (20°C, pF 2) ranged between 0.4 and 2.8 days.

Best-fit $DegT_{50}$ values (trigger endpoints) for metabolite EU could only be derived from one soil incubated at two different soil moisture regimes, with a $DegT_{50}$ of 0.1 days and $DegT_{90}$ of 0.3 and 0.4 days. A normalized modeling endpoint (20°C, pF 2) of 0.1 days could be derived from one only experiment.

Peer reviewed studies (Summaries taken from the Monograph or its Addenda):

Report:	*CA 7.1.2.1.2/9* *Vette H.Q.M. de, 2002a* *A study on the rate of degradation of Carbimide (metabolite of BAS 222 F, Metiram) in three aerobic soils* *2002/1000223*
Guidelines:	*SETAC Europe, EEC 95/36, Dutch General Instructions for the Submission of Applications for Registration of Pesticides March 1995, BBA IV 4-1, OECD 307 (draft)*
GLP:	*yes* *(certified by Ministry of Health, Welfare and Sport, Rijswijk, The Netherlands)*

Report:	*CA 7.1.2.1.2/10* *Vette H.Q.M. de, 2002b* *Amendment I to report V4012/01: A study on the rate of degradation of Carbimide (metabolite of BAS 222 F, Metiram) in three aerobic soils* *2002/1004290*
Guidelines:	*SETAC Europe, CTB Guideline (1999), BBA IV 4-1, Dutch General Instructions for the Submission of Applications for Registration of Pesticides March 1995, EEC 95/36, OECD 307 (draft)*
GLP:	*yes* *(certified by Ministry of Health, Welfare and Sport, Rijswijk, The Netherlands)*

Report:	*CA 7.1.2.1.2/11* *Vette H.Q.M. de,Cremers R.K.H., 2002a* *A study on the rate of degradation of EBIS (Metabolite of BAS 222 F, Metiram) in three aerobic soils (SETAC-Europe, OECD 307 draft document, CTB guideline section G.1.1, BBA Guideline IV, 4-1)* *2002/1005335*
Guidelines:	*BBA IV 4-1, SETAC Europe, EEC 95/36, OECD 307, CTB Guideline (1999)*
GLP:	*yes* *(certified by Ministry of Health, Welfare and Sport, Rijswijk, The Netherlands)*

Materials and methods:

The aerobic degradation of metiram metabolites EBIS and carbimid was investigated in three different soils from the Netherlands (BASF DocID 2002/1005335, BASF DocID 2002/1004290 and BASF DocID 2002/1000223). The soil characteristics are summarized in Table 7.1.2.1.2-14. The same soils, however from different batches were used in these two studies.
Non-radiolabeled EBIS or carbimid was applied at a concentration of 2 mg kg^{-1} dry soil. The incubation conditions were: aerobic, in the dark, 20°C, 50% maximum water holding capacity. A system with continuous passive aeration and trapping of volatiles was used.
The soils were extracted by methanol and the concentration of the test substance was determined by means of HPLC with UV detection. After preliminary tests for range-finding, the final incubations could be limited to only one day. The loss of the test substances was fitted to a first order decay equation using non-linear regression. Regression analyses were performed using Jandel TableCurve^{TM} 2D (version 4) software.

Table 7.1.2.1.2-14: Soils used to investigate the degradation of EBIS and carbimid, metabolites of Metiram.

	EBIS studies		
Soil designation	HW020115	HZ020115	ZV020115
Origin	Heerewarden The Netherlands	Wageningen The Netherlands	Lelystad The Netherlands
Textural class (German scheme)	loamy sand	sand	sandy loam
Textural class (USDA scheme)	sandy loam	sand	loam
Particle size distribution [%] (German scheme): 0.063 - 2 mm 0.002 - 0.063 mm < 0.002 mm (USDA scheme): 0.05 - 2 mm 0.002 - 0.05 mm < 0.002 mm	 57.20 28.46 14.34 59.10 27.27 13.63	 88.82 7.48 3.69 89.36 6.92 3.72	 35.43 41.39 23.18 40.36 36.48 23.16
Organic carbon [%]	1.4	1.8	1.4
Organic matter [%]	2.4*	3.1*	2.4*
Microbial biomass [mg C/100g dry soil]	47.1	51.9	53.5
CEC [mVal/100 g]	17.6	16.4	20.2
pH [$CaCl_2$]	7.4	4.8	7.5
pH [H_2O]	7.7	5.9	7.9
MWC [g H_2O/100 g dry soil]	42.2	35.7	39.6

	EBIS studies		
Soil designation	HW020115	HZ020115	ZV020115
	Carbimid studies		
Soil designation	HW010927	HZ010927	ZV010927
Origin	Heerewarden The Netherlands	Wageningen The Netherlands	Lelystad The Netherlands
Textural class (German scheme)	loamy sand	loamy sand	sandy loam
Textural class (USDA scheme)	Sandy loam	Loamy sand	loam
Particle size distribution [%] (German scheme): 0.063 - 2 mm 0.002 - 0.063 mm < 0.002 mm (USDA scheme): 0.05 - 2 mm 0.002 - 0.05 mm < 0.002 mm	 61.45 25.13 13.41 62.88 22.44 14.68	 80.44 12.50 7.06 81.73 11.20 7.07	 38.83 38.13 23.04 42.43 35.71 21.86
Organic carbon [%]	1.7	2.0	1.3
Organic matter [%]	2.9*	3.4*	2.2*
Microbial biomass [mg C/100g dry soil]	15.9	11.2	30.6
CEC [mVal/100 g]	12.3	12.6	15.2
pH [$CaCl_2$]	7.6	6.6	7.5
pH [H_2O]	8.5	6.7	8.5
MWC [g H_2O/100 g dry soil]	21.1	17.8	19.8

*recalculated from organic carbon by a factor of 1.72

Findings:

The half-lives and DT_{90} values (1st order) of metiram metabolites EBIS and carbimid in soil are shown in Table 7.1.2.1.2-15. Very short half-lives were calculated for these metabolites.

Table 7.1.2.1.2-15: Half-lives and DT_{90} values of metabolites EBIS and Carbimid of Metiram

Compound	BASF DocID	Soil	DT_{50} 1st order [days]	DT_{90} [days]
EBIS	2002/1005335 2002/1005335 2002/1005335	Heerewarden Wageningen Lelystad	0.09 0.13 0.15	0.29 0.43 0.50
Carbimid	2002/1000223 2002/1000223 2002/1000223	Heerewarden Wageningen Lelystad	0.015 0.010 0.008	0.051 0.034 0.026

Conclusion:

The estimated half-lives for the metabolites EBIS and carbimid of metiram in soil are very short.

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Budde E., 2015h
Kinetic evaluation of a laboratory soil degradation study with Carbimide according to FOCUS Degradation Kinetics
2014/1227922

Guidelines: FOCUS Degradation Kinetics (2006) SANCO/10058/2005 version 1.1 of December 2014

GLP: no

Executive Summary

The degradation of carbimide (metabolite of metiram) has been investigated in the laboratory under aerobic conditions in three soils in one study. The purpose of this evaluation was to analyze the degradation kinetics observed in this study, taking into account the current guidance of the FOCUS workgroup on degradation kinetics.

Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints) and degradation parameters for environmental fate models (modeling endpoints).

Best-fit $DegT_{50}$ values (trigger endpoints) for carbimide were between 0.007 to 0.012 days, and $DegT_{90}$ values were between 0.031 and 0.088 days. Normalized modeling endpoints (20°C, pF 2) ranged from 0.007 to 0.015 days.

I. MATERIAL AND METHODS

The degradation of carbimide (metabolite of metiram) has been investigated in the laboratory in three soils in one study [*CA 7.1.2.1.2/9, BASF DocID 2002/1000223*]. The resulting data were analyzed taking into account the current guidance of the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*].

Kinetic modeling strategy

Kinetic evaluation was performed considering the procedures and kinetic models proposed by the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*]. According to FOCUS, degradation endpoints were derived for use as triggers for future work and for use as modeling inputs.

The following kinetic models were employed for the evaluation: SFO, FOMC and DFOP.

Trigger endpoints were derived from the kinetic models that provided the best fit to the measured data, generally indicated by the χ^2 – error. Modeling endpoints were derived preferably from SFO model. If the SFO model was not appropriate, pragmatic procedures were used to derive conservative pseudo-SFO degradation rates from the appropriate bi-phasic model.

The appropriateness of the distinct kinetic model to describe soil degradation was tested by visual assessment. Furthermore, a kinetic model was considered appropriate for deriving trigger or modeling endpoints of χ^2 – error value was low (ideally below 15%) and the t-test for the degradation parameters passed at 5% error level.

Data handling

The software package KinGUI version 2.2012.320.1629 [*SCHÄFER et al. (2007); SCHMITT et al. (2011).*] was used for parameter fitting.

The experimental data were derived from the study reports and adjusted according to FOCUS [FOUCS (2006)].

Experimental data used for kinetic analysis

In the study BASF DocID 2002/1000223 [*see Ca 7.1.2.1.2/9,*], the test soils were treated with the test substance carbimide at a nominal application rate of 2 mg kg^{-1}. Portions of 50 g soil (dry weight) were incubated under aerobic conditions at 20 ± 2°C and 50% of the soil maximum water holding capacity (MWHC) in the dark for 24 hours.

The measured data as well as resulting datasets submitted to kinetic analysis are summarized in Table 7.1.2.1.2-16.

Table 7.1.2.1.2-16: Data for three soils for kinetic evaluation

Time [h]	Residue data [% AD]	Input data according to FOCUS [% AD]	Residue data [% AD]	Input data according to FOCUS [% AD]	Residue data [% AD]	Input data according to FOCUS [% AD]
	HW010927 (Heerewaarden)		HZ010927 (Wageningen)		ZV010927 (Lelystad)	
0	64.14	64.14	53.34	53.34	71.63	71.63
0	55.44	55.44	58.07	58.07	70.51	70.51
0.25	30.49	30.49	24.97	24.97	26.14	26.14
0.25	33.30	33.30	26.83	26.83	25.69	25.69
0.5	21.26	21.26	15.06	15.06	12.38	12.38
0.5	22.75	22.75	13.86	13.86	12.15	12.15
1	10.69	10.69	3.18	3.18	4.72	4.72
1	13.23	13.23	3.77	3.77	6.05	6.05
2	6.65	6.65	1.93	1.93	2.07	2.07
2	6.69	6.69	1.73	1.73	2.06	2.06
4	1.73	1.73	0.41	0.41	1.85	1.85
4	1.72	1.72	0.26	0.26	2.43	2.43
6	0.83	0.83	0.26	0.26	0.41	0.41
6	0.88	0.88	0.22	0.22	0.52	0.52
24	0.15	0.15	0.10	0.10	0.11	0.11
24	0.12	0.12	0.10	0.10	0.20	0.20

AD Applied Dose

Normalization to reference conditions

The normalization was performed using the moisture dependency equations by Walker, with a Walker coefficient of 0.7. The actual soil moisture was used as specified in the study report. The corresponding water content at pF 2 for each soil was derived from FOCUS [*FOCUS (2002): Generic Guidance for FOCUS groundwater scenarios, version 1.1*].

Since the study was performed at 20°C a temperature correction was not necessary.

II. RESULTS AND DISCUSSION

Prior to deriving modeling endpoints for carbimide, the $DegT_{50}$ values suitable for modeling obtained under different incubation conditions were normalized to a reference moisture of pF 2. Parameters included in the normalization are summarized in Table 7.1.2.1.2-17.

Table 7.1.2.1.2-17: Normalization of carbimide $DegT_{50}$ values to reference conditions

Soil, soil type (USDA)	Kinetic Model	θ_{act} [g/100g]	θ_{ref} [g/100g]	f_{moist} [-]	$DegT_{50act}$ [d]	$DegT_{50ref}$ [d]
HW010927, (Heerewaarden), sandy loam	SFO	21.1	19	1.000	0.015	0.015
HZ010927, (Wageningen), loamy sand	SFO	17.8	14	1.000	0.010	0.010
ZV010927, (Lelystad), loam	SFO	19.8	25	0.849	0.008	0.007

θ_{act} Actual soil moisture (50 % of MWHC) [g / 100 g dry soil]
θ_{ref} Reference soil moisture at field capacity (pF 2) according FOCUS (2002), Table 5.2 [g / 100 g dry soil]
f_{moist} Moisture correction factor [-]
$DegT_{50act}$ $DegT_{50}$ at study conditions [d]
$DegT_{50ref}$ $DegT_{50}$ at reference conditions [d]

A summary of trigger and modeling endpoints of carbimide calculated for three soils is given in Table 7.1.2.1.2-18 and Table 7.1.2.1.2-19. For the evaluated soils, visual assessment, goodness-of-fit statistics and t-test for the respective models indicate plausible fit and, therefore, the resulting $DegT_{50}$ values can be considered reliable.

Table 7.1.2.1.2-18: Degradation parameters and trigger endpoints for carbimide

Soil	Best-fit model	χ^2 error [%]	Trigger endpoints	
			$DegT_{50}$ [d]	$DegT_{90}$ [d]
HW010927 (Heerewaarden)	DFOP	2.5	0.012	0.088
HZ010927 (Wageningen)	SFO	5.4	0.010	0.034
ZV010927 (Lelystad)	DFOP	3.0	0.007	0.031

Table 7.1.2.1.2-19: Degradation parameters and modeling endpoints for carbimide

Soil	Kinetic model	χ^2 error [%]	Modeling endpoints	
			$DegT_{50}$ [d]	Normalized $DegT_{50}$ [d] [a]
HW010927 (Heerewaarden)	SFO	13.4	0.015	0.015
HZ010927 (Wageningen)	SFO	5.4	0.010	0.010
ZV010927 (Lelystad)	SFO	10.0	0.008	0.007

[a] Reference conditions: 20°C, pF 2

III. CONCLUSION

Trigger and modeling endpoints were derived for carbimide (metabolite of metiram) in one laboratory degradation study with three different soils. The kinetic evaluation showed that with one exception (HZ010927 – Wageningen; SFO) the bi-phasic model DFOP provided the best fit to the measured data for carbimide, while the SFO model was appropriate for derivation of modeling endpoints in all soils.

Best-fit $DegT_{50}$ values (trigger endpoints) for carbimide were between 0.007 to 0.012 days, and $DegT_{90}$ values were between 0.031 and 0.088 days. Normalized modeling endpoints (20°C, pF 2) ranged from 0.01 to 0.015 days.

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Budde E., 2015g
Kinetic evaluation of a laboratory soil degradation study with EBIS according to FOCUS Degradation Kinetics
2014/1227797
Guidelines: FOCUS Degradation Kinetics (2006) SANCO/10058/2005 version 1.1 of December 2014
GLP: no

Executive Summary

The degradation of EBIS (metabolite of metiram) has been investigated in the laboratory under aerobic conditions in three soils in one study. The purpose of this evaluation was to analyze the degradation kinetics observed in this study, taking into account the current guidance of the FOCUS workgroup on degradation kinetics.

Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints) and degradation parameters for environmental fate models (modeling endpoints).

Best-fit $DegT_{50}$ values (trigger endpoints) for EBIS were between 0.1 and 0.2 days, and $DegT_{90}$ values were between 0.3 and 0.5 days. Normalized modeling $DegT_{50}$ (20°C, pF 2) for each soil were 0.1 days.

I. MATERIAL AND METHODS

The degradation of EBIS (metabolite of metiram) has been investigated in the laboratory in three soils in one study [see *CA 7.1.2.1.2/11, BASF DocID 2002/1005335*]. The resulting data were analyzed taking into account the current guidance of the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*].

Kinetic modeling strategy

Kinetic evaluation was performed considering the procedures and kinetic models proposed by the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*]. According to FOCUS, degradation endpoints were derived for use as triggers for future work and for use as modeling inputs.

The following kinetic models were employed for the evaluation: single first order kinetics (SFO) and the Gustafson-Holden model (FOMC).

Trigger endpoints were derived from the kinetic models that provided the best fit to the measured data, generally indicated by the lowest χ^2 – error. Modeling endpoints were derived preferably from SFO model. If the SFO model was not appropriate, pragmatic procedures were used to derive conservative pseudo-SFO degradation rates from the appropriate bi-phasic model.

The appropriateness of the distinct kinetic model to describe soil degradation was tested by visual assessment. Furthermore, a kinetic model was considered appropriate for deriving trigger or modeling endpoints of χ^2 – error value was low (ideally below 15%) and the t-test for the degradation parameters passed at 5% error level.

Data handling

The experimental data were derived from the study reports and adjusted according to FOCUS [FOUCS (2006)].

The software package KinGUI version 2.2012.320.1629 was used for parameter fitting.

Experimental data used for kinetic analysis

In the study BASF DocID 2002/1005335 [*see Ca 7.1.2.1.2/11*], the test soils were treated with the test substance EBIS at a nominal application rate of 2 mg kg^{-1}. Portions of 50 g soil (dry weight) were incubated under aerobic conditions at 20 ± 2°C and 50% of the soil maximum water holding capacity (MWHC) in the dark for 24 hours.

The measured data as well as resulting datasets submitted to kinetic analysis are summarized in Table 7.1.2.1.2-20.

Table 7.1.2.1.2-20: Data for three soils for kinetic evaluation

Time [h]	Residue data [%AD]	Input data according to FOCUS [% AD]	Residue data [%AD]	Input data according to FOCUS [% AD]	Residue data [%AD]	Input data according to FOCUS [% AD]
	HW020115 (Heerewaarden)		HZ020115 (Wageningen)		ZV020115 (Lelystad)	
0	69.5	69.5	85.2	85.2	61.0	61.0
0	69.9	69.9	80.9	80.9	72.4	72.4
0.25	55.8	55.8	59.9	59.9	73.1	73.1
0.25	53.9	53.9	61.2	61.2	64.5	64.5
0.5	51.6	51.6	60.2	60.2	59.8	59.8
0.5	51.9	51.9	54.4	54.4	62.9	62.9
1	41.5	41.5	51.5	51.5	59.6	59.6
1	45.9	45.9	50.8	50.8	55.9	55.9
2	35.3	35.3	46.2	46.2	48.9	48.9
2	29.9	29.9	50.2	50.2	50.1	50.1
4	20.5	20.5	28.3	28.3	34.1	34.1
4	19.5	19.5	30.3	30.3	32.4	32.4
6	7.8	7.8	21.4	21.4	19.7	19.7
6	8.1	8.1	18.7	18.7	18.5	18.5
24	<3.7	1.9 [a]	<3.7	1.9 [a]	2.3	2.3
24	<3.7	1.9 [a]	2.5	2.5	1.9	1.9

AD Applied Dose
[a] Set to ½ LOQ

Normalization to reference conditions

Since the study was performed at 20°C a temperature correction was not necessary. The normalization was performed using the moisture dependency equations by Walker, with a Walker coefficient of 0.7. The actual soil moisture was used as specified in the study report. The corresponding water content at pF 2 for each soil was derived from FOCUS [*FOCUS (2002)*].

II. RESULTS AND DISCUSSION

Prior to deriving modeling endpoints for EBIS, the $DegT_{50}$ values suitable for modeling obtained under different incubation conditions were normalized to a reference moisture of pF 2. Parameters included in the normalization are summarized in Table 7.1.2.1.2-21.

Table 7.1.2.1.2-21: Normalization of EBIS $DegT_{50}$ values to reference conditions

Soil, soil type (USDA)	pH ($CaCl_2$)	Kinetic Model	θ_{act} [g 100g^{-1}]	θ_{ref} [g 100g^{-1}]	f_{moist} [-]	$DegT_{50act}$ [d]	$DegT_{50ref}$ [d]
HW020115 (Heerewaarden), sandy loam	7.4	SFO	21.1	19	1.000	0.1	0.1
HZ020115 (Wageningen), sand	4.8	SFO	17.8	12	1.000	0.1	0.1
ZV020115 (Lelystad), loam	7.5	SFO	19.8	25	0.849	0.2	0.1

θ_{act} Actual soil moisture (50 % of MWHC) [g 100 g^{-1} dry soil]
θ_{ref} Reference soil moisture at field capacity (pF 2) according to FOCUS [FOCUS (2012)] [g 100 g^{-1} dry soil]
f_{moist} Moisture correction factor [-]
$DegT_{50act}$ $DegT_{50}$ at study conditions [d]
$DegT_{50ref}$ $DegT_{50}$ at reference conditions [d]

A summary of trigger and modeling endpoints of EBIS calculated for three soils is given in Table 7.1.2.1.2-22. For the evaluated soils, visual assessment, goodness-of-fit statistics and t-test for the respective models indicate plausible fit and, therefore, the resulting $DegT_{50}$ values can be considered reliable.

Table 7.1.2.1.2-22: Trigger and modeling endpoints for EBIS

Soil	Best-fit model	χ^2 error [%]	Trigger endpoints		Modeling endpoints	
			$DegT_{50}$ [d]	$DegT_{90}$ [d]	$DegT_{50}$ [d]	Normalized $DegT_{50}$ [d] [a]
HW020115 (Heerewaarden)	SFO	6.8	0.1	0.3	0.1	0.1
HZ020115 (Wageningen)	SFO	10.6	0.1	0.4	0.1	0.1
ZV020115 (Lelystad)	SFO	3.6	0.2	0.5	0.2	0.1

[a] Reference conditions: 20°C, pF 2

III. CONCLUSION

Trigger and modeling endpoints were derived for EBIS (metabolite of metiram) in one laboratory degradation study with three different soils. The kinetic evaluation showed that the SFO model provided the best fit to the measured data for EBIS and was also appropriate for derivation of modeling endpoints.

Best-fit $DegT_{50}$ values (trigger endpoints) for EBIS were between 0.1 and 0.2 days, and $DegT_{90}$ values were between 0.3 and 0.5 days. Normalized modeling $DegT_{50}$ (20°C, pF 2) for each soil were 0.1 days.

Three ETU DT_{50} values derived from a single metabolism study with the active substance mancozeb were clearly outside the range of ETU DT_{50} values that were observed in a couple of studies with the parent metiram or ETU itself as test item. These DT_{50} values are considered for the calculation of PEC values (CP 9.1 and CP 9.2).
Because of this apparent discrepancy it was decided to investigate the degradation of ETU in the respective soils (Senozan, Speyer 2.2 (= LUFA 2.2) and Speyer 2.3 (= LUFA 2.3)) in order to check if the respective DT_{50} values in these soils can be verified. Along with these soils three further soils were tested.

Report: CA 7.1.2.1.2/14
Heinz N., 2014c
ETU (metabolite of Metiram, BAS 222 F): Study on aerobic soil degradation
2014/1189253

Guidelines: OECD 307 (2002), BBA IV 4-1, EPA 835.4100, SANCO/3029/99 rev. 4 (11 July 2000)

GLP: yes
(certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

Executive Summary

The objective of this study was to examine the aerobic degradation of ETU (metabolite of metiram, BAS 222 F) in six soils.

According to the USDA scheme, the soils were characterized as sandy loam (LUFA 2.3, LUFA 5M), loamy fine sand (LUFA 2.2, Li 10), silty clay loam (Senozan), and silt loam (Am Fischteich). Prior to application, the soil samples were acclimatized and adjusted to 40% of their maximum water holding capacities.

The application rate (based on dry soil weight) of ETU dosed to soil was 0.75 mg kg^{-1}. Assuming a soil depth of 2.5 cm and a soil density of 1.5 g cm^{-3} this corresponds to a theoretical field application rate of about 280 g ha^{-1}. After application, incubation flasks were loosely covered with plugs of paper tissue to minimize loss of moisture but to allow air exchange and then placed in thermostated cabinet set to 20°C in the dark. Loss of soil water was controlled by weighing and re-adjusted with distilled water if necessary. The dosed soil samples were incubated for various intervals up to 48 hours or 72 hours prior to extraction.

The analytical method was validated and revealed a limit of quantification (LOQ) of 0.05 mg kg^{-1} (6.7% of the initially applied residue (AR)) and a limit of detection (LOD) of 0.015 mg kg^{-1} (2% AR) for ETU.

For soils LUFA 2.2, LUFA 2.3, Li 10, and Senozan, extractable ETU residues were in the range of 96% to 108% AR at the beginning of the incubation and < LOQ (LUFA 2.2) and < LOD (LUFA 2.3, Li10, Senozan) at the end of the incubation time (2 days). For the soils LUFA 5M and Am Fischteich, the extractable residues were in the range of 102% to 103% AR at the beginning of the incubation and between LOQ and LOD (LUFA 5M) and < LOD (Am Fischteich) at the end of the incubation period after 3 days.

The residues observed for the analyte (expressed as mg kg^{-1}) in the incubated soil samples were fitted by using the software package KinGUI. The kinetic models employed for this evaluation were described by FOCUS Kinetics workgroup. The trigger endpoints for ETU in the six soils examined were in the range of 0.1 to 0.5 days ($DegT_{50}$) and 0.3 to 1.5 days ($DegT_{90}$). The modeling endpoints for ETU were in the range of 0.1 to 0.5 days ($DegT_{50}$).

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Test compound: ETU (ethylene thiourea)
Chemical name (IUPAC): Imidazolidine-2-thione
Batch No.: WF19352
Molar mass: 102.2 g mol^{-1}
Molecular formula: $C_3H_6N_2S$
Purity: 99.7%

2. Soil

Six field-fresh soils were used in the study. The soils were kept under aerobic conditions at about 5°C between 26 to 48 days and the evaporated water was added if necessary. The soil characteristics are summarized in Table 7.1.2.1.2-23.

Table 7.1.2.1.2-23: Characteristics of six soils

Soil designation	LUFA 2.2 (14/736/03)	LUFA 2.3 14/570/03	LUFA 5M (14/1651/03)
DIN Particle size distribution [%]			
Sand 0.063 – 2 mm	85.0	62.9	54.5
Silt 0.002 – 0.063 mm	10.0	29.5	32.1
Clay < 0.002 mm	5.0	7.6	13.4
Textural class	loamy sand (Sl2)	silty sand (Su3)	loamy sand (Sl4)
USDA Particle size distribution [%]			
Sand 0.050 – 2 mm	87.4	64.1	60.1
Silt 0.002 – 0.050 mm	7.7	28.3	26.5
Clay < 0.002 mm	5.0	7.6	13.4
Textural class	loamy fine sand	sandy loam	sandy loam
Total carbon [%]	1.72	0.67	2.07
Total organic carbon [%]	1.72	0.67	1.09
pH [H_2O]	6.4	6.1	7.9
pH [$CaCl_2$]	5.8	5.4	7.0
Cation exchange capacity [$cmol^+ kg^{-1}$]	4.2	6.2	11.2
Max. water holding capacity [g per 100g dry weight]	33.1	23.9	26.3
pF 2.0 [$g_{soil\ moisture}\ g_{dry\ soil}^{-1}$]	0.163	0.108	0.169
pF 2.5 [$g_{soil\ moisture}\ g_{dry\ soil}^{-1}$]	0.130	0.106	0.163
Microbial biomass [mg C per 100g dry soil]	52.6	24.8	33.6

Soil designation	Li 10 (14/1680/03)	Senozan (14/1809/01)	Am Fischteich (14/1808/01)
DIN Particle size distribution [%]			
Sand 0.063 – 2 mm	81.5	13.2	11.1
Silt 0.002 – 0.063 mm	13.3	58.3	70.2
Clay < 0.002 mm	5.2	28.5	18.6
Textural class	loamy sand (Sl2)	silty loam (Lu)	clay silt (Ut4)
USDA Particle size distribution [%]			
Sand 0.050 – 2 mm	83.4	16.7	11.6
Silt 0.002 – 0.050 mm	11.4	54.8	69.8
Clay < 0.002 mm	5.2	28.5	18.6
Textural class	loamy fine sand	silty clay loam	silt loam
Total carbon [%]	0.95	1.13	1.63
Total organic carbon [%]	0.95	1.13	1.63
pH [H_2O]	7.0	6.5	6.1
pH [$CaCl_2$]	6.3	5.6	5.5
Cation exchange capacity [$cmol^+ kg^{-1}$]	7.6	11.4	8.0
Max. water holding capacity [g per 100g dry weight]	24.4	26.6	33.0
pF 2.0 [$g_{soil\ moisture}\ g_{dry\ soil}^{-1}$]	0.106	0.279	0.310
pF 2.5 [$g_{soil\ moisture}\ g_{dry\ soil}^{-1}$]	0.092	0.230	0.269
Microbial biomass [mg C per 100g dry soil]	25.7	27.3	35.7

B. STUDY DESIGN

1. Experimental conditions

Soil samples were adjusted to 40% of their maximum water holding capacities and incubated at room temperature in the dark between 12 and 23 days prior to application.

The application rate (based on dry soil weight) of the analyte dosed to soil was 0.75 mg kg^{-1}. Assuming a soil depth of 2.5 cm and a density of 1.5 g cm^{-3} this rate correspond to a theoretical field application rate of about 280 g ha^{-1}. This rate was chosen under consideration of both, the percentage of ETU observed in degradation studies of the active substance and a reasonable good detectability in the present study.
A stock solution of ETU analytical standard was prepared by accurately weighing and dissolving 7.53 mg in 10 mL of methanol to obtain a concentration of 0.75 mg mL^{-1}. This stock solution was used to dose 50 g soil incubation units and dose level concurrent recoveries.

The incubation flasks were loosely covered with plugs of paper tissue to minimize loss of moisture but to allow air exchange and then placed in a thermostated cabinet set to 20 ± 1°C and kept in the dark. Loss of soil water was controlled by weighing and was re-adjusted with distilled water if necessary.
The treated soil samples were incubated for various intervals up to 72 hours (3 days) prior to extraction. Additionally, untreated moist bulks of soil were kept in the thermostated cabinet.

2. Sampling

Soil samples were removed from the thermostated cabinet for subsequent extraction after 0, 1, 2, 4, 6, 24 and 48 hours (2 days), and additionally for soil samples LUFA 5M and Am Fischteich after 72 hours (3 days) of incubation.

3. Description of analytical procedures

The soil samples were extracted with 125 mL of 0.1% formic acid and 1% thiourea in methanol/water (1/1, v/v) for 30 min on a horizontal shaker, followed by 10 min sonication. After centrifugation, the supernatant was decanted through a glass wool plug. The extraction was repeated twice and the extracts were combined. After diluting with methanol/water (2/8, v/v), aliquots were analyzed by LC-MS/MS.

The analytical method achieves a limit of quantification (LOQ) of 0.05 mg kg^{-1} and a limit of detection (LOD) of 0.015 mg kg^{-1} for ETU.

4. Calculation of the degradation rate

Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints) as well as modeling endpoints. Kinetic analysis and calculation of $DegT_{50}$ and $DegT_{90}$ values was performed following the recommendations of the FOCUS Kinetics workgroup [*FOCUS (2006)*].

The software package KinGUI (version 2.2012.320.1629) was used for parameter fitting [*SCHÄFER et al. (2007)*].

Modeling strategy

The kinetic models employed for this evaluation were described by FOCUS [*FOCUS (2006)*].

Trigger endpoints were derived from the kinetic model that provides the best fit to the measured data, generally indicated by the lowest χ^2 - error. Modeling endpoints were derived preferably from the SFO model. If the SFO model is not appropriate, pragmatic procedures were used to derive conservative pseudo-SFO degradation rates from the appropriate bi-phasic model.

The appropriateness of the distinct kinetic model to describe soil degradation was tested by visual assessment. Furthermore, a kinetic model was considered appropriate for deriving trigger or modeling endpoints of χ^2 – error value was low (ideally below 15%) and the t-test for the degradation parameters passed at 5% error level.

Experimental data used for kinetic analysis

The experimental data were derived from the study reports and adjusted according to FOCUS [*FOUCS (2006)*].

The datasets submitted to kinetic analyses are given in Table 7.1.2.1.2-24.

Table 7.1.2.1.2-24: Input data of ETU expressed in mg kg^{-1} included in the kinetic analysis

Time [h]	LUFA 2.2 [mg kg^{-1}]	LUFA 2.3 [mg kg^{-1}]	Senozan [mg kg^{-1}]	LUFA 5M [mg kg^{-1}]	Li10 [mg kg^{-1}]	Am Fischteich [mg kg^{-1}]
0	0.790	0.760	0.722	0.770	0.778	0.772
	0.812	0.762	0.734	0.776	0.754	0.766
1	0.670	0.640	0.540	0.702	0.648	0.712
	0.620	0.566	0.516	0.720	0.658	0.720
2	0.318	0.418	0.378	0.596	0.482	0.550
	0.352	0.400	0.436	0.610	0.478	0.568
4	0.204	0.234	0.296	0.538	0.304	0.450
	0.226	0.248	0.302	0.540	0.364	0.446
6	0.170	0.147	0.262	0.506	0.268	0.400
	0.178	0.123	0.242	0.512	0.266	0.440
24	0.028	0.035	0.059	0.177	0.029	0.108
	0.028	0.028	0.048	0.210	0.035	0.107
48	0.0166	0.0075*	0.0075*	0.031	0.0075*	0.047
	0.0163	0.0075*	0.0075*	0.030	0.0075*	0.054
72				0.0075*		0.0075*
				0.021		0.0075*

*half of LOD

Normalization

The modeling endpoints were normalized to reference moisture conditions at pF 2 in a separate study [*see CA 7.1.2.1.2/15, BASF DocID 2014/1311118*].

II. RESULTS AND DISCUSSION

For soils LUFA 2.2, LUFA 2.3, Li 10, and Senozan, extractable ETU residues were in the range of 96% to 108% of the initially applied residue (% AR) at the beginning of the incubation and < LOQ (LUFA 2.2) and < LOD (LUFA 2.3, Li10, Senozan) at the end of the incubation period after 2 days.

For soils LUFA 5M and Am Fischteich, extractable ETU residues were in the range of 102% to 103% AR at the beginning of the incubation and between LOQ and LOD (LUFA 5M) and < LOD (Am Fischteich) at the end of the incubation period after 3 days.

Residues of the test item extracted from soil after given incubation periods are summarized in Table 7.1.2.1.2-25 and Table 7.1.2.1.2-26.

Table 7.1.2.1.2-25: Extractable ETU residues from soils LUFA 2.2, LUFA 2.3, and LUFA 5M

Hours [a]	LUFA 2.2		LUFA 2.3		LUFA 5M	
	[mg kg^{-1}]	[% AR] [b]	[mg kg^{-1}]	[% AR] [b]	[mg kg^{-1}]	[% AR] [b]
0	0.790	105	0.760	101	0.770	103
0	0.812	108	0.762	102	0.776	103
1	0.670	89	0.640	85	0.702	94
1	0.620	83	0.566	75	0.720	96
2	0.318	42	0.418	56	0.596	79
2	0.352	47	0.400	53	0.610	81
4	0.204	27	0.234	31	0.538	72
4	0.226	30	0.248	33	0.540	72
6	0.170	23	0.147	20	0.506	67
6	0.178	24	0.123	16	0.512	68
24	0.028	< LOQ	0.035	< LOQ	0.177	24
24	0.028	< LOQ	0.028	< LOQ	0.210	28
48	0.017	< LOQ	0.0075	< LOD	0.031	< LOQ
48	0.016	< LOQ	0.0075	< LOD	0.030	< LOQ
72	-	-	-	-	0.014	< LOD
72	-	-	-	-	0.021	< LOQ

LOQ < 0.05 mg kg^{-1} (< 7% of nominal concentration), LOD < 0.015 mg kg^{-1} (< 2% of nominal concentration)
[a] Hours after application
[b] Percent of the nominally applied residue (0.75 mg kg^{-1})

Table 7.1.2.1.2-26: Extractable ETU residues from soils Li 10, Senozan, and Am Fischteich

Hours [a]	Li 10 Soil		Senozan Soil		Am Fischteich (Soil)	
	[mg kg^{-1}]	[% AR] [b]	[mg kg^{-1}]	[% AR] [b]	[mg kg^{-1}]	[% AR] [b]
0	0.778	104	0.722	96	0.772	103
0	0.754	101	0.734	98	0.766	102
1	0.648	86	0.540	72	0.712	95
1	0.658	88	0.516	69	0.720	96
2	0.482	64	0.378	50	0.550	73
2	0.478	64	0.436	58	0.568	76
4	0.304	41	0.296	39	0.450	60
4	0.364	49	0.302	40	0.446	59
6	0.268	36	0.262	35	0.400	53
6	0.266	35	0.242	32	0.440	59
24	0.029	< LOQ	0.059	7.9	0.108	14
24	0.035	< LOQ	0.048	6.4	0.107	14
48	0.0075	< LOD	0.0075	< LOD	0.047	6.3
48	0.0075	< LOD	0.0075	< LOD	0.054	7.1
72	-	-	-	-	0.0075	< LOD
72	-	-	-	-	0.0075	< LOD

LOQ < 0.05 mg kg^{-1} (< 7% of nominal concentration), LOD < 0.015 mg kg^{-1} (< 2% of nominal concentration)
[a] Hours after application
[b] Percent of the nominally applied residue (0.75 mg kg^{-1})

The calculated degradation parameters and kinetic endpoints of ETU for use as triggers for additional work and for modeling are summarized in the following tables (Table 7.1.2.1.2-27 and Table 7.1.2.1.2-28).

For the evaluated soil degradation experiments, the visual assessment and the goodness-of-fit statistics show plausible fits. The low t-test values of the degradation rate constants ($p<0.05$) indicate that the parameters were estimated significantly different from zero. Therefore, the resulting $DegT_{50}$ / DT_{50} values can be considered reliable.

Table 7.1.2.1.2-27: ETU trigger endpoints

Soil	Kinetic model	χ^2 error [%]	$DegT_{50}$ [d]	$DegT_{90}$ [d]
LUFA 2.2	SFO	12.0	0.1	0.3
LUFA 2.3	SFO	4.6	0.1	0.3
Senozan	DFOP	1.1	0.1	0.9
LUFA 5M	SFO	5.2	0.5	1.5
Li 10	SFO	5.1	0.1	0.5
Am Fischteich	FOMC	5.6	0.2	1.3

Table 7.1.2.1.2-28: ETU modeling endpoints

Soil	Kinetic model	$\chi2$ error [%]	$DegT_{50}$ [d]
LUFA 2.2	SFO	12.0	0.1
LUFA 2.3	SFO	4.6	0.1
Senozan	SFO	9.3	0.1
LUFA 5M	SFO	5.2	0.5
Li 10	SFO	5.1	0.1
Am Fischteich	SFO	7.8	0.3

III. CONCLUSION

The objective of the study was to examine aerobic degradation of ETU (metabolite of metiram) in six soils. The trigger endpoints for ETU in the six soils examined were in the range of 0.1 to 0.5 days ($DegT_{50}$) and 0.3 to 1.5 days ($DegT_{90}$). The modeling endpoints for ETU were in the range of 0.1 to 0.5 days ($DegT_{50}$).
For the soils Senozan, LUFA 2.2 (= Speyer 2.2) and LUFA 2.3 (= Speyer 2.3) much shorter ETU half-lives as in the previous study with the parent mancozeb were observed.

Report: CA 7.1.2.1.2/15
Maassen K., 2015c
Normalization of laboratory degradation half-lives of ETU in soil to reference temperature and moisture conditions
2014/1311118

Guidelines: FOCUS Groundwater (2014) Generic Guidance for Tier 1 FOCUS Ground Water Assessments v 2.2.

GLP: no

Executive Summary

$DegT_{50}$ values of ETU derived from a laboratory study on aerobic soil degradation [*see CA 7.1.2.1.2/14, BASF DocID 2014/1189253*] were normalized to reference moisture conditions at pF = 2 following FOCUS (2014) in order to derive modeling endpoints suitable for the use in environmental fate models.

The normalization was performed using the moisture dependency equations recommended by FOCUS (2014). The actual soil moisture (40% at MWHC) and the reference moisture at field capacity at pF 2 were taken from the study report.

No temperature correction was performed as the study was already conducted at reference conditions (20°C).

The normalized $DegT_{50}$ values ($DegT_{50,ref}$) ranged between 0.1 and 0.3 days.

I. MATERIAL AND METHODS

The modeling endpoints reported in BASF DocID 2014/1189253 [*see CA 7.1.2.1.2/14*] of the metiram metabolite ETU in six different soils were normalized following the recommendations of FOCUS [*FOCUS (2014)*] as soil $DegT_{50}$ values for environmental fate modeling are required at reference conditions (temperature of 20°C and soil moisture at field capacity at pF 2).

A temperature correction of the data was not necessary as the laboratory study was performed at 20°C. The soil moisture normalization was conducted using a Walker coefficient of 0.7. The actual soil moisture and the water content at pF 2 for each soil are shown in Table 7.1.2.1.2-29.

Table 7.1.2.1.2-29: Soil properties needed for the normalization process

Soil	Soil type USDA	T_{act}	T_{ref}	MWHC [g $100g^{-1}$]	θ_{act} [% MWHC]	θ_{act} [g $100g^{-1}$]	θ_{re} [g $100g^{-1}$]	f_{moist}
LUFA 2.2	loamy fine sand	20	20	33.1	40	13.2	16.3	0.86
LUFA 2.3	sandy loam	20	20	23.9	40	9.6	10.8	0.92
Senozan	silty clay loam	20	20	26.6	40	10.6	27.9	0.51
LUFA 5M	sandy loam	20	20	26.3	40	10.5	16.9	0.72
Li10	loamy fine sand	20	20	24.4	40	9.8	10.6	0.94
Am Fischteich	silt loam	20	20	33.0	40	13.2	31.0	0.55

T_{act} actual temperature [°C]
T_{ref} reference temperature [°C]
θ_{act} actual soil moisture [g 100 g^{-1} dry soil]
θ_{ref} reference soil moisture at field capacity (pF 2) as stated in study report [g 100 g^{-1} dry soil]
f_{moist} moisture correction factor [-]

II. RESULTS AND DISCUSSION

The actual and the normalized laboratory $DegT_{50}$ for ETU are presented in Table 7.1.2.1.2-30.

Table 7.1.2.1.2-30: Normalization of ETU $DegT_{50}$ values to reference conditions

Soil	Soil type USDA	Kinetic model	k_{act} [1 d^{-1}]	$DegT_{50,act}$ [d]	f_{moist}	k_{ref} [1 d^{-1}]	$DegT_{50,ref}$ [d]
LUFA 2.2	loamy fine sand	SFO	7.807	0.1	0.86	9.030	0.1
LUFA 2.3	sandy loam	SFO	6.996	0.1	0.92	7.619	0.1
Senozan	silty clay loam	SFO	4.877	0.1	0.51	9.577	0.1
LUFA 5M	sandy loam	SFO	1.500	0.5	0.72	2.090	0.3
Li10	loamy fine sand	SFO	4.666	0.1	0.94	4.944	0.1
Am Fischteich	silt loam	SFO	2.496	0.3	0.55	4.537	0.2

k_{act} rate constant at study conditions [1 d^{-1}]
$DegT_{50,act}$ $DegT_{50}$ at study conditions [d]
f_{moist} moisture correction factor [-]
k_{ref} rate constant at reference conditions [1 d^{-1}]
$DegT_{50,ref}$ $DegT_{50}$ at reference conditions [d]

III. CONCLUSION

The soil degradation rates at study conditions estimated under aerobic conditions in the laboratory were normalized to reference soil moisture (pF 2) in order to derive modeling endpoints for environmental fate models.

The normalized $DegT_{50}$ values ($DegT_{50,ref}$) ranged between 0.1 and 0.3 days.

The following study study has been reported in the Monograph for the active substance mancozeb and is therefore considered as peer reviewed. The summary was taken was taken from the Monograph for mancozeb.

Report:	*CA 7.1.2.1.2/16* *Vette H.Q.M. de et al., 1999a* *Determination of the rate of degradation of N,N-ethylene urea (EU; metabolite of Mancozeb) in three soils. (CTB guideline section G.1.1.3, BBA Guideline IV, 4-1, and OECD draft guidline)* *1999/1014017*
Guidelines:	*BBA IV 4-1, EEC 95/36*
GLP:	*yes* *(certified by Netherlands Ministry of Agriculture, Nature Management and Fisheries, The Hague, Netherlands)*

Materials and methods:

A mixture of uniformly [^{14}C] labeled N,N'-ethylene urea (EU) (specific activity: 24 mCi mmol^{-1}, radiochemical purity: >98%) and unlabeled substance (radiochemical purity: >95%) was applied to a humic sand soil, a sandy loam soil and a low humic content sand soil to study its rate of degradation.

Table 7.1.2.1.2-31: Soil characteristics

Soil description	Sandy loam soil	Humic sand soil	Low humic content sand soil
Organic matter (%)	2.7	3.3	1.3
$CaCO_3$ (%)	7.5	0.1	0.1
pH (KCl)	7.7	5.3	7.8
Nitrogen content (g N 100 g^{-1} dry soil)	0.14	0.107	0.048
Granulation (%) clay (< 2 µm) silt (2-50 µm) sand (> 50 µm)	 9.8 22.3 57.7	 2.8 9.5 84.3	 1.3 2.8 94.5
Moisture content at 0.32 bar (= pF 2.5) (g H_2O 100 g^{-1} dry soil)	12.7	12.7	4.3.
Microbial biomass (0 day) (mg C kg^{-1} dry soil)	n.d.	n.d.	n.d.
Microbial biomass (29 day) (mg C kg^{-1} dry soil)	282	35	34
CEC (C mol^{+} kg^{-1} dry soil)	11.4	3.8	1.8

The incubation was carried out in conical flasks provided with soda lime CO_2 absorption trap. The application rate was 8 mg kg^{-1} on dry soil basis. The soils were incubated under aerobic conditions in the dark at 20 ± 2°C. CO_2 evolution in humic and sand soil only, (methanol) extractable radioactivity in the solids, and distribution of radioactivity between parent compound and metabolites by TLC were performed at 0, 1 ,3 ,7 ,14 and 28 days. The radioactivity was determined by LSC.

Findings:

The microbial biomass determination showed that the soils were microbially active at day 29 of the experiment. The distribution of radioactivity between glass wool (volatiles), carbon dioxide and soil extracts as percentage of the initial radioactivity was carried out only in humic sand soil. The average are reported in Table 7.1.2.1.2-32.

Table 7.1.2.1.2-32: Distribution of radioactivity in humic sand soil (% of initial radioactivity)

Time (days)	Glass wool	CO_2	Soil extracts
0	n.d.	n.d.	91.9
1	0.0	0.1	84.8
3	0.0	0.2	73.7
7	0.0	1.1	63.7
14	0.0	24.8	20.7
21	0.0	41.7	2.1
28	0.0	47.2	1.3

n.d. = not determined

The amount of extractable radioactivity decreases from 91.9% of the initial value at the start to 1.3% after 28 days. The activity which was not recovered as $^{14}CO_2$ or in the extract is assumed to remain as bound residue in the soil. In this test system the radiolabel recovery is ≥ 90%. The radioactivity in the other two soil extracts as percentage of the initial radioactivity is presented in the table below.

Table 7.1.2.1.2-33: Distribution of radioactivity in methanol extract of three soils (% of initial radioactivity)

Time (days)	Sandy loam soil	Humic sand soil	Low humic content sand soil
0	91.1	91.7	91.9
1	78.5	86.4	84.8
3	62.0	76.3	73.7
7	39.6	42.6	63.7
14	21.1	7.3	20.7
21	8.1	6.2	2.1
28	0.8	3.5	1.3

The metabolites identified by TLC are shown in the table below.

Table 7.1.2.1.2-34: Amount of [^{14}C] EU and metabolites as % of initial radioactivity in different soil types

Time	Sandy loam soil			Humic sand soil			Low humic content sand soil		
(days)	EU	Met 1	Met 2	EU	Met 1	Met 2	EU	Met 1	Met 2
0	77.9	1.0	10.0	76.7	1.7	11.0	78.6	1.4	10.6
1	66.6	0.8	9.7	70.9	1.2	12.4	70.3	1.4	11.5
3	52.2	0.8	7.9	62.5	1.1	11.1	61.1	1.2	10.2
7	34.2	0.5	4.0	31.5	0.7	7.3	50.6	0.9	10.9
14	18.6	0.3	1.0	0.6	0.4	4.8	12.5	0.6	5.4
21	6.9	0.1	0.3	1.0	0.4	2.9	0.1	0.2	0.8

In all three soils a rapid degradation of EU was observed. The DT_{50} and DT_{90}, evaluated with first linear regression, for disappearance of [^{14}C] N,N'-ethylene urea are reported in the table below.

Table 7.1.2.1.2-35: DT_{50} and DT_{90} of [^{14}C] N,N'-ethylene urea in the three soils

Soil	r^2	DT_{50} (days)	DT_{90} (days)
Sandy loam soil	0.9946	6.2	20.7
Humic sand soil	0.9407	7.6	25.2
Low humic content sand soil	0.9527	4.8	16.0

Moreover, the calculation of the DT_{50} and DT_{90} values of the parent compound and metabolite 2, a 3/2 order function was used. Results are reported in the table below.

Table 7.1.2.1.2-36: DT_{50} and DT_{90} of [^{14}C] N,N'-ethylene urea in the three soils

Soil	compound	r2	DT_{50} (days)	DT_{90} (days)
Sandy loam soil	EU	0.9990	3.3	19.1
	met 2	0.9836	5.0	14.9
Humic sand soil	EU	0.9875	6.6	18.3
	met 2	0.8927	14.8	20.6
Low humic content sand soil	EU	0.9655	3.8	13.4
	met 2	0.8944	10.8	> 22

It appears that the first order curve fitted the measurements less well than the 3/2 order function.

Conclusion:

EU degrades rapidly in soils. Its DT_{50} ranges from 4.8 to 7.6 days and the DT_{90} from 16 to 25 days. The metabolite 2 degrades with a DT_{50} ranging from 15 to 22 days according a 3/2 order decay function.

Report: CA 7.1.2.1.2/17
Hardy I., 2015a
Mancozeb: Kinetic modelling evaluation of EU from an aerobic soil degradation study according to Focus degradation kinetics
2015/1134752
Guidelines: EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009
GLP: no

Executive Summary

The degradation of EU (a metabolite of mancozeb) in soil under aerobic conditions in the laboratory has been investigated in three soils as reported in a study by de Vette et al. [*see CA 7.1.2.1.2/16, BASF DocID 1999/1014017*].
The purpose of this evaluation was to analyze the degradation kinetics observed in the studies, taking into account the current guidance of the FOCUS workgroup on degradation kinetics and using a compartment modelling approach.
Kinetic evaluation was performed in order to derive
i) Degradation parameters as triggers for additional work (trigger endpoints)
ii) Degradation parameters for environmental fate models (modeling endpoints).
The calculated degradation parameters and kinetic endpoints of EU for use as triggers for additional work and for modeling are summarized in the following tables. For the evaluated soil degradation experiments, the visual assessment and the goodness-of-fit statistics show plausible fit. The low t-test values of the degradation rate constants ($p<0.05$) indicate that the parameters were estimated significantly different from zero. Therefore, the resulting $DegT_{50}$ values can be considered reliable.

Materials and methods

Kinetic modeling following the appropriate FOCUS Kinetics (2006) flowchart was carried out using CAKE v2.0. Modeling endpoint DT_{50} values were normalized to 20°C and pF2 using FOCUS (2014) procedures and an activation energy of 65.4 KJ mol^{-1} (Q_{10} 2.58).

Results and discussion

The calculated trigger and modeling endpoint DT_{50} values are summarized in Table 7.1.2.1.2-37 to Table 7.1.2.1.2-38 respectively.

Table 7.1.2.1.2-37: EU trigger DT_{50} and DT_{90} endpoints in soil

Soil	Best-fit model	Chi² (%)	Model parameters	Trigger endpoints	
				$DegT_{50}$ (days)	$DegT_{90}$ (days)
Maasdijk, Sandy loam	DFOP	2.0	k_1 [d^{-1}]: 1.894 k_2 [d^{-1}]: 0.1015	4.5	20.4
Droevendaal, Humic sand	SFO	11.5	k [d^{-1}]: 0.1143	6.1	20.1
Lisse, Sand	SFO	9.9	k [d^{-1}]: 0.1610	4.3	14.3

Table 7.1.2.1.2-38: EU modeling DT_{50} and DT_{90} endpoints in soil

Soil	Best-fit model	Chi² (%)	Model parameters	Modeling endpoints	
				$DegT_{50}$ (days)	Normalized $DegT_{50}$ (days)[a]
Maasdijk, Sandy loam	SFO	8.1	k [d^{-1}]: 0.1267	5.5	4.1
Droevendaal, Humic sand	SFO	11.5	k [d^{-1}]: 0.1143	6.1	5.7
Lisse, Sand	SFO	9.9	k [d^{-1}]: 0.1610	4.3	2.1

[a] Reference conditions: 20°C, pF2

At the time of the conduct of following two studies, hydantoin was considered to be a major metabolite in soil based on the results of old studies. Therefore DT_{50} values were deemed necessary for the soil compartment and these studies were initiated. As explained above, these old studies are meanwhile considered no longer valid. In contrast in the new, valid studies hydantoin was - if at all - found only in very low amounts and is no longer considered a major metabolite of metiram. Consequently, no DT_{50} values for hydantoin are required. However, since the DT_{50} values of hydantoin have been determined in the context of metiram, these results are reported here.

Metabolite EU was found to be major also in one of the new studies, therefore DT_{50} values are required.

The following degradation study on hydantoin and EU was first conducted under non-GLP conditions at BASF's facilities and was later on repeated to GLP at another test facility.

Report: CA 7.1.2.1.2/18
Reinhard K.,Harder U., 2009b
Aerobic degradation of the Metiram metabolites Ethylene Urea and Hydantoin in soil
2009/1093835

Guidelines: OECD 307, BBA IV 4-1, SETAC Procedures for assessing the environmental fate and behaviour and ecotoxicity of pesticides (March 1995)

GLP: no

Executive Summary

The objective of the study was to investigate the aerobic degradation of the metiram metabolites ethylene urea (EU) and hydantoin in soil under laboratory conditions.

Since no radiolabeled material was available, the residues of the two test compounds in soil were determined using an analytical method based on HPLC-MS. The validity of the method was examined by spiking untreated soil with the test compounds at concentration levels corresponding to 100% and 5% (limit of quantification) of the initially applied amount. The recoveries were in the range of 85-102% and of 69-93%, respectively.

EU and hydantoin were applied to soil in separate experiments at a rate of 0.4 mg kg^{-1} (150 g a.s. ha^{-1}) each. The test soils, one sandy loam (Bruch West) and two loamy sand soils (Li 10 and LUFA 2.2), were incubated in small plastic bags in the dark under aerobic conditions at 20°C and 40% of the maximum water holding capacity. Soil samples were taken at 0, 1, 2, 3 and 7 days after treatment (DAT). Extraction of samples was carried out with methanol/water (1/1). The extracts were analyzed for the respective test compounds by HPLC coupled to an MS detector.

EU degraded rapidly in the three test soils. As early as 1 DAT, less than 53% of the initially applied amount of test compound was recovered. At 2-3 DAT, the residues decreased below the quantification limit of 5% in the two loamy sand soils; in the sandy loam soil, the test compound degraded more slowly reaching a residual amount of about 6% at the end of the incubation (7 DAT).

Hydantoin degraded even more rapidly in soil than EU. As early as 1 DAT, the residues in the two loamy sand soils decreased to concentrations below the quantification limit of 5%. In the sandy loam soil, degradation was slightly slower with 6% of the initially applied amount of hydantoin being recovered at 1 DAT. Due to interferences with constituents of the soil matrix in the range of the quantification limit, the apparent residue levels in the sandy loam soil did not decrease further, but remained around 6% until the end of the incubation (7 DAT).

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Test compound 1: ethylene urea (EU)
Chemical name (IUPAC): 2-imidazolidinone = N,N'-ethylene urea
Molar mass: 86.1 g mol^{-1}
Molecular formula: $C_3H_6N_2O$
Purity: 98%

Test compound 2: hydantoin
Chemical name (IUPAC): imidazolidine-2,4-dione
Molar mass: 100.1 g mol^{-1}
Molecular formula: $C_3H_4N_2O_2$
Purity: 98%

2. Soil

Three different soils (sieved to a particle size < 2 mm) were used in the experiments. The soils were sampled from agricultural land and stored for less than 3 months at about 4°C. The three sites had not been treated with pesticides during the last 5 years. The soil characteristics are summarized in Table 7.1.2.1.2-39.

Table 7.1.2.1.2-39: Characteristics of soil Li 10, LUFA 2.2 and Bruch West

Soil designation	**Li 10 (09/1680/03)** Limburgerhof, Germany	**LUFA 2.2 (09/736/03)** Speyer, Germany	**Bruch West (08/060/04)** Limburgerhof, Germany
DIN Particle size distribution [%]			
Sand 0.063 – 2 mm	81.5	81.6	61.5
Silt 0.002 – 0.063 mm	13.1	12.3	27.2
Clay < 0.002 mm	5.3	6.0	11.2
Textural class	loamy sand	loamy sand	loamy sand
USDA Particle size distribution [%]			
Sand 0.050 – 2 mm	82.5	82.2	63.8
Silt 0.002 – 0.050 mm	12.1	11.8	24.9
Clay < 0.002 mm	5.3	6.0	11.2
Textural class	loamy sand	loamy sand	sandy loam
Total carbon [%]	0.94	1.62	2.92
Total organic carbon [%]	0.94	1.62	1.23
pH [H_2O]	7.0	6.5	7.9
pH [$CaCl_2$]	6.3	5.9	7.3
Cation exchange capacity [$cmol^+ kg^{-1}$]	5.3	6.7	12.2
Max. water holding capacity [g per 100g dry weight]	25.4	30.9	23.6
pF 2.0 [$g_{soil\ moisture}\ g_{dry\ soil}^{-1}$]	0.104	0.186	0.204
pF 2.5 [$g_{soil\ moisture}\ g_{dry\ soil}^{-1}$]	0.091	0.128	0.116
Microbial biomass [mg C per 100g dry soil]	46.7	38.7	37.0

B. STUDY DESIGN

1. Experimental conditions

The soils were treated with 0.4 mg test compound per kg dry soil, which corresponds to a field application rate of 150 g ha^{-1}, calculated on the basis of an equal distribution in the top 2.5 cm soil layer and a soil density of 1.5 g cm^{-3}. These rates were chosen under consideration of both, the percentage of the metabolites observed in degradation studies of the active substance and a reasonable detectability in the present study. The test compound was dissolved in a small volume of acetonitrile and mixed with the calculated amount of water to adjust to a soil moisture of 40% of the maximum water holding capacity (MWHC). After adding the aqueous solution of the test compound, the soils were slowly stirred for some minutes to homogeneously distribute the test item.

The treated soils were filled into small plastic bags (25 g per bag), which were loosely closed with a rubber band and then incubated at 20°C. Two replicates per sampling date were prepared.

2. Sampling

Samples were taken at 0, 1, 2, 3 and 7 days after treatment (DAT). The 0 DAT samples were directly prepared in plastic centrifuge tubes and extracted immediately after adding the test compound.

3. Description of analytical procedures

The soil was extracted once with 100 mL methanol / water (1/1, v/v) by shaking on a rotary shaker for 30 min. An aliquot of the extract was analyzed by HPLC-MS.

In order to validate the applied method of analysis, untreated soil was spiked with 0.4 mg kg^{-1} dry soil corresponding to 100% of the applied amount of test compound and with 0.02 mg kg^{-1} dry soil (limit of quantification – LOQ) corresponding to 5% of the applied amount of test compound. One blank sample of each soil and two replicates per concentration and soil were extracted and analyzed by HPLC-MS.

4. Calculation of the degradation rate

No degradation rates or DT_{50} values were calculated in this study. Instead, a kinetic evaluation was performed and reported in a separate study [*see CA 7.1.2.1.2/20, BASF DocID 2014/1227795*], considering the newest guidelines and model versions.

II. RESULTS AND DISCUSSION

For EU, the recoveries in the three soils ranged from 85.4-98.2% (0.4 mg kg^{-1} level) and from 69.2-73.1% (0.02 mg kg^{-1} level). The corresponding recoveries of hydantoin were 84.8-101.7% (0.4 mg kg^{-1} level) and 71.4-93.1% (0.02 mg kg^{-1} level).

At 1 DAT, about half of the applied amount of EU had disappeared from Bruch West and Li 10 soil and even about 2/3 from LUFA 2.2 soil. At 2 DAT and 3 DAT, the residues decreased below 5% in LUFA 2.2 and Li 10 soil, respectively. In Bruch West soil, a residual amount of 5.5% was reached at 7 DAT (Table 7.1.2.1.2-40). This means that EU is degraded very fast in soil.

Table 7.1.2.1.2-40: Degradation of EU in three soils

Soil	DAT	Rep. 1	Rep. 2	Mean	Mean
		[% of applied test compound]			0 d = 100%
Li 10	0	98.6	95.4	97.0	100.0
	1	47.1	43.6	45.4	46.8
	2	5.7	5.8	5.7	5.9
	3	<LOQ	<LOQ	<LOQ	<LOQ
	7	<LOQ	<LOQ	<LOQ	<LOQ
LUFA 2.2	0	90.5	87.0	88.8	100.0
	1	27.6	28.4	28.0	31.6
	2	<LOQ	<LOQ	<LOQ	<LOQ
	3	<LOQ	<LOQ	<LOQ	<LOQ
	7	<LOQ	<LOQ	<LOQ	<LOQ
Bruch West	0	83.1	99.5	91.3	100.0
	1	49.2	45.9	47.6	52.1
	2	19.8	20.1	20.0	21.8
	3	11.0	10.9	11.0	12.0
	7	5.1	5.1	5.1	5.5

LOQ = 5% (0.02 mg kg^{-1})

The test compound hydantoin degraded even faster in all three soils. As early as 1 DAT, the residual amounts of the test compound had decreased to levels near or below the LOQ of 5% (0.02 mg kg-1) of the initially applied amount of hydantoin. The apparent residue levels in Bruch West soil did not decrease further, but remained around 6% until the end of the incubation (7 DAT, Table 7.1.2.1.2-41). With the given reduced sensitivity of the HPLC-MS detection for hydantoin (compared to that of ethylene urea) this is rather ascribed to interferences with constituents of the soil matrix in the range of the quantification limit than to non-degraded residues of hydantoin. In any case, the data clearly indicate that hydantoin is very unstable in soil.

Table 7.1.2.1.2-41: Degradation of hydantoin in three soils

Soil	DAT	Rep. 1	Rep. 2	Mean	Mean
		[% of applied test compound]			(0d = 100%)
Li 10	0	96.8	100.9	98.8	100.0
	1	<LOQ	<LOQ	<LOQ	<LOQ
	2	<LOQ	<LOQ	<LOQ	<LOQ
	3	<LOQ	<LOQ	<LOQ	<LOQ
	7	<LOQ	<LOQ	<LOQ	<LOQ
LUFA 2.2	0	95.9	93.9	94.9	100.0
	1	<LOQ	<LOQ	<LOQ	<LOQ
	2	5.1	5.2	5.2	5.5
	3	<LOQ	<LOQ	<LOQ	<LOQ
	7	<LOQ	<LOQ	<LOQ	<LOQ
Bruch West	0	92.1	89.4	90.7	100.0
	1	5.6	5.4	5.5	6.1
	2	6.7	6.1	6.4	7.1
	3	5.3	5.3	5.3	5.8
	7	6.0	5.8	5.9	6.5

LOQ = 5% (0.02 mg kg^{-1})

III. CONCLUSION

The objective of the study was to examine aerobic degradation of EU and hydantoin, both metabolites of metiram, in 3 different soils.
Degradation of EU was very fast in all 3 soils. At 2 and 3 DAT, the residues of EU decreased below LOQ of 5% (0.02 mg kg^{-1}) in LUFA 2.2 and Li 10 soil, respectively. In Bruch West soil a residual amount of 5.5% was reached at 7 DAT. Hydantoin degraded even faster in all three soils. As early as 1 DAT, the residual amounts had decreased to levels near or below the LOQ of 5% in Li 10 and LUFA 2.2 soils. Apparent residue levels in Bruch West soil remained until 7 DAT around 6% which is attributed to interferences with constituents of the soil matrix in the range of the quantification limit rather than to non-degraded residues of hydantoin

Report:	CA 7.1.2.1.2/19 Class T., 2010c Ethylene Urea and Hydantoin (metabolites of Metiram, BAS 222 F): Study on aerobic soil degradation 2010/1056131
Guidelines:	OECD 307 (2002), BBA IV 4-1, EPA 835.4100, SANCO/3029/99 rev. 4 (11 July 2000)
GLP:	yes (certified by Umweltministerium Baden-Wuerttemberg, Stuttgart)

Executive Summary

The objective of the study was to examine aerobic degradation of ethylene urea (EU) and hydantoin, both metabolites of metiram (BAS 222 F), in three different soils.

Three field-fresh soils were provided with characterizations: LUFA 2.2 (loamy sand), Li 10 (loamy sand), and Bruch West (sandy loam). The soils were acclimatized with soil moistures adjusted to 45% of their maximum water holding capacities.

The application rates of the two test items dosed separately to bulk soils were 0.4 mg kg^{-1} for EU and 0.8 mg kg^{-1} for hydantoin. Assuming a soil depth of 2.5 cm and a soil density of 1.5 g cm^{-3} these rates correspond to theoretical field application rates of 0.15 kg ha^{-1} for EU and 0.3 kg ha^{-1} for hydantoin. Due to the short half-lives expected for both substances, bulk soils were sampled for extraction during the day of the application (i.e. 2 and 6 hours after dosing). For subsequent sampling during the following days, 25 g soil aliquots were incubated in the dark at 20 ± 2°C.

After given incubation periods, replicate soil aliquots were collected and extracted with methanol/water for subsequent LC/MS/MS determination. The methods were validated with acceptable average recoveries in a range of 87% to 105% (relative standard deviations RSD always < 20%). The methods achieve limits of quantitation (LOQ) of 0.02 mg kg^{-1} for EU and 0.04 mg kg^{-1} for hydantoin (i.e. 5% of the respective dose levels).

At 22 h after application more than 60% of EU in soil Li 10, about 60% in soil LUFA 2.2 and about 85% in soil Bruch West was degraded. At 52 h in soil Li10 and at 48 h in soil Bruch West the residues decreased below LOQ. In soil LUFA 2.2 a residual amount of 6% was reached after 70 h.

The test compound hydantoin degraded even faster, reaching amounts below LOQ in soil Li 10 after 24 h and in soil LUFA 2.2 as well as soil Bruch West after 20 h

The evaluation of the aerobic degradation of both metiram metabolites applying FOCUS kinetics analyses and single first order (SFO) kinetics resulted in degradation times DT_{50} for EU of about 0.5 to 0.7 days, and for hydantoin of about 0.1 to 0.3 days.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Test compound 1:	ethylene urea (EU)
CAS-No.:	120-93-4
Chemical name (IUPAC):	2-imidazolidinone = N,N'-ethylene urea
Molar mass:	86.1 g mol^{-1}
Molecular formula:	$C_3H_6N_2O$
Purity:	≥ 98%

Test compound 2:	hydantoin
CAS-No.:	461-72-3
Chemical name (IUPAC):	imidazolidine-2,4-dione
Molar mass:	100.1 g mol^{-1}
Molecular formula:	$C_3H_4N_2O_2$
Purity:	98%

2. Soil

Three different soils (sieved to a particle size < 2 mm) were used in the experiments. The soils were sampled from agricultural land and stored for less than 3 months at about 4°C. The three sites had not been treated with pesticides during the last 5 years. The soil characteristics are summarized in Table 7.1.2.1.2-42.

Table 7.1.2.1.2-42: Characteristics of soil Li 10, LUFA 2.2 and Bruch West

Soil designation	Li 10 (09/1680/05) Limburgerhof, Germany	LUFA 2.2 (09/736/05) Speyer, Germany	Bruch West (09/060/05) Limburgerhof, Germany
DIN Particle size distribution [%]			
Sand 0.063 – 2 mm	80.7	83.4	62.9
Silt 0.002 – 0.063 mm	13.8	12.4	25.4
Clay < 0.002 mm	5.5	4.2	11.7
Textural class	loamy sand	silt sand	loamy sand
USDA Particle size distribution [%]			
Sand 0.050 – 2 mm	81.4	84.0	65.7
Silt 0.002 – 0.050 mm	13.2	11.8	22.6
Clay < 0.002 mm	5.5	4.2	11.7
Textural class	loamy sand	loamy sand	sandy loam
Total carbon [%]	0.86	1.95	3.11
Total organic carbon [%]	0.86	1.95	1.37
pH [H_2O]	6.8	6.2	8.0
pH [$CaCl_2$]	6.2	5.7	7.5
Cation exchange capacity [$cmol^+ kg^{-1}$]	4.1	6.4	10.1
Max. water holding capacity [g per 100g dry weight]	28.3	38.2	31.3
pF 2.0 [$g_{soil\ moisture}\ g_{dry\ soil}^{-1}$]	0.110	0.189	0.163
pF 2.5 [$g_{soil\ moisture}\ g_{dry\ soil}^{-1}$]	0.154	0.168	0.192
Microbial biomass [mg C per 100g dry soil]	20.6	42.5	29.4

B. STUDY DESIGN

1. Experimental conditions

The soils were acclimatized with soil moistures adjusted to 45% of their maximum water holding capacities.
The application rates (based on dry soil weight) of the two test items dosed separately to bulk soils were 0.4 mg kg^{-1} for EU and 0.8 mg kg^{-1} for hydantoin. Assuming a soil depth of 2.5 cm and a density of 1.5 g cm^{-3} these rates correspond to theoretical field application rates of 150 g ha^{-1} for EU and 300 g ha^{-1} for hydantoin. These rates were chosen under consideration of both, the percentage of the metabolites observed in degradation studies of the active substance and a reasonable good detectability in the present study. A higher concentration was chosen for hydantoin since it is more difficult to analyze compared to EU.

EU and hydantoin were applied as 0.40 mg mL^{-1} and 0.80 mg mL^{-1} solutions in acetonitrile, respectively, i.e. 1.08 to 1.14 mL were applied in small doses of 0.25 mL to about 1 kg (dry-weight equivalent) of soil bulk mass.

The bulk soils were weighed in stainless-steel vessels. After 15 minutes of homogenization, the water content was measured / re-adjusted and the soils were again mixed.

25 g of dry soil mass equivalents were measured from the dosed homogenized soils into 0.25 L sized incubation flasks and were covered to prevent excessive loss of water. Then, the samples were incubated at 20 ± 2°C in the dark.

2. Sampling

For the EU treated soil, replicate soil samples were taken after 0.25, 2.0 and 6.0 hours from dosed bulk soils and 22, 25/26, 28, 44, 48, 52 and 70 hours from soil incubations for subsequent extraction.

For the hydantoin treated soil, replicate soil samples were taken after 0.25, 2.0 and 6.0 hours from dosed bulk soils and 20, 24 and 28 (only Li 10 soil) hours from soil incubations for subsequent extraction.

3. Description of analytical procedures

For soil extraction, soil aliquots (dry weight) were weighed into PE centrifuge bottles and methanol/water (1/1, v/v) were added. Then the samples were shaken for about 30 minutes. Thereafter, the samples were centrifuged and an aliquot of the supernatant was analyzed by LC/MS/MS. For EU the limit of quantification (LOQ) was 0.02 mg kg^{-1} and for hydantoin the LOQ was 0.04 mg kg^{-1} (i.e. 5% of the respective dose levels).

4. Calculation of the degradation rate

Kinetic analysis and calculation of $DegT_{50}$ and $DegT_{90}$ values was performed following the recommendations of the FOCUS Kinetics workgroup [*FOCUS (2006)*].

The software package KinGUI (version 1.1) was used for parameter fitting [*Kinetic Evaluation with MATLAB: Developed by Bayer Technology Services for Bayer CropScience (2006): User Interface for Kinetic Evaluations; MIKOLASCH, B., SCHÄFER, D. (2006) Bayer CropScience, Kinetic Evaluation with MATLAB: Introduction to the Use of KinGUI, version 1.1*]. Results below the respective LOQ (equivalent to 5% applied dose) were set to 0.5xLOQ or 2.5% applied dose, for the first incubation interval where LOQ was reached. Results < LOQ for the subsequent intervals were not considered for modeling calculations.

A new kinetic evaluation was performed and reported in a separate study [*see CA 7.1.2.1.2/20, BASF DocID 2014/1227795*], considering the newest guidelines and model versions.

II. RESULTS AND DISCUSSION

The two test compounds degraded rapidly in soil. The analytical results of the degradation experiments in the three soils are shown as percentages of the initially applied amount of test compound.

At 22 h after application more than 60% of EU in soil Li 10, about 60% in soil LUFA 2.2 and about 85% in soil Bruch West was degraded (Table 7.1.2.1.2-43). At 52 h in soil Li 10 and at 48 h in soil Bruch West the residues decreased below LOQ. In soil LUFA 2.2 a residual amount of 5.2% was reached after 70 h.

The test compound hydantoin degraded even faster, reaching amounts below LOQ in soil Li 10 after 24 h and in soil LUFA 2.2 as well as soil Bruch West after 20 h (Table 7.1.2.1.2-44).

Table 7.1.2.1.2-43: Degradation of EU in three soils (% applied dose)

Hours	Li 10	LUFA 2.2	Bruch West
0.25	95.8	91.2	97.9
2.0	97.6	92.8	94.5
2.0	97.1	91.6	96.0
6.0	88.4	76.3	85.1
6.0	89.4	75.2	84.1
22	36.9	39.9	22.6
22	35.1	40.2	25.4
26	22.2	34.6	16.6
26	22.0	33.1	16.6
28	15.8	26.6	19.5
28	16.4	28.2	17.8
44	13.6	20.3	9.2
44	13.7	19.3	9.1
48	5.6	11.7	<LOQ
48	5.4	11.6	<LOQ
52	<LOQ	8.0	<LOQ
52	<LOQ	7.8	<LOQ
70	<LOQ	6.9	<LOQ
70	<LOQ	5.2	<LOQ

LOQ (EU) = 5% (0.02 mg kg^{-1})

Table 7.1.2.1.2-44: Degradation of hydantoin in three soils (% applied dose)

Hours	Li 10	LUFA 2.2	Bruch West
0.25	99.8	90.2	88.0
2.0	86.0	70.0	58.5
2.0	85.8	72.3	59.8
6.0	76.5	38.8	25.8
6.0	76.0	39.8	26.5
20	10.4	<LOQ	<LOQ
20	9.9		
24	<LOQ	<LOQ	<LOQ
24			
28	<LOQ	-	-
28			

LOQ (Hydantoin) = 5% (0.04 mg kg^{-1})

The evaluation of the aerobic degradation of both metiram metabolites applying FOCUS kinetics analyses and single first order (SFO) kinetics resulted in DT_{50} values for EU of about 0.5 to 0.7 days and for hydantoin of about 0.1 to 0.3 days (Table 7.1.2.1.2-45).

Table 7.1.2.1.2-45: DT_{50} and DT_{90} values of EU and hydantoin by SFO

	EU		hydantoin	
	DT_{50} [d]	DT_{90} [d]	DT_{50} [d]	DT_{90} [d]
Li 10	0.71	2.37	0.20	0.65
LUFA 2.2	0.56	1.87	0.33	1.08
Bruch West	0.48	1.60	0.13	0.45

III. CONCLUSION

The objective of the study was to examine aerobic degradation of ethylene urea (EU) and hydantoin, both metabolites of metiram, in 3 different soils.
The two test compounds degraded rapidly in soil. At 52 h in soil Li 10 and at 48 h in soil Bruch West the residues of EU decreased below LOQ. In soil LUFA 2.2 a residual amount of 6% was reached after 70 h. Hydantoin degraded even faster, LOQ was reached in all three soils after 24 h. DT_{50} values (SFO) of about 0.5 – 0.7 days for EU and about 0.1 to 0.3 days for hydantoin were calculated.

Report: CA 7.1.2.1.2/20
Budde E., 2015e
Kinetic evaluation of laboratory soil degradation studies with Ethylene urea and Hydantoin according to FOCUS Degradation Kinetics
2014/1227795
Guidelines: FOCUS Degradation Kinetics (2006) SANCO/10058/2005 version 1.1 of December 2014
GLP: no
(certified by <none>)

Executive Summary

The degradation of ethylene urea (EU) and hydantoin (metabolites of metiram) has been investigated in the laboratory in three soils in two studies. The purpose of this evaluation was to analyze the degradation kinetics observed in these studies, taking into account the current guidance of the FOCUS workgroup on degradation kinetics.

Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints) and degradation parameters for environmental fate models (modeling endpoints).

Best-fit $DegT_{50}$ values (trigger endpoints) for EU were between 0.5 and 1.0 days, and $DegT_{90}$ values were between 1.6 and 3.3 days. Normalized modeling endpoints (20°C, pF 2) ranged between 0.4 and 0.7 days.

Best-fit $DegT_{50}$ values (trigger endpoints) for hydantoin were between 0.1 and 0.3 days, and $DegT_{90}$ values were between 0.4 and 1.1 days. Normalized modeling endpoints (20°C, pF 2) ranged between 0.1 and 0.3 days.

I. MATERIAL AND METHODS

The degradation of ethylene urea (EU) and hydantoin (metabolites of metiram) has been investigated in the laboratory in three soils in two studies. One study [*see CA 7.1.2.1.2/18, BASF DocID 2009/1093835*] was performed non-GLP, and the other study [*see CA 7.1.2.1.2/19, BASF DocID 2010/1056131*] was executed under GLP regulations. The resulting data were analyzed taking into account the current guidance of the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*].

Kinetic modeling strategy

Kinetic evaluation was performed considering the procedures and kinetic models proposed by the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*]. According to FOCUS, degradation endpoints were derived for use as triggers for future work and for use as modeling inputs.

The following kinetic models were employed for the evaluation: single first order kinetics (SFO) and the Gustafson-Holden model (FOMC).

Trigger endpoints were derived from the kinetic model that provided the best fit to the measured data, generally indicated by the lowest χ^2 - error. Modeling endpoints were derived preferably from the SFO model. If the SFO model was not appropriate, pragmatic procedures were used to derive conservative pseudo-SFO degradation rates from the appropriate biphasic model.

The appropriateness of the distinct kinetic model to describe soil degradation was tested by visual assessment. Furthermore, a kinetic model was considered appropriate for deriving trigger or modeling endpoints of χ^2 – error value was low (ideally below 15%) and the t-test for the degradation parameters passed at 5% error level.

Data handling

The experimental data were derived from the study reports and adjusted according to FOCUS [*FOUCS (2006)*].

The software package KinGUI version 2.2012.320.1629 [*SCHÄFER et al. (2007); SCHMITT et al. (2011)*] was used for parameter fitting.

Experimental data used for kinetic analysis

In the non-GLP study [*see CA 7.1.2.1.2/18, BASF DocID 2009/1093835*], the test soils were treated with the test compounds at a rate of 0.4 mg per kg dry soil. The degradation experiments were conducted at a soil moisture of 40% of the maximum water holding capacity (MWHC) and a temperature of 20°C.

The test soils used in GLP study [*CA 7.1.2.1.2/19, BASF DocID 2010/1056131*] were treated with the test compounds at a rate of 0.4 mg per kg dry soil for EU and 0.8 mg per kg dry soil for hydantoin. The degradation experiments were conducted at a soil moisture of 45% of MWHC and a temperature of 20±2°C.

The measured data as well as resulting datasets submitted to kinetic analysis are summarized in the Table 7.1.2.1.2-46 to Table 7.1.2.1.2-51.

Table 7.1.2.1.2-46: Data for soil Lufa 2.2 for kinetic evaluation [non GLP study, *BASF DocID 2009/1093835*]

Time [d]	Residue data [% AD]		Input data according to FOCUS [% AD]	
	EU	Hydantoin	EU	Hydantoin
0	90.5	95.9	90.5	95.9
0	87.0	93.9	87.0	93.9
1	27.6	< LOQ	27.6	2.5 [a]
1	28.4	< LOQ	28.4	2.5 [a]
2	< LOQ	5.1	2.5 [a]	5.1
2	< LOQ	5.2	2.5 [a]	5.2
3	< LOQ	< LOQ		2.5 [a]
3	< LOQ	< LOQ		2.5 [a]
7	< LOQ	< LOQ		
7	< LOQ	< LOQ		

AD Applied Dose
[a] Set to ½ LOQ (LOQ = 5% AD)

Table 7.1.2.1.2-47: Data for soil Li 10 for kinetic evaluation [non GLP study, *BASF DocID 2009/1093835*]

Time [d]	Residue data [% AD]		Input data according to FOCUS [% AD]	
	EU	Hydantoin	EU	Hydantoin
0	98.6	96.8	98.6	Dataset not evaluated
0	95.4	100.9	95.4	
1	47.1	< LOQ	47.1	
1	43.6	< LOQ	43.6	
2	5.7	< LOQ	5.7	
2	5.8	< LOQ	5.8	
3	< LOQ	< LOQ	2.5 [a]	
3	< LOQ	< LOQ	2.5 [a]	
7	< LOQ	< LOQ		
7	< LOQ	< LOQ		

AD Applied Dose
[a] Set to ½ LOQ (LOQ = 5% AD)

Table 7.1.2.1.2-48: Data for soil Bruch West for kinetic evaluation [non GLP study, *BASF DocID 2009/1093835*]

Time [d]	Residue data [% AD]		Input data according to FOCUS [% AD]	
	EU	**Hydantoin**	**EU**	**Hydantoin**
0	83.1	92.1	83.1	92.1
0	99.5	89.4	99.5	89.4
1	49.2	5.6	49.2	5.6
1	45.9	5.4	45.9	5.4
2	19.8	6.7	19.8	6.7
2	20.1	6.1	20.1	6.1
3	11.0	5.3	11.0	5.3
3	10.9	5.3	10.9	5.3
7	5.1	6.0	5.1	6.0
7	5.1	5.8	5.1	5.8

AD Applied Dose

Table 7.1.2.1.2-49: Data for soil Lufa 2.2 for kinetic evaluation [GLP study, *BASF DocID 2010/1056131*]

Time [h]	Residue data [% AD]		Input data according to FOCUS [% AD]	
	EU	**Hydantoin**	**EU**	**Hydantoin**
0.25	89.0	91.3	89.0	91.3
0.25	95.3	87.0	95.3	87.0
0.25	93.0	89.0	93.0	89.0
0.25	89.3	90.8	89.3	90.8
0.25	92.0	91.8	92.0	91.8
0.25	89.5	90.0	89.5	90.0
0.25	90.0	91.5	90.0	91.5
2	92.8	70.0	92.8	70.0
2	91.6	72.3	91.6	72.3
6	76.3	38.8	76.3	38.8
6	75.2	39.8	75.2	39.8
22 [b] / 20 [c]	39.9	<LOQ	39.9	2.5 [a]
22 [b] / 20 [c]	40.2	<LOQ	40.2	2.5 [a]
25 [b] / 24 [c]	34.6	<LOQ	34.6	
25 [b] / 24 [c]	33.1	<LOQ	33.1	
28	26.6		26.6	
28	28.2		28.2	
44	20.3		20.3	
44	19.3		19.3	
48	11.7		11.7	
48	11.6		11.6	
52	8.0		8.0	
52	7.8		7.8	
70	6.9		6.9	
70	5.2		5.2	

AD Applied Dose
[a] Set to ½ LOQ (LOQ = 5% AD)
[b] Sampling date for EU
[c] Sampling date for hydantoin

Table 7.1.2.1.2-50: Data for soil Li 10 for kinetic evaluation [GLP study, *BASF DocID 2010/1056131*]

Time [h]	Residue data [% AD]		Input data according to FOCUS [% AD]	
	EU	Hydantoin	EU	Hydantoin
0.25	95.0	101.0	95.0	101.0
0.25	95.8	100.3	95.8	100.3
0.25	94.5	98.8	94.5	98.8
0.25	97.0	99.5	97.0	99.5
0.25	97.8	100.0	97.8	100.0
0.25	97.3	98.8	97.3	98.8
0.25	93.0	100.5	93.0	100.5
2	97.6	86.0	97.6	86.0
2	97.1	85.8	97.1	85.8
6	88.4	76.5	88.4	76.5
6	89.8	76.0	89.8	76.0
22 [b] / 20 [c]	36.9	10.4	36.9	10.4
22 [b] / 20 [c]	35.1	9.9	35.1	9.9
26 [b] / 24 [c]	22.2	<LOQ	22.2	2.5 [a]
26 [b] / 24 [c]	22.0	<LOQ	22.0	2.5 [a]
28	15.8	<LOQ	15.8	
28	16.4	<LOQ	16.4	
44	13.6		13.6	
44	13.7		13.7	
48	5.6		5.6	
48	5.4		5.4	
52	<LOQ		2.5 [a]	
52	<LOQ		2.5 [a]	
70	<LOQ			
70	<LOQ			

AD Applied Dose
[a] Set to ½ LOQ (LOQ = 5% AD)
[b] Sampling date for EU
[c] Sampling date for hydantoin

Table 7.1.2.1.2-51: Data for soil Bruch West for kinetic evaluation [GLP study, *BASF DocID 2010/1056131*]

Time [h]	Residue data [% AD]		Input data according to FOCUS [% AD]	
	EU	Hydantoin	EU	Hydantoin
0.25	100.0	91.0	100.0	91.0
0.25	102.0	87.5	102.0	87.5
0.25	96.0	86.0	96.0	86.0
0.25	95.8	88.5	95.8	88.5
0.25	99.5	88.5	99.5	88.5
0.25	94.8	85.8	94.8	85.8
0.25	97.5	88.5	97.5	88.5
2	94.5	58.5	94.5	58.5
2	96.0	59.8	96.0	59.8
6	85.1	25.8	85.1	25.8
6	84.1	26.5	84.1	26.5
22 [b] / 20 [c]	22.6	<LOQ	22.6	2.5 [a]
22 [b] / 20 [c]	25.4	<LOQ	25.4	2.5 [a]
25 [b] / 24 [c]	16.6	<LOQ	16.6	
25 [b] / 24 [c]	16.6	<LOQ	16.6	
28	19.5		19.5	
28	17.8		17.8	
44	9.3		9.3	
44	9.1		9.1	
48	<LOQ		2.5 [a]	
48	<LOQ		2.5 [a]	
52	<LOQ			
52	<LOQ			
70	<LOQ			
70	<LOQ			

AD Applied Dose
[a] Set to ½ LOQ (LOQ = 5% AD)
[b] Sampling date for EU
[c] Sampling date for hydantoin

Normalization to reference conditions

The normalization was performed using the moisture dependency equations by Walker, with a Walker coefficient of 0.7. The actual soil moisture and the corresponding water content at pF2 for each soil was taken from the study reports. Since the study was performed at 20°C a temperature correction was not necessary.

II. RESULTS AND DISCUSSION

The kinetic evaluation showed that the SFO kinetic model is appropriate for deriving modeling and trigger endpoints for EU and hydantoin for all soils.

Prior to deriving modeling endpoints for EU and hydantoin, the $DegT_{50}$ values suitable for modeling obtained under different incubation conditions were normalized to a reference moisture of pF 2. Parameters included in the normalization procedure and the resulting $DegT_{50}$ values for modeling are summarized in the tables below (Table 7.1.2.1.2-52 and Table 7.1.2.1.2-53).

Table 7.1.2.1.2-52: Normalization of EU $DegT_{50}$ values to reference conditions

DocID	Soil	Soil Type (USDA)	Kinetic Model	θ_{act}	θ_{ref}	f_{moist}	$DegT_{50act}$	$DegT_{50ref}$
2009/1093835 non-GLP study	Lufa 2.2	Loamy sand	SFO	12.4	18.6	0.751	0.6	0.5
	Li 10	Loamy sand	SFO	10.2	10.4	0.984	0.7	0.7
	Bruch West	Sandy loam	SFO	9.4	20.4	0.583	1.0	0.6
2010/1056131 GLP study	Lufa 2.2	Loamy sand	SFO	17.2	18.9	0.936	0.7	0.7
	Li 10	Loamy sand	SFO	12.7	11.0	1.000	0.6	0.6
	Bruch West	Sandy loam	SFO	14.1	16.3	0.903	0.5	0.4

θ_{act} Actual soil moisture (40% of MWHC [2009/1093835], 45% of MWHC [2010/1056131]) [g / 100 g dry soil]
θ_{ref} Reference soil moisture at field capacity (pF 2) according to study reports [g / 100 g dry soil]
f_{moist} Moisture correction factor [-]
$DegT_{50act}$ $DegT_{50}$ at study conditions [d]
$DegT_{50ref}$ $DegT_{50}$ at reference conditions [d]

Table 7.1.2.1.2-53: Normalization of hydantoin $DegT_{50}$ values to reference conditions

DocID	Soil	Soil Type (USDA)	Kinetic Model	θ_{act}	θ_{ref}	f_{moist}	$DegT_{50act}$	$DegT_{50ref}$
2009/1093835 non-GLP study	Lufa 2.2	Loamy sand	SFO	12.4	18.6	0.751	0.2	0.2
	Bruch West	Sandy loam	SFO	9.4	20.4	0.583	0.3	0.2
2010/1056131 GLP study	Lufa 2.2	Loamy sand	SFO	17.2	18.9	0.936	0.2	0.2
	Li 10	Loamy sand	SFO	12.7	11.0	1.000	0.3	0.3
	Bruch West	Sandy loam	SFO	14.1	16.3	0.903	0.1	0.1

θ_{act} Actual soil moisture (40% of MWHC [2009/1093835], 45% of MWHC [2010/1056131]) [g / 100 g dry soil]
θ_{ref} Reference soil moisture at field capacity (pF 2) according to study reports [g / 100 g dry soil]
f_{moist} Moisture correction factor [-]
$DegT_{50act}$ $DegT_{50}$ at study conditions [d]
$DegT_{50ref}$ $DegT_{50}$ at reference conditions [d]

A summary of trigger and modeling endpoints of EU and hydantoin calculated from the six soils is given in Table 7.1.2.1.2-54 and Table 7.1.2.1.2-55. The calculated kinetic endpoints of EU and hydantoin for modeling and for use as triggers are summarized in the following tables. For the evaluated soil degradation experiments the visual assessment and the goodness-of-fit statistics show plausible fit. The low t-test values of the degradation rate constants ($p < 0.01$) indicate that the parameters were estimated significantly different from zero. Therefore, the resulting $DegT_{50}$ values can be considered reliable.

Table 7.1.2.1.2-54: Trigger and modeling endpoints for EU

DocID	Soil	Best-fit model	χ^2 error [%]	Trigger endpoints		Modeling endpoints	
				$DegT_{50}$ [d]	$DegT_{90}$ [d]	$DegT_{50}$ [d]	Normalized $DegT_{50}$ [d] [a]
2009/1093835 non-GLP study	LUFA 2.2	SFO	7.0	0.6	1.8	0.6	0.5
	Li 10	SFO	13.1	0.7	2.4	0.7	0.7
	Bruch West	SFO	5.7	1.0	3.3	1.0	0.6
2010/1056131 GLP study	LUFA 2.2	SFO	5.6	0.7	2.4	0.7	0.7
	Li 10	SFO	12.8	0.6	1.9	0.6	0.6
	Bruch West	SFO	10.5	0.5	1.6	0.5	0.4

[a] Reference conditions: 20°C, pF 2

Table 7.1.2.1.2-55: Trigger and modeling endpoints for hydantoin

DocID	Soil	Best-fit model	χ^2 error [%]	Trigger endpoints		Modeling endpoints	
				$DegT_{50}$ [d]	$DegT_{90}$ [d]	$DegT_{50}$ [d]	Normalized $DegT_{50}$ [d] [a]
2009/1093835 non-GLP study[c]	LUFA 2.2	SFO	8.8	0.2	0.7	0.2	0.2
	Li 10	not evaluated [b]					
	Bruch West	SFO	15.6	0.3	0.9	0.3	0.2
2010/1056131 GLP study	LUFA 2.2	SFO	2.2	0.2	0.7	0.2	0.2
	Li 10	SFO	12.9	0.3	1.1	0.3	0.3
	Bruch West	SFO	1.7	0.1	0.4	0.1	0.1

[a] Reference conditions: 20°C, pF 2
[b] Not enough data points for kinetic evaluation (very fast decline of the test item)
[c] non-GLP study, endpoints not further considered for the risk assessment

III. CONCLUSION

Trigger and modeling endpoints were derived for EU and hydantoin in two laboratory degradation studies with three different soils. The kinetic evaluation showed that the SFO model provided the best fit to the measured data for EU and hydantoin as well as for derivation of modeling endpoints. The degradation times of the metabolites in the respective soils derived from the non-GLP and GLP studies are in a good agreement.

Best-fit $DegT_{50}$ values (trigger endpoints) for EU were between 0.5 and 1.0 days, and $DegT_{90}$ values were between 1.6 and 3.3 days. Normalized modeling endpoints (20°C, pF 2) ranged between 0.4 and 0.7 days.

Best-fit $DegT_{50}$ values (trigger endpoints) for hydantoin were between 0.1 and 0.3 days, and $DegT_{90}$ values were between 0.4 and 1.1 days. Normalized modeling endpoints (20°C, pF 2) ranged between 0.1 and 0.3 days.

Finally, according to current guidance, only the degradation endpoints derived from the GLP study were considered for the risk assessment.

Report:	CA 7.1.2.1.2/21 Heinz N., 2014d TDIT (metabolite of Metiram, BAS 222 F): Study on aerobic soil degradation 2014/1189254
Guidelines:	OECD 307 (2002), BBA IV 4-1, EPA 835.4100, SANCO/3029/99 rev. 4 (11 July 2000)
GLP:	yes (certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

For metabolite TDIT, DT_{50} values in two soils could be calculated from studies performed with the active substance metiram. In order to fulfil current requirements, the DT_{50} of TDIT was determined in one additional soil treated with the metabolite TDIT.

Executive Summary

The objective of the study was to examine the aerobic degradation of TDIT (metabolite of metiram, BAS 222 F) in one soil.

According to the USDA scheme, the soil was characterized as sandy loam (LUFA 5M). The soil was acclimatized with soil moisture adjusted to 40% of its maximum water holding capacity.

The application rate (based on dry soil weight) of TDIT dosed to soil was 0.75 mg kg^{-1}. Assuming a soil depth of 2.5 cm and a soil density of 1.5 g cm^{-3} this corresponds to a theoretical field application rate of about 280 g ha^{-1}.

Incubation flasks with 50 g of dosed dry soil equivalents were loosely covered with plugs of paper tissue to minimize loss of moisture but to allow air exchange and then placed in thermostated cabinet set to 20°C in the dark. Loss of water was controlled by weighing and re-adjusted with distilled water if necessary.

The dosed soil samples were incubated for various intervals up to 14 days prior to extraction.

The analytical method was validated and achieves a limit of quantification (LOQ) of 0.05 mg kg^{-1} and a limit of detection (LOD) of 0.008 mg kg^{-1} for TDIT.

TDIT degraded in the soil from close to 100% of the initially applied residue (% AR) at the beginning to 37.5% AR at the end of the incubation period of 14 days.

The kinetic evaluation revealed that the first-order-multi-compartment (FOMC) kinetic fit approach is suitable to derive trigger endpoints for TDIT. The single-first-order (SFO) kinetic model is appropriate for deriving modeling endpoints. The kinetic models employed for this evaluation were described by FOCUS Kinetics workgroup.

The trigger endpoints for TDIT derived from FOMC modeling resulted in degradation times $DegT_{50}$ of 8.0 days and $DegT_{90}$ of 113.0 days. The modeling endpoint for TDIT derived from SFO modeling resulted in degradation time $DegT_{50}$ of 8.3 days.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Test compound:	TDIT
Chemical name (IUPAC):	2,3,7,8-tetrahydrodiimidazo[2,1-b:1',2'-e][1,3,5]thiadiazine-5-thione
Batch No.:	L83-138
Molar mass:	212.3 g mol^{-1}
Molecular formula:	$C_7H_8N_4S_2$
Purity:	91.4%

2. Soil

One field-fresh soil was used in the study. The soil was kept under aerobic conditions at about 5°C for about 34 days, then water was adjusted to 40% of maximum water holding capacity (mwhc) and the soil was acclimatized 14 days at room temperature in the dark with soil moisture adjusted to 40% of its mwhc. The soil characteristics are summarized in Table 7.1.2.1.2-56.

Table 7.1.2.1.2-56: Characteristics of soil LUFA 5M

Soil designation	**LUFA 5M (14/1651/03)**
DIN Particle size distribution [%] Sand 0.063 – 2 mm Silt 0.002 – 0.063 mm Clay < 0.002 mm Textural class	 54.5 32.1 13.4 loamy sand (Sl4)
USDA Particle size distribution [%] Sand 0.050 – 2 mm Silt 0.002 – 0.050 mm Clay < 0.002 mm Textural class	 60.1 26.5 13.4 sandy loam
Total carbon [%] Total organic carbon [%]	2.07 1.09
pH [H_2O] pH [$CaCl_2$]	7.9 7.0
Cation exchange capacity [$cmol^+ kg^{-1}$]	11.2
Max. water holding capacity [g per 100g dry weight]	26.3
pF 2.0 [$g_{soil\ moisture}\ g_{dry\ soil}^{-1}$] pF 2.5 [$g_{soil\ moisture}\ g_{dry\ soil}^{-1}$]	0.169 0.163
Microbial biomass [mg C per 100g dry soil]	33.6

B. STUDY DESIGN

1. Experimental conditions

The soil was acclimatized with soil moisture adjusted to 40% of its maximum water holding capacity.

The application rate (based on dry soil weight) of the analyte dosed to soil was 0.75 mg kg^{-1}. Assuming a soil depth of 2.5 cm and a density of 1.5 g cm^{-3} this rate correspond to a theoretical field application rate of about 280 g ha^{-1}. This rate was chosen under consideration of both, the percentage of TDIT observed in degradation studies of the active substance and a reasonable good detectability in the present study.
A stock solution of TDIT analytical standard was prepared by accurately weighing and dissolving 8.22 mg in 10 mL of methanol to obtain a concentration of 0.75 mg mL^{-1}. This stock solution was used to dose 50 g soil incubation units and dose level concurrent recoveries.

Incubation flasks with 50 g of dosed dry soil equivalents were loosely covered with plugs of paper tissue to minimize loss of moisture but to allow air exchange and then placed in a thermostated cabinet set to 20 ± 1°C and kept in the dark. Loss of soil water was controlled by weighing and was re-adjusted with distilled water if necessary.

The dosed soil samples were incubated for various intervals up to 14 days (336 hours) prior to extraction. Additionally, untreated moist bulks of soil were kept in the thermostated cabinet.

2. Sampling

Soil samples were removed after 0, 2, 4 hours and 1, 2, 3, 4, 7 and 14 days from the thermostated cabinet for subsequent extraction.

3. Description of analytical procedures

50 g of soil were extracted with 125 mL of 0.1% formic acid and 1% thiourea in methanol/water (1/1, v/v) for 30 min on a horizontal shaker, followed by 10 min sonication. After centrifugation, the supernatant was decanted through a glass wool plug. The extraction was repeated twice and the extracts were combined. After diluting with methanol/water (2/8, v/v), aliquots were analyzed by LC-MS/MS.

The analytical method achieves a limit of quantification (LOQ) of 0.05 mg kg^{-1} and a limit of detection (LOD) of 0.008 mg kg^{-1} for TDIT.

4. Calculation of the degradation rate

Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints) as well as modeling endpoints. Kinetic analysis and calculation of $DegT_{50}$ and $DegT_{90}$ values was performed following the recommendations of the FOCUS Kinetics workgroup [*FOCUS (2006)*].

The software package KinGUI (version 2.2012.320.1629) was used for parameter fitting [*SCHÄFER et al. (2007)*].

Modeling strategy

The kinetic models employed for this evaluation were described by FOCUS [*FOCUS (2006)*].

Trigger endpoints were derived from the kinetic model that provides the best fit to the measured data, generally indicated by the lowest χ^2 - error. Modeling endpoints were derived preferably from the SFO model. If the SFO model is not appropriate, pragmatic procedures were used to derive conservative pseudo-SFO degradation rates from the appropriate bi-phasic model.

The appropriateness of the distinct kinetic model to describe soil degradation was tested by visual assessment. Furthermore, a kinetic model was considered appropriate for deriving trigger or modeling endpoints of χ^2 – error value was low (ideally below 15%) and the t-test for the degradation parameters passed at 5% error level.

The modeling endpoint was normalized to reference moisture conditions at pF 2 and reported in a separate study [*see CA 7.1.2.1.2/22, BASF DocID 2014/1311119*], considering the newest guidelines and model versions.

Experimental data used for kinetic analysis

The experimental data were derived from the study reports and adjusted according to FOCUS [*FOUCS (2006)*].

The data used for kinetic analysis are given in Table 7.1.2.1.2-57.

Table 7.1.2.1.2-57: Model Input Data

DAT (days after treatment)	TDIT [mg kg^{-1}]
0	0.701
0	0.647
0.083	0.742
0.083	0.713
0.167	0.671
0.167	0.677
1	0.626
1	0.624
2	0.534
2	0.566
3	0.544
3	0.518
4	0.447
4	0.415
7	0.356
7	0.347
14	0.282
14	0.280

II. RESULTS AND DISCUSSION

TDIT degraded in the soil from close to 100% of the initially applied residue (% AR) at the beginning (0 – 2 h) to 37.5% AR at the end of the incubation period of 14 days.

Residues of the analyte remaining and extracted after given incubation periods are summarized in Table 7.1.2.1.2-58.

Table 7.1.2.1.2-58: Extractable TDIT residues from soil LUFA 5M

Hours [a]	[mg kg^{-1}]	[% AR] [b]
0	0.701	93
0	0.647	86
2	0.742	99
2	0.713	95
4	0.671	89
4	0.677	90
24	0.626	84
24	0.624	83
48	0.534	71
48	0.566	76
72	0.544	73
72	0.518	69
96	0.447	60
96	0.415	55
168	0.356	47
168	0.347	46
336	0.282	38
336	0.280	37

LOQ < 0.05 mg kg^{-1}, LOD < 0.008 mg kg^{-1}
[a] Hours after application
[b] Percent of the nominally applied residue (0.75 mg kg^{-1})

The calculated degradation parameters and kinetic endpoints of TDIT for use as triggers for additional work and for modeling are summarized in the following table (Table 7.1.2.1.2-59). The visual assessment and the goodness-of-fit statistics indicate plausible fits. The t-test was passed for the respective model parameters. Therefore, the resulting $DegT_{50}$ / DT_{50} values can be considered reliable.

Table 7.1.2.1.2-59: TDIT trigger and modeling enpoints

	Kinetic model	χ^2 error [%]	$DegT_{50}$ [d]	$DegT_{90}$ [d]
Trigger endpoints	FOMC	3.5	8.0	113.0
Modeling endpoints	SFO	5.5	8.3	27.7

III. CONCLUSION

The objective of the study was to examine aerobic degradation of TDIT (metabolite of metiram) in one soil.

The trigger endpoints for TDIT derived from FOMC modeling resulted in degradation times $DegT_{50}$ of 8.0 days and $DegT_{90}$ of 113.0 days. The modeling endpoint for TDIT derived from single-first-order (SFO) modeling resulted in degradation time $DegT_{50}$ of 8.3 days.

Report: CA 7.1.2.1.2/22
Maassen K., 2015d
Normalization of a laboratory degradation half-live of TDIT in soil to reference temperature and moisture conditions
2014/1311119

Guidelines: FOCUS Groundwater (2014) Generic Guidance for Tier 1 FOCUS Ground Water Assessments v 2.2.

GLP: no
(certified by <none>)

Executive Summary

A $DegT_{50}$ value of TDIT derived from a laboratory study on aerobic soil degradation [*see CA 7.1.2.1.2/21, BASF DocID 2014/1189254*] was normalized to reference moisture conditions at pF = 2 following FOCUS (2014) in order to derive a modeling endpoint suitable for the use in environmental fate models.

The normalization was performed using the moisture dependency equations recommended by FOCUS (2014). The actual soil moisture (40% at MWHC) and the reference moisture at field capacity at pF 2 were taken from the study report.

No temperature correction was performed as the study was already conducted at reference conditions (20°C).

The normalized $DegT_{50}$ value ($DegT_{50,ref}$) was 6.0 days.

I. MATERIAL AND METHODS

The modeling endpoint reported in BASF DocID 2014/1189254 [*see CA 7.1.2.1.2/21*] of the metiram metabolite TDIT in one soil was normalized following the recommendations of FOCUS [*FOCUS (2014)*] as soil $DegT_{50}$ values for environmental fate modeling are required at reference conditions (temperature of 20°C and soil moisture at field capacity at pF 2).

A temperature correction of the data was not necessary as the laboratory study was performed at 20°C. The soil moisture normalization was conducted using a Walker coefficient of 0.7. The actual soil moisture and the water content at pF 2 for each soil are shown in Table 7.1.2.1.2-60.

Table 7.1.2.1.2-60: Soil properties needed for the normalization process

Soil	Soil type USDA	T_{act}	T_{ref}	MWHC [g 100g^{-1}]	θ_{act} [% MWHC]	θ_{act} [g 100g^{-1}]	θ_{ref} [g 100g^{-1}]	f_{moist}
LUFA 5M	sandy loam	20	20	26.3	40	10.5	16.9	0.72

T_{act}	actual temperature	[°C]
T_{ref}	reference temperature	[°C]
θ_{act}	actual soil moisture	[g 100 g^{-1} dry soil]
θ_{ref}	reference soil moisture at field capacity (pF 2) as stated in study report	[g 100 g^{-1} dry soil]
f_{moist}	moisture correction factor	[-]
$DT_{50,act}$	DT_{50} at study conditions	[d]
$DT_{50,ref}$	DT_{50} at reference conditions	[d]

II. RESULTS AND DISCUSSION

The actual and the normalized $DegT_{50}$ for TDIT are presented in Table 7.1.2.1.2-61.

Table 7.1.2.1.2-61: Normalization of TDIT $DegT_{50}$ value to reference conditions

Soil	Soil type USDA	Kinetic model	$DegT_{50,act}$ [d]	f_{moist}	$DegT_{50,ref}$ [d]
LUFA 5M	sandy loam	SFO	8.3	0.72	6.0

$DegT_{50,act}$	$DegT_{50}$ at study conditions	[d]
f_{moist}	moisture correction factor	[-]
$DegT_{50,ref}$	$DegT_{50}$ at reference conditions	[d]

III. CONCLUSION

The soil degradation rate at study conditions estimated under aerobic conditions in the laboratory was normalized to reference soil moisture (pF 2) in order to derive a modeling endpoint for environmental fate models.

The normalized $DegT_{50}$ value ($DegT_{50,ref}$) was 6.0 days.

Summary on metabolite occurrence and degradation rates in aerobic soil

Table 7.1.2.1.2-62: Maximum occurrence of metiram metabolites in laboratory aerobic soil studies with metiram

Metabolite	BASF DocID	Soil	Maximum [% TAR]
TDIT	2002/1011913	Cashmere	13.5
	2002/1012954	Li 35b	4.6
	2013/1095967	Am Fischteich	not detected
		Speyer 2.2	not detected
EBIS	2002/1011913	Cashmere	1.7
	2002/1012954	Li 35b	11.4
	2013/1095967	Am Fischteich	25.7
		Speyer 2.2	15.0
ETU	2002/1011913	Cashmere	11.2
	2002/1012954	Li 35b	10.1
	2013/1095967	Am Fischteich	1.0
		Speyer 2.2	0.7
EU	2002/1011913	Cashmere	3.0
	2002/1012954	Li 35b	0.1
	2013/1095967	Am Fischteich	6.9
		Speyer 2.2	3.0

TAR = Total applied radioactivity

Table 7.1.2.1.2-63: Trigger endpoints of metiram metabolites in aerobic soil studies (laboratory, 20-25°C)

Metabolite	Data source BASF DocID	Test item	Soil	Trigger DT_{50}/DT_{90} [d]	Method of calculation
TDIT	2002/1011913 2014/1228382	metiram	Cashmere	0.6 / 2.1	SFO[a]
	2002/1012954 2014/1228382	metiram	Li 35b	0.2 / 0.5	SFO[b]
	2014/1189254	TDIT	LUFA 5M	8.0 / 113.0	FOMC
EBIS	2002/1011913 2014/1228382	metiram	Cashmere	1.7 / 5.7	SFO[a]
	2002/1012954 2014/1228382	metiram	Li 35b	0.1 / 0.2	SFO[b]
	2013/1095967 2014/1311117	metiram	Am Fischteich Speyer 2.2	0.3 / 1.0 0.4 / 1.3	SFO[a] SFO[b]
	2002/1005335 2014/1227797	EBIS	Heerewaarden Wageningen Lelystad	0.1 / 0.3 0.1 / 0.4 0.2 / 0.5	SFO SFO SFO
ETU	2002/1011913 2014/1228382	metiram	Cashmere	0.6 / 1.9	SFO[a]
	2002/1012954 2014/1228382	metiram	Li 35b	2.4 / 8.0	SFO[b]
	2013/1095967 2014/1311117	metiram	Am Fischteich	1.7 / 5.7	SFO[a]
	1995/10772 2014/1227796	ETU	St. Maartensbrug	0.4 / 1.3	SFO
	2001/5001155 2014/1227796	ETU	New York silt loam (40% NMHC) New York silt loam (70% NMHC) New York sand (70% NMHC)	3.1 / 10.2 1.0 / 4.7 1.8 / 6.0	SFO DFOP SFO
	2014/1189253	ETU	LUFA 2.2 LUFA 2.3 Senozan LUFA 5M Li 10 Am Fischteich	0.1 / 0.3 0.1 / 0.3 0.1 / 0.9 0.5 / 1.5 0.1 / 0.5 0.2 / 1.3	SFO SFO DFOP SFO SFO FOMC
EU	2013/1095967 2014/1311117	metiram	Am Fischteich	0.7 / 2.5	SFO[a]
	2000/5001155 2014/1227796	ETU	New York silt loam (40% NMHC) New York silt loam (70% NMHC)	0.1 / 0.3 0.1 / 0.4	SFO SFO
	2009/1093835[c] 2014/1227795[c]	EU	LUFA 2.2 Li 10 Bruch West	0.6 / 1.8 0.7 / 2.4 1.0 / 3.3	SFO SFO SFO
	2010/1056131 2014/1227795	EU	LUFA 2.2 Li 10 Bruch West	0.7 / 2.4 0.6 / 1.9 0.5 / 1.6	SFO SFO SFO
	1999/1014017 2015/1134752	EU	Wageningen humic sand soil Heerewaarden sandy loam soil Lisse low humic content sand soil	6.1 / 20.1 4.5 / 20.4 4.3 / 14.3	SFO DFOP SFO

Metabolite	Data source BASF DocID	Test item	Soil	Trigger DT_{50}/DT_{90} [d]	Method of calculation
Carbimide	2002/1000223 2014/1227922	Carbimide	Heerewaarden Wageningen Lelystad	0.012 / 0.088 0.010 / 0.034 0.007 / 0.031	DFOP SFO DFOP
Hydantoin	2009/1093835 [c] 2014/1227795 [c]	Hydantoin	LUFA 2.2 Bruch West	0.2 / 0.7 0.3 / 0.9	SFO SFO
	2010/1056131 2014/1227795	Hydantoin	LUFA 2.2 Li 10 Bruch West	0.2 / 0.7 0.3 / 1.1 0.1 / 0.4	SFO SFO SFO

[a] DFOP kinetics for parent
[b] FOMC kinetics for parent
[c] non GLP study, endpoints not considered for the risk assessment

Table 7.1.2.1.2-64: Modeling endpoints for metiram metabolites in aerobic soil studies (laboratory, 20-25°C)

Metabolite	Data source BASF DocID	Test item	Soil	DT_{50} at study conditions [d]	Method of calculation	DT_{50} normalized to 20°C / pF2 [d]	Formation fraction
TDIT	2002/1011913 2014/1228382	metiram	cashmere	0.6	SFO[a]	0.4	0.416[c]
	2002/1012954 2014/1228382	metiram	Li 35b	0.2	SFO[b]	0.2	0.187[c]
	2014/1189254 2014/1311119	TDIT	LUFA 5M	8.3	SFO	6.0	--
EBIS	2002/1011913 2014/1228382	metiram	cashmere	1.7	SFO[a]	1.1	0.041[c]
	2002/1012954 2014/1228382	metiram	Li 35b	0.1	SFO[b]	0.1	0.713[c]
	2013/1095967 2014/1311117	metiram	Am Fischteich Speyer 2.2	0.3 0.4	SFO[a] SFO[b]	0.3 0.4	0.952[c] 0.608[c]
	2002/1005335 2014/1227797	EBIS	Heerewaarden Wageningen Lelystad	0.1 0.1 0.2	SFO SFO SFO	0.1 0.1 0.1	-- -- --

Metabolite	Data source BASF DocID	Test item	Soil	DT_{50} at study conditions [d]	Method of calculation	DT_{50} normalized to 20°C / pF2 [d]	Formation fraction
ETU	2002/1011913 2014/1228382	metiram	cashmere	0.6	SFO[a]	0.4	1[d]
	2002/1012954 2014/1228382	metiram	Li 35b	2.4	SFO[b]	2.4	0.806[d]
	2013/1095967 2014/1311117	metiram	Am Fischteich	1.7	SFO[a]	1.7	0.024[e]
	1995/10772 2014/1227796	ETU	St. Maartensbrug	0.4	SFO	0.4	--
	2001/5001155 2014/1227796	ETU	New York silt loam (40% NMHC)	3.1	SFO	2.8	--
			New York silt loam (70% NMHC)	1.1	SFO	1.5	--
			New York sand (70% NMHC)	1.8	SFO	1.8	--
	2014/1189253 2014/1311118	ETU	LUFA 2.2 LUFA 2.3 Senozan LUFA 5M Li 10 Am Fischteich	0.1 0.1 0.1 0.5 0.1 0.3	SFO SFO SFO SFO SFO SFO	0.1 0.1 0.1 0.3 0.1 0.2	-- -- -- -- -- --
EU	2013/1095967 2014/1311117	metiram	Am Fischteich	0.7	SFO[a]	0.7	0.268[e]
	2000/5001155 2014/1227796	ETU	New York silt loam (40% NMHC)	0.1	SFO	0.1	--
	2009/1093835[f] 2014/1227795[f]	EU	LUFA 2.2 Li 10 Bruch West	0.6 0.7 1.0	SFO SFO SFO	0.5 0.7 0.6	-- -- --
	2010/1056131 2014/1227795	EU	LUFA 2.2 Li 10 Bruch West	0.7 0.6 0.5	SFO SFO SFO	0.7 0.6 0.4	-- -- --
	1999/1014017 2015/1134752	EU	Wageningen humic sand soil	6.1	SFO	5.7	--
			Heerewaarden sandy loam soil	4.5	SFO	4.1	--
			Lisse low humic content sand soil	4.3	SFO	2.1	--
Carbimide	2002/1000223 2014/1227922	carbimide	Heerewaarden Wageningen Lelystad	0.015 0.010 0.008	SFO SFO SFO	0.015 0.010 0.007	-- -- --
Hydantoin	2009/1093835[f] 2014/1227795[f]	hydantoin	LUFA 2.2 Bruch West	0.2 0.3	SFO SFO	0.2 0.2	-- --
	2010/1056131 2014/1227795	hydantoin	LUFA 2.2 Li 10 Bruch West	0.2 0.3 0.1	SFO SFO SFO	0.2 0.3 0.1	-- -- --

[a] DFOP kinetics for parent
[b] FOMC kinetics for parent
[c] From metiram
[d] From TDIT
[e] From EBIS
[f] non GLP study, endpoints not considered for the risk assessment

CA 7.1.2.1.3 Anaerobic degradation of the active substance

Studies presented in the first Annex I inclusion process:

The two presented anaerobic soil metabolism studies [*Keller E., Huber R., 1985, BASF DocID 1985/10059; Ruedel H., 1990, BASF DocID 1990/0014*] are considered invalid as discussed in CA 7.1.1.2.

Submission of not yet peer-reviewed study in this AIR3-Dossier:

A new anaerobic soil metabolism study with metiram was performed.

Report:	CA 7.1.2.1.3/1 Voelkel W., 2015c 14C-Metiram - Degradation and metabolism in one soil incubated under anaerobic conditions 2013/1095969
Guidelines:	OECD 307 (2002), EPA 835.4100
GLP:	yes (certified by Swiss Federal Office of Public Health, Berne, Switzerland)

The experimental study including the kinetic evaluation is already summarized in detail in CA 7.1.1.2/1.
The $DegT_{50}$ value according DFOP kinetics is 1.5 days, the $DegT_{90}$ is 77.5 days.

CA 7.1.2.1.4 Anaerobic degradation of metabolites, breakdown and reaction products

No separate studies on the anaerobic degradation of metabolites, breakdown and reaction products were performed. The degradation of metabolites is estimated based on the study with the active substance.

Report:	CA 7.1.2.1.4/1 Voelkel W., 2015c 14C-Metiram - Degradation and metabolism in one soil incubated under anaerobic conditions 2013/1095969
Guidelines:	OECD 307 (2002), EPA 835.4100
GLP:	yes (certified by Swiss Federal Office of Public Health, Berne, Switzerland)

The experimental study including the kinetic evaluation is already summarized in detail in CA 7.1.1.2/1.
The $DegT_{50}$ values of metabolites EBIS, ETU and EU according to SFO kinetics are 1.4, 187.7 and >1000 days. The corresponding $DegT_{90}$ vales are 4.6, 623.5 and >1000 days.

CA 7.1.2.2 Field studies

CA 7.1.2.2.1 Soil dissipation studies

The laboratory rate studies indicate that metiram and its metabolites are degraded rapidly in soil and that there is no risk of persistence in the soil. The DT_{50} values in the laboratory were all far below the trigger value of 60 days.
Consequently, no new data on field soil dissipation have been generated. An existing field soil dissipation study is briefly summarized that investigated the degradation of metiram and the determination the concentrations of the metabolite ETU in soil under field conditions.

Peer reviewed study:

Report:	*CA 7.1.2.2.1/1* *Novak R.A., 1986a* *Polyram soil dissipation: Determination of Polyram and Ethylenethiourea residues* *1986/10204*
Guidelines:	*none*
GLP:	*no*

Materials and Methods:

Polyram WP (about 10 kg ha^{-1}; original data in the study: 10 lb $acre^{-1}$) was applied to bare field plots in Princeton, NJ, and Champaign, IL, USA. The soil textures at the test sites were: Princeton, NJ: Loam (42% sand, 42% silt, 16% clay); Champaign, IL: Silt loam (30.4% sand, 44% silt, 25.6% clay). Soil samples were taken at 0, 7, 14, 30, and 90 DAT at two depths (0 – 15 cm and 15 - 30 cm). Total precipitation over the 90 day period was 427 mm in Princeton and 255 mm in Champaign.
The samples were analyzed for metiram (CS_2 method, limit of determination: 0.1 mg kg^{-1}) and for ETU (GC with flame photometric detector (sulfur mode) after derivatization with 1-bromobutane, limit of determination: 0.01 mg kg^{-1}). Recovery was 96±2 and 86±9%, respectively.

Findings:

The residue data for metiram and ETU are shown in Table 7.1.2.2.1-1.

Table 7.1.2.2.1-1: Field dissipation of metiram and ETU in Princeton, NJ, and Champaign, IL. Data in mg kg^{-1} (wet soil weight).

DAT	Princeton[a]		Champaign[a]	
	metiram	ETU	metiram	ETU
0	1.4	0.13	0.24	0.07
7	0.36	0.07	< 0.1	< 0.01
14[b]	< 0.1	< 0.01	< 0.1	< 0.01

[a] Mean of duplicate samples of the 0 - 15 cm soil layers. In the 15 - 30 cm depth, no residues above the determination limits were detected at any sampling.
[b] Beyond the 14 DAT sampling all residues were below the determination limits.

The results have shown that metiram and ETU were easily degraded at both locations. Due to the fast degradation, a calculation of DT_{50} was not sensible; however, it can be estimated that DT_{50} is far below 7 days for both metiram and ETU.

Conclusion:

Metiram and ETU are very fast degraded in the field as well.

CA 7.1.2.2.2 Soil accumulation studies

Metiram and its metabolites are degraded very fast in soil. No soil accumulation studies are required and no such studies have been performed.

CA 7.1.3 Adsorption and desorption in soil

CA 7.1.3.1 Adsorption and desorption

CA 7.1.3.1.1 Adsorption and desorption of the active substance

Metiram is practically insoluble in water and dissolves only under decomposition. It is rather unstable in water and soil due to hydrolysis and other degradation processes. It can be concluded that there is no risk for leaching of the active substance.
Its characteristics make the active substance metiram unsuitable for conducting standard adsorption/desorption studies. For this reason the leaching behavior of Metiram has in the past been evaluated by laboratory column leaching studies which confirmed that there is no risk of leaching.

For modeling purposes a Koc value of 1000 mL g^{-1} has been assumed as a conservative approach. In order to support this assumption a study has been set up that intended to follow as far as possible the procedures of the adsorption study according to the OECD guideline 106.
There are however limitations given by the characteristics of metiram: The amount of test substance actually adsorbed cannot be distinguished from solid undissolved test substance. Results are therefore considered as indicative and should not be taken as fully reliable sorption data. Because of the indicative character of this study, the sorption values determined were not used for modeling. Instead, the conservative default Koc value of 1000 mL g^{-1} was used further.

Report:	CA 7.1.3.1.1/1 Heinz N., 2015a Determination of adsorption behavior of Metiram (BAS 222 F) in 5 soils 2013/1095966
Guidelines:	OECD 106 (2000)
GLP:	yes (certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

Executive Summary

The adsorption behavior of metiram was investigated on five different soils. The five soils covered a range of pH (in $CaCl_2$) from 5.3 to 7.5, a range of organic carbon content from 0.66% to 1.97%, and five different USDA textural classes: sand, sandy loam, loamy sand, silt loam, and loam.

The test item was determined in the 0.01 M $CaCl_2$ soil water phase and in soil pellet extracts by LC-MS/MS after methylation (using iodomethan) as methyl-ethylenebis(dithiocarbamate) derivative (DiMe-EBDC).

For all adsorption experiments, soil was pre-equilibrated with aqueous 0.01 M $CaCl_2$ solution, then the analyte in 0.01 M $CaCl_2$ suspension was added to the soil/solution mixture resulting in a nominal concentration of 100 ng mL^{-1} in the water phase.
The mixture was agitated for two hours and then the soil and water phase was separated by centrifugation. The aqueous phase was analyzed by LC-MS/MS. Subsequently, the soil pellet was extracted to analyze the test item adsorbed to the soil (direct method).

Soil adsorption tests exhibited acceptable mass balances for the soils LUFA 2.1, LUFA 2.3, and Li 10, but insufficient mass balances for the soils Nierswalde Wildacker (80%) and Poggio Renatico (55%). This indicates that metiram has limited stability in some soil/water systems.
For all soil/water samples, the concentration of metiram in the aqueous phase was extremely low, representing only about 0.3% or less of amount dosed. Thus, nearly complete adsorption of metiram after two hours of equilibration was observed for all five soils.

The K_{OC} values calculated using the direct method were in the range of 392044 to 1801689 mL g^{-1}, thus, indicating extremely high adsorption.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

BAS Code:	BAS 222 F
Common name:	metiram
Reg. No.:	250284
Chemical name:	zinc ammoniate ethylenebis(dithiocarbamate)-poly(ethylenethiuram disulfide)
Molecular weight:	1088.7 g mol^{-1}
Molecular formula:	$(C_4H_9N_3S_4Zn)_3$; $C_4H_6N_2S_4$
Batch No.:	CP052744
Chemical Purity:	93.6%

2. Soils

The study was conducted with five soils originating from different locations in Europe. The soils were air-dried and sieved to ≤ 2mm particle size. The physico-chemical properties of the soils are provided in Table 7.1.3.1.1-1.

Table 7.1.3.1.1-1: Soil characteristics

Soil designation	LUFA 2.1 (14/735/01)	LUFA 2.3 (14/570/04)	Li 10 (14/1680/04)	Nierswalde Wildacker (13/1709/01)	Poggio Renatico (14/1719/01)
Textural class (USDA)	sand	sandy loam	loamy sand	silt loam	loam
USDA Soil texture [%]					
Sand 0.050 – 2 mm	89.8	63.4	82.3	21.4	51.1
Silt 0.002 – 0.050 mm	7.7	28.1	12.2	69.2	31.7
Clay < 0.002 mm	2.5	8.5	5.5	9.4	17.3
Textural class (DIN)	sand	loamy sand	loamy sand	clay silt	sandy loam
DIN Soil texture [%]					
Sand 0.063 – 2 mm	88.9	62.4	81.4	17.7	46.9
Silt 0.002 – 0.063 mm	8.6	29.0	13.2	72.9	35.8
Clay < 0.002 mm	2.5	8.5	5.5	9.4	17.3
Total Organic carbon [%]	0.72	0.66	0.89	1.97	0.82
Total carbon [%]	0.72	0.66	0.89	1.97	2.70
CEC [$cmol^+ kg^{-1}$]	1.7	4.5	3.5	7.6	10.2
pH ($CaCl_2$)	5.6	5.3	6.1	5.8	7.5
pH (H_2O)	6.2	6.3	6.9	6.8	8.3
MWHC [g 100 g^{-1} dry soil]	24.5	21.5	25.1	34.5	29.6
Water content [%]	0.077	0.66	0.35	1.31	1.32

CEC = Cation Exchange capacity
MWHC = Maximum water holding capacity

B. STUDY DESIGN

1. Experimental conditions

Preliminary test and adsorption kinetics
Adsorption was assumed to be high and the analyte was expected to be possibly stable during equilibration times of ≤ 2 hours. The direct (i.e. determination in 0.01 M $CaCl_2$ aqueous phase and in soil by appropriate solvent extraction) and the parallel (i.e. individual soil/solution samples) method procedures were used.

A soil/solution ratio of 1/25 was used, testing 1 g of sterilized soil and 25 mL of sterilized 0.01 M $CaCl_2$ aqueous solution.

Nominally 2.5 µg of the analyte (for dose and adsorption controls) were dosed to each of the samples, corresponding to an initial concentration of 100 ng mL^{-1} in the water phase.

1.0 g of the air-dried and sterilized soils were weighed into glass centrifuge vials and equilibrated with 24 mL of sterilized aqueous 0.01 M $CaCl_2$ solution over night by shaking. Then the analyte dose suspension was added to the soil water to obtain a nominal amount of 2.5 µg (m_0) in the aqueous phases.

The samples were shaken on a horizontal shaker at 20.0 to 22.0°C. After equilibration time intervals of 2 hours, the soil/water systems were analyzed.

All experiments were performed at least in duplicates. Control specimens with only the analyte in 0.01 M $CaCl_2$ solution (no soil) were used as dose and adsorption controls.

Stability of the test item
The stability of metiram in soil/$CaCl_2$ solution equilibrated for two hours was tested for all five soils.

2. Description of analytical procedures

The soil and water phases were separated by centrifugation. Soil water was methylated to obtain the amount in the aqueous phase by LC-MS/MS. Soil pellets were extracted and methylated to obtain the amount adsorbed (direct method) by LC-MS/MS and to examine stability. In both phases, stability was examined by establishing mass balances.

For all five soils, the soil water method was concurrently validated at two fortification levels (1.0 ng mL^{-1} (limit of quantification; LOQ) and 10 ng mL^{-1}) and the soil extraction method was concurrently validated at two fortification levels (0.20 µg g^{-1} (LOQ) and 2.0 µg g^{-1}).

II. RESULTS AND DISCUSSION

A. MASS BALANCE

Soil adsorption tests gave acceptable mass balances between 89 to 104% for the soils LUFA 2.1, LUFA 2.3, and Li 10, but insufficient mass balances for the soils Nierswalde Wildacker (80%) and Poggio Renatico (55%).

B. FINDINGS

The stability of metiram in soil/$CaCl_2$ solution equilibrated for two hours was demonstrated for three soils (LUFA 2.1, LUFA 2.3, and Li 10) by complete mass balance of 89 to 104%. For soil Nierswalde Wildacker, a reduced mass balance of 80% was observed indicating a possibly limited stability. For soil Poggio Renatico, a significantly reduced mass balance of 55% indicated possibly hydrolysis and degradation.
In contrast, recovery of metiram in $CaCl_2$ solution (no soil present) left for two hours in the test vials was only 65% (adsorption controls), indicating a possible adsorption to the vessel surfaces in the absence of soil or degradation.

The analytical methods for the determination of the analyte were concurrently validated, resulting in acceptable recoveries and relative standard deviations for the water method (except for relative standard deviation of 26 and 30% for the lower soil water fortification level of 1 ng mL^{-1}) and for the soil method.

For all soil/water samples the concentration of metiram in the aqueous phase was extremely low, representing only about 0.3% or less of amount dosed. Thus, nearly complete adsorption of metiram after two hours of equilibration was observed for all five soils.

The K_{OC} values calculated using the direct method were in the range of 392044 to 1801689 mL g^{-1}, thus, indicating extremely high adsorption (Table 7.1.3.1.1-2).

Table 7.1.3.1.1-2: Results from the adsorption experiments with metiram

Soil	Soil Type (USDA)	Adsorption [%]	K_d [mL g^{-1}]	K_{OC} [mL g^{-1}]
LUFA 2.1	sand	100	9438	1310767
LUFA 2.3	sandy loam	100	11891	1801689
Li 10	loamy sand	100	10395	1168002
Nierswalde Wildacker	silt loam	100	7723	392044
Poggio Renatico	loam	99	4553	555243

III. CONCLUSION

The adsorption behavior of metiram was determined on five different soils, which covered a range of pH (in $CaCl_2$) from 5.3 to 7.5, a range of organic carbon content from 0.66% to 1.97%, and five different USDA textural classes: sand, sandy loam, loamy sand, silt loam and loam.

Soil adsorption tests gave acceptable mass balances for the soils LUFA 2.1, LUFA 2.3 and Li10, but insufficient mass balances for the soils Nierswalde Wildacker (80%) and Poggio Renatico (55%). This indicates that metiram has limited stability in some soil/water systems.
For all soil/water samples the concentration of metiram in the aqueous phase was extremely low, representing only about 0.3% or less of amount dosed. Thus, nearly complete adsorption of metiram after two hours of equilibration was observed for all five soils.

The K_{OC} values calculated using the direct method were in the range of 392044 to 1801689 mL g^{-1}, thus, indicating extremely high adsorption.

CA 7.1.3.1.2 Adsorption and desorption of metabolites, breakdown and reaction products

Studies presented in the first Annex I inclusion process:
Five studies on sorption of metabolites of metiram have been presented in the first Annex I inclusion process.
In the study of Yeh, S., 1986 [*BASF DocID 1986/5201*] on sorption of ETU, degradation was observed during the study and considerable amounts of metabolites other than ETU were detected. This was however not taken into account. The results are therefore considered not valid. Already during the first Annex I process, the use of the comparably high adsorption values (average Koc: 70 L kg^{-1}) determined in this study for modeling purposes was rejected.
In the study of Cornelese, A., 1995 [*BASF DocID 1995/10772*] the K_d of ETU was estimated from the ^{14}C-breakthrough curve of a column leaching experiment. In the same study an aerobic soil half life of ETU of 3.8 h was determined in the topsoil whereas the travel time of the residues in the soil column was approx. ≥1.3 half lives. Therefore, significant amounts of degradation products including $^{14}CO_2$ may have been detected as part of the total ^{14}C. For that reason these results are considered not suitable to derive a K_d value for ETU. Remark: The soil degradation experiments performed in the same study are considered valid and are reported in CA 7.1.2.1.2/6.
The report of Dressel, J., 2001 [*BASF DocID 2001/1010672*] is no experimental study but a compilation and evaluation of endpoints. This compilation is outdated since many new endpoints have been generated since this time.
A new compilation of current endpoints for EBDC common metabolites has been developed for the calculation of PEC values. This document [*Platz, K. and Hardy, I., 2015, BASF DocID 2015/1093409*] is referred to in CP 9.1 and CP 9.2.

The studies of Richter, T., 2002 [*CA 7.1.3.1.2/3, BASF DocID 2002/1005329* and *CA 7.1.3.1.2/4, BASF DocID 2002/1005327*] are considered still valid.

Submission of not yet peer-reviewed study in this AIR3-Dossier:

New studies on the sorption of metabolites were performed in order to fulfill current data requirements for soil metabolites: One study for each, M222F002 (ETU) [CA 7.1.3.1.2/1, BASF DocID 2014/1111056], M222F003 (EU) [CA 7.1.3.1.2/2, BASF DocID 2003/1016761], M222F007 (TDIT) [CA 7.1.3.1.2/6, BASF DocID 2014/1000103] and M222F008 (Hydantoin) [CA 7.1.3.1.2/5, BASF DocID 2010/1065127].

Since the existing sorption studies for M222F002 (ETU) are considered not valid, a new study was performed.

Report:	CA 7.1.3.1.2/1 Harder U., 2014b Adsorption / Desorption - Study of Ethylenethiourea (ETU, Reg. No. 146099) on two acidic, two neutral and one alkaline soil 2014/1111056
Guidelines:	OECD 106, EPA 835.1220
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Report:	CA 7.1.3.1.2/2 Harder U., 2016 a Report Amendment No. 1: Adsorption / Desorption - Study of Ethylenethiourea (ETU, Reg. No. 146099) on two acidic, two neutral and one alkaline soil 2016/1322724
Guidelines:	OECD 106, EPA 835.1220
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

The adsorption behavior of radiolabeled ETU (metabolite of metiram) was investigated on five different European soils. The five soils covered a range of pH (in $CaCl_2$) from 5.6 to 7.4, and five different USDA textural classes: sand, silt loam, loamy sand, sandy loam and loam.

For the determination of the adsorption isotherms, five different concentrations (nominal 50.0, 10.0, 5.0, 1.0, and 0.5 µg mL^{-1}) of the test item in 0.01 M $CaCl_2$ solutions were used.

The soil to solution ratio was approx. 1:1 and the measurements were performed at the adsorption equilibrium times of 5 h (with Li 10 soil, Nierswalde Wildacker soil and LUFA 2.1 soil) and 2 h (with LUFA 2.3 soil and Fiorentino Poggio Renatico 1 soil).

Degradation products were observed in almost all samples, especially at low concentration levels in LUFA 2.3 and Fiorentino Poggio Renatico 1 soil extracts. In all cases, the radiobalance from liquid phase, soil extract and soil residue at the individual concentration level was above 90% of the initially applied amount of radioactivity.

The following adsorption parameters were derived for the test item in each of the tested soils: The Freundlich adsorption coefficient K_F, the Freundlich exponent 1/n and the corresponding K_{FOC} values. The Freundlich adsorption coefficient K_F covered a range from 0.0275 to 0.0670 mL g^{-1}. The K_{FOC} values ranged from 3.41 to 4.58 mL g^{-1} and the 1/n values ranged from 0.632 to 1.041.

Summary of adsorption isotherm tests of ETU on five soils

Soil	Soil Type (USDA)	K_F [mL g^{-1}]	1/n	K_{FOC} [mL g^{-1}]
LUFA 2.1	sand	0.027	0.743	4.6
Nierswalde Wildacker	silt loam	0.067	0.884	3.6
Li 10	loamy sand	0.037	0.632	3.8
LUFA 2.3	sandy loam	0.037	0.817	3.8
Fiorentino Poggio Renatico 1	loam	0.034	1.041	3.4

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Common name: Ethylene thiourea (ETU)
Reg. No.: 146099
Chemical name: Imidazolidine-2-thione
Molecular weight: 102.2 g mol^{-1}
Molecular formula: $C_3H_6N_2S$

<u>Labeled compound:</u>
Label: Ethylene-1,2-^{14}C
Batch No.: 807-1302
Specific activity of A.S.: 9.88 MBq mg^{-1}
Radiochemical purity: 97.2%
Chemical purity: 99.3%

<u>Non-Labeled compound:</u>
Batch No.: 01815-177
Chemical purity: 99.9% (± 1.0%)

2. Soils

The study was conducted with five different soils (0-20 cm depth) originating from different locations in Europe. The soils were sieved to a particle size < 2 mm and were air dried at room temperature. The physico-chemical properties of the soils are provided in Table 7.1.3.1.2-1.

Table 7.1.3.1.2-1: Characteristics of soils used in adsorption study with ETU

Soil designation Origin	LUFA 2.1 (11/735/01)	Nierswalde Wildacker (10/1709/01)	Li 10 (11/1680/02)	LUFA 2.3 (11/570/02)	Fiorentino Poggio Renatico 1 (10/1719/01)
Textural class (USDA)	sand	silt loam	loamy sand	sandy loam	loam
USDA Soil texture [%] Sand 0.050 – 2 mm Silt 0.002 – 0.050 mm Clay < 0.002 mm	90.8 6.9 2.3	17.7 73.5 8.8	83.5 12.2 4.3	68.6 23.1 8.3	49.4 33.9 16.7
Textural class (DIN)	sand	clay silt	silty sand	loamy sand	loamy sand
DIN Soil texture [%] Sand 0.063 – 2 mm Silt 0.002 – 0.063 mm Clay < 0.002 mm	89.5 8.2 2.3	15.1 76.2 8.8	81.7 13.9 4.3	66.9 24.8 8.3	41.7 41.6 16.7
Total organic carbon [%]	0.60	1.85	0.95	0.99	1.00
CEC [$cmol^+ kg^{-1}$]	-0.7[a)]	3.1	5.5	7.5	11.8
pH ($CaCl_2$) pH (water)	5.6 6.5	5.7 6.5	6.2 6.9	6.7 7.4	7.4 8.2
MWHC [g 100 g^{-1} dry soil]	23.1	36.1	23.2	28.2	29.7
Bulk density [g L^{-1}]	1381	1236	1384	1226	1403

a) Should be regarded as 0 since cation exchange capacity cannot be below 0

B. STUDY DESIGN

1. Experimental conditions

Prior to the tests, the soils were sterilized at 120°C for 30 min in an autoclave in order to reduce very fast soil degradation velocity to ensure a reasonable execution of the sorption experiment. The experiments were performed in 5 mL Luer lock syringes equipped with a 20 µm disposable frit and closed at one end with a cock and at the other end with a piston. With this set-up the liquid phase was separated from the solid phase by centrifugation-driven filtration. This resulted in a considerably lower amount of residual soil water compared to normal centrifugation which allowed for a more precise determination of the actual amount adsorbed to soil.

Tier 1: Preliminary tests

Soil to solution ratio:
Since a previous study indicated that the test item has a very weak tendency to adsorb to soil, a soil/solution ratio of 1:1 was chosen without further pretests and applied to all five soils.

Stability of the test item and adsorption on the vessel surface:
A test for adsorption of ETU to the surfaces of the test vessel and for stability of the test item in 0.01M $CaCl_2$ solution was performed at a nominal test item concentration of 50.0 µg mL^{-1}. The test syringes filled with 2 mL of the $CaCl_2$ solution were shaken on a mechanical shaker for 5 h.

Adsorption equilibrium test:
To ensure that equilibrium conditions have been met, an experiment with a series of 5 samples (10 samples soil LUFA 2.3) of each of the sterile soils was performed at a test item concentration of 5.0 µg mL^{-1}. The experiments were run at a soil / solution ratio of approx. 1:1 (2 g of sterilized soil + 2 mL of 0.01M $CaCl_2$) with one replicate per sampling and soil except for LUFA 2.3 soil, where two replicates per sampling were prepared.

The test syringes were shaken on a mechanical shaker for 0, 0.5, 2, 5 and 14 h.

Blank runs of soil suspensions without test item
Blank runs of the soil suspensions were carried out to check for background radioactivity. The syringes were filled with 2 g of sterilized soil and 2 mL 0.01M $CaCl_2$ solution each and shaken on a mechanical shaker for 5 h.

In all preliminary tests the test vessels were covered with towels to protect the test item from the influence of light.

Tier 2: Screening test

The screening test was not performed, as it was essentially covered by the adsorption isotherm determination. The K_d and K_{OC} values were calculated for each concentration level.

Tier 3: Freundlich adsorption isotherm determination

The adsorption isotherm determination was performed with five concentration levels (nominal concentrations: 0.5, 1.0, 5.0, 10.0 and 50.0 µg mL^{-1}) and the five soils. Each single adsorption experiment was done in duplicates. The soil to solution ratio was approx. 1:1.

After adding 2 mL of the $CaCl_2$ solutions with different concentrations of test item (nominal concentrations: 0.5, 1.0, 5.0, 10.0 and 50.0 µg mL^{-1}), the syringes were closed and shaken for 5 h (Li 10, Nierswalde Wildacker and LUFA 2.1) and 2 h (LUFA 2.3 and Fiorentino Poggio Renatico 1). Because of the very fast ETU degradation behaviour the shaking time has kept as short as possible and as long as necessary for the different soil experiments. To protect the test item from the influence of light, the vessels were covered with towels.

Due to the very low adsorption of the test item and the necessity to extract the soils after adsorption, no desorption steps were performed.

2. Description of analytical procedures

Stability of the test item and adsorption on the vessel surface
After shaking an aliquot of the $CaCl_2$ solution was radioassayed and analyzed by HPLC to determine the concentration of the test item and potential degradation products in the $CaCl_2$ solution.

Blank runs of soil suspensions without test item
After shaking the soil suspension was centrifuged in the syringe through the frit and the filtrate (in the report erroneously described as supernatant) was isolated for analysis. The volume of the filtrate was determined by weighing, and an aliquot was radioassayed.

Adsorption equilibrium test and Freundlich adsorption isotherm determination
After shaking, the soil / solution suspension was centrifuged in the syringe through the frit and the filtrate was isolated for analysis. The volume of the filtrate was determined by weighing and an aliquot was radioassayed to measure the concentration of the test item in the $CaCl_2$ solution. The total liquid phase which has to be considered comprised the filtrate and the portion of the solution retained in the soil. The latter part was determined by weighing the soil and taking its dry mass into account for further calculations. The mass of the test item in the total liquid phase was calculated from the measured concentration and the weight of the complete liquid phase.

To ascertain the proportion of adsorbed test item, the soils were extracted after the adsorption step. A solvent mixture of 2 mL acetonitrile/water (4/1, v/v) adjusted to pH 7.5 with 1N NaOH was used. The extraction of the test item was performed twice by shaking the soils with the solvent mixture on a mechanical shaker for 30 min. The extracts were separated from the soil by centrifugation through the frit, weighed and combined. Aliquots were withdrawn for LSC analysis.

To check for soluble degradation products, the liquid phases after adsorption and the soil extracts of each concentration level were checked by radio-HPLC after a concentration procedure. To increase precision (for Freundlich adsorption isotherm determination), the same sample was injected up to 3 times and the results averaged. Significant degradation of the test item could be detected.

The amounts of radioactivity in the liquid phase and in the soil extracts after the adsorption step were added up in order to provide a mass balance. In those cases where the total recovery was less than 90%, the soil residue after extraction was combusted and the resulting radioactivity added to that from the liquid phase and the soil extract.

II. RESULTS AND DISCUSSION

A. MASS BALANCE

In all five soils, the total radioactivity from the liquid phases after adsorption, extraction and combustion was generally above 90% (90.2 to 101.9%).

There was an increase of the non-extractable portion of radioactivity with decreasing concentration levels in each soil, which could be an indicator that to some extent the test item underwent transformation processes during the test period.

B. FINDINGS

A soil to solution ratio of 1:1 was chosen, since a previous study indicated that the test item has a very weak tendency to adsorb to soil.

Despite of the soil sterilization, degradation products of the test item in significant amounts were observed in almost all soil samples, especially at low concentration levels in LUFA 2.3 and Fiorentino Poggio Renatico 1 soil extracts. Therefore, the equilibration times for the Freundlich adsorption isotherm determination were chosen in a way that adsorption of the test item was maximum with minimum degradation during the experiment. The measurements were performed at the adsorption equilibrium times of 5 h (with Li 10 soil, Nierswalde Wildacker soil and LUFA 2.1 soil) and 2 h (with LUFA 2.3 soil and Fiorentino Poggio Renatico 1 soil).
The test item was stable in 0.01M $CaCl_2$ solution at a concentration of 50.0 µg mL^{-1} (without soil) over a period of at least 5 h when analyzed by LSC and radio-HPLC. No appreciable adsorption to the surface of the syringe was observed.

The counting rate of blank runs without test item was for all soils within the normal range of background radioactivity and far below the lowest concentration level.

The Freundlich adsorption coefficient K_F covered a range from 0.0275 to 0.0670 mL g^{-1}. The K_{FOC} values ranged from 3.4 to 4.6 mL g^{-1} and the 1/n values ranged from 0.632 to 1.041. Adsorption of ETU to soil was low and did not differ significantly between the individual soils as indicated by low coefficient of variations of 11.5% and 18.7% for the average K_{FOC} value and the freundlich exponent 1/n, respectively.

Freundlich adsorption coefficient K_F, the Freundlich exponent 1/n, and the corresponding K_{FOC} values, are presented in Table 7.1.3.1.2-2.

Table 7.1.3.1.2-2: Results from the adsorption experiments with ETU

Soil	Soil Type (USDA)	K_F [mL g^{-1}]	1/n	K_{FOC} [mL g^{-1}]
LUFA 2.1	sand	0.027	0.743	4.6
Nierswalde Wildacker	silt loam	0.067	0.884	3.6
Li 10	loamy sand	0.037	0.632	3.8
LUFA 2.3	sandy loam	0.037	0.817	3.8
Fiorentino Poggio Renatico 1	loam	0.034	1.041	3.4
Arithmetic mean			0.82	3.9
CV [%]			11.5	18.7

Reliability of the regression method and the estimated parameters (log K_F, 1/n):
Because of the refined study design and the weak and non-linear sorption behavior of ETU different statistical indices were given in the study report in order to investigate the reliability and appropriatness of the the model and the estimated parameters.

The Freundlich equation (adsorption isotherm) describes the sorbed compound on soil as a function of the concentration in the liquid phase. The high determination coefficients (R^2 from 0.93 – 0.98) indicate low scattering between the regression lines and the observations. Hence, the Freundlich isotherm was considered to be an appropriate regression model for the concentration series of the different data sets.

The reliability of the estimated parameters (adsorption constant log K_F and freundlich exponent 1/n) was confirmed by very low standard errors. The standard error of the log K_F ranged from 0.046 to 0.110, the standard error of the 1/n ranged from 0.049 to 0.092 (Table 7.1.3.1.2-3).

Table 7.1.3.1.2-3: Estimated parameters and statistical indices

Soil	Parameter	Value	Standard error	R^2
LUFA 2.1	log K_F [mL g^{-1}]	-1.561	0.069	0.926
	1/n	0.743	0.074	
Nierswalde Wildacker	log K_F [mL g^{-1}]	-1.174	0.064	0.953
	1/n	0.884	0.069	
Li 10	log K_F [mL g^{-1}]	-1.437	0.046	0.954
	1/n	0.632	0.049	
LUFA 2.3	log K_F [mL g^{-1}]	-1.426	0.110	0.963
	1/n	0.817	0.092	
Fiorentino Poggio Renatico 1	log K_F [mL g^{-1}]	-1.467	0.077	0.983
	1/n	1.041	0.068	

Data amended in Report Amendment No. 1:
- HPLC area percentages of the aqueous phases and soil extracts derived from unreported raw data. The values reported as % ROI (ROI = region of interest) are representing the test item specific quantification of both phases analysed by radio-HPLC. The individual values are required to assess the overall test item specific mass balance.

- Mass of dry soil and residual water after centrifugation derived from unreported raw data: The dry mass and amount of residual water entrained in the soil pellet after centrifugation is required to determine the p-value.

III. CONCLUSION

The adsorption behavior of ETU was determined. Five European soils were used, which covered a range of pH (in $CaCl_2$) from 5.6 to 7.4, a range of total organic carbon content from 0.60% to 1.85% and five different USDA textural classes: sand, silt loam, loamy sand, sandy loam and loam.

ETU shows only a very weak adsorption to soil with K_{FOC} values in a narrow range of 3.4 to 4.6 mL g^{-1}. The Freundlich exponents range from 0.632 to 1.041 indicating non-linearity of the adsorption with the concentration in the liquid phase in most soils.

Upon discussion with the RMS and as requested by the RMS, the following evaluation was additionally taken up as well a Report Addendum to the ETU sorption study BASF DocID 2014/1111056, CA 7.1.3.2.1/1, on which this evaluation partly relied.

Report:	CA 7.1.3.1.2/2 Platz K., 2016 a Evaluation of the ETU sorption study of Harder (2014) under consideration of a draft evaluator checklist for soil sorption experiments developed by MS experts 2016/1297349
Guidelines:	none
GLP:	no

Executive Summary

The sorption study for metabolite ETU (Harder (2014b), BASF DocID 2014/1111056) has been subjected to a quality check following the draft evaluator checklist for soil adsorption studies which was circulated to EU Fate and Behaviour experts by the EFSA PRAS Secretariat on 29/10/2015. Where the quality check steps have been followed and the graphical fits to the Freundlich equations are found to be acceptable, atypical results are proposed be accepted for regulatory purposes. However where either some of the quality check steps fail and/or the graphical fitting to the data is poor, the data may need to be excluded from the regulatory database. Care was taken to ensure that excluding individual data points does not lead to biasing the selection in favour of soils where sorption was higher. It is suggested that the reasons behind including or excluding data values shall be clearly reported in the EU evaluation documentation. Applying the above principles only few data points were identified as outliers which should not be considered for the evaluation. Applying linear and non-linear fit approaches to all sorption experiments revealed that mean values of K_F and 1/n derived from the best-fit and the linear fit approaches are nearly identical, which confirmed the endpoints used for the risk assessment.

I. MATERIAL AND METHODS

The evaluation of the ETU sorption study of Harder (2014b) “Adsorption / Desorption - Study of Ethylenethiourea (ETU, Reg. No. 146099) on two acidic, two neutral and one alkaline soil” has been conducted under consideration of a draft evaluator checklist for soil sorption experiments developed by MS experts. The checklist is still under development. It was circulated by the EFSA PRAS Secretariat on 29/10/2015.

As the evaluation report of Platz (2016) considered experimental study raw data that were not provided in the report of Harder (2014b) the experimental study report was amended accordingly (CA 7.1.3.1.2/2, BASF DocID 2016/1322724).

II. RESULTS AND DISCUSSION

Selection of outliers:
ETU is a highly degrading and low sorbing compound; hence, as recommended the direct method was applied in the sorption experiments. At the lower concentration levels, in the sorption experiments LUFA 2.3 and Fiorentino Poggio Renatico 1, ETU was degraded to a large extent. The ETU degradation led to unrealistic low or even negative K_d values. Hence, these values were treated as outliers and not considered for the evaluation. Note that the outliers were already excluded in the original study report, i.e. the K_F values reported in the study of Harder (2014b) for soils LUFA 2.3 and Fiorentino Poggio Renatico 1 do not change.

Reliability of the analytical method:
The analytical methods were appropriate to ensure reliable analytical results over the whole concentration range.

Derivation of appropriate P values:
With the exception of soil experiment Fiorentino Poggio Renatico 1, all tested soils include K_d values provided with P values > 0.3. These experiments confirm significant sorption of ETU. As the K_d values correlate with the P values, excluding individual sorption experiments (out of a series of concentrations for the determination of the Freundlich isotherm) with low P values from evaluation would not be a conservative measure. It would lead to an elimination of low sorption values by trend. Therefore, this was considered not a suitable option to derive reliable sorption parameters. In a practical approach, the overall P values derived from the average soil/liquid ratio and the K_F value are proposed as appropriate indicator values. The overall P value of the soil sorption experiments LUFA 2.1, Nierswalde Wildacker, Li10 and LUFA 2.3 are > 0.3.

Only the soil experiment Fiorentino Poggio Renatico 1 undercut the P value of 0.3 (overall P-value 0.188). But reliable fits are also confirmed for experiment Fiorentino Poggio Renatico 1 by low standard errors of the estimated sorption parameters (Harder (2014b), chapter 3.2.1) and the good visual fits. Furthermore the estimated sorption parameters are in the same range as the results from the sorption experiments conducted in the other soils.

Linear versus non-linear fit approaches:
Linear and non-linear fit approaches were conducted for all soil sorption experiments. Best-fit approaches were selected under consideration of the r^2 values and the visual fit as shown in the tables below.

Table 7.1.3.1.2-4: Results of linear and non-linear fit

Soil experiment	fit	K_F [ml/g]	1/n	r^2	Visual fit (normal form)
LUFA 2.1 (11/735/01), sand	**linear**	**0.027**	**0.743**	**0.926**	**Good**
	non-linear	0.012	1.048	0.855	Good
Nierswalde Wildacker (10/1709/01), silt loam	linear	0.067	0.884	0.953	Good
	non-linear	**0.078**	**0.844**	**0.994**	**Good**
Li10 (11/1680/02), loamy sand	**linear**	**0.037**	**0.632**	**0.954**	**Acceptable**
	non-linear	0.053	0.485	0.951	Good
LUFA 2.3 (11/570/02), sandy loam	linear	0.037	0.817	0.963	Good
	non-linear	**0.033**	**0.859**	**0.973**	**Good**
Fiorentino Poggio Renatico 1 (10/1719/01), loam	**linear**	**0.034**	**1.042**	**0.983**	**Good**
	non-linear	0.036	1.030	0.981	Good

Table 7.1.3.1.2-5: Mean values of K_F and 1/n derived from the best-fit and the linear fit approaches

Best-fit	Geometric mean K_F [ml/g]	0.0388
	Arithmetic mean 1/n [-]	0.8239
Linear fit (considered for PEC calculation)	Geometric mean K_F [ml/g]	0.0386
	Arithmetic mean 1/n [-]	0.8237

The mean values of K_F and 1/n derived from the best-fit and the linear fit approaches are nearly identical (4 decimal places are given to show the difference).

Hence, the sorption parameter K_F and 1/n used for the risk assessment are confirmed considering the linear and non-linear fit approaches.

Non-linear fit of Li10 soil:
The Freundlich exponent of 0.632 estimated in the linear fit of Li10 soil was below the boundary value of 0.7. The statistical indices, the overall P value and the visual fit of the linear fit show reliable results. The non-linear fit delivers an even lower 1/n value of 0.485. Hence the sorption parameters derived from linear fit were considered acceptable for the risk assessment.

III. CONCLUSION

Under consideration of the draft evaluator checklist for soil sorption experiments developed by MS experts, reliable estimations of ETU sorption values derived from Harder (2014b) could be confirmed.

Report: CA 7.1.3.1.2/4
Cooke J., 2003a
(14C)-Ethylene urea, a metabolite of Mancozeb: Adsorption/desorption in soil
2003/1016761
Guidelines: EEC 95/36, OECD 106
GLP: yes

Executive Summary

The adsorption and desorption behavior of ethylene urea (EU) was investigated in four British soils. The soils covered a range of pH ($CaCl_2$) from 4.2 to 7.6, a range of organic carbon content from 0.8% to 4.6% and three different USDA textural classes: two clay loams, one loam and one loamy sand. The soils were sterilized by gamma irradiation in order to avoid microbial degradation of the test item during the adsorption experiments.

For the determination of the adsorption isotherm, five different concentrations (nominal 5.0, 2.5, 0.5, 0.25 and 0.05 µg mL^{-1}) of the test item in 0.01 M $CaCl_2$ (mix of ^{14}C labeled and non-labeled EU) were used. The ratio of soil versus test solution was 1:1, and the measurements were performed at the adsorption equilibrium time of 24 h. Samples were then centrifuged to separate the soil and solution.

The supernatant removed from each sample after the adsorption phase was replaced with an equivalent portion of fresh 0.01M $CaCl_2$. The tubes were mixed continuously for a further 24 h for the desorption phase. Samples were centrifuged to separate the soil and solution. The radioactivity in the supernatant was determined by assaying duplicate aliquots by liquid scintillation counting (LSC). Soil residues were air-dried prior to quantification by combustion. The mean recovery of applied radioactivity for each soil type was in the range 97% to 102%.

Freundlich adsorption coefficients (K_F) of 0.15 to 0.22 mL g^{-1} were determined. Corresponding K_{FOC} values were between 4 and 19 ml g^{-1} indicating that EU is adsorbed rather weak to soil. Freundlich exponents 1/n ranged from 0.9152 to 1.0464.

Desorption coefficients were in a similar range as the adsorption coefficients with K_F of 0.20 to 0.42, K_{FOC} between 5 and 36 mL g^{-1} and 1/n ranged from 0.9622 to 1.0105.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Common name:	Ethylene urea (EU)
Chemical name:	2-Imidazolidone
Molecular weight:	86.1 g mol^{-1}
Molecular formula:	$C_3H_6N_2O$

Labeled compound:

Label:	^{14}C-ethylene
Batch No.:	INV-1891
Specific radioactivity:	0.473 MBq mg^{-1}
Radiochemical purity:	99.5%

Non-Labeled compound:

Batch No.:	06403AO
Purity:	100%

2. Soils

The study was conducted with four different soils originating from locations in Great Britain. The soils were sieved to a particle size 2 mm and were air dried and stored in the dark at room temperature. Soils used were sterilized by gamma irradiation. The physico-chemical properties of the soils are provided in Table 7.1.3.1.2-6.

Table 7.1.3.1.2-6: Characteristics of soils used in adsorption and desorption study with EU

Soil designation Origin	SK 961089 Empingham, Rutland	SK 179618 Middleton, Derbyshire	SK 566696 Warsop, Nottinghamshire	SK920191 South Witham, Lincolnshire
Textural class (USDA)	clay loam	loam	loamy sand	clay loam
USDA Soil texture [%]				
Sand 0.050 – 2 mm	38	34	85	38
Silt 0.002 – 0.050 mm	28	46	4	26
Clay < 0.002 mm	34	20	11	36
Textural class (UK scheme)	clay loam	clay loam	loamy sand	clay
UK & BBA Soil texture [%]				
Sand 0.063 – 2 mm	37	33	84	37
Silt 0.002 – 0.063 mm	29	47	5	27
Clay < 0.002 mm	34	20	11	36
Organic carbon [%]	4.6	3.8	0.8	2.1
Organic matter [%]	7.9	6.6	1.4	3.6
Cation Exchange Capacity [mEq 100 g^{-1}]	38.2	24.9	13.4	23.0
pH (0.01M $CaCl_2$)	7.6	5.6	4.2	7.3
pH (1M KCl)	7.6	5.8	4.2	7.4
pH (water)	8.0	6.0	5.1	8.0
Water Holding Capacity				
pF 0 (0.001 bar) [%]	65.4	75.9	29.0	78.6
pF2.5 (0.33 bar) [%]	29.4	27.3	7.1	34.4

B. STUDY DESIGN

1. Experimental conditions

Tier 1: Preliminary tests

Solubility:
Solubility was assessed by preparation of duplicate solutions of 5 µg mL^{-1} EU in 25 mL of 0.01M $CaCl_2$. The solutions were measured by LSC after centrifugation in order to determine whether complete solubilization had occurred.

Adsorption on test vessel:
The potential for adsorption to containers was assessed by preparation of duplicate solutions of 0.05 µg mL^{-1} EU in 25 mL of 0.01M $CaCl_2$. Polypropylene and Teflon centrifuge tubes were tested. No soil was added to the tubes. The solutions were measured by LSC before and after mixing for 24 h.

Soil to solution ratio:
To determine the appropriate soil to solution ratio, several soil to solution ratios were tested (1:1, 1:5 and 1:25) with each soil type. Duplicate samples of 5 µg mL^{-1} EU in 0.01M $CaCl_2$ were prepared and mixed for 24 h.

Adsorption equilibrium test:
The adsorption equilibrium time for each soil type was determined by preparation of duplicate samples of 5 µg mL^{-1} EU in 0.01M $CaCl_2$ with 10 g of soil (dry weight equivalents).
Samples were centrifuged after 3, 6, 24 and 48 h mixing and the amount of test item in the supernatants was quantified by taking duplicate aliquots for LSC. After each sampling interval, the volume of supernatant removed for LSC was replaced with fresh 0.01M $CaCl_2$.

Desorption equilibrium test:
After mixing for the adsorption equilibrium time of 24 h, samples were centrifuged and the supernatants removed. The supernatants were replaced by equal weights of fresh 0.01M $CaCl_2$. Samples were centrifuged after 3, 24 and 48 h mixing and the amount of test item in the supernatants was quantified by taking duplicate aliquots for LSC. After each sampling interval, the volume of supernatant removed for LSC was replaced with fresh 0.01M $CaCl_2$.

Stability test:
The stability of EU in the test system for each soil type was assessed by preparation of duplicate samples of 5 µg mL^{-1} EU in 0.01M $CaCl_2$ with 10 g of soil.
After mixing for 48 h, the samples were centrifuged. Extra 0.01 M $CaCl_2$ was used to support the recovery of test article from the soil and this was added to the supernatants. The supernatants were quantified by LSC prior to analysis by HPLC.

Tier 3: Sorption isotherm determination

Tests solutions of EU in 0.01M autoclaved aqueous $CaCl_2$ were prepared with nominal concentrations of 0.05, 0.25, 0.5, 2.5 and 5.0 µg mL^{-1}. Tests were initiated by addition of an appropriate amount of a ^{14}C-EU dose formulation.
The appropriate soil to solution ratio and the time for reaching equilibrium conditions were selected from preliminary tests and were applied to all further experiments. Aliquots of 10 mL of the test solution were shaken with 10 g of test soil (dry weight equivalents) for 24 h at 20 ± 2°C in the dark.

Adsorption isotherms
For each soil type, duplicate samples at each concentration were prepared. After mixing for the adsorption equilibrium time of 24 h, each sample was centrifuged and the supernatants quantified by LSC. As much of the adsorption supernatant as possible was then removed.

Desorption isotherms
After replacing the removed volume of adsorption supernatant with fresh 0.01M $CaCl_2$, the samples were shaken and then re-mixed for the desorption equilibrium time of 24 h. Subsequently, each sample was centrifuged and the amount of test item in the supernatants quantified by LSC.
After removal of as much of the desorption supernatant, the soils were air-dried and the amount of radioactivity in the soil quantified by LSC following combustion.

2. Description of analytical procedures

Radioactivity in the aqueous supernatants was determined by LSC. Radio-HPLC was used to show the purity and stability of the test item during the study.

Stability test
Besides investigation of aqueous supernatants soils were extracted by shaking with the following solvents: 3 x acetonitrile / water (1/1, v/v), 3 x acetonitrile, 2 x methanol and 1 x water. In each instance the extracts were separated by centrifugation and quantified by LSC. Soil extracts were combined and concentrated by rotary evaporation prior to analysis by HPLC. Samples of soils SK 961089 and SK 920191 were extracted further by shaking with 3 x aqueous 0.1% trifluoroacetic acid (TFA), due to a recovery of less than 90%. The extracts were separated by centrifugation and the amount of test item quantified by LSC. The extracts were concentrated by rotary evaporation prior to analysis by HPLC.

II. RESULTS AND DISCUSSION

A. MASS BALANCE

For each of the samples, the mass balance was determined from the sum of the radioactivity present in the adsorption supernatant, the desorption supernatant and the combusted residue and ranged from 97% to 102%.

B. FINDINGS

Preliminary tests
Results from the solubility test showed that EU was soluble at 5 µg mL^{-1} in 0.01M $CaCl_2$.

There was no adsorption of EU at 0.05 µg mL^{-1} from 0.01M $CaCl_2$ to centrifuge tubes (polypropylene as well as Teflon).

Results from the ratio of soil to aqueous phase test showed very little adsorption of EU (5 µg mL^{-1} in 0.01M $CaCl_2$) at the soil to solution ratios of 1:1, 1:5 and 1:25 to any of the soil types. A ratio of 1:1 was selected for the remainder of the study to gain maximum adsorption.

Results from the equilibrium time determinations showed that for all soil types, with a soil to aqueous phase ratio of 1:1, EU had reached adsorption and desorption equilibriums by 24 h. After this time the percent change in the amount of EU in the supernatant was < 10%.

Results from the stability test showed that the overall recovery of applied radioactivity ranged from 89% to 96% and chromatography of supernatants and soil extracts showed that the purity of EU was > 98% in all samples. EU was therefore considered stable for the study under the test conditions chosen.

Adsorption and desorption isotherms

Freundlich adsorption and desorption coefficients (K_F, K_{FOC} and K_{FOCdes}) are presented in Table 7.1.3.1.2-7 and Table 7.1.3.1.2-8, respectively, together with the corresponding Freundlich exponents (1/n).

Table 7.1.3.1.2-7: Freundlich adsorption constants of EU in four soils

Soil Name	Soil Type (USDA)	K_F [mL g^{-1}]	1/n [-]	K_{FOC} [mL g^{-1}]
SK 961089	clay loam	0.22	1.0464	5
SK 179618	loam	0.16	1.0099	4
SK 566696	loamy sand	0.15	0.9152	19
SK920191	clay loam	0.22	0.9772	11

Table 7.1.3.1.2-8: Freundlich desorption constants of EU in four soils

Soil Name	Soil Type (USDA)	K_F [mL g^{-1}]	1/n [-]	K_{FOCdes} [mL g^{-1}]
SK 961089	clay loam	0.40	1.0105	9
SK 179618	loam	0.20	1.0068	5
SK 566696	loamy sand	0.29	0.9546	36
SK920191	clay loam	0.42	0.9622	20

III. CONCLUSION

The adsorption behavior of EU (metabolite of metiram) was determined. Four British soils were used, which covered a range of pH (in $CaCl_2$) from 4.2 to 7.6, a range of organic carbon content from 0.8% to 4.6% and three different USDA textural classes: two clay loams, one loam and one loamy sand.

Freundlich adsorption coefficients (K_F) of 0.15 to 0.22 mL g^{-1} were determined. Corresponding K_{FOC} values were between 4 and 19 ml g^{-1} indicating that EU is adsorbed rather weak to soil. Freundlich exponent 1/n ranged from 0.9152 to 1.0464.

Desorption coefficients were in a similar range as the adsorption coefficients with K_F of 0.20 to 0.42, K_{FOC} between 5 and 36 mL g^{-1} and 1/n ranged from 0.9622 to 1.0105.

In the following two studies the adsorption to soil of metabolites Carbimid and EBIS was investigated. Both studies were done with the same soils and in close correlation in the same laboratory and have been summarized together.
Based on old, invalid studies Carbimid was formerly considered a major metabolite in soil and the adsorption to soil was investigated. Based on new, valid studies Carbimid is no longer considered major and no adsorption data are required. However since the adsorption of Carbimid has been investigated in the context of metiram, these data are reported here.
EBIS is a major metabolite also based on new studies and consequently data on sorption to soil are required.

Peer reviewed studies (Summary taken from the Monograph or its Addenda):

Report:	*CA 7.1.3.1.2/5* *Richter T., 2002a* *Study for the adsorption/desorption determination of Metiram-metabolite 243 959 (BF 222-EBIS) on 5 european soils* *2002/1005329*
Guidelines:	*OECD 106, EPA 163-1, EPA 540/9-82-021*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Report:	*CA 7.1.3.1.2/6* *Richter T., 2002b* *Study for the adsorption/desorption determination of Metiram-metabolite 251 072 (BF 222-Carbimide) on 5 european soils* *2002/1005327*
Guidelines:	*OECD 106, EPA 163-1, EPA 540/9-82-021*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

The adsorption/desorption studies with metabolites EBIS and Carbimid are summarized together:

Materials and methods:

For each experiment 2 g of soil were agitated with 4 ml of a 0.01 M $CaCl_2$ solution containing respectively ^{14}C-BF 222-EBIS at a nominal concentration of 2.0 µg mL^{-1} or BF 222-Carbimid at a nominal concentration of 1.0 µg mL^{-1}. Agitation was done for 1, 3, 8, 16 and 24 hours. The solid phase was extracted with methanol by ultrasonication. The $CaCl_2$ solution was determined without further treatment. The measurements were done by HPLC/UV at 276 nm.
The determination of K_d and K_{oc} values were done for five European soils. Details of the soils are provided in Table 7.1.3.1.2-9. In both studies, the same soils were used. The soil : water ratio was 1:2. The test substances in the soil and in the aqueous phase were determined by HPLC/UV.

Table 7.1.3.1.2-9: Soils used to investigate the adsorption of EBIS and Carbimid, metabolites of Metiram

Soil designation	Lufa 2.2 01/736/02	Borgeby 99/1330	Birnbaum 98/1296	Stetten 98/1288	Sora 98/1301
Origin	Germany	Sweden	Germany	Germany	Spain
Textural class (German scheme)	silty sand	loamy sand	loamy sand	sandy clay loam	silty loamy sand
Textural class (USDA scheme)	loamy sand	loamy sand	sandy loam	loam	loam
Particle size distribution [%] (German scheme):					
0.063 - 2 mm	77.4	78	77	40	42
0.002 - 0.063 mm	19.1	15	12	34	42
< 0.002 mm	3.5	7	11	26	16
(USDA scheme):					
0.05 - 2 mm	79.2	80	79	40	44
0.002 - 0.05 mm	17.6	13	10	34	40
< 0.002 mm	3.2	7	11	26	16
Organic carbon [%]	2.21	1.4	1.4	1.0	1.7
Organic matter [%]	3.8*	2.4*	2.4*	1.7*	2.9*
CEC [mVal/100 g]	11.4	13	13	25	18
pH [$CaCl_2$]	6.1	5.6	5.4	7.5	6.5
pH [H_2O]	6.9	6.3	6.1	8.1	7.1

* recalculated from organic carbon by a factor of 1.72

Findings:

A decrease of the test substance concentrations in the aqueous and in the soil phase was observed with longer agitation times. From this it could be stated that EBIS as well as Carbimid was degraded during agitation. Due to this behavior, K_d and K_{oc} values were calculated and related to the corresponding agitation time. The calculated K_{oc} values and related agitation times were fitted to a quadratic function. The maximum of this graph indicates equilibrium concentrations in soil and solution. If no equilibrium was reached the maximum measured K_{oc} value is reported. The results are shown in Table 7.1.3.1.2-10.

For Carbimid with the Stetten soil, the first adsorption effects were observed after 16 hours of agitation. Therefore, equilibrium might not have been reached within the maximum agitation time of 24 hours.

Due to the fast degradation of the test substance during agitation, neither desorption experiments nor adsorption/desorption isotherms were conducted.

Table 7.1.3.1.2-10: Adsorption of Metiram metabolites EBIS and Carbimid in different soil types

Compound	EBIS			Carbimid		
Soil (origin)	K_{oc} [mL g^{-1}]	K_d * [mL g^{-1}]	agitation time [hours]	K_{oc} [mL g^{-1}]	K_d * [mL g^{-1}]	agitation time [hours]
Birnbaum (Germany)	1140**	15.96	24	129	1.81	4.5
Sora (Spain)	445	7.57	16.3	123	2.09	17.8
Stetten (Germany)	615	6.15	13.7		--	--
Borgeby (Sweden)	279**	3.90	24	2268	31.75	6.3
LUFA 2.2 (Germany)	356**	7.87	16	78	1.72	22.6

*K_d values were calculated from K_{OC} values
** Maximum K_{oc} value after related agitation time. Due to still increasing Koc tendency, equilibrium may have not been reached at this time.

Mass balances were established for both metabolites in all five soils. These balances measured after respectively one and 24 hours clearly demonstrated a nearly fully degradation within the first 24 hours of agitation, with loss values higher than 90% except for Carbimid in the Stetten soil with a loss of 86%.

Conclusion:

The studies were performed according to accepted guidelines. The results clearly indicate a nearly total degradation within 24 hours. The measured K_d and K_{oc} values demonstrate that in the beginning the primarily process is the partition of the test substances between the aqueous and the soil phases, followed by a degradation resulting in decreasing K_{oc} values. Overall no risk for leaching to the groundwater is foreseen.

Formerly, hydantoin was considered to be a major metabolite in soil based on the results of old, invalid studies. Based on more recent, valid studies hydantoin is no longer considered a significant metabolite of Metiram. Consequently, no adsorption data for hydantoin are required. However, since the adsorption of hydantoin has been determined in the context of Metiram, these results are reported here.

Report:	CA 7.1.3.1.2/7 Class T., 2010d Hydantoin (metabolite of Metiram, BAS 222 F): Determination of the adsorption / desorption behavior of Hydantoin on soils 2010/1065127
Guidelines:	OECD Guidelines for the Testing of Chemicals - Volume 1 No 106 (21 January 2000)
GLP:	yes (certified by Umweltministerium Baden-Wuerttemberg, Stuttgart)

Executive Summary

In laboratory batch experiments, the adsorption behavior of hydantoin (metabolite of metiram) was investigated on three different soils. The three soils covered a range of pH ($CaCl_2$) from 5.7 to 7.5, a range of organic carbon content from 0.86% to 1.95% and two different USDA textural classes: two loamy sands and one sandy loam.

For the determination of the distribution coefficient K_d, a concentration of 5 µg mL^{-1} of the test item dissolved in 0.01 M $CaCl_2$ solution was used. The applied soil to solution ratio was 1/1. The measurements were performed at the equilibration times of 0.5, 2, 4 and 24 hours for the three soils.

Within the three soils studied K_d values from 0.060 to 0.156 mL g^{-1} were measured. The K_{oc} values ranged from 7.0 to 8.0 mL g^{-1}.

Tier 3 experiments (determination of adsorption isotherms and desorption kinetics/desorption isotherms) were not performed as hydantoin is very labile and difficult to analyze at lower concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Chemical name: Hydantoin
Molecular weight: 100.1 g mol^{-1}
Molecular formula: $C_3H_4N_2O_2$
Lot No. S23814
Purity: 98%

A standard of the test item hydantoin obtained commercially with a Certificate of Analysis was used as test and reference item.

2. Soils

The study was conducted with three different soils originating from Germany. The soils were sieved to a particle size < 2 mm. The physico-chemical properties of the soils are provided in Table 7.1.3.1.2-11.

Table 7.1.3.1.2-11: Characteristics of soils used in adsorption study with hydantoin

Soil designation Origin	LUFA 2.2 Germany	Li 10 Germany	Bruch West Germany
Textural class (USDA scheme)	loamy sand	loamy sand	sandy loam
Soil texture [%] (USDA scheme) Sand Silt Clay	 84.0 11.8 4.2	 81.4 13.2 5.5	 65.7 22.6 11.7
Textural class (German scheme)	silt sand	loamy sand	loamy sand
Soil texture [%] (German scheme) Sand Silt Clay	 83.4 12.4 4.2	 80.7 13.8 5.5	 62.9 25.4 11.7
Organic carbon [%]	1.95	0.86	1.37
CEC [$cmol^+$ kg^{-1}]	6.4	4.1	10.1
pH ($CaCl_2$) pH (water)	5.7 6.2	6.2 6.8	7.5 8.0
MWHC [g $100g^{-1}$ dry soil]	38.2	28.3	31.3
Bulk density [g L^{-1}]	1187	1446	1254

B. STUDY DESIGN

1. Experimental conditions

Tier 1 and Tier 2: Adsorption Kinetics and Determination of distribution coefficients

Adsorption kinetics testing experiments and determination of distribution coefficients were performed for all three soils. Portions of 5 g dry soil were equilibrated on a horizontal shaker with 4.5 mL of 0.01M $CaCl_2$ solution (1/1 soil / solution ratio) in PE centrifuge vials overnight. Then, hydantoin (0.5 mL of a 50 µg mL^{-1} application solution) was added to the soil water to obtain initially a nominal amount of 25 µg in the aqueous phase (total volume 5 mL) and an initial nominal concentration of 5 µg mL^{-1}.
Ten specimens were dosed per soil type (duplicates per sampling event). At least two blanks per soil type were prepared without hydantoin for concurrent soil recoveries. For each sampling event at least two specimens with no soil present were dosed at a nominal concentration of 5 µg mL^{-1} of hydantoin in the aqueous phase to be used as dose and adsorption controls.

Test systems were then equilibrated on a horizontal shaker for various equilibration times (0.5, 2, 4, 6 and 24 h) at 19-21°C.

2. Description of analytical procedures

For analysis, the soil / solution suspension was centrifuged and the supernatant was decanted. Total weights/volumes of the supernatant (density 1 g mL^{-1}) were determined gravimetrically.

For LC/MS/MS analysis, the aqueous phases were diluted by a factor of 20 with methanol/water (1/1, v/v). The mass of the test item in the total liquid phase was calculated from its concentration and the weight of the complete liquid phase.

The remaining soil pellets were extracted with water / methanol (1/1, v/v) – in each case 5 g soil pellet plus 5 mL of solvent. The final soil extract was diluted by a dilution factor in the range of 2 to 20 with methanol / water (1/1, v/v), as appropriate, for LC/MS/MS analysis.
The applicability of the soil extraction method was investigated by fortifying soil pellets of control specimens with hydantoin at two different concentration levels (0.5 and 5.0 µg g^{-1}).
The limit of quantitation (LOQ), defined as the lowest fortification level performed, was 0.5 µg g^{-1} (or 2.5 µg absolute per 5 g soil).

The percent adsorption on soil was calculated by the indirect as well as by the direct method. Calculation of distribution coefficients K_d and organic carbon normalized adsorption coefficients K_{OC} used the percent adsorption obtained by the indirect method.

II. RESULTS AND DISCUSSION

A. MASS BALANCE

The average mass balances were for all three soils higher than 90% and thus considered acceptable.

B. FINDINGS

Controls

Control specimens with only hydantoin in 0.01 M $CaCl_2$ solution (no soil) were subjected to the same steps as the test systems. These controls demonstrated the stability of hydantoin in $CaCl_2$ solution and the absence of adsorption on the surface of the test vessels.

Average recoveries of the soil extraction method were 108% (± 5 % RSD) for soils fortified at 0.5 µg g^{-1} (LOQ) and 109% (± 4% RSD) for soils fortified at 5.0 µg g^{-1}.

Adsorption Kinetics and Determination of distribution coefficients

For loamy sand LUFA 2.2 the indirect method resulted in initial adsorption of up to 18% after 2 hours, whereas the direct method indicated insignificant adsorption of ≤ 4%.
For loamy sand Li 10 the indirect method resulted in initial adsorption of up to 6% after 4 hours, whereas the direct method indicated insignificant adsorption of ≤ 3%.
For sandy loam Bruch West the indirect method resulted in initial adsorption of up to 17% after 2 hours, whereas the direct method indicated insignificant adsorption of ≤ 5%. Adsorption of hydantoin on the soils generally decreased with longer equilibration times of up to 24 hours, thus indicating that adsorption equilibrium was reached fast.

Distribution coefficients K_d and organic carbon normalized adsorption coefficients K_{OC} were calculated for all three soil types using the indirect method using two replicates and results from shorter equilibration times as indicated in Table 7.1.3.1.2-12.

Table 7.1.3.1.2-12: Results from the adsorption experiments with hydantoin

Soil	Soil Type (USDA)	TOC (%)	pH ($CaCl_2$)	K_d (mL g^{-1})	Mean K_d Calculated from K_d after h	K_{oc} (mL g^{-1})
LUFA 2.2	loamy sand	1.95	5.7	0.156	0.5, 2	8.0
Li 10	loamy sand	0.86	6.2	0.060	0.5, 2, 4	7.0
Bruch West	sandy loam	1.37	7.5	0.101	0.5, 4, 6, 24	7.4

TOC = total organic carbon

III. CONCLUSION

Adsorption of hydantoin on soils depends only to a minor extent on the soil used and seems to reach equilibrium within less than 2 hours.
The indirect method generally resulted in higher adsorption results than the direct method; mean mass balances were higher than 90% and thus considered acceptable.
K_d values range from 0.060 to 0.156 mL g^{-1}. K_{OC} values vary only within a small range from 7.0 to 8.0 mL g^{-1} indicating that adsoption of hydantoin to the tested soils was rather weak.

TDIT is a major metabolite in soil but no adsorption data have been available so far. Therefore, the following study on the adsorption of TDIT to soil has been initiated.

Report: CA 7.1.3.1.2/8
Sacchi R.R., 2014b
Adsorption/desorption behavior of TDIT (metabolite of Metiram) on different European soils
2014/1000103
Guidelines: OECD 106 (2000), EPA 835.1230, SOP-PA.1005
GLP: yes
(certified by Instituto Nacional de Metrologia, Normalizacao e Qualidade Industrial - INMETRO, Rio de Janeiro, Brazil)

Executive Summary

The adsorption behavior of TDIT (metabolite of metiram) was investigated on four different European soils. The four soils covered a range of pH (in $CaCl_2$) from 5.5 to 7.1, a range of organic carbon content from 0.83% to 1.97%, and three different USDA textural classes: silt loam, loamy sand, and sandy loam.

The methods of analysis were validated for both aqueous phase ($CaCl_2$ 0.01 mol L^{-1}) and soil. The results demonstrated the efficiency of the methods of analysis.

During the preliminary study, the equilibrium test was conducted based on the direct method. Direct determination was chosen because the mass balance was below 90%. The main study was also based on direct determination.

For the determination of the adsorption isotherms, five different concentrations (nominal 1.0, 0.5, 0.1, 0.05 and 0.01 µg mL^{-1}) of the test item in 0.01 mol L^{-1} $CaCl_2$ solutions were used. The ratio of mass of soil per test solution volume was 1:5, and the adsorption tests were performed at the adsorption equilibrium time of 48 h for all the soils.

The following adsorption parameters were derived for the test items in each of the tested soil: the Freundlich adsorption coefficient K_F, the Freundlich exponent 1/n, and the corresponding K_{FOC} values. The Freundlich adsorption coefficient K_F covered a range from 1.58 mL g^{-1} to 9.00 mL g^{-1} for the four soils. The K_{FOC} values ranged from 191 mL g^{-1} to 457 mL g^{-1} and the 1/n values ranged from 0.79 to 0.88.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Common name: TDIT
Reg. No.: 4670450
Chemical name: 2,3,7,8-tetrahydroiimidazo[2,1-b:1',2'-e][1,3,5]thiadiazine-5-thione
Molecular weight: 212.3 g mol^{-1}
Molecular formula: $C_7H_8N_4S_2$
Batch No.: L83-138
Chemical Purity: 91.4%

2. Soils

The study was conducted with four different soils (0-20 cm) originating from different locations in Germany. The soils were sieved to a particle size < 2 mm and were air dried at room temperature. The physico-chemical properties of the soils are provided in Table 7.1.3.1.2-13.

Table 7.1.3.1.2-13: Characteristics of soils used in adsorption study with TDIT

Soil designation Origin BASF Trial No.	NW Nierswalde Wildacker (13/1709/01)	SW2 Speyerer Wald 2 (10/1715/03)	L2.2 LUFA 2.2 (12/736/02)	L5M LUFA 5M (10/1651/03)
Textural class (USDA)	silt loam	loamy sand	sandy loam	sandy loam
USDA Soil texture [%]				
Sand 0.050 – 2 mm	21.4	82.6	77.0	56.8
Silt 0.002 – 0.050 mm	69.2	13.3	15.6	31.1
Clay < 0.002 mm	9.4	4.1	7.5	12.0
Textural class (DIN)	clay silt	silty sand	loamy sand	loamy sand
DIN Soil texture [%]				
Sand 0.063 – 2 mm	17.7	79.4	76.1	51.5
Silt 0.002 – 0.063 mm	72.9	16.5	16.5	36.5
Clay < 0.002 mm	9.4	4.1	7.5	12.0
Organic carbon [%]	1.97	0.83	1.74	1.19
CEC [$cmol^+ kg^{-1}$]	7.6	4.3	8.6	11.3
pH ($CaCl_2$)	5.8	5.5	5.5	7.1
pH (water)	6.8	6.6	6.1	8.1
MWHC [g 100 g^{-1} dry soil]	34.5	22.9	30.9	26.7
Microbial biomass [mg C 100 g^{-1} dry soil]	n.a.	16.4	47.5	35.3
Water content [%]	1.72	2.21	0.90	2.48

n.a. not analyzed

B. STUDY DESIGN

1. Experimental conditions

Tier 1: Preliminary tests

Adsorption to the test vessels:
An experiment was run for 24 h with different centrifuge tubes (Polypropylene Co Polymer (PPCO), Teflon, and glass) in order to find the optimal material to be used for each substance with the least interference on the experiment.

Soil to solution ratio:
To determine the appropriate soil to solution ratio, several soil to solution ratios were tested (1:1, 1:5 and 1:10) with two non-sterilized soils (Lufa 2.2 and Lufa 5M).

Adsorption equilibrium test:
To determine the minimum shaking time required to reach adsorption equilibrium in each soil, an experiment was performed at a test item concentration of 1.0 µg mL^{-1} in 0.01 mol L^{-1} $CaCl_2$ solution. Series of 5 replicates of each soil were prepared and the experiments were run at a soil to solution ratio of 1:5.

1 g of each soil was weighed into centrifuge tubes and suspended with 5 mL of the treatment solution. Sampling times were for 4, 8, 24, 32 and 48 h. In addition to the treated samples, a control run per soil was carried out in centrifuge tubes. Therefore, 1 g of each soil and 5 mL of 0.01 mol L^{-1} aqueous $CaCl_2$ solution were mixed in centrifuge tubes and suspensions were shaken for 48 h. The Teflon tubes, containing either soil or control, were closed after application of the proper solution and shaken horizontally on a mechanical shaker at 250 rpm at 20 ± 2°C for the indicated test period.

The equilibrium test was conducted by the direct method, therefore, both, solution and soil, were analyzed.

Tier 2: Screening test

The screening test was not performed, as it was essentially covered by the adsorption isotherm determination.

Tier 3: Freundlich adsorption isotherm determination

The adsorption isotherm determination was performed with five concentration levels (nominal concentrations: 1.0, 0.5, 0.1, 0.05 and 0.01 µg mL^{-1}) and the four soils. Each experiment (one soil and one solution) was done in duplicate. The soil to solution ratio was 1:5 and the test equilibration time was 48 h.

Since the mass balance was below 90%, the direct method was chosen for determination of the adsorption isotherms as proposed by the guideline.

For all experiments, 1 g of soil was weighed into Teflon tubes, and then 5 mL solution of the respective application solution was added to reach the proposed nominal concentration levels. The tubes were closed with screw caps and shaken horizontally on a mechanical shaker at 250 rpm for the determined equilibrium time in a temperature controlled dark room at 20 ± 2°C.

Desorption experiments were not conducted. The soil was extracted with organic solvents after the adsorption phase.

Stability of the test item and standard solution

In order to prove the stability of the test item, the solutions to be investigated were compared against solutions freshly prepared at the same conditions and concentrations. Aliquots of a treatment solution (1.0 µg mL^{-1} test item in 0.01 M aqueous $CaCl_2$) were shaken without soil for 48 hours. Both, original solution and control after 48 h shaking were analyzed by LC-MS/MS.

Additionally, the stability of TDIT during a period of 30 days dissolved in the standard solutions was investigated. Three solutions, one stock solution (0.5 ng mL^{-1}) and two standard solutions (0.1 ng mL^{-1} and 0.5 ng mL^{-1}), all prepared in methanol, were measured by LC-MS/MS. After 1, 2, 3, 7, 10, 15 and 30 days, the same solutions were prepared again. Both series, stored and freshly prepared, were injected together in the same run.
As a result, the test item was considered to be stable in the standard solutions.

2. Description of analytical procedures

After the respective equilibration time, the soil suspensions were centrifuged at 9000 rpm for 10 min and the supernatants were isolated. The aqueous $CaCl_2$ solution was collected for analysis. The tubes with the soil including the residual $CaCl_2$ solution were weighed after centrifugation in order to determine the volume of the remaining $CaCl_2$ solution.

Aliquots of the supernatants and the applied treatment solution were diluted and analyzed by LC-MS/MS in order to determine the initial concentration as well as the concentration of the test item in the aqueous $CaCl_2$ solution after adsorption.
The remaining soil samples in the centrifuge tubes were extracted three times with 3 mL methanol. The tubes were shaken at 250 rpm for 30 min at room temperature and then centrifuged at 9000 rpm for 10 min. The supernatants were decanted and made up to 10 mL.

Aliquots of the soil extracts were diluted in methanol and analyzed by LC-MS/MS to determine the amount of test item adsorbed to the soil phase.

The method of analysis of the aqueous phase and soil was validated. For validation of the test substance in aqueous solution, aqueous solutions (10 mL of $CaCl_2$ 0.01 mol L^{-1}) were fortified with TDIT at the limit of quantification (0.5 ng mL^{-1}) and at a level of 1000 ng mL^{-1}. For validation of the test substance in soil, soil samples were fortified with TDIT at levels at the LOQ (0.0005 mg kg^{-1}) and 1.0 mg kg^{-1}. In addition, an aliquot of the untreated aqueous solution and untreated soil sample was analyzed to demonstrate that matrix components do not interfere with the detection and quantification of TDIT. The fortified aqueous solutions were diluted with methanol and analyzed by LC-MS/MS. The fortified soil samples were extracted 3 times with 3 mL methanol by shaking at 250 rpm for 30 min and then centrifuged at 9000 rpm for 10 min. The samples were diluted with methanol and then analyzed by LC-MS/MS.

II. RESULTS AND DISCUSSION

A. MASS BALANCE

For the equilibrium test, the mass balance ranged from 51.8% to 94.5% of the applied test item. For the determination of the isotherms, the mass balance ranged from 49.0% to 106.8%.

B. FINDINGS

No relevant adsorption of the test item on the tested centrifuge tubes could be observed. Teflon tubes were chosen to conduct the study.

A soil to solution ratio of 1:5 was chosen, as it provided adsorption of around 50% of the applied test item, enabling enough test item in both phases (soil and solution phase) for accurate measurements.

The soils exhibited constant adsorption of the test item throughout the 48 h test period of the adsorption equilibrium test. Only in soil Lufa 5M adsorption increased during the test period. Hence, the equilibration time chosen for the conduct of the isotherm experiment was 48 hours.

Solutions were proven to be stable during the time they were used. In addition, the test item was also proven to be stable in 0.01 M $CaCl_2$ for at least 48 hours and constant shaking. However, the mass balance below 90% of samples from equilibrium test and main study indicate that degradation may have occurred.

The Freundlich adsorption coefficient K_F covered a range from 1.58 mL g^{-1} to 9.00 mL g^{-1}. The K_{FOC} values ranged from 191 mL g^{-1} to 457 mL g^{-1}. The 1/n values ranged from 0.79 to 0.88. The calculated adsorption parameters for the test items in each soil, namely the Freundlich adsorption coefficient K_F, the Freundlich exponent 1/n, and the corresponding K_{FOC} values, are presented in Table 7.1.3.1.2-14.

Table 7.1.3.1.2-14: Results from the adsorption experiments with TDIT

Soil	Soil Type (USDA)	K_F [mL g^{-1}]	1/n	K_{FOC} [mL g^{-1}]
NW	silt loam	9.00	0.79	457
SW2	loamy sand	1.58	0.83	191
L2.2	sandy loam	4.04	0.88	232
L5M	sandy loam	2.63	0.83	221

III. CONCLUSION

The adsorption behaviour of TDIT (metabolite of metiram) was determined in four European soils using the direct method. The Freundlich adsorption coefficient K_F covered a range from 1.58 mL g^{-1} to 9.00 mL g^{-1}. The K_{FOC} values ranged from 191 mL g^{-1} to 457 mL g^{-1} and the 1/n values ranged from 0.79 to 0.88. The data indicate medium to high adsorption of TDIT to the four tested soils.

CA 7.1.3.2 Aged sorption

No studies on aged sorption were performed.

CA 7.1.4 Mobility in soil

CA 7.1.4.1 Column leaching studies

No new column leaching studies have been performed since the first Annex I inclusion process. The existing studies are considered still valid and are summarized briefly below. From the study of Keller E., Huber R., 1995 [*see CA. 7.1.4.1.1/4, BASF DocID 1985/10059*], only the part on column leaching is considered still valid and is reported in this dossier whereas the metabolism performed in the same study is considered invalid (compare *CA 7.1.1.1*).

CA 7.1.4.1.1 Column leaching of the active substance

Peer reviewed study (Summary taken from the Monograph or its Addenda):

Report:	*CA 7.1.4.1.1/1* *Anonymous, 1974a* *Leaching behaviour of pesticides* *1974/10253*
Guidelines:	*BBA Merkblatt Nr. 37*
GLP:	*no*

Materials and Methods:

Three soils were used in the study: A sand (standard soil 2.1), a loamy sand (standard soil 2.2), and a sandy loam (standard soil 2.3). The soils were filled into glass columns (5 cm diameter). 1 mg BAS 222 01 F per 20 cm^2 soil (corresponds to 4.0 kg metiram ha^{-1}) was applied on the top of each of the column. The columns were eluted with a volume of water corresponding to 200 mm rainfall within two days. Metiram was analyzed for by using the CS_2 method (limit of determination: 0.3 mg kg^{-1}).

Findings:

Metiram did not leach out of the laboratory soil columns as shown in Table 7.1.4.1.1-1.

Table 7.1.4.1.1-1: Metiram (including CS_2 yielding degradates) in percolate water of laboratory soil columns (in % of amount applied).

Soil	Metiram
Sand	0*
Loamy sand	0
Sandy loam	0

* 0 stands for below determination limit

Conclusion:

Metiram and its CS_2-yielding degradates are not mobile in soil.

Peer reviewed study (Summary taken from the Monograph or its Addenda):

Report:	*CA 7.1.4.1.1/2* *Anonymous, 1974b* *Leaching behaviour of pesticides* *1974/10203*
Guidelines:	*BBA Merkblatt Nr. 37*
GLP:	*no*

Materials and Methods:

The same soil types and experimental details were used as in CA 7.1.4.1.1/1. The following application rates were used:
0.75 mg BAS 379 80 F (→ 0.45 mg metiram) per 20 cm^2 soil corresponding to 3.75 kg BAS 379 80 F ha^{-1} (→ 2.25 kg metiram ha^{-1}). The determination limit was 300 µg L^{-1}.

Findings:

Metiram did not leach out of the laboratory soil columns (see Table 7.1.4.1.1-2).

Table 7.1.4.1.1-2: Metiram including CS_2 yielding degradates in percolate water of laboratory soil columns (in % of amount applied).

Soil	Metiram
Sand	0*
Loamy sand	0
Sandy loam	0

* 0 stands for below determination limit (1.85% of applied rate)

Conclusion:

Metiram and its CS_2-yielding degradates are not mobile in soil.

Peer reviewed study (Summary taken from the Monograph or its Addenda):

Report:	*CA 7.1.4.1.1/3* *Keller W., 1986a* *Leaching behaviour of pesticides* *1986/10809*
Guidelines:	*BBA Merkblatt Nr. 37*
GLP:	*no*

Materials and Methods:

The same soil types and experimental details were used as in CA 7.1.4.1.1/1 (except for a higher rainfall of 400 mm). The following application rates were used:
0.784 mg BAS 222 28 F (→ 0.6272 mg metiram) per 20 cm^2 soil corresponding to 4 kg BAS 222 28 F ha^{-1} (→ 3.2 kg metiram ha^{-1}).
Limit of determination of the CS_2 method in this study was 2 µg L^{-1}.

Findings:

Metiram did not leach out of two laboratory soil columns. Out of a column filled with standard soil 2.2 (loamy sand) low amounts of metiram or CS_2 yielding degradates did leach (see Table 7.1.4.1.1-3).

Table 7.1.4.1.1-3: Metiram including CS_2 yielding degradates in percolate water of laboratory soil columns (in % of amount applied).

Soil	Metiram
Sand	0*
Loamy sand	1.9
Sandy loam	0

* 0 stands for below determination limit (0.4%)

Conclusion:

Metiram and its CS_2-yielding metabolites were not mobile except in one soil where amounts of CS_2-producing residues were detected in the leachate.

Peer reviewed study (Summary taken from the Monograph or its Addenda):

Report:	*CA 7.1.4.1.1/4* *Keller E.,Huber R., 1985a* *Soil metabolism of Metiram under aerobic and anaerobic conditions - Mineralization - Leaching of 30 days aerobically aged residues* *1985/10059*
Guidelines:	*none*
GLP:	*no*

Report:	CA 7.1.4.1.1/5 Klein W.,Koerdel W. et al., 1986a Addendum to lab.-report No. 2208: Aerobic biodegradation of the EBDC fungicide Metiram-complex in soil 1986/10134
Guidelines:	none
GLP:	no

Materials and Methods:

A loamy sand soil spiked with 10 mg [^{14}C]-metiram/kg soil was incubated for 30 days at 20 °C ± 2 °C. After incubation, 100 g of aged soil was applied onto the top of a glass column (5 cm inner diameter) which had been filled previously with untreated soil (5 segments of 5 cm height each) and at the bottom segment (outlet) with quartz sand. The column was percolated with one liter of water within approximately one day. Radioactivity in the column segments was determined by combustion of soil samples, radioactivity in the percolates was measured by LSC.

Findings:

A recovery of applied radioactivity of 102% was found. Only 1.9% of the initially applied radioactivity was detected in the total percolate (approximately 1 L water).

Conclusion:

Aged residues of metiram (i.e. soil metabolites) are not prone to leaching; consequently, there is no risk of groundwater contamination by aged residues of metiram.

Peer reviewed study (Summary taken from the Monograph or its Addenda):

Report:	*CA 7.1.4.1.1/6* *Specht W., 1991a* *Leaching behavior of Metiram (Polyram Combi) in soil after aging in accordance with Guideline Part IV, 4-2, Section 5.2, of the German Federal BBA with simultaneous determination of leaching behavior of Ethylenethiourea formed during aging* *1991/11675*
Guidelines:	*BBA IV 4-2*
GLP:	*yes* *(certified by Freie und Hansestadt Hamburg, Behoerde fuer Arbeit, Gesundheit und Soziales, Hamburg, Germany)*

Report:	*CA 7.1.4.1.1/7* *Specht W., 1991b* *Leaching behavior of Metiram (Polyram Combi) in the soil after aging with the simultaneous determination of the leaching behavior of the metabolite Ethylene thiourea (ETU) formed during the aging* *1991/11676*
Guidelines:	*BBA IV 4-2*
GLP:	*yes* *(certified by Freie und Hansestadt Hamburg, Behoerde fuer Arbeit, Gesundheit und Soziales, Hamburg, Germany)*

Materials and Methods:

2.2 mg metiram per 20 cm^2 soil corresponding to 12 kg metiram ha^{-1} (nominal) which was a 3x exaggerated rate was applied to a sand soil (standard soil 2.1) aerobically aged for 17 hours. The exaggerated rate was necessary to reach the needed determination limit (determination limits: 0.1 mg L^{-1} for metiram (0.05 mg L^{-1} CS_2) and 0.02 mg L^{-1} ETU). Metiram was analyzed by the CS_2 method (DFG method 515) and ETU on GC with an ECD. The leaching experiment was carried out in parallel soil columns with an irrigation rate of 200 mm. Percolate volume was 370 mL.

Findings:

Neither metiram (or CS_2-yielding metabolites) nor ETU could be detected in the percolates of the soil columns filled with sandy soil.

CA 7.1.4.1.2 Column leaching of metabolites, breakdown and reaction products

No column leaching studies on metabolites, breakdown or reaction products have been performed. The leaching of degradation products of metiram can be reliably estimated from their half-lives and their sorption to soil.

CA 7.1.4.2 Lysimeter studies

The leaching risk of metiram and its metabolites is addressed by PEC_{gw} calculations using results from degradation rate and adsorption/desorption studies. Neither the active substance nor its metabolites reveal a risk for groundwater contamination. Lysimeter studies are considered not necessary and no lysimeter studies were conducted.

CA 7.1.4.3 Field leaching studies

Neither the active substance nor its metabolites reveal a risk for groundwater contamination. Field leaching studies are considered not necessary and no field leaching studies were conducted.

CA 7.2 Fate and behaviour in water and sediment

CA 7.2.1 Route and rate of degradation in aquatic systems (chemical and photochemical degradation)

CA 7.2.1.1 Hydrolytic degradation

Studies presented in the first Annex I inclusion process:

Three hydrolysis studies were presented, two with metiram and one with its metabolite ETU. An overview is given in Table 7.2.1.1-1.

Table 7.2.1.1-1: Studies on hydrolytic degradation performed with metiram and ETU

Reference	BASF DocID	pH	Incubation temperature [°C]	Incubation period [days]	Remark
Anonymous, 1981	1981/10030	5, 7, 9	22	10	Relevant
Klein W., 1985	1985/0500	3, 5, 7, 9	25	30/40	Invalid
Carpenter M., 1987	1987/10154	5, 7, 9	25	30	Relevant

The old hydrolysis study of Anonymous [*see CA 7.2.1.1/1, BASF DocID 1981/10030*] is considered in principle valid for the rate of hydrolytical degradation of the active substance. However, more recent data show that the degradation rate was underestimated due to the presence of the hydrolytic degradation product EBIS that interferes with measurement. Also, no radiolabeled test item was used in this study and no information on hydrolytical degradation products was generated.

The study of Klein W. [*Klein W., 1985, BASF DocID 1985/0500*] is considered invalid for the following reasons:
In “experiment A” metiram was pre-dissolved in DMSO before application. This results in fast decomposition to various degradates and therefore, not the active substance but its degradates (solvolysis products) were applied in this part of the study.
In “experiment B” metiram was suspended in water or buffer. While this is the correct approach, major deficiencies were observed in the further conduct of the study. No material balance was established for any sampling time. Instead, only dissolved radioactivity was measured and related to the initial applied radioactivity. Effects like adsorption to the vial surface were not investigated but must be assumed from the unexpected slow increase of the dissolved radioactivity. Therefore, the results of this part of the study are considered not reliable.
Generally, the quantification of metabolites relied solely on radio-TLC analysis which was later on shown to be not a suitable method for the investigation of degradation products of metiram [*see CA 7.1.1.1/5, BASF DocID 1993/10578*]. Although HPLC is mentioned in the report, this was not used for quantification.

The study of Carpenter M. [*CA 7.2.1.1/3, BASF DocID 1987/10154*] is considered still valid.

Submission of not yet peer-reviewed studies in this AIR3-Dossier:

A new hydrolysis study was performed with radiolabeled metiram [*see CA 7.2.1.1/2, BASF DocID 2013/1095965*].

Peer reviewed study (Summary taken from the Monograph or its Addenda) :

Report:	*CA 7.2.1.1/1* *Anonymous, 1981a* *Metiram - Behaviour of pesticides in water* *1981/10030*
Guidelines:	*none*
GLP:	*no*

Materials and Methods:

The hydrolytic degradation of metiram was studied at pH 5, 7 and 9 at 22°C. The application rate was 16.7 mg L^{-1} (as metiram is insoluble in water an aqueous suspension was produced by ultrasonification). The sampling schedule was: pH 5: 0, 6, 24, 48, 72 and 96 hours; pH 7: 0, 4, 8, 24, 48, 72 and 96 hours; pH 9: 0, 4, 8, 24, 48, 72, 120, 144, 168 and 240 hours. The CS_2 method was used with a determination limit of 40 µg L^{-1} (BASF method no. 135).

Findings:

The half life of metiram was calculated to be 22.5 h at pH 5, 17.4 h at pH 7 and 115.1 h at pH 9.

Conclusion:

Metiram was degraded fastest at pH 7 and slowest at pH 9.

Report: CA 7.2.1.1/2
Class T., 2015a
Aqueous hydrolysis of 14C-Ethylene-labelled Metiram at pH 4, 5, 7, and 9
2013/1095965
Guidelines: OECD 111, EPA 835.2120
GLP: yes
(certified by Landesamt fuer Umwelt, Messungen und Naturschutz Baden Wuerttemberg, Karlsruhe, Germany)

Executive Summary

The objective of the study was to assess the abiotic hydrolytic transformation of metiram in water at four different pH levels (pH 4, 5, 7, and 9). Experiments were performed to estimate the rate of hydrolysis of metiram as a function of pH. Furthermore, the purpose of the study was to examine formation, decline, and identity of metiram hydrolysis products.

The rate of hydrolysis of metiram was determined by adding metiram to aqueous pH 4, 5, 7, and 9 buffer solutions. The amount of metiram was determined by quantification of the evolved CS_2 after acid digestion.

The results indicate short half-lives ($DegT_{50}$) between 0.6 days (pH 4), and 2 days (pH 5) for slightly acidic conditions. Half-lives of 3 days and 2 days were calculated for pH 7 and pH 9. However, the latter are considered to be effected by the presence of the degradation product EBIS that is known to form also CS_2 after digestion. EBIS was present in substantial amounts at early sampling time points at pH 7 (25-35% TAR) and pH 9 (26% TAR). Thus, it can be assumed that hydrolysis of polymeric metiram at pH 7 and 9 is much faster than derived from the digestion and CS_2 formation method.

In a separate experiment, radio-labeled metiram was added to aqueous pH 4, 5, 7 and 9 buffer solutions and the mixtures were kept in a thermostated cabinet at 25°C in the dark over 96 hours. After filtration of the suspension, the samples were analyzed by LSC and radio-HPLC. Degradates formed were identified and confirmed by structure elucidation work using high resolution mass spectrometry (HRMS) and co-chromatography.

Several degradates of metiram were detected. ETU (M222F002) is a major and stable hydrolysis product of metiram at all pHs examined. EBIS (M222F004) is a significant hydrolysis product of metiram at all pHs except at pH 4. EBIS degrades fast at pH 7 and even faster at pH 5 and 9. EU (M222F003) was formed under all pH conditions except at pH 4, however only at max 6% TAR. M222F011 was only observed in relevant amounts at pH 4 and increased to about 13% TAR after 4 days. Thus, M222F011 is considered a major hydrolysis product formed under acidic conditions.

Shorter half-lives of metiram were determined in a kinetic evaluation based on the experiments with radiolabeled metiram. $DegT_{50}$ values of 1.0 day (pH 4), 0.9 day (pH 5), 0.1 day (pH 7) and 0.4 day (pH 9) were estimated.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

BAS-Code:	BAS 222 F (metiram)
Registry No.:	250284
Chemical name:	Zinc ammoniate ethylenebis(dithiocarbamate)-poly-(ethylenethiuramdisulfide)
Molecular formula:	$(C_4H_9N_3S_4Zn)_3$; $C_4H_6N_2S_4$
Molar mass:	1088.7 g mol^{-1}

<u>Labeled test item:</u>

Label:	^{14}C-ethylene
Batch No.:	153-6001
Specific activity of a.s.:	7.64 MBq mg^{-1}
Releasable CS_2:	89.0%

<u>Non-labeled test item:</u>

Batch No.:	CP052744
Purity:	93.6%

2. Test system

All buffer solutions were sterilized and depleted of oxygen. The following buffer solutions were used:
pH 4: citric acid / sodium hydroxide / sodium chloride solution
pH 5: citric acid / sodium hydroxide
pH 7: potassium dihydrogen phosphate / disodium hydrogen phosphate
pH 9: borax / hydrochloric acid

B. STUDY DESIGN

1. Experimental conditions

<u>Rate of hydrolysis</u>
To determine the rate of hydrolysis, non-radiolabeled metiram was added to aqueous buffer solutions to obtain a concentration of about 40 µg mL^{-1} and suspended by sonication and stirring in a thermostated cabinet at 20°C for up to 142 hours. Samples were prepared in duplicates.

<u>Formation of degradates</u>
^{14}C-labeled metiram was suspended in 4 mL buffer solution using an IKA-Turrax-Tube-Drive with grinding balls and diluted then to 40 mL with buffer solution to obtain a concentration of about 100-150 µg mL^{-1}. The samples were stored under continuous magnetic stirring in a thermostated cabinet at 25°C in the dark over ≥ 96 hours (about 4 days).

2. Sampling

At various time points (0, 1, 2, 4, 24, 48, 72 and 142 hours), samples were taken for determination of the rate of hydrolysis of non-radiolabeled metiram.

For determination of degradates, aliquots were taken at various time points (0.5/1, 2/2.5, 6/6.5, 24/24.5, 48/48.5, 96/96.5, 168/168.5 (only pH 9) hours).

3. Description of analytical procedures

Rate of hydrolysis
Samples of the suspensions with non-radiolabeled metiram were taken and dosed into 10 mL headspace vials. A solution of 20% (w/v) $SnCl_2$ in 5 N HCl was added, the headspace vial was sealed and the solution was digested at 80°C in a shaking water bath for 20 min. After sonication, aliquots of the hydrolysis mixtures were analyzed by HS-GC/MS.

Formation of degradates
Samples of the suspension were filtered (0.22 µm, Nylon centrifuge filter) and analyzed by LSC and radio-HPLC. The filters were extracted with dimethyl sulfoxide (DMSO) by shaking for 20 min. The activity in the extracts was determined by LSC for calculation of the mass balance.
To identify and confirm the degradates, structure elucidation with high resolution mass spectrometry (HRMS) and co-chromatography was performed.

4. Calculation of half-lives

Peak areas of the CS_2 formed from remaining metiram and apparently including its degradation product EBIS (M222F004) were fitted with single first order kinetics using EXCEL to calculate degradation rate constants K and half-lives (DT_{50}) for metiram + EBIS at various pH.

Additionally, half-lives of metiram were determined in a kinetic evaluation based on the experiments with radiolabeled metiram. Since no specific recommendation is available how to carry out kinetic evaluations for hydrolysis experiments, the recommendations of the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*] have been applied. The software package KinGUI2 (version 2.2014.224.104) was used for parameter fitting [*SCHÄFER et al. (2007)*].

The $DegT_{50}$, $DegT_{90}$ endpoints of the parent compound metiram and its main metabolites EBIS (pH 5, pH 7, pH 9), ETU (pH 4, pH 5, pH 7, pH 9), M222F011 (pH 4), and EU (pH 7) were derived from a compartment modeling approach. The following kinetic models were employed for the evaluation: SFO, FOMC and DFOP.

The observed "not dissolved activity" from the experiments with radiolabeled metiram was used as (undissolved polymeric) metiram residue; the day 0 values for the parent compound were set equal to the mass balance values. The metabolite residues considered for the kinetic evaluation were taken from the radio HPLC determinations.

II. RESULTS AND DISCUSSION

A. MASS BALANCE

The dissolved (buffer solution) and not-dissolved (filter) radioactivity and the resulting mass balances for the aqueous hydrolysis of ^{14}C-labeled metiram are summarized in Table 7.2.1.1-2 to Table 7.2.1.1-5. The calculated mass balances resulted for pH 4 samples in an average of 99% TAR (86 to 109%), for pH 5 in an average of 94% TAR (86 to 103%), for pH 7 in an average of 105% TAR (104 to 106%), and for pH 9 in an average of 107% TAR (range 106 to 111%).

Table 7.2.1.1-2: Mass balance of metiram at pH 4 at 25°C

Hydrolysis time	Dissolved activity (Buffer) [% TAR]		Not dissolved activity (Filter) [% TAR]		Mass Balance [% TAR]	
hours	single sample	mean	single sample	mean	single sample	mean
0.5	20	20	89	89	109	109
1.0	20		89		108	
2.0	27	27	80	81	107	107
2.5	26		81		107	
6.0	36	35	68	69	103	104
6.5	34		70		104	
24.0	46	46	50	53	97	99
24.5	45		55		101	
48.0	51	51	37	40	88	91
48.5	50		44		94	
96.0	55	56	26	31	82	86
96.5	56		35		91	
				Average (n = 12)	99	

TAR = Total applied radioactivity

Table 7.2.1.1-3: Mass balance of metiram at pH 5 at 25°C

Hydrolysis time	Dissolved activity (Buffer) [% TAR]		Not dissolved activity (Filter) [% TAR]		Mass Balance [% TAR]	
hours	single sample	mean	single sample	mean	single sample	mean
0.5	47	47	57	57	104	103
1.0	46		56		102	
2.0	29	30	66	67	95	97
2.5	30		68		99	
6.0	36	37	56	55	92	92
6.5	37		54		91	
24.0	50	51	36	35	86	86
24.5	53		34		87	
48.0	56	57	41	35	98	92
48.5	58		29		87	
96.0	67	67	32	26	99	93
96.5	66		21		87	
				Average (n = 12)	94	

TAR = Total applied radioactivity

Table 7.2.1.1-4: Mass balance of metiram at pH 7 at 25°C

Hydrolysis time	Dissolved activity (Buffer) [% TAR]		Not dissolved activity (Filter) [% TAR]		Mass Balance [% TAR]	
hours	single sample	mean	single sample	mean	single sample	mean
0.5	38	37	68	69	106	106
1.0	37		70		106	
2.0	69	77	36	28	106	105
2.5	85		20		105	
6.0	88	90	17	14	105	104
6.5	92		12		104	
24.0	93	95	12	10	105	105
24.5	97		9		105	
48.0	96	97	10	9	106	106
48.5	99		8		106	
96.0	98	98	8	7	106	105
96.5	99		6		105	
Average (n = 12)					105	

TAR = Total applied radioactivity

Table 7.2.1.1-5: Mass balance of metiram at pH 9 at 25°C

Hydrolysis time	Dissolved activity (Buffer) [% TAR]		Not dissolved activity (Filter) [% TAR]		Mass Balance [% TAR]	
hours	single sample	mean	single sample	mean	single sample	mean
0.5	29	31	79	77	109	107
1.0	32		74		106	
2.0	34	36	75	70	108	106
2.5	39		66		104	
6.0	45	55	61	52	107	107
6.5	65		42		107	
24.0	94	101	6	5	100	106
24.5	108		5		112	
48.0	101	103	3	3	104	106
48.5	106		3		109	
96.0	104	106	2	2	106	108
96.5	108		2		110	
168.0	107	109	2	2	109	111
168.5	111		2		112	
Average (n = 14)					107	

TAR = Total applied radioactivity

B. FINDINGS

Metiram

The degree of hydrolysis (in percent of applied) for experiments with non-radiolabeled metiram is summarized in Table 7.2.1.1-6 to Table 7.2.1.1-9. At pH 4, a degree of 70-79% after 24 hours was observed and increased to 96-97% after 72 hours, indicating a fast hydrolysis. With increasing pH, a slightly reduced hydrolysis rate was observed. After 24 hours, degrees of hydrolysis of 51-53%, 8-36%, and 8-15% were determined for pH 5, pH 7, and pH 9, respectively. After 142 hours, the degree of hydrolysis increased to ≥ 82% for pH 5, 7, and 9.

Table 7.2.1.1-6: Hydrolysis of non-radiolabeled metiram at pH 4 and 25°C

Hydrolysis time	Degree of Hydrolysis [%]	
	Repl. 1	Repl. 2
0	n.a.	n.a.
1	0	0
2	6	13
4	24	30
24	70	79
48	92	93
72	96	97

n.a. = not analyzed

Table 7.2.1.1-7: Hydrolysis of non-radiolabeled metiram at pH 5 and 25°C

Hydrolysis time	Degree of Hydrolysis [%]	
	Repl. 1	Repl. 2
0	n.a.	0
1	0	3
2	0	10
4	8	29
24	51	53
48	--	--
72	56	69
142	86	86

n.a. = not analyzed

Table 7.2.1.1-8: Hydrolysis of non-radiolabeled metiram at pH 7 and 25°C

Hydrolysis time	Degree of Hydrolysis [%]	
	Repl. 1	Repl. 2
0	n.a.	n.a.
1	0	n.a.
2	0	0
4	10	3
24	8	36
48	23	65
72	38	71
142	82	--

n.a. = not analyzed

Table 7.2.1.1-9: Hydrolysis of non-radiolabeled metiram at pH 9 and 25°C

Hydrolysis time	Degree of Hydrolysis [%]	
	Repl. 1	Repl. 2
0	n.a.	n.a.
1	0	0
2	1	-1
4	2	2
24	8	15
48	45	51
72	65	66
142	88	93

n.a. = not analyzed

Degradates

The results of the determination of the degradates are summarized in
Table 7.2.1.1-10 to Table 7.2.1.1-13.

At pH 4, the main degradate was M222F002 (ETU) which increased continuously up to 43% TAR after 4 days. A second major degradate was M222F011 which increased up to 13% TAR after 4 days. All other hydrolysis products were observed only at very low percentages far below 5% TAR.

At pH 5, the main degradate was M222F002 (ETU) which increased continuously up to 53% TAR after 4 days. M222F004 (EBIS) was present at levels around 4-6% TAR throughout the hydrolysis period. All other hydrolysis products were observed only at percentages at or below 5% TAR, except for UK3 with 6.7% TAR after one hour.

At pH 7, the main degradate was M222F002 (ETU) which increased continuously up to 59% TAR after 4 days. M222F004 (EBIS) increased to 35% TAR within the first day of hydrolysis, then declined to 27% TAR after 4 days. M222F003 (EU) increased to 6% TAR after 4 days. All other hydrolysis products were observed only at very low percentages far below 5% TAR.

At pH 9, the main degradate was M222F002 (ETU) which increased continuously up to 105% TAR after 4 days. M222F004 (EBIS) increased to 26% TAR within the first day of hydrolysis, then declined to 1% TAR after 4 days. All other hydrolysis products were observed only at low percentages below 5% TAR.

The presence of ETU, EBIS and EU was confirmed in the structure elucidation phase by high-resolution mass spectrometry (HRMS) and co-chromatography with the corresponding reference item.
Structure elucidation assigned for the very polar degradates UK1 the formula $C_3H_6N_2$, which was finally assigned based on co-chromatography with 4,5-dihydro-1*H*-imidazole as M222F011.
For the minor degradate UK6 found at pH 9, a formula with $C_4H_7N_3OS$ was derived. The structure of UK6 was tentatively assigned as M222F009.
The very transient degradate UK3 was tentatively assigned as M222F018 by comparison to a compound in the aqueous photolysis study run in parallel *[BASF DocID 2014/1189252, CA 7.2.1.2/3])*.

Table 7.2.1.1-10: Formation of degradates by hydrolysis of ^{14}C-metiram at pH 4 and 25°C [% TAR]

hours	Assigned peak retention time [min]												Sum of areas [a] not assigned	Dissolved but not detected activity [b]
	M222F011 (1.6 UK1)		M222F002 (4.8 ETU)		7.8 UK2		M222F018 [c] (11.7 UK3)		12.3 UK4		M222F004 (18.8 EBIS)			
	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean		
0.5	1.0	1.3	1.6	2.5	0.2	0.2	0.5	0.5	0.6	0.6	0.7	0.7	0.4	15
1.0	1.6		3.4		0.3		0.6		0.6		0.6		0.6	12
2.0	3.3	3.7	8.0	8.6	0.6	0.7	0.6	0.6	0.7	0.7	1.0	0.7	1.6	11
2.5	4.0		9.2		0.8		0.6		0.6		0.4		1.7	9.3
6.0	7.2	7.5	17	17	0.6	0.6	--	--	--		0 1	0.1	0.7	10
6.5	7.8		16		0.6		--		--		--		1.0	8.2
24.0	12	12	30	30	0.1	0.0	0.3	0.4	--		--	--	1.6	insignificant
24.5	12		30		0.0		0.4		--		--		1.0	
48.0	13	12	37	37	0.0	0.1	0.4	0.4	--		--	--	1.1	
48.5	12		37		0.1		0.5		--		--		0.8	
96.0	13	13	43	43	0.0	0.0	--	--	--		--	--	2.1	
96.5	12		43		0.1		--		--		--		0.9	

s.s.= Single sample
[a] Activity of peaks < 1% TAR and/or increased background
[b] Detected by direct LSC but not by radio HPLC (potentially metiram particles that do not pass the column/precolumn)
[c] tentatively assigned

Table 7.2.1.1-11: Formation of degradates by hydrolysis of ^{14}C-metiram at pH 5 and 25°C [% TAR]

hours	Assigned peak retention time [min]														Sum of areas [a] not assigned	Dissolved but not detected activity [b]
	M222F011 (1.6 UK1)		M222F003 (2.2 EU)		M222F002 (4.8 ETU)		6.4		7.8 UK2		M222F018 [c] (11.7 UK3)		M222F004 (18.8 EBIS)			
	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean		
0.5	0.3	0.4	--	--	6.7	11	--	--	0.3	0.3	7.5	6.7	6.7	5.4	8.5	17
1.0	0.4		--		14		--		0.3		5.9		4.2		6.2	15
2.0	0.2	0.2	0.2	0.4	16	18	1.3	1.4	0.1	0.3	5.1	4.5	4.3	3.7	1.0	insignificant
2.5	0.3		0.5		19		1.5		0.4		3.8		3.2		0.8	
6.0	0.6	0.6	0.8	0.8	22	23	1.5	1.5	0.2	0.2	4.0	3.6	4.3	4.6	1.3	
6.5	0.6		0.9		24		1.5		0.3		3.2		4.9		0.8	
24.0	1.7	1.9	1.2	1.4	38	39	0.8	0.7	0.4	0.4	1.1	0.9	5.7	5.8	1.1	
24.5	2.1		1.5		40		0.6		0.4		0.8		5.8		0.9	
48.0	2.7	2.8	2.1	2.2	44	45	--	--	0.7	0.7	0.2	0.2	5.7	5.6	1.5	
48.5	2.9		2.3		46		--		0.7		0.1		5.4		1.2	
96.0	3.9	4.0	3.4	2.9	52	53	--	--	1.1	1.1	0.1	0.0	4.8	4.2	1.6	
96.5	4.0		2.4		53		--		1.1		0.0		3.6		2.1	

s.s.= Single sample
[a] Activity of peaks < 1% TAR and/or increased background
[b] Detected by direct LSC but not by radio HPLC (potentially metiram particles that do not pass the column/precolumn)
[c] tentatively assigned

Table 7.2.1.1-12: Formation of degradates by hydrolysis of ^{14}C-metiram at pH 7 and 25°C [% TAR]

hours	Assigned peak retention time [min]														Sum of areas [a] not assigned	Dissolved but not detected activity [b]
	M222F011 (1.6 UK1)		M222F003 (2.2 EU)		M222F002 (4.8 ETU)		7.8 UK2		9.0 UK5		M222F018 [c] (11.7 UK3)		M222F004 (18.8 EBIS)			
	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean		
0.5	0.2	0.1	0.0	0.0	9.0	7.9	0.1	0.0	0.0	0.0	4.0	3.6	9.1	8.3	2.2	13
1.0	0.1		0.0		6.8		0.0		0.0		3.1		7.4		1.5	18
2.0	0.2	0.3	0.4	0.5	24	32	0.1	0.2	0.0	0.1	6.9	7.1	20	25	5.0	12
2.5	0.5		0.6		39		0.2		0.1		7.3		30		4.9	2.8
6.0	0.8	0.9	0.2	0.2	42	44	0.3	0.5	0.2	0.2	5.1	5.0	33	35	1.0	5.1
6.5	1.0		0.2		45		0.7		0.2		4.8		36		1.3	2.9
24.0	1.2	1.2	1.2	1.3	47	49	0.9	0.9	1.7	1.8	2.7	2.6	33	34	1.7	4.0
24.5	1.2		1.4		51		0.9		1.8		2.6		35		1.0	insignifi-cant
48.0	1.4	1.4	2.9	3.0	52	54	0.5	0.6	2.3	2.1	1.5	1.5	31	31	1.1	
48.5	1.4		3.2		56		0.7		1.8		1.4		32		1.4	
96.0	1.4	1.5	6.0	6.2	59	59	0.2	0.3	1.5	1.3	0.4	0.4	26	27	0.6	
96.5	1.5		6.4		60		0.3		1.1		0.4		28		0.4	

s.s.= Single sample
[a] Activity of peaks < 1% TAR and/or increased background
[b] Detected by direct LSC but not by radio HPLC (potentially metiram particles that do not pass the column/precolumn)
[c] tentatively assigned

Table 7.2.1.1-13: Formation of degradates by hydrolysis of ^{14}C-metiram at pH 9 and 25°C [% TAR]

hours	Assigned peak retention time [min]																		Sum of areas [a] not assigned	Dissolved but not detected activity [b]
	M222F011 (1.6 UK1)		M222F003 (2.2 EU)		M222F002 (4.8 ETU)		7.8 UK2		9.0 UK5		M222F009 (9.5 UK6)		M222F018 [c] (11.7 UK3)		13 UK7		M222F004 (18.8 EBIS)			
	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean	s.s.	mean		
0.5	0.1	0.2	0.1	0.2	3.3	3.7	0.1	0.2	0.1	0.1	--	--	2.3	2.3	0.1	0.1	6.1	5.7	1.4	16
1.0	0.2		0.3		4.0		0.4		0.1		--		2.3		0.1		5.4		1.3	18
2.0	0.1	0.1	0.0	0.1	5.5	5.1	0.3	0.4	0.1	0.1	0.0	0.0	2.5	2.1	0.1	0.1	8.2	6.9	1.0	16
2.5	0.2		0.1		4.8		0.4		0.1		--		1.7		0.1		5.5		0.7	25
6.0	0.1	0.1	0.2	0.2	6.7	9.2	0.8	0.8	0.1	0.3	0.0	0.0	1.3	1.5	0.3	0.3	8.8	14	1.0	26
6.5	0.2		0.1		12		0.8		0.5		--		1.6		0.3		19		1.6	28
24.0	0.3	0.4	0.4	0.6	41	50	0.8	0.8	1.1	1.3	1.3	1.9	0.6	0.6	1.9	1.9	27	26	2.2	18
24.5	0.5		0.7		60		0.7		1.5		2.5		0.7		1.9		25		1.4	13
48.0	0.6	0.6	1.0	1.0	75	80	0.4	0.3	0.3	0.4	2.7	3.1	--	0.2	2.5	2.2	7.7	8.1	1.4	10
48.5	0.7		1.1		85		0.2		0.6		3.4		0.2		2.0		8.6		0.7	3.6
96.0	1.3	1.2	0.8	0.9	92	97	0.1	0.1	0.0	0.2	3.6	3.8	0.8	0.8	2.2	1.9	3.2	1.6	2.2	insigni-ficant
96.5	1.1		0.9		102		0.1		0.3		4.1		--		1.5		0.1		0.3	
168.0	2.3	1.9	1.4	1.6	103	105	--	--	0.2	0.2	4.2	4.4	--	--	1.5	1.3	1.3	1.3	1.9	
168.5	1.5		1.8		106		--		--		4.6		--		1.2		--		1.4	

s.s.= Single sample
[a] Activity of peaks < 1% TAR and/or increased background
[b] Detected by direct LSC but not by radio HPLC (potentially metiram particles that do not pass the column/precolumn)
[c] tentatively assigned

C. ESTIMATION OF HALF-LIVES

The results of the experiments with non-radiolabeled metiram by quantification via CS_2 indicate short half-lives (DT_{50}) between 0.6 days (pH 4) and 2 days (pH 5). Half-lives of 3 days and 1.8 days were calculated for pH 7 and pH 9. However, the latter are considered to be effected by the presence of the degradation product EBIS that is known to form also CS_2 after digestion. EBIS was present in substantial amounts at early sampling time points at pH 7 (25-35% TAR) and pH 9 (26% TAR). This interpretation is supported by the observation of fast solubilization of the total applied radioactivity (% TAR) at pH 7 and 9. Thus, it can be assumed that hydrolysis of polymeric metiram at pH 7 and 9 is much faster than derived from the digestion and CS_2 formation method. The half-lives obtained from Single First Order Kinetics of non-radiolabeled metiram + EBIS are summarized in Table 7.2.1.1-14.

Table 7.2.1.1-14: Summary of Single First Order Kinetics of non-radiolabeled metiram+EBIS at pH 4, 5, 7, and 9 at 25°C

pH	Rate constant K [h^{-1}]	DT_{50} [h]	Mean DT_{50} [h]	Mean DT_{50} [d]
4	0.0467	14.8	14	0.6
	0.0533	13.0		
5	0.0139	49.9	49	2.0
	0.0144	48.3		
7	0.0090	77.0	72	3.0
	0.0104	66.4		
9	0.0144	48.2	44	1.8
	0.0174	39.9		

A separate kinetic evaluation was performed including the metabolites that exceed 5% TAR at the different pH levels into the kinetic analysis using a compartment modeling approach as recommended by the FOCUS kinetics workgroup. Whereas, because of the very low water solubility of the parent compound, the observed "not dissolved activity" in the experiment with radiolabeled test item was considered as metiram residue.

From these estimations it can be concluded that metiram has short $DegT_{50}$ of 1.0 day (pH 4), 0.9 day (pH 5), 0.1 day (pH 7) and 0.4 day (pH 9).

The estimated trigger endpoints of metiram and its metabolites at the different pH levels are listed from Table 7.2.1.1-15 to Table 7.2.1.1-18.

Table 7.2.1.1-15: Estimated trigger endpoints for metiram and its metabolites (pH 4)

Compound	Kinetic model	χ^2 error [%]	Trigger endpoints	
			$DegT_{50}$	$DegT_{90}$
Metiram	DFOP	6.9	1.0 d	9.9 d
M222F011	SFO	2.8	4.9 d	16.2 d
ETU	SFO	3.1	The ETU rate constant was not estimated significantly different from zero	

Table 7.2.1.1-16: Estimated trigger endpoints for metiram and its metabolites (pH 5)

Compound	Kinetic model	χ^2 error [%]	Trigger endpoints	
			$DegT_{50}$	$DegT_{90}$
Metiram	DFOP	20.4	0.9 d	4.5 d
EBIS	SFO	24.5	1.3 d	4.2 d
ETU	SFO	4.4	The ETU rate constant was not estimated significantly different from zero	

Table 7.2.1.1-17: Estimated trigger endpoints for metiram and its metabolites (pH 7)

Compound	Kinetic model	χ^2 error [%]	Trigger endpoints	
			$DegT_{50}$	$DegT_{90}$
Metiram	DFOP	20.0	0.1 d	0.7 d
EBIS	SFO	8.0	6.8 d	22.6 d
ETU	SFO	9.2	The ETU rate constant was not estimated significantly different from zero	
EU	SFO	11.1	The EU rate constant was not estimated significantly different from zero	

Table 7.2.1.1-18: Estimated trigger endpoints for metiram and its metabolites (pH 9)

Compound	Kinetic model	χ^2 error [%]	Trigger endpoints	
			$DegT_{50}$	$DegT_{90}$
Metiram	SFO	20.2	0.4 d	1.3 d
EBIS	SFO	28.6	0.6 d	2.0 d
ETU	SFO	11.9	The ETU rate constant was not estimated significantly different from zero	

III. CONCLUSION

The results of hydrolysis experiments indicated short DT_{50} for metiram. Based on experiments with radiolabeled metiram DT_{50} values of 1.0 day (pH 4), 0.9 day (pH 5), 0.1 day (pH 7) and 0.4 day (pH 9) were estimated.

Based on the results with non-radiolabeled metiram slightly higher DT_{50} of 0.6 d, 2.0 d, 3.0 d and 1.8 d were derived, which are however considered to be effected by the presence of the degradate EBIS (M222F004).

Several degradates of metiram were detected. ETU (M222F002) is a major and stable hydrolysis product of metiram at all pHs examined. EBIS (M222F004) is a significant hydrolysis product of metiram at all pHs except at pH 4. EBIS degrades fast at pH 7 and even faster at pH 5 and 9. EU (M222F003) was formed under all pH conditions except at pH 4, however only at max 6% TAR. M222F011 was only observed in relevant amounts at pH 4 and increased to about 13% TAR after 4 days and is considered as major hydrolysis product formed under acidic conditions.

Peer reviewed study (Summary taken from the Monograph or its Addenda):

Report:	*CA 7.2.1.1/3* *Carpenter M., 1987b* *Hydrolysis as a function of pH at 25°C of 14C-Ethylenethiourea* *1987/10154*
Guidelines:	*EPA 161-1*
GLP:	*yes*

Materials and Methods:

The fate of ^{14}C-ETU in sterile water at pH 5, 7 and 9 was investigated at 25°C ± 1°C in the dark. Application rate was 10 mg L^{-1} (nominal). Buffers used: pH 5: 0.05 M acetate buffer; pH 7: 0.05 M HEPES buffer, 0.05 M TRIS buffer; pH 9: 0.1 M borate buffer. The buffers were sterilized by autoclaving.
The samples were incubated up to 30 days. Analytical procedures were LSC and radio-TLC with confirmation by radio-HPLC.

Findings:

Analysis of the samples showed that no detectable degradation of ETU occurred in any of the test samples. The degradation seen on the TLC plates was entirely attributable to photolytic degradation or oxidation during sample application and plate elution. Examination of time zero and subsequent sample points revealed that the longer it took to spot and elute a TLC plate the more degradation appeared in the sample.
Hence, due to the stability of ETU at pH 5, 7, and 9 no degradation rate could be calculated.

Conclusion:

ETU was stable at pH 5, 7 and 9.

For the ETU hydrolysis study described above [see CA 7.2.1.1/3, BASF DocID 1987/10154] a new kinetic evaluation was conducted in order to consider current guidance of the FOCUS kinetics workgroup [FOCUS (2006)]. The kinetic evaluation is summarized in CA 7.2.1.1/4 [BASF DocID 2014/1228265].

Report:	CA 7.2.1.1/4 Pape L., 2015b Kinetic evaluation of two studies on aqueous photolysis and hydrolysis of Ethylenethiourea (ETU) according to FOCUS Degradation Kinetics 2014/1228265
Guidelines:	FOCUS Degradation Kinetics (2006) SANCO/10058/2005 version 1.1 of December 2014
GLP:	no

Executive Summary

The hydrolytic behavior of ethylenethiourea (ETU) has been investigated in four sterile solutions buffered at three different pH values. The purpose of this evaluation was to analyze the degradation kinetics of ETU observed in this study, taking into account the current guidance of the FOCUS workgroup on degradation kinetics. Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints).

The visual assessment of the SFO kinetic fit approaches for the different pH test systems were acceptable. The χ^2 - error values were <15 %. But the ETU degradation rate constants could not be estimated significantly different from zero. Consequently, no reasonable kinetic endpoints could be derived.

I. MATERIAL AND METHODS

The hydrolytic behavior of ETU (metabolite of metiram) has been investigated in four experiments in one study [*CA 7.2.1.1/3, BASF DocID 1987/10154*]. The resulting data were analyzed taking into account the current guidance of the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*].

The degradation endpoints were derived for use as triggers for additional work (trigger endpoints).

Kinetic models included in the evaluations

As the degradation of ETU is expected to follow SFO kinetics, biphasic models were not tested if the SFO kinetic model was considered appropriate to derive reliable endpoints.

The SFO kinetic model was considered appropriate if the residuals ware randomly distributed around zero (visual fit), the χ^2 - error value was low (ideally < 15 %) and the estimated degradation parameters were significantly different from zero as outlined by FOCUS

The software package KinGUI version 2.2012.320.1629 [*SCHÄFER et al. (2007); SCHMITT et al. (2011)*] was used for parameter fitting.

Experimental data

The hydrolytic degradation of ^{14}C-labeled ETU was investigated at 25 ± 1°C for a period of 30 days in four sterile solutions buffered at pH 5, pH 7 (HEPES buffer), pH 7 (Tris buffer), and pH 9 [*see CA 7.2.1.1/3, BASF DocID 1987/10154*].

The measured data as well as resulting datasets submitted to kinetic analysis are summarized in Table 7.2.1.1-19 and Table 7.2.1.1-20.

Table 7.2.1.1-19: Data for kinetic evaluation for pH 5 buffered and pH 7 (HEPES buffer) test systems

Day	Experimental data [% TAR]	Input data according to FOCUS [% TAR]	Experimental data [% TAR]	Input data according to FOCUS [% TAR]
	pH 5 buffered test system		pH 7 (HEPES buffer) test system	
0	86.0	100.0 [a]	81.3	100.0 [a]
0.79	56.4	56.4	63.4	63.4
2.61	86.8	86.8	87.4	87.4
6.67	81.0	81.0	81.3	81.3
14.7	83.9	83.9	86.3	86.3
16.9	68.5	68.5	68.5	68.5
21.8	85.3	85.3	85.0	85.0
29.7	78.9	78.9	83.3	83.3

TAR = Total applied radioactivity
[a] Set to material balance

Table 7.2.1.1-20: Data for kinetic evaluation for pH 7 (Tris buffer) and pH 9 buffered test systems

Day	Experimental data [% TAR]	Input data according to FOCUS [% TAR]	Experimental data [% TAR]	Input data according to FOCUS [% TAR]
	pH 7 (Tris buffer) test system		pH 9 buffered test system	
0	91.5	100.0 [a]	73.9	100.0 [a]
0.79	57.6	57.6	60.6	60.6
2.61	85.5	85.5	83.1	83.1
6.67	80.2	80.2	77.5	77.5
14.7	86.0	86.0	76.5	76.5
16.9	69.2	69.2	67.4	67.4
21.8	85.8	85.8	81.7	81.7
29.7	81.4	81.4	81.4	81.4

TAR = Total applied radioactivity
[a] Set to material balance

II. RESULTS AND DISCUSSION

The visual assessment of the SFO kinetic fit approaches for the different pH test systems were acceptable. The χ^2 - error values were <15 %. But the ETU degradation rate constants could not be estimated significantly different from zero. Consequently, no reasonable kinetic endpoints could be derived.

III. CONCLUSION

The hydrolytic behavior of ETU (metabolite of metiram) has been investigated in four sterile solutions buffered at three different pH values.

For the four experiments of the hydrolysis study no significant degradation of ETU could be observed. Consequently, no kinetic endpoints could be derived.

Special study

Dimethyl sulfoxide (DMSO) is known to solubilize metiram. In older studies metiram was applied after dissolving in DMSO. It is assumed that the dissolved material is no longer intact metiram. This study was set up in order to characterize the material obtained by dissolving metiram in DMSO.

Report:	CA 7.2.1.1/5 Walter W., 2013a HPLC/UV/LSC Pattern of 14C-Metiram degradation products in Dimethyl sulfoxide 2013/1339078
Guidelines:	none
GLP:	yes (certified by Landesamt fuer Umwelt, Messungen und Naturschutz Baden Wuerttemberg, Karlsruhe, Germany)

Executive Summary

The objective of the study was to assess the degradation of ^{14}C-metiram in dimethyl sulfoxide (DMSO). Experiments were performed to determine the degradation of metiram, and to examine the identity or nature of degradation products.

The degradation of metiram in DMSO was determined by adding ^{14}C-labeled metiram to DMSO. The suspension was subsequently stirred continuously and kept at 25°C in the dark. Aliquots of the mixture were taken at various time-points and filtered through 0.22 µm high-speed-centrifuge-filter for subsequent HPLC-UV/LSC determination of the degradation products.

Metiram was immediately dissolved in DMSO and after 0.5 h the entire radioactivity was found in the filtrate. Two known degradation products of metiram, ethylene thiourea (ETU) and ethylene bisisothiocarbamate (EBIS) were formed from the beginning. ETU increased with prolonged degradation time to more than 80% of the total applied radioactivity (TAR). EBIS reaches its maximum of 10 to 18% TAR within the first 6 h, but was present in minor amounts (≤ 1% TAR) at later time points. Several other unknown degradates were formed from the beginning and/or with prolonged degradation time. Except for unknown metabolite 3 (UK3) with maximum of 8% TAR, the other metabolites did not exceed 3% TAR.

A significant amount of activity was detected as increased background which decreased from about 30% to 10% TAR with prolonged degradation time. Another portion of radioactivity passed the filter but not the HPLC column. This portion was quite significant and decreased from about 40% in the beginning to 5% TAR with prolonged degradation time.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

BAS code:	BAS 222 F
Common name:	metiram
Reg. No.:	250284
Chemical name (IUPAC):	zinc ammoniate ethylenebis(dithiocarbamate)-poly(ethylenethiuram disulfide)
Molecular formula:	$(C_{16}H_{33}N_{11}S_{16}Zn_3)_x$
Molecular weight:	1088.6 g mol^{-1} (theoretical value for monomeric compound)
Radiolabel:	ethylene-^{14}C
Batch No.:	153-6001
Chemical Purity:	89.0% (by releasable CS_2)
Specific Activity:	7.64 MBq g^{-1}

B. STUDY DESIGN

1. Experimental conditions

The degradation of metiram was determined by adding about 5 mg of radiolabeled metiram to 5 mL dimethyl sulfoxide (DMSO). After homogenization with stainless steel balls for 5 min, the balls were separated with a stainless steel sieve and both were rinsed with fresh DMSO. The DMSO fractions were combined and filled up to a volume of 40 mL with DMSO and subsequently stirred. Two aliquots of each 15 mL were placed in 20 mL brown-glass vials with a magnetic stir bar and the suspensions were placed on a magnetic stirrer in a thermostated cabinet at 25°C in the dark.

2. Sampling

Sampling times were 0.5, 1, 2/2.5, 6/6.5, 24/24.5 and 96/96.5 hours.

3. Description of the analytical procedures

At various time-points, 0.5 mL aliquots of the mixture were taken and filtered through 0.22 µm high-speed-centrifuge-filters for subsequent HPLC-UV/LSC determination of the degradation products.

II. RESULTS AND DISCUSSION

Metiram was immediately dissolved in DMSO and after 0.5 h the entire radioactivity was found in the filtrate, which was further investigated by radio HPLC analysis.

Ethylene thiourea (ETU) was formed from the beginning and the amount increased with prolonged degradation time resulting in the main formed metabolite with more than 80% of the total applied radioactivity (TAR). Ethylene bisisothiocarbamate (EBIS) was formed with 10% to 18% TAR in the first 6 hours, but was present only in minor amounts (≤ 1% TAR) at the later time points. Several other unknown degradates were formed from the beginning and/or with prolonged degradation time. However, other metabolites did not exceed 3% TAR, except unknown metabolite 3 (UK3) with a temporary maximum of 8% TAR. The summary of the results are shown in Table 7.2.1.1-21.

Another portion of radioactivity in the filtrate did not pass the HPLC column and did not result in a HPLC radio signal. This portion was significant at the beginning with around 40% TAR, but decreased to amounts < 5% TAR after 24 hours. This radioactivity was calculated as the difference between the total dissolved radioactivity and the amount of radioactivity eluting from the HPLC column. It may be interpreted as very small suspended metiram particles or as metiram oligomers being temporarily formed while metiram is disintegrated in DMSO.

Table 7.2.1.1-21: Summary of degradation products of ^{14}C-metiram in DMSO at 25°C

Approx. solvolysis time	**Assigned peak retention time [d]**								**Sum of areas not assigned [b]**	**Dissolved but not detected activity [c]**
	4.5 ETU	7.3 UK8	7.7 UK2	9.1 UK5	10.8 UK9	11.7 UK3	13.7 UK10	18.4 EBIS		
hours	% of TAR (Inj.)									
0.5	14.5	-	-	0.4	0.1	5.5	2.7	10.2	25.9	44.1
1.0	17.3	-	0.8	0.6	0.1	5.8	2.2	13.8	27.5	35.2
2.0	20.1	-	0.5	0.7	0.2	5.5	3.0	17.6	31.0	24.4
2.5	25.7	-	0.4	0.5	0.4	6.0	2.8	17.1	28.1	22.0
6.0	45.5	-	1.0	0.3	0.2	8.0	1.5	15.6	19.3	10.7
6.5	42.8	1.0	1.1	0.6	0.2	7.5	1.6	13.3	19.5	15.6
24.0	83.6	2.0	1.6	0.8	0.2	2.7	0.4	0.7	9.4	1.3
24.5	80.7	1.8	1.9	1.0	-	3.2	1.0	1.1	15.1	0.0 [e]
96.0	88.4	2.4	0.9	1.3	0.9	0.2	0.2	0.2	5.8	3.7
96.5	81.0	1.9	1.6	1.4	0.5	-	0.4	0.1	11.1	5.9

[b] Activity that cannot be assigned as peak and results in increased background
[c] Detected by direct LSC, but not by LC/LSC
[d] Peaks assigned according to hydrolysis study run in parallel
[e] nominal -2.1%
- = not detected

III. CONCLUSION

Solid Metiram was immediately dissolved in DMSO and after 0.5 h the entire radioactivity was found in the filtrate of a 0.22 µm centrifuge filter.

ETU and EBIS were the main degradation products already formed from the beginning with maxima of 80% TAR (ETU) and 10 to 18% TAR (EBIS). Several other unknown degradates were formed from the beginning and/or with prolonged degradation time. However, those metabolites did not exceed 3% TAR, except UK3 with a temporary maximum of 8% TAR.

This study shows that metiram dissolved in DMSO is no longer intact metiram but degradates thereof. Pretreatment with DMSO is not suitable for the investigation of the environmental fate of metiram since degradates instead of the parent compound are applied.
However, the main degradation products are the same that are formed in hydrolysis (ETU, EBIS). For this reason the use of DMSO may be acceptable for other types of investigations.

CA 7.2.1.2 Direct photochemical degradation

Studies presented in the first Annex I inclusion process:

In the first Annex I inclusion process a water photolysis study [*Klein W., 1985, DocID 1985/0499*], an absorption coefficient study [*see CA 7.2.1.2/1, BASF DocID 1991/10727*] and a quantum yield study [*see CA 7.2.1.2/2, BASF DocID 1992/10088*] were submitted. Furthermore, a study on the aqueous photolysis of ^{14}C-ETU was presented.

The aqueous photolysis study [*Klein W., 1985, DocID 1985/0499*] is considered invalid for the following reasons:
In “experiment A” metiram was suspended in water. While this is the correct approach, a wrong light source (mercury lamp) was used for irradiation.
Also, major deficiencies were observed in the further conduct of the study. No material balance is given for any sampling time. Only dissolved radioactivity was measured and related to the initial applied radioactivity. Effects like adsorption to the vial surface were not investigated but must be assumed from the unexpected slow increase of the dissolved radioactivity.
The quantification of the solvolysis products is misleading. Values represent “% radioactivity in the sample”, not “% of applied” (which would be much lower but can hardly be derived from the report).
For these reasons, results of this part of the study are considered not valid.
In “experiment B” and “experiment C” metiram was pre-dissolved in DMSO before application. This results in fast decomposition to various degradates and therefore, not the active substance but its degradates (solvolysis products) were applied in these parts of the study.
Generally, the quantification of metabolites relied solely on radio-TLC analysis which was later on shown to be not a suitable method for the investigation of degradation products of metiram [*see CA 7.1.1.1/5, BASF DocID 1993/10578*]. Although HPLC is mentioned in the report of Klein W., 1985, this was not used for quantification.

Generally, the quantification of metabolites relied solely on radio-TLC analysis which was later on shown to be not a suitable method for the investigation of degradation products of metiram [*see CA 7.1.1.1/5, BASF DocID 1993/10578*]. Although HPLC is mentioned in the report, this was not used for quantification.

In the studies on the absorption coefficient and the quantum yield, the test item metiram was dissolved in DMSO. Since DMSO leads to a decomposition of metiram, these measurements are considered to reflect the characteristics of the degradates of metiram. However, since these are no fate studies but physical measurements and since it is not possible to perform these measurements with suspended instead of dissolved material, these studies are reported here.

The study of Carpenter M. and Fennessey M. [*BASF DocID 1987/10153*] concerning the determination of the photolysis rate of ^{14}C-ETU in aqueous solution is considered still valid and is summarized in CA 7.2.1.2/4.

Submission of not yet peer-reviewed studies in this AIR3-Dossier:

A new aqueous photolysis study was performed with metiram and is summarized in chapter CA 7.2.1.2/3 [BASF DocID 2014/1189252].
For the photolysis study with ETU [see CA 7.2.1.2/4, BASF DocID 1987/10153] a new kinetic evaluation was conducted following the recommendations of FOCUS [FOCUS (2006)] and is summarized in chapter CA 7.2.1.2/5 [BASF DocID 2014/1228265].

Peer reviewed study (Summary taken from the Monograph or its Addenda):

Report:	*CA 7.2.1.2/1* *Sarafin R., 1991a* *Metiram (BAS 222 .. F): Absorption coefficients* *1991/10727*
Guidelines:	*BBA IV 6-1*
GLP:	*yes* *(certified by Ministerium fuer Arbeit, Soziales, Familie und Gesundheit, Postfach 3180, 6500 Mainz)*

Materials and Methods:
Metiram (0.546 mg) was dissolved in DMSO (25 mL). The spectrum of the resulting solution was recorded in the range 290 - 800 nm. The background spectrum (DMSO) was automatically subtracted by the instrument. In order to avoid deviations from linearity at large absorbance values, measurements were repeated with a diluted solution (1:3) where absorbance values exceeded 0.8 (0.182 mg metiram per 25 mL DMSO).
Absorption coefficients were calculated according to the formula

$$\varepsilon = \frac{A \times M \times 100000}{c \times d \times w}$$

A: absorption [AU]
M: molar mass [g mol^{-1}] = 1088.7
c: concentration of test solution [mg L^{-1}]
d: path length of cell [cm]
w: purity of test substance [%]

Findings:

Strong absorption was observed for metiram dissolved in DMSO (i.e. for the solvolysis products) up to about 400 nm leading to extinction coefficients exceeding 10 L mol^{-1} cm^{-1} by orders of magnitude.

Conclusion:
Light quanta in the wave length range up to about 400 nm may be absorbed by metiram which may lead to photolytic disintegration of the molecule.

Peer reviewed study (Summary taken from the Monograph or its Addenda):

Report:	*CA 7.2.1.2/2* *Sarafin R., 1992a* *Metiram - Determination of quantum yield* *1992/10088*
Guidelines:	*BBA IV 6-1, OECD Draft Test Guideline Phototransformation of Chemicals in Water (January 1990)*
GLP:	*yes* *(certified by Ministerium fuer Arbeit, Soziales, Familie und Gesundheit, Postfach 3180, 6500 Mainz)*

Materials and Methods:

[^{14}C]-labeled metiram (label position in the thiocarbonyl moiety) was dissolved in DMSO/H_2O (1/1) at a concentration of 7.5 µg mL^{-1} (nominally). The solution was irradiated at 200 nm for 24 h (first experiment) and 5 h (second experiment). Irradiation and determination of absorbed photons was performed in a Quantacount electronic actinometer. Decomposition of metiram was tentatively followed by radio-TLC.
The quantum yield Φ in mol/Einstein was calculated according to the formula

where $$\mathbf{\Phi} = \frac{N_{lost}}{N_{abs}}$$

and $$\mathbf{N_{lost}} = \frac{C_{dark} - C_{irr}}{MM}$$

and $$\mathbf{N_{abs} = F \times CQ}$$

N_{lost}: Metiram equivalents lost during irradiation in µmol
C_{start}: µg mL^{-1} of metiram equivalents in start solution
C_{final}: µg mL^{-1} of metiram equivalents in final solution
MM: Molar mass in g mol^{-1}
$V_{(irr)}$: Volume of irradiated solution
N_{abs}: Number of absorbed photons
F: Calibration factor in µEinstein/count (determined by calibration with a Fe (III) oxalate actinometer)
CQ: Counts displayed by the Quantacount software

Findings:

Chromatography revealed considerable dark reactions in the case of the first irradiation. In the case of the second irradiation degradation of metiram solution was even more severe, already at the beginning of irradiation as shown by the absorption data (1st irradiation: 0.210, 2nd irradiation: 0.067). The theoretical value should have been in the order of 0.27.
Because of the dark reactions no conclusions could be drawn regarding the initial attack on the parent molecule.
However, the losses of radioactivity during irradiation indicated the formation of a volatile degradation product. This product was most probably $^{14}CS_2$ which was produced by cleavage of the respective C-N bond. So, the losses could be interpreted in terms of a total breakdown of metiram.
Considering the larger uncertainty in the evaluation of the second irradiation, the quantum yield can be assumed to be at least 0.001.
The experiments unambiguously show that metiram degrades in aqueous solution and that this process is promoted under the influence of irradiation with wavelengths present in sunlight reaching the earth's surface.
The quantum yield and the absorption coefficients given allow to calculate the half life of direct photodegradation for locations in Central Europe using a special computer program [*Frank R., Klöpffer W. (1989): A convenient model and program for the assessment of abiotic degradation of chemicals in natural water, Ecotox. Environ. Safety 17, Pp. 323 - 332*]. With this program a half life of < 6.3 h upon continuous irradiation or < 0.6 calendar days was calculated. This data is based on a scenario when metiram is applied in march with a day length of 11.6 h.

Conclusion:

The data seem to indicate that the degradation process of metiram in water is promoted under the influence of light.

Report:	CA 7.2.1.2/3 Class T., 2015b Aqueous photolysis of 14C-Ethylene-labelled Metiram at pH 5, 7, and 9 2014/1189252
Guidelines:	OECD 316 (Photodegradation in Water)
GLP:	yes (certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

Executive Summary

The objective of the study was to assess the aqueous photolysis and transformation of metiram in water at three different pH levels (pH 5, 7, and 9).
The rate of photolysis of metiram was determined by adding non-radiolabeled metiram to aqueous pH 5, 7, and 9 buffer solutions. Irradiated samples were kept in quartz containers in a Suntest CPS^+ artificial sunlight apparatus thermostated at 22±1°C. Samples were exposed continuously to artificial sunlight from a xenon arc lamp equipped with a UV-filter system for up to 7 days allowing transmission from 290 to 800 nm with a light intensity of ≈ 3 mW cm^{-2} (≈ 30 W m^{-2}) in the relevant ≈ 300 – 400 nm region. Dark controls were kept in a thermostated cabinet at 22°C in the dark.
The results indicate short half-lives for direct aqueous photolysis of 0.9 days (pH 5), 0.8 days (pH 7) and 0.5 days (pH 9).

In a separate experiment, radiolabeled metiram was added to aqueous pH 5, 7, and 9 buffer solutions to examine potential photolysis products. The irradiated and dark control samples were exposed/kept for 15 days as described above. Samples were filtered to remove suspended polymeric metiram. Radioactive material retained by the filter (i.e. polymeric metiram) was dissolved with DMSO and quantified by liquid scintillation counting (LSC). Not retained radioactivity was analyzed by LSC and radio-HPLC. Degradates were identified and confirmed by structure elucidation work using high-resolution mass spectrometry (HRMS) and co-chromatography.

Several degradates of metiram were detected. In the aqueous photolysis, ETU is a major transformation product of metiram at all pH levels examined. EU is a major product at pH 7 and 9. As further major hydrolysis product of metiram, EBIS is photolyzed rapidly.
Additionally, a very polar major peak containing M222F011, M222F012 and M222F017 is formed in considerable amounts at all pH levels.
Furthermore, degradates M222F013/M222F015, M222F016 and M222F018 were observed as intermediate photolysis products. However, all of them amounted to ≤ 6% TAR after 15 days of light exposure.

The experiments with radiolabeled metiram were subjected to a kinetic evaluation using a compartment model. $DegT_{50}$ values of 0.3 days (pH 5), 0.1 days (pH 7), and 0.2 days (pH 9) were estimated for metiram.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

BAS-Code: BAS 222 F (metiram)
Registry No.: 250284
Chemical name: Zinc ammoniate ethylenebis(dithiocarbamate)-poly-(ethylenethiuramdisulfide)
Molecular formula: $(C_4H_9N_3S_4Zn)_3$; $C_4H_6N_2S_4$
Molar mass: 1088.7 g mol^{-1}

Labeled test item:
Label: ^{14}C-ethylene
Batch No.: 153-6001
Specific activity of a.s.: 7.64 MBq mg^{-1}
Releasable CS_2: 89.0%

Non-labeled test item:
Batch No.: 300014
Purity: 92.7%

2. Test system

Photolysis was investigated in buffer at three different pH values. All buffer solutions were sterilized and saturated with air. The following buffer solutions were used:

pH 5: citric acid / sodium hydroxide
pH 7: potassium dihydrogen phosphate / disodium hydrogen phosphate
pH 9: borax / hydrochloric acid

B. STUDY DESIGN

1. Experimental conditions

Rate of aqueous photolysis/hydrolysis
For aqueous photolysis/hydrolysis experiments, 20 mL of non-radiolabeled metiram stock solutions/suspensions (about 60 µg mL^{-1}) prepared in buffer solutions (pH 5, 7, and 9) were transferred into a quartz photolysis flask with septum-sealed side arm. Irradiated samples were placed into the irradiation chamber (Suntest CPS^+ artificial sunlight apparatus) thermostated at 22 ± 1°C and exposed continuously to artificial sunlight from the xenon arc lamp equipped with a UV-filter system allowing transmission from 290 to 800 nm with a light intensity of ≈ 3 mW cm^{-2} in the relevant ≈ 300 – 400 nm region. Dark control samples were placed into a thermostated cabinet at 22°C. Samples were prepared in duplicates and incubated for up to 168 hours.

Formation of degradates
For the determination of aqueous photolysis/hydrolysis degradates, a 20 mL suspension of ^{14}C-labeled metiram (about 60 µg mL^{-1} ^{14}C-labeled metiram, 200 µg mL^{-1} total metiram) prepared in various buffer solutions (pH 5, 7, and 9) were transferred into a quartz photolysis flask with septum-sealed side arm and placed into the irradiation chamber (thermostated with a water bath at 22°C) for photolysis rate determination or into a thermostated cabinet (tempered at 22°C) for dark control samples. Samples were incubated for up to 360 hours under the same conditions as described above.

2. Sampling

At various time points (0, 2, 6, 24, 48, 72 and 168 hours), samples were taken for determination of the rate of aqueous photolysis/hydrolysis of non-radiolabeled metiram.

For determination of degradates, aliquots were taken at various time points (0.5, 2, 6, 24, 72, 168 and 360 hours).

3. Description of analytical procedures

Rate of hydrolysis
Samples of the suspensions with non-radiolabeled metiram were taken and dosed into 20 mL headspace vials. A solution of 20% (w/v) $SnCl_2$ in 5 N HCl and isooctane was added, the headspace vial was sealed, and the solution was digested at 80°C in a shaking water bath for 20 min. Then, the samples were shaken for 10 min on a horizontal shaker and an aliquot of the isooctane phase was analyzed by GC-MS/MS.

Formation of degradates
Samples of the suspension were filtered (0.22 µm, Nylon centrifuge filter) and analyzed by LC/UV/LSC and radio-HPLC.
The filters were extracted with dimethyl sulfoxide (DMSO) by shaking for 20 min in order to dissolve solid polymeric metiram. The activity in the DMSO extracts was determined by LSC for quantification of metiram and calculation of the mass balance.
To identify and confirm the degradates, structure elucidation with high-resolution mass spectrometry (HRMS) and co-chromatography was performed.

Trapping of volatiles
The trapping of volatiles was performed after each sampling interval by purging the headspace of the photolysis flask with synthetic air with subsequently bubbling the air flow through three volatile traps filled with trapping solutions in-line.
The first trap was filled with 0.5 M $NaOH_{aq}$ to trap $^{14}CO_2$, the second trap was filled with 1.0 M KOH in methanol for trapping of (non-radioactive) CS_2 and the third trap hold ethylene glycol for trapping of organic volatiles.
Aliquots of all traps were counted by LSC for the presence of ^{14}C volatiles. Aliquots of the second trap containing 1.0 M KOH were processed for CS_2 determination by adding an aliquot of the 1.0 M KOH into a 20 mL headspace vial. After addition of isooctane and 2.0 M HCl, the headspace vial was sealed and shaken for 30 min. An aliquot of the isooctane phase was analyzed by GC-MS/MS.

4. Calculation of half-lives

Peak areas of the CS_2 formed from remaining metiram and apparently from its degradation product ethylene bisisothiocarbamate (M222F004, EBIS) were evaluated via single first order kinetics to calculate (using Excel) rate constants k for dark control and irradiated samples. Subsequently, the difference k_d was used to estimate the half-lives $t_{1/2}$ for photolysis of metiram at various pH.

Additionally, half-lives of metiram were determined in a kinetic evaluation based on the experiments with radiolabeled metiram. Since no specific recommendation is available how to carry out kinetic evaluations for aqueous photolysis experiments, the recommendations of the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*] have been applied. The software package KinGUI2 (version 2.2012.320.1629) was used for parameter fitting [*SCHÄFER et al. (2007)*].

The $DegT_{50}$ and $DegT_{90}$ endpoints of the parent compound metiram and its main metabolites EBIS (pH 7, pH 9), ETU (pH 5, pH 7, pH 9), EU (pH 5, pH 7, pH 9), M222F016 (pH 5, pH 7), and M222F018 (pH 5, pH 7) were derived from a compartment modeling approach. In the case that no reliable endpoints for the metabolites could be derived from the compartment model but a clear dissipation of the metabolite was observed, metabolite decline fits were performed, applying SFO kinetics only, in order to derive conservative endpoint estimates for the metabolite. The following kinetic models were employed for the evaluation: SFO, FOMC, and DFOP.

The observed “not dissolved activity” from the experiments with radiolabeled metiram was considered as metiram residue; the day 0 values for the parent compound were set equal to the mass balance values. As no limit of quantification (LOQ) was provided, it was conservatively assumed to be 0.1% TAR, and the limit of detection (LOD) was set equal to this value. The metabolite residues considered for the kinetic evaluation were taken from the LC/LSC determinations.

II. RESULTS AND DISCUSSION

A. MASS BALANCE

Dissolved radioactivity in the buffer solutions, not-dissolved radioactivity on the filter, formation of volatiles and the resulting mass balances for the aqueous photolysis of ^{14}C-labeled metiram and dark control are summarized in Table 7.2.1.2-1 to Table 7.2.1.2-3. The calculated mass balances for the irradiated samples resulted in averages between 94% TAR and 101% TAR.
Amounts of radioactivity between 0.01 to 0.6% TAR were detected within the alkaline CO_2 trapping for pH 5 photolysis samples only. Insignificant radioactivity (≤ 0.02% TAR) was detected in alkaline (1.0 M KOH in methanol) and ethylene glycol volatile traps after purging of the headspace of the photolysis and dark control samples.
The 1.0 M KOH in methanol intended for trapping of (non-radioactive) CS_2 was examined - after liquid/liquid extraction - by GC/MS/MS. The results indicated that non-radiolabeled CS_2 formed in irradiated samples was well below 2% TAR (total applied residue) equivalent. For the dark controls the CS_2 formed was <5% TAR.

Table 7.2.1.2-1: Non-dissolved/dissolved activity, volatile traps, and mass balance of metiram at pH 5 at 22°C [% TAR]

Photolysis time	Dissolved activity (Buffer)		Not dissolved activity (Filter)		Volatiles Traps						Mass Balance	
					0.5M $NaOH_{aq}$ (presumably CO_2)		1M KOH in methanol		Ethylene glycol			
Irradiated samples												
hours	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean
0.5	38	39	63	61	--	--	--	--	--	--	101	100
0.5	40		60		--		--		--		100	
2.0	29	30	75	72	0.00	0.00	--	--	--	--	103	102
2.0	31		69		0.00		--		--		100	
6.0	39	40	68	63	0.00	0.00	--	--	--	--	107	103
6.0	40		58		0.01		--		--		99	
24	82	81	21	21	0.01	0.03	0.00	0.00	--	--	103	102
24	81		20		0.05		0.00		--		101	
72	95	95	7.4	7.3	0.12	0.23	0.01	0.01	--	--	102	102
72	94		7.1		0.34		0.01		--		102	
168	97	96	3.4	2.7	0.31	0.47	0.02	0.02	--	--	101	99
168	94		1.9		0.63		0.02		--		97	
360	92	91	1.4	1.3	0.05	0.12	0.01	0.01	--	--	94	92
360	90		1.1		0.19		0.02		--		91	
					Total	0.9			Average (n = 14)		100	
Dark control												
hours	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean
0.5	38	39	65	63	--	--	--	--	--	--	103	102
0.5	40		61		--		--		--		101	
2.0	45	48	52	50	0.00	0.00	--	--	--	--	96	98
2.0	52		47		n.d.		--		--		100	
6.0	58	52	44	49	0.00	0.00	--	--	--	--	101	101
6.0	46		55		0.00		--		--		101	
24	54	51	47	50	0.00	0.00	--	--	--	--	101	100
24	48		52		0.01		--		--		100	
72	62	59	39	42	0.01	0.00	--	--	--	--	101	101
72	55		45		0.00		--		--		100	
168	71	68	32	34	0.00	0.00	--	--	--	--	102	102
168	65		36		0.00		--		--		101	
360	81	79	22	24	0.00	0.00	--	--	--	--	103	103
360	76		26		0.00		--		--		103	
					Total	0.0			Average (n = 14)		101	

TAR = Total applied radioactivity
n.d. < 100 dpm

Table 7.2.1.2-2: Non-dissolved/dissolved activity, volatile traps, and mass balance of metiram at pH 7 at 22°C [% TAR]

Photolysis time	Dissolved activity (Buffer)		Not dissolved activity (Filter)		Volatiles Traps: 0.5M $NaOH_{aq}$ (presumably CO_2)		Volatiles Traps: 1M KOH in methanol		Volatiles Traps: Ethylene glycol		Mass Balance	
Irradiated samples												
hours	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean
0.5	11	11	90	89	--	--	--	--	--	--	100	100
0.5	11		89		--		--		--		100	
2.0	46	49	53	49	0.00	0.00	--	--	--	--	99	98
2.0	52		45		0.01		--		--		97	
6.0	67	68	29	30	0.01	0.01	0.00	0.00	--	--	96	98
6.0	69		31		0.01		0.00		--		100	
24	88	89	10	9.8	0.01	0.02	0.00	0.00	--	--	99	98
24	89		9.3		0.02		0.00		--		98	
72	94	94	4.2	3.7	0.02	0.02	0.00	0.00	--	--	98	98
72	95		3.3		0.02		0.00		--		98	
168	97	96	3.6	2.8	0.08	0.07	0.00	0.00	--	--	101	99
168	95		2.0		0.06		0.00		--		97	
360	97	93	1.4	1.5	0.07	0.07	0.00	0.00	--	--	98	94
360	89		1.7		0.07		0.00		--		90	
					Total	0.2	Average (n = 14)				98	
Dark control												
hours	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean
0.5	10	11	82	84	--	--	--	--	--	--	92	95
0.5	12		87		--		--		--		98	
2.0	22	23	73	72	--	--	--	--	--	--	95	95
2.0	24		72		--		--		--		95	
6.0	25	27	60	64	--	--	--	--	--	--	85	91
6.0	28		68		--		--		--		97	
24	32	34	59	59	--	--	--	--	--	--	91	93
24	36		60		--		--		--		95	
72	38	41	52	50	0.00	0.00	--	--	--	--	89	92
72	45		49		0.00		--		--		94	
168	39	41	54	54	0.00	0.00	--	--	--	--	93	95
168	42		54		0.00		--		--		97	
360	46	47	47	47	0.00	0.00	--	--	--	--	93	94
360	49		47		0.00		--		--		96	
					Total	0.0	Average (n = 14)				94	

TAR = Total applied radioactivity
n.d. < 100 dpm

Table 7.2.1.2-3: Non-dissolved/dissolved activity, volatile traps and mass balance of metiram at pH 9 at 22°C [% TAR]

Photolysis time	Dissolved activity (Buffer)		Not dissolved activity (Filter)		Volatiles Traps: 0.5M $NaOH_{aq}$ (presumably CO_2)		Volatiles Traps: 1M KOH in methanol		Volatiles Traps: Ethylene glycol		Mass Balance	
Irradiated samples												
hours	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean
0.5	21	21	70	73	--	--	--	--	--	--	92	93
0.5	20		75		--		--		--		95	
2.0	31	33	61	61	0.01	0.00	0.00	0.00	--	--	92	94
2.0	35		61		0.00		0.00		--		96	
6.0	45	51	48	43	0.00	0.00	0.00	0.00	--	--	93	94
6.0	57		38		0.00		0.00		--		95	
24	80	84	12	9.3	0.00	0.00	0.00	0.00	--	--	92	93
24	88		6.7		0.01		0.00		--		95	
72	90	92	1.5	1.5	0.00	0.00	--	--	--	--	92	93
72	93		1.5		0.00		--		--		94	
168	91	92	1.4	1.5	0.01	0.01	--	--	--	--	92	93
168	93		1.6		0.01		--		--		94	
360	92	93	1.3	1.3	0.01	0.01	0.00	0.00	--	--	93	94
360	94		1.4		0.01		--		--		95	
					Total	0.0			Average (n = 14)		94	
Dark control												
hours	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean
0.5	22	21	71	74	--	--	--	--	--	--	93	95
0.5	20		76		--		--		--		96	
2.0	23	23	70	70	0.00	0.00	--	--	--	--	93	93
2.0	24		70		0.00		--		--		94	
6.0	24	25	70	69	0.00	0.00	--	--	--	--	94	94
6.0	25		68		0.00		--		--		93	
24	35	36	59	59	0.00	0.00	--	--	--	--	94	95
24	37		58		0.00		--		--		95	
72	53	54	39	39	0.00	0.00	--	--	--	--	92	93
72	54		39		0.00		--		--		94	
168	83	84	7.2	8.1	0.00	0.00	--	--	--	--	91	93
168	85		9.0		0.00		--		--		94	
360	89	90	2.7	2.8	0.00	0.00	--	--	--	--	92	93
360	92		2.9		0.00		--		--		94	
					Total	0.0			Average (n = 14)		94	

TAR = Total applied radioactivity
n.d. <100 dpm

B. FINDINGS

Metiram

The degree of aqueous photolysis/hydrolysis (in percent of applied) for experiments with non-radiolabeled metiram is summarized in Table 7.2.1.2-4 to Table 7.2.1.2-6. Already after 72 hours, a degree of > 90% was observed for the irradiated samples at all pH levels, indicating a fast photolysis. The degree of hydrolysis after 168 hours of the dark control samples ranged from 45 to 54% at pH 5, 31 to 34% at pH 7 and 45% at pH 9.

Table 7.2.1.2-4: Aqueous photolysis/hydrolysis of non-radiolabeled metiram at pH 5 and 22°C for irradiated sample and dark control

Time [hours]	Irradiated, Degree of Photolysis [%]		Dark control, Degree of Hydrolysis [%]	
	Repl. 1	Repl. 2	Repl. 1	Repl. 2
0	0	0	0	0
2	38	37	4	1
6	60	61	28	21
24	80	77	49	39
72	94	94	51	41
168	99	n.d.	54	45

n.d. = no remaining metiram detected

Table 7.2.1.2-5: Aqueous photolysis/hydrolysis of non-radiolabeled metiram at pH 7 and 22°C for irradiated sample and dark control

Time [hours]	Irradiated, Degree of Photolysis [%]		Dark control, Degree of Hydrolysis [%]	
	Repl. 1	Repl. 2	Repl. 1	Repl. 2
0	0	0	0	0
2	26	20	4	3
6	64	63	14	20
24	87	87	21	22
72	98	99	24	26
168	99	100	31	34

Table 7.2.1.2-6: Aqueous photolysis/hydrolysis of non-radiolabeled metiram at pH 9 and 22°C for irradiated sample and dark control

Time [hours]	Irradiated, Degree of Photolysis [%]		Dark control, Degree of Hydrolysis [%]	
	Repl. 1	Repl. 2	Repl. 1	Repl. 2
0	0	0	0	0
2	17	13	-1	2
6	45	38	21	21
24	87	79	5	6
72	99	99	9	12
168	n.d.	n.d.	45	45

n.d. = no remaining metiram detected

<u>Degradates</u>
The results of the formation of degradates are summarized in
Table 7.2.1.2-7 to
Table 7.2.1.2-9.

At pH 5, a major polar peak was formed containing M222F011, M222F012 and M222F017, which increased to 26% TAR after 7 days; after 15 days 19% TAR were still present. M222F002 (ETU) increased continuously up to 36% TAR after 1 day and subsequently declined to 6% TAR. M222F003 (EU) increased to 5% TAR after 3 days, then declined to < 2 % TAR after 15 days. M222F016 reached about 11% TAR after 1 day and subsequently was no longer detectable after day 3. M222F018 was observed at about 7% TAR on the initial sampling but decreased to non-detectable levels already after >1 day. All other photolysis products were observed only at percentages at or below 5% TAR.

At pH 7, a major polar peak was formed containing M222F011, M222F012, and M222F017 reaching 31% TAR after 15 days. M222F002 (ETU) increased continuously up to 57% TAR after 3 days and subsequently declined to 32% TAR. M222F004 (EBIS) increased to 8% TAR after 2 hours and subsequently disappeared completely in samples >1 day after treatment. M222F003 (EU) increased to 18% TAR after 15 days. M222F016 reached about 9% TAR after 1 day, the n declined to about 3% TAR on day 3 and values < 2% TAR on longer irradiation times. M222F018 reached about 21% TAR after 6 hours of irradiation, declined to about 5% TAR on day 3 and was no longer detected on longer irradiation times. All other degradation products were observed only at percentages at or below 5% TAR.

At pH 9, a polar peak was formed containing M222F011, M222F012, and M222F017 that increased to 7% TAR at the end of the incubation. Less polar degradates were M222F013 and M222F015 occurring in sum in a maximum amount of 15% TAR after 3 days of irradiation and subsequently decreasing to about 5% after 15 days of irradiation. M222F002 (ETU) increased to 35% TAR after 1 day and subsequently declined to 15% TAR after 15 days of irradiation. M222F003 (EU) increased continuously to 49% TAR after 15 days. All other degradation products were observed only at percentages at or below 5% TAR.

Additionally, the formation of a multitude of minor unknown products was observed. Radio-chromatograms showed to varying extent non-assigned minor peaks respectively background signals. The sum of the increased background areas consisting presumably of a multitude of non-resolved metiram degradation products, which significantly increased for pH 5 irradiated (360 h) sample aliquots, reaching about 40% TAR after 360 hours of irradiation. pH 7 irradiated (360 h) sample aliquots resulted in a sum of background areas of about 13% TAR; pH 9 irradiated (360 h) sample aliquots resulted in a sum of background areas of about 11% TAR.

HPLC recovery was indicated by the difference between the % TAR nominally injected (based on direct LSC of non-retained radioactivity passing the syringe filter), and the total radio-activity counted by the radio-chromatographic detector. In
Table 7.2.1.2-7 to
Table 7.2.1.2-9 this is represented as “dissolved but not detected activity”.
This difference is presumably caused by extremely fine ^{14}C-metiram particles which passed the syringe filter but not the HPLC injector/column system. For pH 7 and pH 9 samples (irradiated and dark), the differences were always <10% TAR, thus indicating acceptable HPLC recoveries. Only at pH 5 (which also showed increased background with prolonged irradiation times) for several but not all samples the differences were significantly above the 10% TAR threshold, supporting the assumption that oligomeric metiram products passing the filter prior to HPLC were partially retained at the pre-column, causing rather incomplete HPLC recovery (as difference between nominally injected and actually detected after chromatography). A potential explanation for this observation could be that the pH 5 assay was not perfectly homogenized and coarser particles of the suspended test item were not completely photolyzed/hydrolyzed at the time of sampling.

The presence of ETU, EBIS, and EU was confirmed in the structure elucidation phase by high-resolution mass spectrometry (HRMS) and co-chromatography with the corresponding reference item. Furthermore the chemical structure of metabolites M222F011, M222F012, M222F013, M222F014, M222F015, M222F016, M222F017, M222F018, M222F019 and M222F020 was elucidated by high-resolution mass spectrometry.

Table 7.2.1.2-7: Formation of degradates by aqueous photolysis of ^{14}C-metiram irradiated at pH 5 and 22°C [% TAR]

hours	Assigned peak retention time [min]																		Sum of areas [a] not assigned	Dissolved but not detected activity [b]
	M222F011 / M222F012 / M222F017 (1:20)		M222F003 (2:00 EU)		M222F019 / M222F020 (2:10)		M222F019 / M222F020 (2:20)		M222F002 / M222F014 (3:40 ETU)		M222F013 / M222F015 (6:30)		M222F016 (8:40)		M222F018 (11:50)		M222F004 (20:00 EBIS)			
	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean		
Irradiated samples																				
0.5	0.4	0.4	--	--	--	--	--	--	2.0	5.2	--	--	--	--	6.4	7.4	2.0	2.4	13.8	13.5
0.5	0.4		--		--		--		8.5		--		--		8.3		2.8		25.7	insigni-ficant <10% TAR
2.0	1.4	1.5	1.1	1.2	--	--	--	--	13.8	15.0	--	--	0.8	1.0	3.5	3.4	1.0	1.3	9.2	
2.0	1.6		1.2		--		--		16.3		--		1.1		3.3		1.6		11.4	
6.0	1.1	1.6	0.8	1.2	--	--	--	--	9.9	15.0	--	--	1.3	2.0	3.1	2.9	1.2	1.0	5.0	15.9
6.0	2.2		1.6		--		--		20.1		--		2.6		2.8		0.8		8.8	insigni-ficant <10% TAR
24	8.4	8.5	4.3	4.0	--	--	--	--	38.6	35.7	--	--	10.1	10.5	3.6	2.5	2.0	2.0	13.4	
24	8.7		3.7		--		--		32.9		--		10.9		1.4		--		14.4	
72	30.9	23.7	5.3	4.6	2.9	2.3	2.1	1.5	5.2	8.9	6.3	4.6	4.3	5.5	--	--	--	--	17.7	
72	16.5		3.9		1.7		1.0		12.5		3.0		6.7		--		--		14.0	27.6
168	27.3	25.7	3.1	3.8	2.3	2.2	2.4	2.3	5.6	5.8	6.1	6.3	--	--	--	--	--	--	30.3	14.1
168	24.2		4.6		2.2		2.1		5.9		6.5		--		--		--		32.0	14.2
360	20.8	18.5	2.0	1.7	3.5	2.3	--	2.3	7.0	6.2	4.6	4.5	--	--	--	--	--	--	41.3	9.2
360	16.2		1.4		1.0		2.3		5.4		4.4		--		--		--		34.2	21.3
Dark control (hydrolysis) samples																				
0.5	0.2	0.2	--	--	--	--	--	--	2.8	7.6	--	--	--	--	7.0	7.0	2.2	2.3	12.4	13.3
0.5	0.3		--		--		--		12.4		--		--		7.0		2.3		15.3	insigni-ficant <10% TAR
2.0	0.6	0.5	--	--	--	--	--	--	15.6	20.8	--	--	--	--	5.7	5.6	1.3	1.3	18.0	
2.0	0.5		--		--		--		26.1		--		--		5.5		1.3		14.4	
6.0	0.2	0.4	--	--	--	--	--	--	14.6	23.1	--	--	--	--	2.7	2.2	0.8	0.8	9.9	29.4
6.0	0.6		--		--		--		31.7		--		--		1.7		0.7		10.4	insignificant <10% TAR
24	1.1	1.1	--	--	--	--	--	--	47.8	43.6	--	--	0.5	0.5	0.8	0.8	0.7	0.8	7.4	
24	1.0		--		--		--		39.4		--		0.5		0.7		0.8		7.7	
72	1.8	1.9	--	--	--	--	--	--	52.1	49.0	--	--	0.8	0.8	0.5	0.5	0.8	0.9	10.1	
72	2.0		--		--		--		45.9		--		0.8		0.4		1.0		8.3	
168	1.2	2.0	--	--	--	--	--	--	30.0	41.8	--	--	0.4	0.5	--	--	0.4	0.6	7.1	31.5
168	2.9		--		--		--		53.6		--		0.7		--		0.9		11.8	insigni-ficant <10% TAR
360	3.5	3.5	1.6	1.8	--	--	--	--	72.9	68.7	--	--	1.4	1.3	--	--	0.4	0.6	19.7	
360	3.6		1.9		--		--		64.6		--		1.2		--		0.7		19.1	

[a] Activity of peaks < 2% TAR and/or increased background; [b] Detected by direct LSC but not by LC/LSC (particles that do not pass the column/precolumn, difference between total LSC and LC/LSC Run-Area)

Table 7.2.1.2-8: Formation of degradates by aqueous photolysis of ^{14}C-metiram irradiated at pH 7 and 22°C [% TAR]

hours	Assigned peak retention time [min]																		Sum of areas [a] not assigned	Dissolved but not detected activity [b]
	M222F011 / M222F012 / M222F017 (1:20)		M222F003 (2:00 EU)		M222F019 / M222F020 (2:10)		M222F002 (3:40 ETU)		M222F013 / M222F015 (6:30)		UK (7:10)	M222F016 (8:40)		M222F018 (11:50)		M222F004 (20:00 EBIS)			
	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean	mean	single sample	mean	single sample	mean	single sample	mean		
Irradiated samples																				
0.5	0.2	0.2	--	--	--	--	1.2	1.5	--	--	--	--	--	1.7	1.9	2.1	2.1	4.9	Insignificant, <10% TAR
0.5	0.3		--		--		1.8		--			--		2.0		2.2		4.6	
2.0	1.1	1.5	1.0	0.8	--	--	8.8	10.0	0.3	0.3	1.3	1.5	1.9	12.6	13.2	7.9	7.8	7.0	
2.0	2.0		0.6		--		11.3		0.3			2.3		13.7		7.7		9.3	
6.0	3.1	3.4	2.0	1.9	--	--	17.2	17.5	1.0	1.1	3.0	4.5	4.6	21.7	20.5	3.0	3.2	1.2	
6.0	3.7		1.7		--		17.8		1.1			4.7		19.4		3.5		8.5	
24	6.7	6.8	3.6	3.6	--	--	36.4	38.4	2.9	3.0	1.6	9.1	8.9	16.9	15.9	1.3	1.0	6.8	
24	6.8		3.7		--		40.4		3.0			8.7		14.9		0.8		8.2	
72	8.3	8.4	5.6	5.2	1.3	1.1	54.8	57.1	4.2	4.4	--	4.2	3.4	6.4	5.1	--	--	7.9	
72	8.5		4.7		0.9		59.3		4.6			2.5		3.8		--		9.8	
168	19.1	18.7	12.1	11.4	1.7	1.7	47.5	47.8	4.8	4.8	--	0.8	0.8	--	--	--	--	10.8	
168	18.3		10.8		1.7		48.0		4.9			0.7		--		--		9.2	
360	30.0	30.7	2.3 [c]	18.0	1.5	1.5	30.5	31.6	4.9	5.0	2.1	2.8	1.7	--	--	--	--	15.3	
360	31.4		18.0		--		32.7		5.2			0.7		--		--		10.1	
Dark control (hydrolysis) samples																				
0.5	0.3	0.3	--	--	--	--	1.4	1.7	--	--	--	--	--	1.6	2.0	1.6	2.0	4.1	Insignificant, <10% TAR
0.5	0.3		--		--		2.0		--			--		2.3		2.3		5.4	
2.0	0.3	0.3	--	--	--	--	5.4	6.0	--	--	--	--	--	2.3	2.4	8.9	8.1	6.0	
2.0	0.3		--		--		6.7		--			--		2.5		7.4		6.4	
6.0	0.5	0.5	0.2	0.2	--	--	7.5	8.2	--	--	0.4	0.3	0.2	1.6	1.4	12.1	11.7	4.2	
6.0	0.4		0.2		--		8.9		--			0.2		1.3		11.3		5.9	
24	0.8	0.7	0.3	0.3	--	--	8.9	9.8	--	--	1.5	1.0	0.9	1.4	1.3	13.4	13.6	2.5	
24	0.7		0.4		--		10.8		--			0.8		1.2		13.9		6.6	
72	0.9	1.0	1.2	1.2	--	--	12.2	13.5	--	--	1.7	2.8	2.8	0.8	0.9	12.8	12.9	5.8	
72	1.1		1.2		--		14.7		--			2.7		1.0		13.0		7.0	
168	1.2	1.3	2.5	2.6	--	--	17.9	19.0	--	--	1.2	3.1	3.4	0.3	0.3	6.1	6.3	6.9	
168	1.3		2.6		--		20.2		--			3.8		0.2		6.4		6.4	
360	1.5	1.6	4.1	4.4	--	--	26.4	27.2	--	--	0.6	2.0	2.0	0.4	0.4	5.4	5.9	4.6	
360	1.6		4.7		--		28.0		--			2.0		0.4		6.3		6.1	

[a] Activity of peaks < 2% TAR and/or increased background; [b] Detected by direct LSC but not by LC/LSC (particles that do not pass the column difference between total LSC and LC/LSC Run-Area); [c] excluded

Table 7.2.1.2-9: Formation of degradates by aqueous photolysis of ^{14}C-metiram irradiated at pH 9 and 22°C [% TAR]

hours	Assigned peak retention time [min]																Sum of areas [a] not assigned	Dissolved but not detected activity [b]
	M222F011 / M222F012 / M222F017 (1:20)		M222F003 (2:00 EU)		M222F019 / M222F020 (2:20)		M222F002 / M222F014 (3:40 ETU)		M222F013 / M222F015 (6:30)		UK (9:00)	M222F018 (11:50)		M222F004 (20:00 EBIS)			
	single sample	mean	single sample	mean	single sample	mean	single sample	mean	single sample	mean	mean	single sample	mean	single sample	mean		
Irradiated samples																	
0.5	0.3	0.3	--	0.3	--	--	3.2	3.1	--	--	--	2.1	2.0	4.6	4.6	7.4	insignificant, <10% TAR
0.5	0.3		0.3		--		3.1		--			2.0		4.6		8.3	
2.0	2.1	1.8	1.1	1.4	0.2	0.1	10.9	10.6	1.9	1.6	0.6	0.3	1.2	2.2	1.5	5.8	
2.0	1.5		1.7		0.1		10.4		1.4			2.1		0.8		9.1	
6.0	3.2	3.2	3.2	3.6	0.2	0.2	17.1	17.9	5.4	6.1	1.1	0.2	1.3	1.7	1.7	7.7	
6.0	3.1		4.0		0.2		18.7		6.8			2.5		--		12.9	
24	3.9	4.3	14.9	15.8	1.3	1.4	34.6	35.2	13.4	14.0	2.8	1.6	1.6	--	--	12.5	
24	4.6		16.7		1.6		35.7		14.6			1.7		--		13.1	
72	4.2	4.7	29.3	31.3	2.5	2.3	26.8	27.2	13.2	14.5	3.6	--	--	--	--	9.4	
72	5.3		33.3		2.2		27.7		15.8			--		--		13.8	
168	5.2	5.9	41.3	41.1	2.3	2.5	18.9	21.7	9.4	9.8	3.5	--	--	--	--	9.7	
168	6.6		40.9		2.8		24.5		10.1			--		--		9.0	
360	6.6	7.0	49.7	49.0	2.0	2.3	12.6	15.3	5.1	5.4	3.4	--	--	--	--	11.0	
360	7.3		48.2		2.6		18.0		5.6			--		--		10.6	
Dark control (hydrolysis) samples																	
0.5	0.4	0.3	0.0	0.1	--	--	2.8	2.9	--	--	--	1.9	1.8	5.0	5.1	7.6	insignificant, <10% TAR
0.5	0.2		0.3		--		3.0		--			1.8		5.2		7.8	
2.0	0.4	0.4	0.0	0.2	--	--	4.0	4.4	--	--	--	1.2	1.1	3.8	4.1	9.1	
2.0	0.4		0.3		--		4.8		--			1.1		4.5		9.2	
6.0	0.4	0.4	0.3	0.3	--	--	5.1	5.4	--	--	0.2	1.0	1.1	3.7	3.8	8.3	
6.0	0.3		0.3		--		5.6		--			1.2		3.9		9.9	
24	0.5	0.5	0.4	0.5	--	--	8.8	10.4	--	--	0.4	1.1	1.1	2.7	2.7	10.5	
24	0.5		0.5		--		12.0		--			1.1		2.8		11.8	
72	0.7	0.8	0.7	0.6	--	--	19.4	20.6	--	--	0.6	--	--	2.4	2.1	13.4	
72	0.9		0.5		--		21.8		--			--		1.9		12.7	
168	1.3	1.3	1.7	1.5	--	--	54.3	55.3	0.7	0.7	2.3	--	--	2.8	3.0	10.1	
168	1.3		1.4		--		56.3		--			--		3.3		10.9	
360	2.4	2.4	2.0	2.1	--	--	64.7	65.7	0.7	0.7	3.0	--	--	0.0	0.0	10.5	
360	2.4		2.1		--		66.7		0.6			--		0.0		9.7	

[a] Activity of peaks < 2% TAR and/or increased background; [b] Detected by direct LSC but not by LC/LSC (particles that do not pass the column/precolumn, difference between total LSC and LC/LSC Run-Area)

C. ESTIMATION OF HALF-LIVES

The results of the experiments with non-radiolabeled metiram were evaluated via single first order kinetics to calculate rate constants k for dark control and irradiated samples. Subsequently, the difference k_d was used to estimate the photolytical DT_{50}. The results of the experiments with quantification via CS_2 indicate short half-life values (DT_{50}) of 22 hours (pH 5), 19 hours (pH 7), and 11 hours (pH 9). However, it should be considered that the estimated DT_{50} is affected to some extent by the presence of the degradation product EBIS that is known to form also CS_2 after digestion. EBIS was present at early sampling time points in irradiated and in dark dark control samples also at later time points. The half-lives obtained from single first order kinetics of non-radiolabeled metiram+EBIS are summarized in Table 7.2.1.2-10.

Table 7.2.1.2-10: Summary of Single First Order Kinetics of non-radiolabeled metiram+EBIS at pH 5, 7, and 9 at 22°C

	Irradiated samples			Dark control samples			Difference (irradiated minus dark control)	
pH	k_{obs} [h^{-1}]	DT_{50} [h]	Mean DT_{50} [h]	k_{obs} [h^{-1}]	DT_{50} [h]	Mean DT_{50} [h]	k_d [h^{-1}]	DT_{50} [h]
5	0.0320	22	19	0.0059	117	137	0.0320	22 [a]
	0.0424	16		0.0045	156			
7	0.0353	20	18	0.0026	268	254	0.0367	19 [a]
	0.0435	16		0.0029	240			
9	0.0729	10	11	0.0032	216	212	0.0629	11 [a]
	0.0595	12		0.0033	209			

$k_d = k_{irradiated} - k_{dark}$
[a] $DT_{50} = \ln2 / k_d$

A separate kinetic evaluation was performed including the metabolites that exceed 5% TAR at the different pH levels into the kinetic analysis using a compartment modeling approach as recommended by the FOCUS kinetics workgroup. Metabolite peaks, which represented more than one metabolite and have not been measured and reported separately exceeding 5% TAR were not included in the kinetic evaluation. Whereas, because of the very low water solubility of the parent compound, the observed “not dissolved activity” in the experiment with radiolabeled test item was considered as metiram residue.

From these estimations of irradiated samples, it can be concluded that metiram has short $DegT_{50}$ of 0.3 days (pH 5), 0.1 days (pH 7), and 0.2 days (pH 9).

The estimated trigger endpoints of metiram and its metabolites in irradiated and dark control samples at the different pH levels are listed in Table 7.2.1.2-11 to Table 7.2.1.2-16.

Table 7.2.1.2-11: Estimated endpoints for metiram and its metabolites (pH 5, irradiated)

Compound	Kinetic model	χ^2 error [%]	Trigger endpoints	
			DT_{50}	DT_{90}
Metiram	DFOP	13.7	0.3 d	1.5 d
ETU	No reliable endpoints derived			
EU	SFO	14.6	6.1 d	20.2 d
M222F016	SFO [a]	11.1	1.8 d	5.9 d
M222F018	SFO	22.8	0.1 d	0.2 d

[a] Decline fit

Table 7.2.1.2-12: Estimated endpoints for metiram and its metabolites (pH 5, dark control)

Compound	Kinetic model	χ^2 error [%]	Trigger endpoints	
			DT_{50}	DT_{90}
Metiram	DFOP	2.9	0.7 d	29.5 d
ETU	The ETU rate constant was not estimated significantly different from zero			
M222F018	SFO	10.4	0.1 d	0.3 d

Table 7.2.1.2-13: Estimated endpoints for metiram and its metabolites (pH 7, irradiated)

Compound	Kinetic model	χ^2 error [%]	Trigger endpoints	
			DT_{50}	DT_{90}
Metiram	FOMC	13.2	0.1 d	0.8 d
EBIS	SFO	31.3	<0.1 d	0.1 d
ETU	SFO [a]	0.8	14.2 d	47.1 d [b]
EU	SFO	7.4	>1000 d	>1000 d
M222F016	SFO [a]	19.5	1.5 d	4.9 d
M222F018	SFO	10.1	0.8 d	2.7 d

[a] Decline fit
[b] Calculated

Table 7.2.1.2-14: Estimated endpoints for metiram and its metabolites (pH 7, dark control)

Compound	Kinetic model	χ^2 error [%]	Trigger endpoints	
			$DegT_{50}$	$DegT_{90}$
Metiram	DFOP	3.6	13.9 d	104.3 d
EBIS	SFO	11.1	6.0 d	19.8 d
ETU	The ETU rate constant was not estimated significantly different from zero			

Table 7.2.1.2-15: Estimated endpoints for metiram and its metabolites (pH 9, irradiated)

Compound	Kinetic model	χ^2 error [%]	Trigger endpoints	
			$DegT_{50}$	$DegT_{90}$
Metiram	DFOP	4.8	0.2 d	1.0 d
ETU	SFO	13.8	6.9 d	22.9 d
EU	The EU rate constant was not estimated significantly different from zero			

Table 7.2.1.2-16: Estimated endpoints for metiram and its metabolites (pH 9, dark control)

Compound	Kinetic model	χ^2 error [%]	Trigger endpoints	
			$DegT_{50}$	$DegT_{90}$
Metiram	DFOP	4.3	1.8 d	8.4 d
EBIS	No acceptable visual fit could be derived			
ETU	No acceptable visual fit could be derived			

III. CONCLUSION

The results of aqueous photolysis experiments indicated a very fast degradation of metiram.

Several degradates of metiram were detected. In the aqueous photolysis, ETU is a major transformation product of metiram at all pH levels examined. EU is a major product at pH 7 and 9. As further major hydrolysis product of metiram, EBIS is photolyzed rapidly.
Additionally, a very polar major peak containing M222F011, M222F012 and M222F017 is formed in considerable amounts at all pH levels.
Furthermore, degradates M222F013/M222F015, M222F016 and M222F018 are observed as intermediate photolysis products. However, all of them amounted to ≤ 6% TAR after 15 days of light exposure.
Based on experiments with radiolabeled metiram DT_{50} values of 0.3 days (pH 5), 0.1 days (pH 7), and 0.2 days (pH 9) were estimated.

Peer reviewed study (Summary taken from the Monograph or its Addenda):

Report:	*CA 7.2.1.2/4* *Carpenter M.,Fennessey M., 1987a* *Determination of the photolysis rate of 14C-Ethylenethiourea in pH 7 aqueous solution* *1987/10153*
Guidelines:	*EPA 161-2*
GLP:	*yes*

Materials and Methods:

ETU dissolved in 0.05 M TRIS buffer pH 7 at a concentration of 10 µg L^{-1} (nominal) was irradiated with a Xe lamp continuously at about one-half the intensity of the sun. This was to simulate the diurnal cycle of light and dark. Unsensitized and sensitized (0.75% (v/v) acetone as photosensitizer) conditions were studied. Duplicate samples were taken at each sampling time (from 0 up to 30 days) and analyzed by LSC, radio-TLC and radio-HPLC. A device to trap volatiles was available (ethylene glycol, H_2SO_4, KOH).

Findings:

ETU was degraded fast when irradiated with acetone as sensitizer. The half life calculated by first order kinetics was about two days. The half lives of the unsensitized exposed and dark samples were instead much higher and lay in the range between 76 days and 97 days.
TLC analysis of the sensitized exposed samples showed EU and an unidentified component called "ui C" being the main photolytic degradation products. However, it also shown that the method mainly used for quantitative and qualitative characterization, i.e. TLC, yielded decomposition during the application of the aqueous solution onto the TLC plate (see also the hydrolysis study with ETU).

Conclusion:

ETU is very fast degraded by light when photosensitizers are present which is common in nature.

Report: CA 7.2.1.2/5
Pape L., 2015b
Kinetic evaluation of two studies on aqueous photolysis and hydrolysis of Ethylenethiourea (ETU) according to FOCUS Degradation Kinetics
2014/1228265

Guidelines: FOCUS Degradation Kinetics (2006) SANCO/10058/2005 version 1.1 of December 2014

GLP: no

Executive Summary

The aqueous photolytic behavior of ethylenethiourea (ETU) has been investigated in two test systems in one study. The purpose of this evaluation was to analyze the degradation kinetics of ETU observed in this study, taking into account the current guidance of the FOCUS workgroup on degradation kinetics. Kinetic evaluation was performed in order to derive degradation parameters as triggers for additional work (trigger endpoints).

The $DegT_{50}$ values for the non-sensitized test system were 69.7 days and 77.7 days for the irradiated sample and dark control, respectively. $DegT_{90}$ were 231.5 days and 258.3 days, respectively.

The $DegT_{50}$ values for the test system sensitized with acetone were 1.9 days and 79.4 days for the irradiated sample and dark control, respectively. $DegT_{90}$ were 6.5 days and 263.8 days, respectively.
Although the degradation observed under non-sensitized conditions and in the dark control are mainly due to decomposition on the TLC plate, it is obvious that the degradation in the presence of photosensitizers is much accelerated.

I. MATERIAL AND METHODS

The aqueous photolytic behavior of ETU (metabolite of metiram) has been investigated in two test systems in one study [*see CA 7.2.1.2/4, BASF DocID 1987/10153*]. The resulting data were analyzed taking into account the current guidance of the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*].

Details of the kinetic modeling strategy, models included in the evaluation and data handling are already described in CA 7.2.1.1/4 [*see chapter CA 7.2.1.1 Hydrolytic degradation*].

Experimental data

The photolytic degradation of ^{14}C-labeled ETU was investigated for a period of 30 days at pH 7 in a buffered sterile aqueous solution (non-sensitized test system) or in a buffered sterile aqueous solution sensitized with 0.75% v/v acetone solution (sensitized test system) [*see 7.2.1.2/4, BASF DocID 1987/10153*]. ETU was applied at a nominal rate of 10 µg mL^{-1}.

The volatility of the test substance was evaluated in a separate test. No loss of the activity of the test compound due to volatilization was observed.

The measured data as well as resulting datasets submitted to kinetic analysis are summarized in Table 7.2.1.2-17 and Table 7.2.1.2-18.

Table 7.2.1.2-17: Data for kinetic evaluation for non-sensitized test system

Day	Experimental data [%TAR]		Input data according to FOCUS [%TAR]	
	Dark control	Irradiated	Dark control	Irradiated
0	88.0	88.0	100.0 [a]	100.0 [a]
0.89	81.4	81.8	81.4	81.8
2.78	87.7	87.2	87.7	87.2
6.7	77.9	79.3	77.9	79.3
13.8	84.2	81.2	84.2	81.2
20.7	73.8	76.0	73.8	76.0
29.8	69.1	65.6	69.1	65.6

TAR = Total applied radioactivity
[a] Set to material balance

Table 7.2.1.2-18: Data for kinetic evaluation for sensitized test system

Day	Experimental data [%TAR]		Input data according to FOCUS [%TAR]	
	Dark control	Irradiated	Dark control	Irradiated
0	90.3	90.3	100.0 [a]	100.0 [a]
0.89	81.8	73.8	81.8	73.8
2.78	89.6	42.1	89.6	42.1
6.7	79.4	0.0	79.4	0.2 [b]
13.8	83.4	7.8	83.4	7.8
20.7	79.0	4.2	79.0	4.2
29.8	67.8	0.0	67.8	0.2 [b]

TAR = Total applied radioactivity
[a] Set to material balance
[b] set to 1/2LOQ

II. RESULTS AND DISCUSSION

The SFO model was appropriate to derive kinetic endpoints for ETU (Table 7.2.1.2-19). For the evaluated test systems, the visual assessment and the goodness-of-fit statistics show plausible fit. The low t-test values of the degradation rate constants ($p<0.05$) indicate that the parameters were estimated significantly different from zero. Therefore, the resulting $DegT_{50}$ values can be considered reliable.

Table 7.2.1.2-19: Kinetic endpoints for the degradation of ETU in the aqueous photolysis study

Test system	Incubation	Kinetic model	χ^2 error [%]	Kinetic endpoints	
				$DegT_{50}$ [d]	$DegT_{90}$ [d]
Non-sensitized	dark control	SFO	5.6	77.7	258.3
	irradiated	SFO	5.2	69.7	231.5
Sensitized with acetone	dark control	SFO	5.3	79.4	263.8
	irradiated	SFO	12.1	1.9	6.5

III. CONCLUSION

The aqueous photolytic behavior of ETU (metabolite of metiram) has been investigated in two test systems in one study.

The $DegT_{50}$ values (trigger endpoints) for the non-sensitized test system were 69.7 days and 77.7 days for the irradiated sample and dark control, respectively. $DegT_{90}$ were 231.5 days and 258.3 days, respectively.

The $DegT_{50}$ values (trigger endpoints) for the test system sensitized with acetone were 1.9 days and 79.4 days for the irradiated sample and dark control, respectively. $DegT_{90}$ were 6.5 days and 263.8 days, respectively.

Although the degradation observed under non-sensitized conditions and in the dark control are mainly due to decomposition on the TLC plate, it is obvious that the degradation in the presence of photosensitizers is much accelerated.

CA 7.2.1.3 Indirect photochemical degradation

No studies on indirect photochemical degradation were performed.

CA 7.2.2 Route and rate of biological degradation in aquatic systems

CA 7.2.2.1 "Ready biodegradability"

Studies presented in the first Annex I inclusion process:

In the first Annex I inclusion process the study of Lungershausen, R. [see CA 7.2.2.1/1, BASF DocID 1992/10646] was presented which is considered still valid.

Submission of not yet peer-reviewed studies in this AIR3-Dossier:

In the new aqueous photolysis study, M222F017 (= imidazole) was reported as a significant degradation product. However, this study type is considered to represent highly artificial abiotic conditions. Under biotic conditions, the environmental behavior of imidazole is quite different, i.e. it will be fast degraded which can be derived from the study of Schwarz, H. [see CA 7.2.2.1/2, BASF DocID 2003/1034559].

Peer reviewed study (Summary taken from the Monograph or its Addenda):

Report:	*CA 7.2.2.1/1* *Lungershausen R., 1992a* *Determination of biodegradability of Metiram premix - Closed bottle test* *1992/10646*
Guidelines:	*OECD 301 D*
GLP:	*yes* *(certified by Ministerium fuer Umwelt und Gesundheit, Postfach 3160, 6500 Mainz)*

Materials and Methods:

Metiram, a mineral salt solution and sewage (concentration: 2 mg L^{-1}) were mixed and incubated at 20°C – 25°C in BOD-bottles up to 28 DAT (closed bottle test). The oxygen consumption was measured with an oxygen electrode. Control and blank tests were run simultaneously.

Findings:

The biodegradability of metiram was 34% of COD after 28 days, i.e. the test substance was moderately or only partially biodegradable. Metiram exhibited at the test concentration no inhibition on the biodegradation of the control substance sodium benzoate, i.e. metiram had no toxic effect.

Conclusion:

Metiram was moderately or only partially biodegradable under the test conditions used.

Report: CA 7.2.2.1/2
Schwarz H., 2003a
Imidazol - Determination of the biodegradability in the DOC Die-Away test
2003/1034559
Guidelines: ISO 7827 (1994), OECD 301 A, EEC 92/69 C 4 A
GLP: yes
(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)

Executive Summary

The objective of the study was to determine the ultimate aerobic biodegradability of imidazole in water by measurement of the dissolved organic carbon (DOC). Mixtures of the test substance and activated sludge were incubated and aerated at room temperature for up to 18 days. Samples were taken after 0, 1, 3, 5, 7, 10, 14, 17 and 18 days.

According to the OECD criteria, the results indicate that the test substance imidazole is readily biodegradable with a biodegradation degree (DOC removal) of 90-100% after 18 days.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Substance

Test substance: imidazole
CAS No.: 288-32-4
Molecular mass: 68.08 g mol^{-1}
Molecular formula: $C_3H_4N_2$
Batch No.: 400 544 47 G0
Water-solubility: 633 g L^{-1}
Purity: 99.76%

2. Test system

Activated sludge from laboratory wastewater plants fed with municipal sewage. The concentration of the dry substance was 30 mg L^{-1}.

B. STUDY DESIGN

1. Experimental conditions

Mixtures of the test substance imidazole, a defined inorganic medium, and a non pre-adapted inoculum (activated sludge) were incubated and aerated at room temperature (22 ± 2°C) for up to 18 days. The test concentration was 37.5 mg L^{-1}, which is equivalent to 20 mg L^{-1} dissolved organic carbon (DOC).

Two blank control, one reference substance, one inhibition control, one abiotic control, one adsorption control and two test substance solutions were investigated. In all test solution an inoculum concentration of 30 mg L^{-1} dry matter were added, except in the abiotic control. The pH values in the test vessels were measured and adjusted to 7.4, if necessary.

At day 14 of the test, 1 mL mercury chloride solution was added to the abiotic control assay.

2. Sampling

Samples were taken after 0, 1, 3, 5, 7, 10, 14, 17 and 18 days.

3. Description of analytical procedures

Samples were taken at regular intervals to determine the DOC concentration. The DOC removal at the end of the test was compared with the measured initial concentration, which was calculated as a percent value and evaluated in accordance to the OECD Test Guideline 301A (DOC Die-away Test).

II. RESULTS AND DISCUSSION

According to the OECD criteria, the test substance was readily biodegradable with a DOC removal between 90-100% after a test period of 18 days. The duration of the adaption phase was 5 days, and the duration of the degradation phase 2 days.

The degradation degree of the reference substance aniline and in the inhibition control was between 90-100% DOC after 14 days, respectively.
The physico-chemical (abiotic) elimination of imidazole was < 10% DOC at the end of the test, and the elimination by adsorption < 10% DOC after 5 days.

A summary of the degree of biodegradation is presented in Table 7.2.2.1-1.

Table 7.2.2.1-1: Degree of biodegradation [DOC-degree %]

Days	Reference substance	Inhibition control	Abiotic control	Adsorption control	Test substance (replicate 1)	Test substance (replicate 2)	Test substance mean
0	0	0	0	0	0	0	0
1	2	1	4	-2	-7	-9	-8
3	58	20	-3	-7	-9	-14	-12
5	96	45	0	-5	27	-8	10
7	93	70	1	-	95	96	96
10	95	95	-1	-	97	97	97
14	95	95	2	-	95	98	97
17	96	96	2	-	96	97	97
18	99	97	5	-	98	97	98

III. CONCLUSION

The DOC Die-away Test was used to determine the ultimate aerobic biodegradability of imidazole in water.
According to the OECD criteria the results indicate that the test substance imidazole is readily biodegradable, with a biodegradation degree (DOC removal) of 90-100% after 18 days.

CA 7.2.2.2 Aerobic mineralisation in surface water

Intact polymeric metiram is almost insoluble in water as well as in other solvents. It dissolves only under decomposition and the dissolved material is no longer intact metiram.
In water metiram decomposes fast by purely abiotic hydrolytical processes leading to a variety of degradates including ETU (M222F002), EBIS (M222F004) and EU (M222F003).
In studies using the CS_2 method for quantification (e.g. for measuring water solubility), a certain amount of metiram appeared to be dissolved in water that could potentially be subject to other degradation processes. However this was reported as the potential maximum dissolved that included the contribution of dissolved CS_2-forming degradates like EBIS.
Recent studies reported in the e-fate section of this dossier using radiolabeled test item, like the new hydrolysis study [*CA 7.2.1.1/2*] or studies that involved detection based on derivatization of the “metiram-monomer” like the adsorption study with metiram [*CA 7.1.3.1.1/1*], clearly show that there is virtually no metiram dissolved in water and that all dissolved material is no longer intact metiram.
That means that the fast decomposition of metiram in water including the formation of hydrolytic degradates is driven by abiotic mechanisms and is appropriately described by the hydrolysis study. The further degradation is no longer a degradation of the active substance itself but of its hydrolytic degradates.

Therefore, no study on the aerobic mineralization of metiram in surface water was performed. The hydrolysis study describes the fate of metiram in water whereas the subsequent fate of the hydrolysis products is described by the water/sediment studies.

CA 7.2.2.3 Water/sediment studies

Studies presented in the first Annex I inclusion process:

In the first Annex I inclusion process the study of Voelkl, S. [*CA 7.2.2.3/1, BASF DocID 1993/10415*] was presented. The results of this study are still considered valid for metiram and ETU.
However since the water and the extracts of the sediment were not subjected to radio-HPLC analysis, no information on metabolites other than ETU was generated. Also the amount of bound residues was overestimated in this study since the sediment was extracted only once, following the "cold" residue analytical method for ETU.
In order to complete the picture of formation and decline of metabolites, another water/sediment study was performed [*CA 7.2.2.3/2, BASF DocID 2003/1001011*]. The latter study was already submitted during the first Annex I inclusion process and summarized in the 3rd Addendum to the Monograph (May 2003). This summary is provided below. However it is mentioned in the 3rd Addendum to the Monograph that the study is "under evaluation".

Peer reviewed study (Summary taken from the Monograph or its Addenda):

Report:	*CA 7.2.2.3/1* *Voelkl S., 1993a* *Metiram: Degradation and metabolism in aquatic systems* *1993/10415*
Guidelines:	*BBA IV 5-1*
GLP:	*yes* *(certified by Eidgenoessisches Departement des Inneren, Bern, Schweiz)*

Materials and Methods:

Two natural water/sediment systems collected from river Rhine about 35 km above Basel ("Mumpf", system I) or from a pond ("Froschteich/Möhlin", system II) were used in the study. Relevant water and sediment data are presented in Table 7.2.2.3-1 and Table 7.2.2.3-2.

Table 7.2.2.3-1: Parameters of water from Rhine (system I) and pond (system II)

	System I	System II
Temperature (°C)*	17.8	17.7
pH*	8.2	7.7
Redox potential (mV)*	188	192
O_2-content (mg L^{-1})* Surface 5 cm above sediment	 9.7 9.3	 6.2 5.8
Total N (mg L^{-1}) Beginning End	 0.90 0.27	 0.68 0.41
Total P (mg L^{-1}) Beginning End	 0.13 0.19	 0.21 0.04
TOC (mg L^{-1})	82.4	69.8
Total hardness (° dH)	13.0	16.0

*Determined immediately before collecting

Table 7.2.2.3-2: Parameters of sediments from Rhine (system I) and pond (system II)

	System I	System II
Particle size distribution (%) ISSS nomenclature: sand (> 0.02 mm) silt (0.002 - 0.02) clay (< 0.002 mm)	 82.8 11.0 6.2	 61.2 21.3 17.5
USDA nomenclature*: sand (> 0.05 mm) silt (0.002 - 0.05 mm) clay (< 0.002 mm)	 69.0 24.8 6.2	 38.6 44.0 17.4
Organic C (%)	1.0	1.3
CEC (meq/100 g soil)	8.9	13.3
PH (KCl)	6.5	7.0
Biomass (mg C/100 g dry soil)	153.4	192.5
Total N (%)	1.4	1.7
Total P (%)	0.78	0.51

*Applying the USDA classification system, system I was a sandy loam sediment and system II a loam sediment

Metiram was applied to both systems at a rate of 1.06 mg ai per flask containing 550 mL water and 250 g sediment which was equivalent to 6 kg ai ha^{-1}, and incubated at 20°C ± 2°C up to 105 DAT. The fate of metiram itself, ETU and CS_2 was followed in an experiment by using "cold" metiram and conventional analytical methods ("treatment A"). Another experiment was set up with [^{14}C]-metiram labeled in the ethylene moiety designed to estimate the balance and to follow the mineralization rate to CO_2 and the incorporation into "bound" residues in the sediments ("treatment B").

During incubation, practically no differences in pH and oxygen concentration were observed between treated and untreated aquatic systems. The values obtained for oxygen concentration and redox potential indicated aerobic conditions in the water whereas in the sediment samples anaerobic conditions prevailed.

On each sampling, duplicates of each system were worked up.

Treatment A

Water and sediment samples were analyzed for metiram according to a modified DFG method for the determination of dithiocarbamate and thiuramdisulfide fungicides (CS_2-method, limit of determination: 0.06 mg kg^{-1} (sediment) and 0.03 mg kg^{-1} (water)) and for ETU according to DFG method no. 389 (limit of determination: 0.02 mg kg^{-1} (sediment) and 0.005 mg kg^{-1} (water)). Both methods use GC/S-FPD for final detection.

Volatile CS_2 was trapped in methanolic KOH solutions (1 M) and analyzed for CS_2 according to the parent substance method.

Treatment B

Water and sediment were analyzed by radioanalytical methods, i.e. LSC for liquid samples and combustion on an oxidizer for solid samples.

Findings:

Treatment A

The amount of metiram, ETU and CS_2 along the incubation period is shown in Table 7.2.2.3-3.

Table 7.2.2.3-3: Metiram, ETU and volatile CS_2 in aquatic metabolism. Data (mean of duplicates) in % IRR (initial radioactive residue)

DAT	0	0.25	1	2	7	14	28	59	105
System I									
Water:									
Metiram	54.6	31.7	11.3	9.3	-*	-	-	-	-
ETU	20.6	49.1	32.7	31.5	25.9	12.7	1.8	-	-
Sediment:									
Metiram	23.7	16.6	15.3	14.3	13.1	9.7	6.7	4.9	2.3
ETU	-	-	3.8	4.9	7.6	5.1	1.8	-	-
Total:									
Metiram	78.2	48.2	26.7	23.7	13.1	9.7	6.7	5.2	2.3
ETU	20.6	49.1	36.5	36.4	33.4	17.8	3.6	-	-
Volatile CS_2	-	1.1	3.1	2.3	0.8	-	-	-	-
System II									
Water:									
Metiram	56.2	35.1	9.6	5.0	-	-	-	-	-
ETU	24.6	41.3	40.8	38.2	31.2	15.8	1.5	-	-
Sediment:									
Metiram	21.4	14.0	11.3	12.2	12.6	9.2	7.9	4.9	4.5
ETU	1.7	-	2.0	-	6.4	5.7	2.0	3.4	-
Total:									
Metiram	77.6	49.1	20.8	17.2	12.6	9.2	7.9	4.9	4.5
ETU	26.3	41.3	42.8	38.2	37.7	21.5	3.4	3.4	-
Volatile CS_2	-	0.9	3.9	4.4	1.8	-	-	-	-

*- = not detectable (< determination limit)

These data were used to calculate the degradation rates of metiram and ETU. The data were evaluated according to the best fit approach of Timme et al. and according to first order kinetics. Degradation rates of ETU were calculated beginning computing when metiram was degraded (7 DAT) in order not to impair the kinetics by superimposing degradation of ETU by its new formation from metiram.

$DT_{50/90}$ are shown for water phases and whole systems (water and sediment) in Table 7.2.2.3-4 (metiram) and Table 7.2.2.3-5 (ETU).

Table 7.2.2.3-4: $DT_{50/90}$ of metiram in water/sediment (aquatic) degradation. Data in days

	DT_{50}		DT_{90}	
	First order	**Best fit**	**First order**	**Best fit**
System I:				
Water	0.8 (r = 0.9169)[2]	0.3 (r = 0.9912)[3]	2.7	2.9
Total[1]	26 (r = 0.5844)	1.2 (r = 0.9565)[4]	86	34
System II:				
Water	0.6 (r = 0.969)	0.4 (r = 0.9919)[4]	1.9	1.9
Total	34 (r = 0.4752)	1.0 (r = 0.8603)[5]	114	82

1: Total = whole system (water and sediment)
2: r = correlation coefficient
3: Best fit = first order root function
4: Best fit = 1.5th order root function
5: Best fit = second order root function

Table 7.2.2.3-5: $DT_{50/90}$ of ETU in water/sediment (aquatic) degradation. Data in days

	DT_{50}		DT_{90}	
	First order	**Best fit**	**First order**	**Best fit**
System I:				
Water	5 (r = 0.9897)	5[1]	18	18
Total	7 (r = 0.9951)	7	22	22
System II:				
Water	5 (r = 0.9632)	5	16	16
Total	15 (r = 0.8471)	3.4 (r = 0.9581)[2]	51	38

1: Best fit = first order
2: Best fit = first order root function

Treatment B

The distribution of radioactivity along the incubation period is shown in Table 7.2.2.3-6.

Table 7.2.2.3-6: Distribution and balance of radioactivity in aquatic degradation of metiram. Data in % IRR (mean of duplicates).

DAT	System I (Rhine)				System II (Pond)			
	Water	Sediment	CO_2[1]	Balance	Water	Sediment	CO_2	Balance
0	81.0	16.1	-[2]	97.0	85.6	13.1	-[2]	98.7
30	18.2	69.6	19.7	107.5	15.1	70.6	19.4	105.1
59	4.9	59.7	43.6	108.1	4.1	54.8	40.1	99.0
105	3.2	56.7	50.5	110.4	2.4	56.2	49.8	108.3

1: Any other volatiles except CO_2 could not be trapped (H_2SO_4 and ethyleneglycol traps)
2: - = not determined

The data make evident that metiram was degraded primarily to CO_2 and non-extractable residues which arose most probably from recycled radiocarbon incorporated into natural organic soil constituents.
The non-extractable radioactivity of the 59 and 105 DAT samplings was further submitted to soil organic matter fractionation: results are shown in Table 7.2.2.3-7.

Table 7.2.2.3-7: Soil organic matter fractionation of non-extractable sediment residues. Data in % IRR.

DAT	Fulvic acids[1]		Humic acids[2]		Humins[3]	
	Rhine	Pond	Rhine	Pond	Rhine	Pond
59	27.8	24.6	13.9	13.4	18.3	19.5
105	24.6	21.7	7.6	9.7	25.1	25.9

1: Fulvic acid fraction: extractable with 0.5 M NaOH, soluble after acidification of the extract
2: Humic acid fraction: extractable with 0.5 M NaOH, insoluble after acidification of the extract
3: Humin fraction: non-extractable with 0.5 M NaOH

The NaOH-extractable "bound" residues decreased with time, the NaOH-non-extractable "bound" residues increased indicating strong binding to and incorporation in soil humin. It can be excluded, therefore, that relevant (bio-active) metabolites may be released to soil solution in the long range.

In conclusion, the two water/sediment systems have shown that metiram was rapidly eliminated from aquatic systems ($DT_{50} < 1$ day) via degradation to ETU followed by mineralization to CO_2 and adsorption to the sediment phase. ETU was mainly present in the water phase in both systems and was rapidly (DT_{50} 5 days) eliminated, it could be assumed - on the basis of study results - through mineralization to CO_2.

Report: CA 7.2.2.3/2
Ebert D., 2003b
Degradation of BAS 222 F (Metiram) in water/sediment-systems under aerobic conditions
2003/1001011

Guidelines: BBA IV 5-1, EPA 162-4, SETAC Europe Part 8.2, OECD 308

GLP: yes
(certified by Landesamt fuer Umweltschutz und Gewerbeaufsicht, Mainz, Germany)

Materials and methods:

The distribution and degradation of BAS 222 F (Metiram) was studied in two natural systems of water and sediment taken from a pond (Kellmetschweiher) and a small stream (Ranschgraben) both in Rhineland-Palatinate, Germany. The specific radioactivity of the ethylene-^{14}C labeled active substance was 4.68 MBq mg^{-1} with a radiochemical purity of > 98%.
Characteristics of the water/sediment systems are given in Table 7.2.2.3-8. BAS 222 F was applied to the water at a rate of about 196 µg active substance (as) per test vessel which slightly exceeded the maximum field application rate of 1.8 kg as ha^{-1} when related to a 30 cm deep water body. Experiments under sterile conditions were also carried out in both water/ sediment systems. The test vessels were incubated in the dark at a temperature of 20 ± 2 C for up to 100 days. Aeration was achieved by a stream of air over the water surface.
Samples were taken (in replicates) up to 100 DAT (Days after treatment).
Metiram was determined by conventional residue analysis using the indirect CS_2-method. Material balance and metabolite determinations were done using radio-analytical techniques. Metabolites were analyzed by radio-HPLC and the compound identities were verified by co-chromatography with reference substances and HPLC/MS.

Table 7.2.2.3-8: Characterization of the water/sediment systems

Designation Origin		Kellmetschweiher (pond) Rhineland-Palatinate, FRG	Ranschgraben (small stream) Rhineland-Palatinate, FRG
Sediment	sand [%]	81	82
	silt [%]	11	14
	clay [%]	8	5
	textural class (German scheme)	loamy sand	silty sand
	pH ($CaCl_2$)	7.7	6.4
	organic C [%]	0.5	0.6
	total N [%]	0.04	0.05
	total P [mg kg^{-1}]	83.7	143
	CEC [mVal 100 g^{-1}]	10.1	6.9
	ATP [µg kg^{-1}]	369	132
	plate counts [cfu g^{-1}]		
	bacteria	4.7×10^6	7.8×10^6
	actinomycetes	3.9×10^3	2.9×10^5
	fungi	5.8×10^2	1.1×10^4
Water	pH	8.4	7.6
	hardness [mmol L^{-1}]	1.98	1.02
	TOC [mg L^{-1}]	11.4	3.1
	total N [mg L^{-1}]	< 1	2.1
	total P [mg L^{-1}]	< 0.05	0.12

Findings:

The distribution and recovery of radioactivity from the two water/sediment systems are shown in Table 7.2.2.3-9.

Table 7.2.2.3-9: Material balance and distribution of radioactivity after application of ^{14}C-metiram to water/sediment systems (% TAR)

DAT	water	sediment					CO_2	material balance
		extractable residues						
		H_2O	EDTA	acetone	bound residues	total		
Kellmetschweiher								
0	101.7	n.p.	n.p.	n.p.	n.p.	-	n.p.	101.7
1	82.8	3.2	10.2	1.7	7.3	22.5	0.0	105.3
2	80.5	4.2	9.3	1.4	12.6	27.5	0.0	108.0
7	60.0	7.3	15.2	1.7	18.2	42.3	0.1	102.4
14	51.5	9.1	13.3	2.1	18.9	43.3	0.4	95.2
30	50.6	10.6	14.3	1.5	25.7	52.1	1.8	104.6
62	48.3	10.3	11.3	1.5	27.4	50.5	4.6	103.3
100	45.3	10.3	10.2	1.3	25.6	47.4	7.7	100.4
101 (s)	53.3	19.3	12.4	1.4	21.0	54.1	n.p.	107.4

Table 7.2.2.3-9: Material balance and distribution of radioactivity after application of ^{14}C-metiram to water/sediment systems (% TAR)

DAT	water	sediment					CO_2	material balance
		extractable residues						
		H_2O	EDTA	acetone	bound residues	total		
Ranschgraben								
0	103.8	n.p.	n.p.	n.p.	n.p.	-	n.p.	103.8
1	83.6	4.5	6.2	1.1	7.2	19.0	0.0	102.6
2	80.7	6.1	10.4	0.6	9.4	26.4	0.1	107.3
7	62.5	6.9	11.2	1.2	14.7	34.0	1.0	97.5
14	52.5	8.0	10.3	1.3	23.1	42.7	4.3	99.5
30	39.2	7.4	10.3	1.2	27.4	46.2	13.1	98.6
62	28.9	6.1	9.4	1.1	31.2	47.7	20.2	96.9
100	21.0	5.0	8.2	0.8	26.2	40.2	32.8	94.0
101 (s)	28.1	13.3	24.6	2.2	37.4	77.5	n.p.	105.5

s = sterilized
n.p. = not performed

The results of the HPLC analysis of the water and of the extracts of sediment is shown in Table 7.2.2.3-10 for system Kellmetschweiher and in Table 7.2.2.3-11 for the system Ranschgraben.

Table 7.2.2.3-10: HPLC analysis of water and sediment extracts after application of ^{14}C-metiram to water sediment system Kellmetschweiher (%TAR)

DAT	Metiram	EU	ETU	EBIS	others [a]
	determined by CS_2-method	determined by radio-HPLC after filtration of water or H_2O-extraction of sediment			
water					
0	75.0	0.4	4.0	7.3	2.0
1	22.5	6.3	63.3	1.9	8.5
2	16.6	5.0	58.5	1.8	9.3
7	4.4	5.2	47.6	0.3	4.8
14	2.2	4.1	42.1	0.4	3.5
30	0.4	3.5	41.5	0.2	4.1
62	0.5	3.9	41.2	0.1	2.3
100	0.6	3.6	39.1	0.0	1.2
101 (s)	1.0	4.1	47.8	0.0	0.0
sediment					
0	n.p.	n.p.	n.p.	n.p.	n.p.
1	7.4	0.5	1.4	0.2	1.1
2	4.9	0.8	2.7	0.1	0.6
7	3.6	1.1	5.2	0.2	0.7
14	3.4	1.8	6.4	0.2	0.7
30	2.7	2.5	6.9	0.1	1.1
62	2.6	2.2	7.4	0.0	0.7
100	2.1	2.2	7.7	0.0	0.4
101 (s)	3.4	2.1	16.8	0.1	0.4

s = sterilized
n.p. = not performed
[a] sum of up to 18 peaks (each < 5% TAR)

Table 7.2.2.3-11: HPLC analysis of water and sediment extracts after application of metiram to system Ranschgraben (%TAR)

DAT	Metiram	EU	ETU	EBIS	others [a]
	determined by CS_2-method	determined by radio-HPLC after filtration of water or H_2O-extraction of sediment			
water					
0	78.0	0.1	2.3	5.5	1.5
1	12.8	4.1	51.1	8.1	12.2
2	nd	3.8	44.4	16.3	10.3
7	6.1	4.9	44.4	4.0	6.9
14	2.2	3.5	42.0	0.8	4.7
30	0.7	2.2	32.7	0.3	3.1
62	0.2	2.4	24.8	0.0	1.2
100	0.6	3.2	15.0	0.1	2.0
101 (s)	1.4	5.8	18.1	0.4	2.8
sediment					
0	np	np	np	np	Np
1	6.7	0.9	2.7	0.2	0.7
2	5.4	1.0	3.0	0.2	1.5
7	4.7	1.0	4.2	0.2	1.5
14	3.3	1.3	6.1	0.2	0.4
30	1.8	1.0	6.1	0.1	0.1
62	3.9	1.2	4.8	0.0	0.2
100	2.6	1.6	3.4	0.0	0.0
101 (s)	5.0	1.9	9.5	0.5	1.4

s = sterilized
n.p. = not performed
[a] sum of up to 17 peaks (each < 4% TAR, except one peak reaching 6.6% TAR at day 1)

Metiram was quickly transformed to ETU and EBIS with less than 1% of the applied active substance in the water phase after 30 days. After 100 days, only 0.6% Metiram was still found in the water of both water/sediment systems. In the sediment, Metiram reached the highest amounts of about 7% of applied substance at the early sampling times and then slowly degraded to 2-3% at the end of the study.
ETU was the most significant metabolite reaching maximum amounts of 63 and 51% of TAR in the water phase after 1 day degrading to about 39% (Kellmetschweiher) and 15% (Ranschgraben) after 100 days. In the sediment the max value found was 7.7%.
The metabolite EBIS degraded to below 1% after 7 days in the Kellmetschweiher system and after 14 days in the Ranschgraben system. Immediately after treatment (0 day) it was 5-7% of the TAR in water samples increasing to 16% TAR after 2 days in the Ranschgraben system before degrading. In the sediment, the amounts of EBIS are neglectable (≤ 0.2% TAR at any sampling time).
A range of minor metabolites (up to 18 peaks) were detected in the HPLC chromatograms of the water samples, none of them appearing in amounts >6.6% TAR and most of them never exceeding 1% TAR during the whole incubation period.

The water extractable residues were in the range 3.2% to 10.6% for the Kellmetschweiher sediment and up to 8% for Ranschgraben sediment. The residues extracted with EDTA were slightly higher. Bound residues reached a maximum of 27.4% after 30 days (Kellmetschweiher) and 31.2% after 62 days (Ranschgraben). The radioactivity recovered as CO_2 (mineralization) was low for the Kellmetschweiher system with a max of 7.7%, for the Ranschgraben system the max value was 32.8%, both found at DAT 100. The HPLC analysis of the water extracts showed ETU at a maximum of 8% TAR and 6% TAR in the two systems. All other metabolites never exceeded 2.5% TAR. NaOH extraction showed that a considerable portion of the radioactivity could be released. HPLC analysis of the fulvic acids (max. 14% TAR) revealed that up to 8-12% TAR could be attributed to ETU.
The estimated DT_{50} values are presented in Table 7.2.2.3-12 and the proposed route of degradation in Figure 7.2.2.3-1.

Table 7.2.2.3-12: Half-lives of Metiram and its metabolites in water/sediment systems (first order if not stated otherwise)

SYSTEM		DT_{50} [days]	DT_{90} [days]	r^2
Kellmetschweiher				
metiram	water	0.5	1.5	0.95
EU	water	12.7	42.2	0.95
ETU	water	140.0 [a]	>300	0.95
EBIS	water	0.7	2.2	0.95
metiram	sediment	6.8	22.4	0.95
EU	sediment	n.c.	n.c.	0.95
ETU	sediment	176.5 [a]	> 300	0.95
EBIS	sediment	1.9	6.2	0.95
Ranschgraben				
metiram	water	0.4	1.3	0.96
EU	water	n.c.	n.c.	0.96
ETU	water	52.6	174.9	0.96
EBIS	water	3.0	10.0	0.96
metiram	sediment	6.0	20.0	0.96
EU	sediment	n.c.	n.c.	0.96
ETU	sediment	51.2	170.0	0.96
EBIS	sediment	n.c.	n.c.	0.96

n.c. = not calculated due to the generally very low concentration level
[a] best fit values

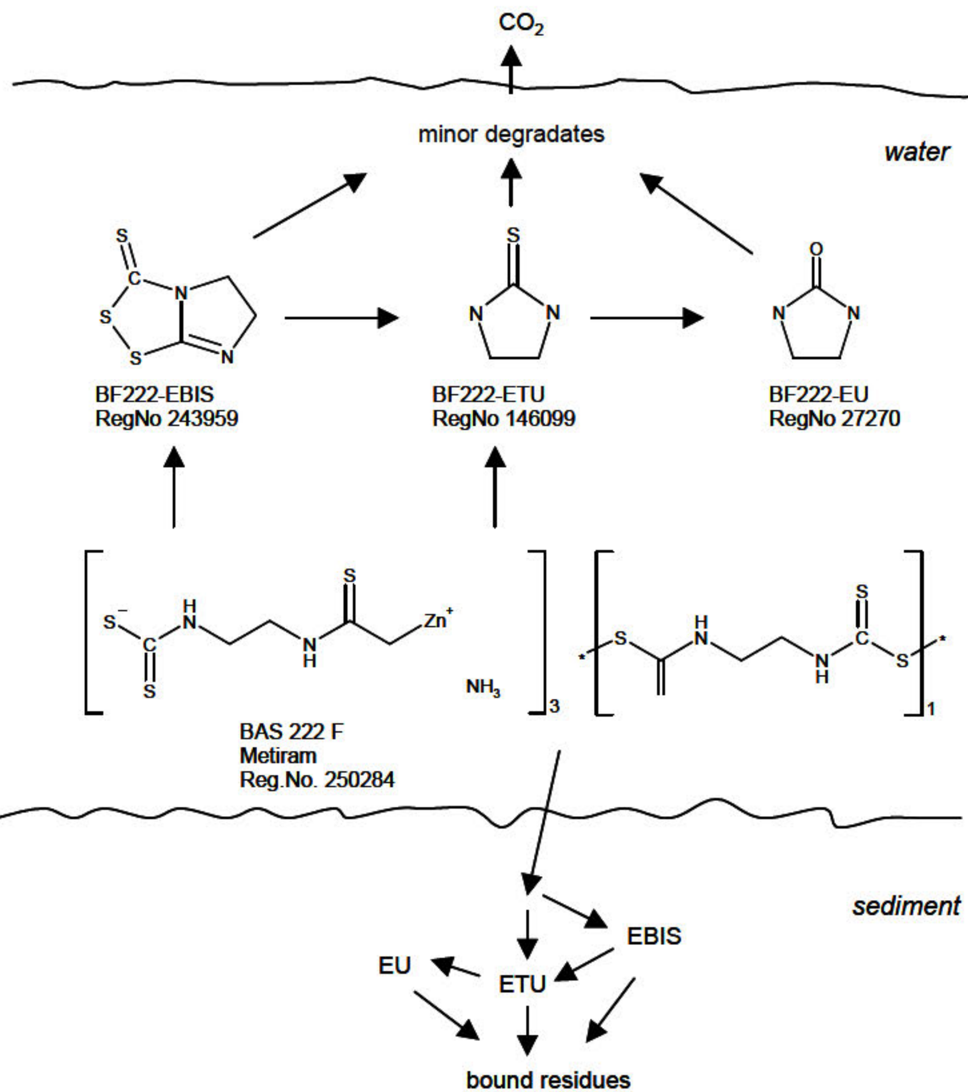

Figure 7.2.2.3-1: Proposed route of degradation of metiram in water/sediment systems

Note added by the applicant: The recent kinetic evaluations of the degradation in both, soil and water revealed that there is additionally a degradation pathway directly from EBIS to EU (i.e. bypassing ETU).

III. CONCLUSION (added by the applicant)

The distribution and degradation of metiram was studied in two natural systems of water and sediment taken from a pond and a small stream.
Metiram disappeared fast from the water and in the sediment metiram never exceeded 7% of applied.
The active substance was quickly transformed to ETU and EBIS with ETU being the most significant metabolite in the water phase. EBIS reached its maximum already at 0 – 2 days after treatment and thereafter degraded fast. EU was formed only in moderate amounts. Additionally a range of minor metabolites (up to 18 peaks) were detected in the water. In the sediment, ETU was the only significant metabolite.
Final degradation resulted in substantial mineralization with up to 33% TAR CO_2 and the formation of bound residues.

Report: CA 7.2.2.3/3
Pape L., 2015d
Kinetic evaluation of two water-sediment studies with BAS 222 F - Metiram according to FOCUS Degradation Kinetics
2014/1228264
Guidelines: FOCUS Kinetics (2006) SANCO/10058/2005 version 1.1 of Dec. 2014
GLP: no

Executive Summary

The aim of the study was to evaluate the kinetics of metiram and its metabolites EBIS, ETU and EU in four water/sediment systems in two studies in order to derive trigger endpoints (persistence endpoints) and modeling endpoints according to the recommendations of the FOCUS workgroup on degradation kinetics. In the first study, non-labeled metiram was applied to two water/sediment systems (System I – Rhine River and System II – Froschteich) and incubated in the dark for up to 105 days. Samples were analyzed for metiram and its metabolite ETU. In the second study, ethylene-^{14}C labeled metiram was applied to two water/sediment systems (System A – Kellmetschweiher and System B – Ranschgraben) and incubated in the dark for up to 104 days. The metabolites EBIS, ETU and EU were determined by radio-HPLC.

Kinetic evaluation at Level P-I (one-compartment approach) was performed for metiram in order to derive DegT50 values for degradation in the total system as well as DT50 values for the water and DT50 values for the sediment phase of the test systems. At Level P-II (two-compartment approach: water and sediment), the kinetic analysis considered the degradation in water and sediment and the sorption desorption processes between both phases.

Kinetic evaluation at Level M-I dissipation (one-compartment approach) was performed for the metabolites EBIS, ETU and EU for metabolite decline from the maximum observed concentration in order to derive DT50 values in the total system as well as DT50 values for the water and DT50 values for the sediment phase of the test systems, where appropriate.

In addition, the Level M-I degradation times (DegT50) of the metabolites in the total system were estimated under consideration of the actual degradation pathway.

For all modelling approaches that were considered appropriate to derive kinetic endpoints the visual assessment and goodness-of-fit statistics indicated plausible fit. For some fits, χ^2 error values above 15% were obtained which was attributed to the scattering of the data caused by the rapid transformation processes that are symptomatic for the degradation of metiram and its metabolites in water/sediment systems. As the observations were generally well described by the fitted curves the high χ^2 error values were considered acceptable. The t-test was passed for the respective model parameters. Therefore, the resulting endpoints were considered reliable.

In cases, where for the metabolites no reliable endpoints could be derived from the fit and clearly no dissipation was observed the use of the default value for modeling of 1000 days was recommended. In cases where no reliable endpoints could be derived although dissipation was clearly observed or where a conclusion on the dissipation behavior could not be drawn due to highly scattered data in combination with low residues no endpoints were reported.

At Level P-I for metiram, trigger $DegT_{50}$ in the total system from 0.1 to 0.3 days and trigger DT_{50} in the water and sediment phase from 0.1 to 0.3 days and from 2.8 to 25.8 days were calculated.

Reliable modeling endpoints at Level P-I were $DegT_{50}$ values from 1.4 to 3.9 days for the total system and DT_{50} values from 0.3 to 1.2 days and from 30.7 to 81.7 days for the water and sediment phase, respectively.

At Level P-II, no trigger or modeling endpoints could be calculated.

For metabolite EBIS at Level M-I dissipation, trigger DT_{50} of 0.3 and 2.6 days for the total system and of 0.3 and 2.4 days for the water phase were calculated. Modeling DT_{50} were 2.3 and 2.6 days for the total system and 1.6 and 2.4 days for the water phase. Dissipation kinetics for the sediment phase were not evaluated, as the observed residues were very low (max. 0.2% TAR). At Level M-I degradation, a $DegT_{50}$ of 3.0 days and a formation fraction from metiram of 0.032 were calculated for test system Kellmetschweiher.

For metabolite ETU at Level M-I dissipation, trigger and modeling DT_{50} of 10.4 to 72.1 days for the total system were calculated. A default modeling DT_{50} of 1000 d for the total system Kellmetschweiher was reported as the dissipation pattern was biphasic and visually no dissipation occurred in slow phase.

In the water phase, trigger DT_{50} of 7.7 to 441.6 days and modeling DT_{50} of 7.7 to 1000 days were obtained. In the sediment phase, trigger and modeling endpoints ranged from 10.7 to 107.7 days.

At Level M-I degradation, $DegT_{50}$ of 6.9 to 59.7 days and formation fractions from metiram of 0.569 to 0.654 were calculated. A default modeling $DegT_{50}$ of 1000 d for test system Kellmetschweiher was reported as the dissipation pattern was biphasic and visually no dissipation occurred in slow phase.

For metabolite EU the evaluation at Level M-I dissipation revealed no reliable endpoints for the total system. For test system Kellmetschweiher, a default modeling DT_{50} of 1000 days was recommended as visually no dissipation occurred. In the water phase, a trigger DT_{50} of 467.8 days and a modeling DT_{50} of 138.4 days were obtained for test system Kellmetschweiher. Dissipation kinetics for the sediment phase were not evaluated, as the observed residues were very low (max. 2.6% TAR). At Level M-I degradation, $DegT_{50}$ of 254.8 and 209.4 days and formation fractions from metiram of 0.070 and 0.054 were determined.

I. MATERIAL AND METHODS

The kinetic evaluation of metiram and its metabolites was conducted for four water/sediment systems in two studies [*see CA 7.2.2.3/1, BASF DocID 1993/10415 and CA 7.2.2.3/2, BASF DocID 2003/1001011*] in order to derive trigger and modeling endpoints.

Kinetic modeling

The appropriate kinetic model for deriving trigger and modeling endpoints was identified considering the procedures and kinetic models proposed by the FOCUS workgroup on degradation kinetics [*FOCUS (2006)*].

Kinetic evaluation was performed for the parent substance metiram and for metabolites observed at amounts above 5% AD (applied dose of metiram), i.e., ETU in both studies and EBIS and EU observed in study BASF DocID 2003/1001011 [*see CA 7.2.2.3/2*] at the following levels recommended by FOCUS:

Parent substance:

- Level P-I (one-compartment approach): estimation of parent degradation times ($DegT_{50}$) in the total system as well as dissipation times (DT_{50}) in the water and DT_{50} in the sediment phase.
- Level P-II: (two-compartment approach water and sediment), estimation of parent degradation times (DegT50's) in the water and sediment phase under consideration of sorption desorption processes between both phases.

Metabolites:

- Level M-I dissipation (one-compartment approach): estimation of metabolite DT_{50}'s from the maximum observed concentration in the total system, in the water and sediment phase, respectively.
- Level M-I degradation (multi-compartment approach): estimation of metabolite degradation times (DT_{50}) and formation fractions in the total system in a combined fit of parent and metabolites.

Level M-I degradation (multi-compartment approach):
At Level M-I degradation, the fitting procedure for metiram, EBIS, ETU and EU was performed in a stepwise approach. Initially, the most reasonable compartment model as provided in the study report, i.e. degradation of metiram to EBIS and ETU with possible formation of ETU from EBIS and further degradation of ETU to EU was applied. Based on the visual and statistical results of the individual fitting steps the compartment model was modified by including a flow metiram → EU and removing the flow EBIS → ETU, and, finally, removing the flow ETU → EU. The final compartment model that was considered for the estimation of the $DegT_{50}$'s is shown in Figure 7.2.2.3-2.

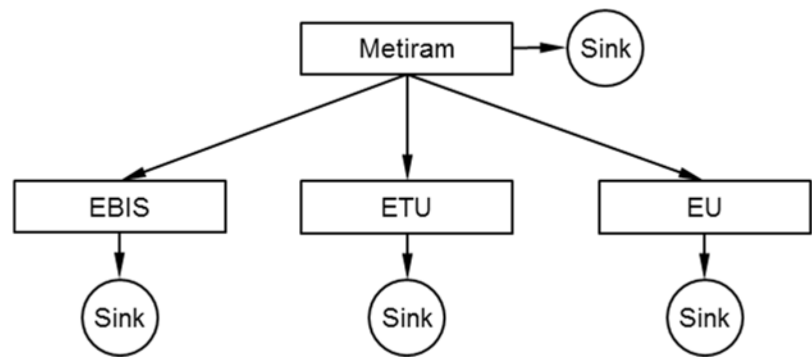

Figure 7.2.2.3-2: Final degradation pathway of metiram

Kinetic models included in the assessment

The following kinetic models were employed for the evaluation:
Level P-I: SFO, FOMC, DFOP, HS
Level P-II**:** SFO
Level M-I dissipation: SFO, FOMC, DFOP
Level M-I degradation: Parent: best-fit model from Level P-I, Metabolites: SFO kinetics

The appropriateness of the distinct kinetic model to describe soil degradation was tested by visual assessment, estimation of the error percentage at which the χ^2 test was passed (ideally below 15%, but may be larger if the overall pattern of decline in pesticide concentrations was represented adequately by the model and the distribution of the residuals was random) and the t-test to evaluate whether estimated degradation parameters differ from zero (at a 10% error level).

At Level P-I and M-I dissipation, trigger endpoints were derived from the kinetic models that provided the best fit to the measured data, generally indicated by the lowest χ^2 - error. Modeling endpoints were derived preferably from the SFO model. If the SFO model was not appropriate, pragmatic procedures were used to derive conservative pseudo-SFO degradation rates from the appropriate bi-phasic model.

Data handling

At Level P-I and P-II of the analysis as well as at Level M-I degradation, the kinetic evaluation started on the day of treatment (i.e. 0 DAT). For BASF DocID 2003/1001011 [*see CA 7.2.2.3/2*], the initial concentration of the parent substance in the total system or in water was set to the material balance recovered at DAT 0. For BASF DocID 1993/10415 [*see CA 7.2.2.3/1*] the initial concentration of the parent substance was set to 100% AD as the material balance, determined in a separate experiment, was very close to this value. Accordingly, the initial concentration in the sediment phase was assumed to be zero at Level P-II.

The assessment of dissipation in sediment at Level P-I and in all compartments at Level M-I dissipation only requires kinetics to be fitted to the corresponding decline data, starting from the maximum observed concentration in the compartment. The dissipation of the respective compound was thus evaluated starting at the day of maximum occurrence.

Values below the quantification or detection limit for parent compound and degradation products were treated as recommended by FOCUS [*FOCUS (2006)*].

Software for kinetic evaluation

The software package KinGUI (version 2.2012.320.1629) was used for parameter fitting [*SCHÄFER et al. (2007); SCHMITT et al. (2011)*].

**Expe
rimental data**

In the first study [*see CA 7.2.2.3/1, BASF DocID 1993/10415*] the distribution and degradation of metiram was studied in two natural systems of water and sediment, which were taken from the Rhine (System I) and pond Froschteich (System II), both located in Aargau, Switzerland. Both systems were treated with non-labeled metiram at a target concentration of 6 kg a.s. ha^{-1}. The systems were incubated in the dark at 20°C for up to 105 days.

In the second study [*see CA 7.2.2.3/2, BASF DocID 2003/1001011*] the distribution and degradation of metiram was studied in two natural systems of water and sediment and were taken from the pond Kellmetschweiher (System A) and the small stream Ranschgraben (System B), both located in Rhineland-Palatinate, Germany. Both systems were treated with ethylene-^{14}C labeled metiram at a target concentration of 1.8 kg a.s. ha^{-1}. The systems were incubated in the dark at 20°C for up to 104 days.

The measured data as well as resulting datasets submitted to kinetic analysis are summarized in Table 7.2.2.3-13 to Table 7.2.2.3-16.

Table 7.2.2.3-13: Experimental data of metiram and ETU in System Rhine River used for kinetic evaluation [*BASF DocID 1993/10415*]

DAT [d]	Metiram residues [% AD]			ETU residues [% AD]		
	Total system	Water	Sediment	Total system	Water	Sediment
0	100 [a]	100 [a]	0 [b] / **25.3**	0 [c]	22.9	<LOD
0	100 [a]	100 [a]	0 [b] / **22.0**	0 [c]	18.4	<LOD
0.25	49.0	28.2	20.8	**51.3**	**51.3**	<LOD
0.25	47.4	35.1	12.3	**46.8**	**46.8**	<LOD
1	27.9	12.0	15.9	37.5	34.0	3.5
1	25.4	10.6	14.8	35.6	31.4	4.2
2	30.6	14.0	16.6	40.6	36.7	3.9
2	16.8	4.7	12.1	32.2	26.3	5.9
7	11.6	0.75 [d]	11.6	29.6	21.9	**7.7**
7	14.7	0.75 [d]	14.7	37.3	29.9	**7.4**
14	10.7	<LOD	10.3	19.9	14.5	5.4
14	9.7	<LOD	9.2	15.7	11.0	4.8
28	6.7	<LOD	6.7	2.8	1.4	1.4
28	6.8	<LOD	6.8	4.4	2.3	2.1
59	6.5	<LOD	6.1	0.33 [d]	0.33 [d]	0.33 [d]
59	4.0	<LOD	3.6	0.33 [d]	0.33 [d]	0.33 [d]
105	4.5	<LOD	4.5	<LOD	<LOD	<LOD
105	0.75 [d]	<LOD	0.75 [d]	<LOD	<LOD	<LOD

AD applied dose of metiram

Bold numbers: peak concentration considered for single-compartment evaluation; previous values were omitted; sampling dates were adjusted accordingly

[a] Set to the nominal application amount (100% AD; the material balance, determined in a separate experiment, was 98.7 to 108.3% AD); original values: total system 79.2 and 77.3% AD, water 53.9 and 55.3% AD

[b] Set to zero for kinetic evaluation at Level P-II

[c] Set to zero for kinetic evaluation at Level M-I degradation

[d] Set to ½ LOD (LOD = 0.03 mg kg^{-1} for metiram corresponding to 1.5% AD and 0.005 mg kg^{-1} for ETU corresponding to 0.67% AD)

Table 7.2.2.3-14: Experimental data of metiram and ETU in System Froschteich used for kinetic evaluation [*BASF DocID 1993/10415*]

DAT [d]	Metiram residues [% AD]			ETU residues [% AD]		
	Total system	Water	Sediment	Total system	Water	Sediment
0	100 [a]	100 [a]	0 [b] / **16.6**	0 [c]	21.4	<LOD
0	100 [a]	100 [a]	0 [b] / **26.1**	0 [c]	27.9	3.4
0.25	38.8	24.8	14.0	38.4	**38.4**	<LOD
0.25	59.4	45.4	14.0	44.2	**44.2**	<LOD
1	21.1	10.3	10.9	**48.1**	44.1	4.0
1	20.5	8.9	11.6	**37.4**	37.4	<LOD
2	17.7	6.8	10.9	38.5	38.5	<LOD
2	16.6	3.2	13.5	37.9	37.9	<LOD
7	10.4	0.75 [d]	10.4	43.9	37.0	**6.9**
7	14.8	0.75 [d]	14.8	31.4	25.5	**5.9**
14	9.8	<LOD	9.8	20.9	15.5	5.4
14	8.5	<LOD	8.5	22.1	16.1	6.1
28	7.9	<LOD	7.9	4.1	1.9	2.1
28	7.8	<LOD	7.8	2.8	1.0	1.8
59	4.4	<LOD	4.4	3.5	0.33 [d]	3.5
59	5.4	<LOD	5.4	3.4	0.33 [d]	3.4
105	4.7	<LOD	4.7	0.33 [d]	<LOD	0.33 [d]
105	4.3	<LOD	4.3	0.33 [d]	<LOD	0.33 [d]

AD applied dose of metiram
Bold numbers: peak concentration considered for single-compartment evaluation; previous values were omitted; sampling dates were adjusted accordingly
[a] Set to the nominal application amount (100% AD; the material balance, determined in a separate experiment, was 98.7 to 108.3% AD); original values: total system 60.5 and 94.6% AD, water 43.9 and 68.5% AD
[b] Set to zero for kinetic evaluation at Level P-II
[c] Set to zero for kinetic evaluation at Level M-I degradation
[d] Set to ½ LOD (LOD = 0.03 mg kg^{-1} for metiram corresponding to 1.5% AD and 0.005 mg kg^{-1} for ETU corresponding to 0.67% AD)

Table 7.2.2.3-15: Experimental data of metiram, EBIS, ETU and EU in System Kellmetschweiher used for kinetic evaluation [*BASF DocID 2003/1001011*]

DAT [d]	Metiram residues [% TAR]			EBIS residues [% TAR]			ETU residues [% TAR]			EU residues [% TAR]		
	Total system	Water	Sed.	Total system	Water	Sed. [a]	Total system	Water	Sed. [a]	Total system	Water	Sed. [a]
0	103.0 [b]	103.0 [b]	0 [c]	0 [d] / **6.7**	**6.7**	n.p.	0 [d]	4.6	n.p.	0 [d]	0.5	n.p.
0	105.1 [b]	105.1 [b]	0 [c]	0 [d] / **8.0**	**8.0**	n.p.	0 [d]	3.3	n.p.	0 [d]	0.3	n.p.
1	28.7	20.9	**7.9**	2.0	1.8	0.2	**65.5**	**63.9**	1.6	**6.3**	**5.9**	0.4
1	31.2	24.2	**7.0**	2.1	2.0	0.1	**64.0**	**62.7**	1.3	**7.3**	**6.7**	0.6
2	19.8	14.9	4.9	1.6	1.5	0.1	64.4	61.4	3.0	5.9	4.8	1.1
2	22.5	17.5	5.0	2.2	2.0	0.2	58.0	55.6	2.4	5.6	5.1	0.5
7	7.3	3.7	3.6	0.4	0.2	0.2	52.7	47.5	5.2	6.3	5.2	1.1
7	8.7	5.2	3.6	0.6	0.4	0.2	53.0	47.7	5.3	6.3	5.2	1.1
14	6.0	2.5	3.5	0.7	0.5	0.2	49.6	42.8	6.8	5.5	3.6	1.9
14	5.1	1.9	3.3	0.6	0.4	0.2	47.5	41.5	6.0	6.3	4.6	1.7
30	2.6	0.8	1.8	0.3	0.2	0.1	47.9	41.4	6.5	6.0	3.4	2.6
30	3.5	0.05 [e]	3.5	0.4	0.3	0.1	49.0	41.6	7.4	5.9	3.5	2.4
62	5.4	0.8	4.6	0.2	0.2	0.05 [e]	49.8	42.4	7.4	6.5	4.2	2.3
62	0.9	0.2	0.7	0.1	0.05 [e]	0.1	47.3	39.9	7.4	5.7	3.6	2.1
100	2.6	0.6	2.0	0.05 [e]	0.05 [e]	0.05 [e]	47.0	39.1	7.9	6.2	4.2	2.0
100	2.7	0.5	2.2	0.05 [e]	n r.	n r.	46.8	39.2	7.6	5.3	3.0	2.3

TAR total applied radioactivity
n.p. not processed
n r. not reported
Sed. Sediment
Bold numbers: peak concentration considered for single-compartment evaluation; previous values were omitted; sampling dates were adjusted accordingly.
[a] Not evaluated, no decline observed
[b] Set to material balance; original values: total system and water 75.4 and74.7% AD
[c] Sample not analyzed; set to zero for kinetic evaluation at Level P-II
[d] Set to zero for kinetic evaluation at Level M-I degradation
[e] Set to ½ LOD. As no LOD was reported it was assumed to correspond to 0.1% TAR, i.e. the lowest significant value according to the number of decimal places reported

Table 7.2.2.3-16: Experimental data of metiram, EBIS, ETU and EU in System Ranschgraben used for kinetic evaluation [*BASF DocID 2003/1001011*]

DAT [d]	Metiram residues [% TAR]			EBIS residues [% TAR]			ETU residues [% TAR]			EU residues [% TAR]		
	Total system	**Water**	**Sed.**	**Total system**	**Water**	**Sed.** [a]	**Total system**	**Water**	**Sed.**	**Total system**	**Water**	**Sed.** [a]
0	102.1 [b]	102.1 [b]	0 [c]	0.0 [d]	4.5	n.p.	0 [d]	2.4	n.p.	0 [d]	0.2	n.p.
0	108.4 [b]	108.4 [b]	0 [c]	0.0 [d]	6.5	n.p.	0 [d]	2.1	n.p.	0 [d]	n r.	n.p.
1	19.6	12.7	**6.9**	8.2	8.0	0.2	**53.3**	**51.0**	2.3	5.0	4.0	1.0
1	19.5	13.0	**6.6**	8.2	8.1	0.1	**54.3**	**51.2**	3.1	5.2	4.3	0.9
2	n.p.	n.p.	5.4	**12.4**	**12.2**	0.2	51.5	49.0	2.5	5.6	4.8	0.8
2	n.p.	n.p.	5.4	**20.5**	**20.3**	0.2	43.3	39.9	3.4	3.9	2.7	1.2
7	9.9	5.2	4.7	3.3	3.1	0.2	48.5	44.6	3.9	**5.7**	**4.9**	0.8
7	11.9	7.1	4.8	5.0	4.9	0.1	48.8	44.2	4.6	**6.2**	**4.9**	1.3
14	5.2	2.0	3.2	0.9	0.8	0.1	49.5	43.3	**6.2**	4.9	3.6	1.3
14	5.7	2.4	3.3	0.9	0.7	0.2	46.8	40.8	**6.0**	4.8	3.5	1.3
30	2.5	0.7	1.8	0.4	0.3	0.1	38.8	32.7	6.1	3.2	2.2	1.0
30	Considered as outlier in study report											
62	5.4	0.3	5.1	0.05 [e]	0.05 [e]	0.05 [e]	25.9	21.5	4.4	4.1	2.8	1.3
62	2.7	0.1	2.6	0.05 [e]	0.05 [e]	0.05 [e]	33.2	28.0	5.2	3.0	1.9	1.1
100	3.5	0.7	2.8	n.r.	n.r.	n.r.	22.3	19.0	3.3	3.3	1.7	1.6
104	3.0	0.6	2.4	n.r.	n.r.	n.r.	14.6	11.1	3.5	6.4	4.7	1.7

TAR total applied radioactivity
n.p. not processed
n r. not reported
Sed. Sediment
Bold numbers: peak concentration considered for single-compartment evaluation; previous values were omitted; sampling dates were adjusted accordingly
[a] Not evaluated, no decline observed.
[b] Set to material balance; original values: total system and water 76.6 and 79.5 % AD
[c] Sample not analyzed; set to zero for kinetic evaluation at Level P-II
[d] Set to zero for kinetic evaluation at Level M-I degradation
[e] Set to ½ LOD. As no LOD was reported it was assumed to correspond to 0.1% TAR, i.e. the lowest significant value according to the number of decimal places reported

II. RESULTS AND DISCUSSION

For some fits, χ^2 error values above 15% were obtained. They can be attributed to the scattering of the data caused by the rapid transformation processes that are symptomatic for the degradation of metiram and its metabolites in water/sediment systems. As the observations were generally well described by the fitted curves, the high χ^2 error values are acceptable.

Level P-I

The evaluation of all four test systems at Level P-I resulted in reliable endpoints for metiram for degradation in the total system as well as for dissipation in the water and sediment phase.

An overview of the estimated trigger and modeling endpoints for metiram from four water/sediment systems is given in Table 7.2.2.3-17 to Table 7.2.2.3-19.

Table 7.2.2.3-17: Summary of trigger and modeling endpoints for metiram, Level P-I, total system

DocID	Test system	Kinetic model	χ^2 error [%]	Trigger endpoints		Modeling endpoint
				$DegT_{50}$ [d]	$DegT_{90}$ [d]	$DegT_{50}$ [d]
1993/10415	Rhine River	FOMC	3.6	0.2	12.8	3.9 [a]
	Froschteich	FOMC	8.1	0.2	8.1	2.4 [a]
2003/1001011	Kellmetschweiher	FOMC	3.0	0.3	5.7	1.7 [a]
	Ranschgraben	FOMC	5.4	0.1	4.7	1.4 [a]

[a] Calculated as $DegT_{50} = DegT_{90}/3.32$

Table 7.2.2.3-18: Summary of trigger and modeling endpoints for metiram, Level P-I, water phase

DocID	Test system	Kinetic model	χ^2 error [%]	Trigger endpoints		Modeling endpoint
				DT_{50} [d]	DT_{90} [d]	DT_{50} [d]
1993/10415	Rhine River	HS	2.2	0.2	1.5	0.5 [a]
	Froschteich	FOMC	0.8	0.1	1.0	0.3 [a]
2003/1001011	Kellmetschweiher	DFOP	2.7	0.3	3.9	1.2 [a]
	Ranschgraben	HS	2.1	0.3	2.6	0.8 [a]

[a] Calculated as $DT_{50} = DT_{90}/3.32$

Table 7.2.2.3-19: Summary of trigger and modeling endpoints for metiram, Level P-I, sediment phase

DocID	Test system	Kinetic model	χ^2 error [%]	Trigger endpoints		Modeling endpoint
				DT_{50} [d]	DT_{90} [d]	DT_{50} [d]
1993/10415	Rhine River	DFOP	6.1	10.4	81.7	30.7 [a]
	Froschteich	HS	7.2	9.5	137.5	55.1 [a]
2003/1001011	Kellmetschweiher	DFOP	4.1	2.8	281.5	-
		SFO	23.4	-		48.0
	Ranschgraben	FOMC	15.0	25.8	>1000	-
		SFO	23.6	-		81.7

[a] Calculated as $DT_{50} = \ln 2/k2$

Level P-II

The kinetic evaluation revealed no reliable fit for any of the evaluated water/sediment systems. Consequently, no trigger or modeling endpoints were calculated.

Level M-I

Metabolite EBIS

A summary of the trigger and modeling endpoints at Level M-I dissipation for metabolite EBIS is given in Table 7.2.2.3-20 and Table 7.2.2.3-21. Dissipation kinetics were evaluated for the total system and for the water phase but not for the sediment phase as the observed residues were very low (max. 0.2% TAR). Trigger and modeling endpoints at Level M-I degradation are shown in Table 7.2.2.3-22.

Table 7.2.2.3-20: Summary of trigger and modeling endpoints for EBIS, Level M-I dissipation, total system

DocID	Test system	Kinetic model	χ^2 error [%]	Trigger endpoints		Modeling endpoint
				DT_{50} [d]	DT_{90} [d]	DT_{50} [d]
2003/1001011	Kellmetschweiher	FOMC	9.5	0.3	7.5	2.3 [a]
	Ranschgraben	SFO	3.9	2.6	8.5	2.6

[a] Calculated as $DT_{50} = DT_{90}/3.32$

Table 7.2.2.3-21: Summary of trigger and modeling endpoints for EBIS, Level M-I dissipation, water phase

DocID	Test system	Kinetic model	χ^2 error [%]	Trigger endpoints		Modeling endpoint
				DT_{50} [d]	DT_{90} [d]	DT_{50} [d]
2003/1001011	Kellmetschweiher	FOMC	10.4	0.3	5.3	1.6 [a]
	Ranschgraben	SFO	2.9	2.4	8.7	2.4

[a] Calculated as $DT_{50} = DT_{90}/3.32$

Table 7.2.2.3-22: Summary of trigger and modeling endpoints for EBIS at Level M-I degradation

DocID	Test system	Kinetic model	χ^2 error [%]	Trigger endpoints		Modeling endpoints	
				$DegT_{50}$ [d]	$DegT_{90}$ [d]	$DegT_{50}$ [d]	Formation fraction
2003/1001011	Kellmetschweiher	SFO [a]	23.8	3.0	9.9	3.0	0.032
	Ranschgraben	Dissipation observed but no reliable endpoints calculated.					

[a] FOMC kinetics for the parent

Metabolite ETU

A summary of the trigger and modeling endpoints at Level M-I dissipation for metabolite ETU is given in Table 7.2.2.3-23 to Table 7.2.2.3-25. Trigger and modeling endpoints at Level M-I degradation are shown in Table 7.2.2.3-26.

Table 7.2.2.3-23: Summary of trigger and modeling endpoints for ETU, Level M-I dissipation, total system

DocID	Test system	Kinetic model	χ^2 error [%]	Trigger endpoints DT50 [d]	Trigger endpoints DT90 [d]	Modeling endpoint DT50 [d]
1993/10415	Rhine River	SFO	12.0	10.4	34.5	10.4
	Froschteich	SFO	13.8	12.1	40.2	12.1
2003/1001011	Kellmetschweiher	No reliable endpoints derived, biphasic pattern, visually no dissipation in slow phase [a].				
	Ranschgraben	SFO	5.4	72.1	239.5	72.1

[a] For modeling purposes a default DT_{50} of 1000 d is recommended

Table 7.2.2.3-24: Summary of trigger and modeling endpoints for ETU, Level M-I dissipation, water phase

DocID	Test system	Kinetic model	χ^2 error [%]	Trigger endpoints DT50 [d]	Trigger endpoints DT90 [d]	Modeling endpoint DT50 [d]
1993/10415	Rhine River	SFO	15.2	7.7	25.7	7.7
	Froschteich	SFO	8.4	9.7	32.1	9.7
2003/1001011	Kellmetschweiher	DFOP	0.8	441.6	>1000	1000 [a]
	Ranschgraben	SFO	5.9	61.0	202.7	61.0

[a] Default value; DT_{50} calculated from k2 of DFOP model was >1000 d

Table 7.2.2.3-25: Summary of trigger and modeling endpoints for ETU, Level M-I dissipation, sediment phase

DocID	Test system	Kinetic model	χ^2 error [%]	Trigger endpoints DT50 [d]	Trigger endpoints DT90 [d]	Modeling endpoint DT50 [d]
1993/10415	Rhine River	SFO	3.8	10.7	35.6	10.7
	Froschteich	SFO	25.2	30.8	102.2	30.8
2003/1001011	Kellmetschweiher	Not evaluated, max. occurrence at end of study.				
	Ranschgraben	SFO	4.4	107.7	357.9	107.7

Table 7.2.2.3-26: Summary of trigger and modeling endpoints for ETU at Level M-I degradation

DocID	Test system	Kinetic model	χ^2 error [%]	Trigger endpoints		Modeling endpoints	
				$DegT_{50}$ [d]	$DegT_{90}$ [d]	$DegT_{50}$ [d]	Formation fraction
1993/10415	Rhine River	SFO [a]	21.6	6.9	23.0	6.9	0.654
	Froschteich	SFO [a]	13.0	8.6	28.6	8.6	0.627
2003/1001011	Kellmetschweiher	No reliable endpoints derived, biphasic pattern, visually no dissipation in slow phase [b].					
	Ranschgraben	SFO [a]	6.2	59.7	198.4	59.7	0.569

[a] FOMC kinetics for the parent
[b] For modeling purposes a default value of 1000 d is recommended

Metabolite EU

A summary of the trigger and modeling endpoints at Level M-I dissipation for metabolite EU is given in Table 7.2.2.3-27 and Table 7.2.2.3-28. Dissipation kinetics were evaluated for the total system and for the water phase but not for the sediment phase as the observed residues were very low (max. 2.6% TAR). The kinetic evaluation for the total system revealed no reliable endpoints as there was either visually no dissipation observed (System Kellmetschweiher) or no acceptable fit derived due to highly scattered data and low residues (System Ranschgraben). Trigger and modeling endpoints at Level M-I degradation are shown in Table 7.2.2.3-29.

Table 7.2.2.3-27: Summary of trigger and modeling endpoints for EU, Level M-I dissipation, total system

DocID	Test system	Kinetic model	χ^2 error [%]	Trigger endpoints		Modeling endpoint
				DT_{50} [d]	DT_{90} [d]	DT_{50} [d]
2003/1001011	Kellmetschweiher	No reliable endpoints derived, visually no dissipation observed [a]				
	Ranschgraben	No reliable endpoints derived, highly scattered data and low residues.				

[a] For modeling purposes a default value of 1000 d should be used

Table 7.2.2.3-28: Summary of trigger and modeling endpoints for EU, Level M-I dissipation, water phase

DocID	Test system	Kinetic model	χ^2 error [%]	Trigger endpoints		Modeling endpoint
				DT_{50} [d]	DT_{90} [d]	DT_{50} [d]
2003/1001011	Kellmetschweiher	FOMC	6.6	467.8	>1000	-
		SFO	11.9	-		138.4
	Ranschgraben	No reliable endpoints derived, highly scattered data and low residues.				

Table 7.2.2.3-29: Summary of trigger and modeling endpoints for EU at Level M-I degradation

DocID	Test system	Kinetic model	χ^2 error [%]	Trigger endpoints		Modeling endpoints	
				$DegT_{50}$ [d]	$DegT_{90}$ [d]	$DegT_{50}$ [d]	Formation fraction
2003/1001011	Kellmetschweiher	SFO [a]	9.3	254.8	846.6	254.8	0.070
	Ranschgraben	SFO [a]	20.5	209.4	695.8	209.4	0.054

[a] FOMC kinetics for the parent

III. CONCLUSION

The dissipation and degradation of metiram and its metabolites EBIS, ETU and EU water/sediment systems was evaluated to determine trigger and modeling endpoints. In two studies, four different natural systems of water and sediment were incubated in the dark under at 20°C for up to 105 days.

For all models considered appropriate to derive kinetic endpoints the visual assessment and goodness-of-fit statistics indicated plausible fit. For some fits, χ^2 error values above 15% were obtained which was attributed to the scattering of the data caused by the rapid transformation processes that are symptomatic for the degradation of metiram and its metabolites in water/sediment systems. As the observations were generally well described by the fitted curves the high χ^2 error values were considered acceptable. The t-test was passed for the respective model parameters. Therefore, the resulting endpoints were considered reliable.

In cases, where for the metabolites no reliable endpoints could be derived from the fit and clearly no dissipation was observed the use of the default value for modeling of 1000 days was recommended. In cases where no reliable endpoints could be derived although dissipation was clearly observed or where a conclusion on the dissipation behavior could not be drawn due to highly scattered data in combination with low residues no endpoints were reported.

At Level P-I for metiram, trigger $DegT_{50}$ in the total system of 0.1 to 0.3 days and trigger DT_{50} in the water and sediment phase of DT_{50} of 0.1 to 0.3 days and 2.8 to 25.8 days were calculated.

Reliable modeling endpoints at Level P-I were $DegT_{50}$ values of 1.4 to 3.9 days for the whole system and DT_{50} values of 0.3 to 1.2 days and 30.7 to 81.7 days for the water and sediment phase, respectively.

At Level P-II, no trigger or modeling endpoints could be calculated.

For metabolite EBIS at Level M-I dissipation, trigger DT_{50} of 0.3 and 2.6 days for the total system and of 0.3 and 2.4 days for the water phase were calculated. Modeling DT_{50} were 2.3 and 2.6 days for the total system and 1.6 and 2.4 days for the water phase. Dissipation kinetics for the sediment phase were not evaluated, as the observed residues were very low (max. 0.2% TAR). At Level M-I degradation, a $DegT_{50}$ of 3.0 days and a formation fraction from metiram of 0.032 were calculated for test system Kellmetschweiher.

For metabolite ETU at Level M-I dissipation, trigger and modeling DT_{50} of 10.4 to 72.1 days for the total system were calculated. As for test system Kellmetschweiher, no endpoints were derived, a default modeling DT_{50} of 1000 d was recommended as dissipation pattern was biphasic and visually no dissipation occurred in slow phase. In the water phase, trigger DT_{50} of 7.7 to 441.6 days and modeling DT_{50} of 7.7 to 1000 days were obtained. In the sediment phase, trigger and modeling endpoints ranged from 10.7 to 107.7 days. At Level M-I degradation, $DegT_{50}$ of 6.9 to 59.7 days and formation fractions from metiram of 0.569 to 0.654 were calculated. As for test system Kellmetschweiher, no endpoints were derived, a default modeling DT_{50} of 1000 d was recommended as dissipation pattern was biphasic and visually no dissipation occurred in slow phase.

For metabolite EU the evaluation at Level M-I dissipation revealed no reliable endpoints for the total system. For test system Kellmetschweiher, a default modeling DT_{50} of 1000 days was recommended as visually no dissipation occurred. In the water phase, a trigger DT_{50} of 467.8 days and a modeling DT_{50} of 138.4 days were obtained for test system Kellmetschweiher. Dissipation kinetics for the sediment phase were not evaluated, as the observed residues were very low (max. 2.6% TAR). At Level M-I degradation, $DegT_{50}$ of 254.8 and 209.4 days and formation fractions from metiram of 0.070 and 0.054 were determined.

Overview on degradation products of metiram in aqueous systems

Metiram is unstable in water and is degrading fast, thereby forming a range of degradates. There are three degradation products (M222F002 (ETU), M222F003 (EU), M222F004 (EBIS)) appearing in aquatic environment under biotic as well as abiotic conditions. These degradates are considered the important metabolites for further aquatic risk assessment.

In addition there are a number degradates that are only observed under the very artificial conditions of abiotic studies like hydrolysis and aqueous photolysis.
The degradates of metiram investigated so far or their precursors are partly very fast degraded by biological degradation. Due to their rather simple structures and structural similarity this can also be expected for the other degradates. Therefore, the artificial abiotic conditions of the hydrolysis and aqueous photolysis studies are of little significance for the environmental behavior of the degradation products of metiram. Degradates - although they may be formed - are not expected to reach under real outdoor conditions comparable levels as in the abiotic studies.
In the following the information on the behavior in the aqueous phase is compiled for each metabolite:

Important aquatic metabolites:

M222F002 (ETU) is a prominent metabolite under most conditions in aquatic environment. It is observed with the highest concentrations under abiotic conditions in hydrolysis.

M222F003 (EU) is a major metabolite in aqueous photolysis whereas the formation in hydrolysis is limited. It is also observed in the water/sediment study under biotic conditions where it exceeds 5% only slightly. High concentrations in photolysis are only observed after extended continuous irradiation under highly artificial conditions in the absence of biotic degradation.

M222F004 (EBIS) is a major metabolite under various conditions with highest concentrations being observed under abiotic conditions in hydrolysis. It is formed immediately and reaches its maximum after 0 – 2 days.

These metabolites or their precursors are subject to biological degradation which is partly very fast (e.g for EBIS). Therefore, the relevant exposure is derived from the water/sediment study.

Other aquatic metabolites:

M222F011 is observed only in aqueous photolysis. It co-elutes as a major polar peak together with M222F012 and M222F017 and quantification is only possible as the sum of these three compounds. The sum of the three compounds reaches rather high concentrations after extended continuous photolysis: 25.7% after 168 h at pH 5 and 30.7% after 360 h at pH 7.
M222F011 was identified as imidazole which was shown to be "readily biodegradable" under biotic conditions. That means that significant concentrations of this compound can only build up under very artificial abiotic conditions. Under biotic conditions it will be fast degraded as it is being formed.

M222F012 is observed only in aqueous photolysis. It co-elutes as a major polar peak together with M222F012 and M222F017 and quantification is only possible as the sum of these three compounds. The sum of the three compounds reaches rather high concentrations after extended continuous photolysis: 25.7% after 168 h at pH 5 and 30.7% after 360 h at pH 7.
A search for chemical synthesis revealed that virtually no realistic synthesis route is described in chemical literature. Given that M222F012 is a quite simple, small molecule this can only mean that this compound is not stable and for this reason no synthesis route is described. Under the artificial conditions of the aqueous photolysis study M222F012 was obviously formed to an extent that it became detectable by LC/MS-MS. However, due to its instability it is unlikely that it will occur in natural aqueous environment in noticeable amounts.

M222F013 is observed only in aqueous photolysis. It co-elutes with M222F015 and quantification is only possible as the sum of these two compounds. The two compounds together form a major peak in aqueous photolysis at pH 9 whereas at pH 5 and pH 7 the sum of both is only once above 5% or just reaches 5%, respectively. The maximum of 14.0/14.5% for the sum of both is reached after 24-72 h.

M222F014 is observed only in aqueous photolysis. It co-elutes with M222F002 (ETU) and cannot be quantified independent of ETU. However it was only detected in pH 5 and pH 9 aqueous photolysis by LC/MS-MS but not at pH 7. Since ETU is clearly the dominant major compound in this peak under the various conditions tested (different pH values, irradiated in aqueous photolysis or not irradiated in hydrolysis) it is concluded that this is a minor compound that becomes only occasionally detectable. Also for that reason only a tentative structure could be assigned.

M222F015 is observed only in aqueous photolysis. It co-elutes with M222F013 and quantification is only possible as the sum of these two compounds. The two compounds together form a major peak in aqueous photolysis at pH 9 whereas at pH 5 and pH 7 the sum of both is only once above 5% or just reaches 5%, respectively. At pH 9 the maximum of 14.0/14.5% for the sum of both is reached after 24-72 h.

M222F016 is detected only in aqueous photolysis at pH 5 and pH 7 where it exceeds once 5% (pH 7) or once 10% (pH 5) of applied. Its maximum of 10.5% is reached after 24 h.

M222F017 is observed in aqueous photolysis and in hydrolysis. In the aqueous photolysis it co-elutes as a major polar peak together with M222F011 and M222F012 and quantification is only possible as the sum of these three compounds. The sum of the three compounds reaches rather high concentrations after extended continuous photolysis: 25.7% after 168 h at pH 5 and 30.7% after 360 h at pH 7. M222F017 is also formed to some extent under the artificial conditions of the hydrolysis study but is not observed under the more realistic conditions of the water/sediment study that include biotic degradation. From this it is concluded that this compound is not of importance for the aqueous environment under realistic outdoor conditions.

M222F018 is observed in aqueous photolysis; in hydrolysis it was tentatively assigned. This is a transient compound that is formed immediately after application. It reaches its maximum already after 0 – 6 h and is fast degraded further on. The highest level is observed at pH 7 in aqueous photolysis at 6 h after treatment with 20.5%. Due to its inherent instability only very low amounts were available for structure elucidation and only a tentative structure could be assigned.

M222F019 is observed only in aqueous photolysis. It co-elutes with M222F020 and quantification is only possible as the sum of these two compounds. The sum of both compounds reaches max. 2.5% and no further consideration is required.

M222F020 is observed only in aqueous photolysis. It co-elutes with M222F019 and quantification is only possible as the sum of these two compounds. The sum of both compounds reaches max. 2.5% and no further consideration is required.

Besides the above described compounds further minor, unidentified degradates were observed.

Overview on degradation products in aqueous systems under different conditions

Metabolite code	Other designation	Evaluation and remarks
M222F002	ETU	major under biotic and abiotic conditions
M222F003	EU	major under biotic and abiotic conditions
M222F004	EBIS	major under biotic and abiotic conditions, formed early, degraded fast under biotic conditions
M222F011	Imidazole	major under photolytic conditions, “readily biodegradable”
M222F012		minor, unstable
M222F013		major under photolytic conditions, formed early with maximum after 24- 72 h
M222F014		minor
M222F015		major under photolytic conditions, formed early with maximum after 24- 72 h
M222F016		major under photolytic conditions, transient with maximum after 24 h
M222F017		major under photolytic conditions; detected in hydrolysis but not detected under biotic conditions
M222F018		major under photolytic conditions, tentatively detected in hydrolysis, transient with maximum at 0 – 6 h
M222F019		minor
M222F020		minor

Conclusion

Important major metabolites of metiram in the aquatic environment are M222F002 (ETU), M222F003 (EU), M222F004 (EBIS).

Further metabolites that were observed in major amounts only under artificial abiotic conditions are M222F011, M222F013, M222F015, M222F016, M222F017 and M222F018.

M222F011 and M222F017 can be expected not to appear in significant amounts under real outdoor conditions.
M222F018 is immediately formed and is inherently transient.
M222F013, M222F015 and M222F016 are formed early upon application and reach their maximum already after 24 – 72 hours.

CA 7.2.2.4 Irradiated water/sediment study

No irradiated water/sediment studies were performed.

CA 7.2.3 Degradation in the saturated zone

Due to its low leaching potential, metiram is not expected to reach deeper soil layers or the saturated zone. Therefore, investigations on the degradation in the saturated zone are considered to be not necessary.

CA 7.3 Fate and behaviour in air

CA 7.3.1 Route and rate of degradation in air

Studies presented in the first Annex I inclusion process:

Volatilization of the a.s. metiram is considered unlikely. Therefore, for the first Annex I inclusion process, the photochemical oxidative degradation in air was calculated for the degradation product EBIS (M222F004) instead for the a.s. [*CA 7.3.1/2, BASF DocID 2000/1000148*]. This calculation is considered still valid.

Submission of not yet peer-reviewed studies in this AIR3-Dossier:

Calculations of the photochemical oxidative degradation in air (Atkinson) of Metiram, ETU (M222F002) and EU (M222F003) according to the newest model are provided.
Furthermore, a laboratory volatility study on ETU is presented [*CA 7.3.1/3*]. This study was already cited in the Monograph for the active substance mancozeb. However, no summary appears to be given, therefore it is unclear if this study can be considered as being peer-reviewed.

Active substance metiram:

Metiram is a polymeric compound and has a very low vapor pressure of $7.4 \cdot 10^{-8}$ Pa at 20 °C [*CA 2.2*]. Volatilization of metiram is therefore unlikely.

Report:	CA 7.3.1/1 Hassink J., 2014c Photochemical oxidative degradation of Metiram (QSAR estimates) 2014/1036828
Guidelines:	EC 1107/2009 of the European Parliament
GLP:	no

Executive Summary

The degradation rates for reactions of metiram with OH-radicals and ozone in the atmosphere were calculated using the AOPWIN program based on ATKINSON's increment method.

Based on the resulting degradation rate of $k_{OH} = 359.7361 \times 10^{-12}$ cm^3 $molecule^{-1}$ s^{-1}, the atmospheric degradation half-life of the substance via this reaction route is $t_{1/2} = 0.03$ d (12 h day). Although O_3 is likely to react with metiram, the degradation rate resulting from ozone attack could not be estimated.

Due to the rapid degradation in air, it can be concluded that there is no risk of long-range transport of metiram.

I. MATERIAL AND METHODS

The degradation rate resulting from attack of OH-radicals was calculated with the AOPWIN Program (Atmospheric Oxidation Program for Microsoft Windows 3.1, Version 1.88, Syracuse Research Corp. 1997) based on ATKINSON's increment method [*Atkinson, R. (1987): A Structure-Activity Relationship for the Estimation of Rate Constants for the Gas-Phase Reactions of OH Radicals with Organic Compounds, Int.J.Chem.Kin. 19, 799*]. The degradation rate resulting from attack of ozone was calculated according to an OECD method [*Anonymous (1992): The rate of photochemical transformation of gaseous organic compounds in air under tropospheric conditions. OECD Environment Monographs No. 61, OECD, Paris*].

The degradation rate of metiram with OH-radicals was estimated based on the structural formula. The SMILE notation used for metiram in AOPWIN was:

S=C(S[Zn](SC(=S)NCCNC(=S)S)N)NCCNC(=S)S

II. RESULTS AND DISCUSSION

Assuming a pseudo-first order reaction, the degradation half-life was calculated by taking into account the diurnally and seasonally averaged concentration of hydroxyl-radicals in the troposphere. The total rate constant was estimated to be k_{OH} = 359.7361 x 10^{-12} cm^3 $molecule^{-1}$ s^{-1}.

Considering a weighted global average tropospheric hydroxyl-radical concentration of 1.5 x 10^6 mol cm^{-3}, the half-life for the degradation of metiram by OH-radicals was calculated according to Equation 7.3.1-1.

Equation 7.3.1-1 Estimation of the atmospheric degradation half-life (t1/2) of metiram

$$t_{1/2} = \ln2 / (359.7361\ 10^{-12} \times 1.5\ 10^{6})\ s$$
$$= 21.4\ min$$
$$= \underline{0.03\ d\ (12\ h\ day)}$$

Although metiram contains reactive sites for an ozone attack, a reasonable approximation by AOPWIN was not possible. Therefore, although O_3 is likely to react with metiram, no degradation estimation could be given.

III. CONCLUSION

Based on the results of the atmospheric degradation half-life of metiram ($t_{1/2}$ = 0.03 d), it can be concluded that the substance will be rapidly degraded by photochemical processes in the troposphere. Hence, due to the rapid degradation in air, it can be concluded that there is no risk of long-range transport of metiram.

Metabolite M222F004 (EBIS):

Metabolite M222F004 (EBIS) has a vapor pressure of $1.6 \cdot 10^{-3}$ Pa at 20 °C [*CA 2.14*], i.e. it is potentially volatile.

Peer reviewed study:

Report:	*CA 7.3.1/2* *Goetz N. von, 2000a* *Photochemical oxidative degradation of Ebis, a degradation product of Metiram (BAS 222 F) (QSAR estimates)* *2000/1000148*
Guidelines:	*EEC 94/37*
GLP:	*no*

Materials and Methods:

Using the computer program AOPWIN which is based on the increment system published by Atkinson (1987), the degradation rate for reactions of EBIS with hydroxyl radicals is calculated based on the structural formula. Assuming a pseudo-first order reaction, the degradation half-life via this reaction route is calculated by taking into account the diurnally and seasonally averaged concentration of hydroxyl radicals in the troposphere.
The degradation of a compound A by OH-radicals can then be calculated by

$-d[A]/dt = k' \cdot [A]$ (1)
with $k' = k \cdot$ [OH-radicals]

The half-life of this process can be calculated by equation (2):

$t_{1/2} = \ln2/k' = \ln2 / (k \cdot [\text{OH-radicals}])$

Findings:

The total rate constant is $\underline{k > 231\ 10^{-12}\ cm^3\ molecule^{-1}s^{-1}}$.
With

$t_{1/2}$ $< \ln2 / (231\ 10^{-12}\ 8\ 10^5)$ s
< 1.04 h
<u>< 0.043 d (24 h day)</u>

The resulting half-life due to OH-radical attack is < 0.043 days on a 24 hour day basis.

Metabolite M222F002 (ETU):

Metabolite M222F002 (ETU) has a very low vapor pressure of $3.1 \cdot 10^{-6}$ Pa at 20 °C [*CA 2.14*]. Volatilization of ETU is unlikely.

Report:	CA 7.3.1/3 Obrist J.J., 1992a Laboratory volatility studies on 14C-ETU 1992/5045
Guidelines:	EPA 163-2
GLP:	yes (certified by United States Environmental Protection Agency)

Summary

The volatilization of ^{14}C-ethylene thiourea (ETU) from a sandy loam soil under sterile and non-sterile conditions was investigated. ^{14}C-ETU (radiochemical purity 98.9%) was applied to 100 g soil at a nominal concentration of 1 ppm which was incubated at 40°C whilst humidified air was passed over at 30-90 mL min^{-1} for up to 30 days (a total of five different systems, 3 non-sterile and 2 sterile).
Any radioactivity that volatilized from the soil was trapped continuously using charcoal (x2), 0.1N sulfuric acid, 2-ethoxyethanol/ethanolamine (1:1) (x2) and potassium hydroxide. The volatile traps were analyzed at days 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30.
Total recovery of radioactivity in the five test systems ranged from 100.0 – 120.1% TAR.
Radioactivity in the traps was measured using liquid scintillation counting (LSC).
The only trap to contain measurable quantities of radioactivity >0.1% TAR was the first 2-ethoxyethanol/ethanolamine trap, which contained 70.4-74.7% TAR after 30 days in the non-sterile systems. All radioactivity in the 2-ethoxyethanol/ethanolamine traps was confirmed as CO_2.
The remainder of the radioactivity (43.0-49.0% TAR after 30 days) was associated with the soil. After 30 days only 3.5% TAR was extractable with acetonitrile/water (80:20) and acetonitrile (no ETU or EU found by 2D-TLC).
In the sterilized soil systems, no radioactivity >0.1% TAR was measured in any of the 6 traps. The recovered radioactivity (100.0-110.0% TAR) was all associated with the soil. 63% TAR was extractable from sterile soil after 30 days, with 23% TAR identified as ETU and 18% TAR as EU by 2D-TLC.

The results show that ETU is not volatilized from soil and that it is rapidly mineralized to CO_2 in biologically active soils.

Materials and methods

^{14}C-ETU (radiochemical purity 98.9%, specific activity 10.08 mCi g^{-1}) was applied to a Californian sandy loam soil (Table 7.3.1-1).

Table 7.3.1-1: Soil characterization

	California
Texture (USDA)	Sandy loam
Sand (%)	68.6
Silt (%)	23.0
Clay (%)	8.4
Organic matter (%)	0.79
pH	8.1
CEC (meq 100 g^{-1} soil)	4.72
pF 2.5 (%w/w)	9.1

Five glass flasks were prepared by weighing 100 g of soil into each container. Two flasks were autoclaved to sterilize the soil.
980 µL of the dose solution in methanol was applied to the 100 g portions of soil, resulting in a nominal concentration of 1 ppm (1 µg test material per g soil). The methanol was allowed to evaporate and then water was added to raise the soil moisture content to 75% field moisture capacity (pF 2.5).

The three flasks containing the biologically active soil were incubated at 40 ºC for 30 days whilst humidified air was passed over the soil at flow rates of 30, 60 and 90 mL min^{-1}.
The two flasks containing the sterilized soil were incubated at 40 ºC for 30 days whilst humidified air was passed over the soil at flow rates of 30 and 90 mL min^{-1}.

- Flask 1: Airflow 30 mL min^{-1} non-sterile
- Flask 2: Airflow 60 mL min^{-1} non-sterile
- Flask 3: Airflow 90 mL min^{-1} non-sterile
- Flask 4: Airflow 30 mL min^{-1} sterile
- Flask 5: Airflow 90 mL min^{-1} sterile

Each of the 5 flasks were connected to a series of 6 traps:

- Trap 1: Charcoal
- Trap 2: Charcoal
- Trap 3: 0.1N Sulfuric acid
- Trap 4: 2-Ethoxyethanol/ethanolamine (1:1)
- Trap 5: 2-Ethoxyethanol/ethanolamine (1:1)
- Trap 6: Potassium hydroxide

Air was continuously pulled through by vacuum.

The traps were changed on days 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 and analyzed by LSC.
The presence of CO_2 in the 2-ethoxyethanol/ethanolamine traps was confirmed by evolution with sulphuric acid and re-trapping in KOH. Barium chloride precipitation was also used to confirm the presence of CO_2 in the KOH solution.

The soil (10 or 20 g) was extracted two times with acetonitrile/water (80:20) followed by acetonitrile for 20 minutes at ambient temperature. The extractable radioactivity was analyzed by LSC and 2D-TLC.

Results and discussion

The total radioactivity recovered in the various matrices are summarized in Table 7.3.1-2.

Table 7.3.1-2: Summary of radioactivity found among the sample matrices

Sample	% Total Applied Radioactivity				
	Flask				
	1	2	3	4	5
Charcoal 1	<0.1	<0.1	<0.1	<0.1	<0.1
Charcoal 2	<0.1	<0.1	<0.1	<0.1	<0.1
Sulfuric acid	<0.1	<0.1	<0.1	<0.1	<0.1
2-Ethoxy ethanol/ ethanolamine 1	71.1	70.4	74.7	<0.1	<0.1
2-Ethoxy ethanol/ ethanolamine 2	<0.1	<0.1	<0.1	<0.1	<0.1
Potassium hydroxide	<0.1	<0.1	<0.1	<0.1	<0.1
Soil	49.0	47.3	43.0	100.0	110.0
Flask rinse	<0.1	<0.1	<0.1	<0.1	<0.1
Total	120.1	117.7	117.6	100.0	110.0

The radioactivity in the 2-ethoxyethanol/ethanolamine trap 1 was confirmed as being CO_2.

At the end of the 30 days, only 3.5% TAR was extractable from the biologically active soil (Flask 2) with the remainder being non-extractable residues. None of the components corresponded to either ETU or EU by 2D-TLC.
In the sterilized soil, 63% TAR was extracted from the soil (Flask 4) with ETU (23% TAR) and EU (18% TAR) confirmed by 2D-TLC.

Conclusions

The results show that ETU is not volatilized from soil and that it is rapidly mineralized to CO_2 in biologically active soils.
The presence of EU in the sterile soil but lack of detection in the traps indicates that EU is also non-volatile.

Report: CA 7.3.1/4
Hassink J., 2014d
Photochemical oxidative degradation of ETU, a degradation product of Metiram (QSAR estimates)
2014/1036829
Guidelines: EC 1107/2009 of the European Parliament
GLP: no

Executive Summary

The degradation rates for reactions of ethylene thiourea (ETU) with OH-radicals and ozone in the atmosphere were calculated using the AOPWIN program based on ATKINSON's increment method.

Based on the resulting degradation rate of $k_{OH} = 139.6756 \times 10^{-12}$ cm^3 $molecule^{-1}$ s^{-1}, the atmospheric degradation half-life of the substance via this reaction route is $t_{1/2} = 0.077$ d (12 h day). Although O_3 is likely to react with ETU, the degradation rate resulting from ozone attack could not be estimated.

Due to the rapid degradation in air, it can be concluded that there is no risk of long-range transport of ETU.

I. MATERIAL AND METHODS

The degradation rate resulting from attack of OH-radicals was calculated with the AOPWIN Program (Atmospheric Oxidation Program for Microsoft Windows 3.1, Version 1.88, Syracuse Research Corp. 1997) based on ATKINSON's increment method [*Atkinson (1987)*]. The degradation rate resulting from attack of ozone was calculated according to an OECD method [*Anonymous (1992)*].

The degradation rate of ETU with OH-radicals was estimated based on the structural formula. The SMILE notation used for ETU in AOPWIN was:

N(C(=S)NC1)C1

II. RESULTS AND DISCUSSION

Assuming a pseudo-first order reaction, the degradation half-life was calculated by taking into account the diurnally and seasonally averaged concentration of hydroxyl-radicals in the troposphere. The total rate constant was estimated to be $k_{OH} = 139.6756 \times 10^{-12}$ cm^3 molecule $^{-1}$ s^{-1}.

Considering a weighted global average tropospheric hydroxyl-radical concentration of 1.5×10^6 mol cm^{-3}, the half-life for the degradation of ETU by OH-radicals was calculated according to Equation 7.3.1-2.

Equation 7.3.1-2 Estimation of the atmospheric degradation half-life (t1/2) of ETU

$$t_{1/2} = \ln 2 / (139.6756\ 10^{-12} \times 1.5\ 10^{6})\ s$$
$$= 0.919\ h$$
$$= \underline{0.077\ d\ (12\ h\ day)}$$

Although ETU contains reactive sites for an ozone attack, a reasonable approximation by AOPWIN was not possible. Therefore, although O_3 is likely to react with ETU, no degradation estimation could be given.

III. CONCLUSION

Based on the results of the atmospheric degradation half-life of ethylene thiourea (ETU) ($t_{1/2} = 0.077$ d), it can be concluded that the substance will be rapidly degraded by photochemical processes in the troposphere. Hence, due to the rapid degradation in air, it can be concluded that there is no risk of long-range transport of ETU.

Metabolite M222F003 (EU):

Metabolite M222F003 (EU) has a vapor pressure of $1.6 \cdot 10^{-3}$ Pa at 20 °C [*CA 2.14*], i.e. it is potentially volatile.

Report:	CA 7.3.1/5 Hassink J., 2015a Photochemical oxidative degradation of EU, a degradation product of Metiram (QSAR estimates) 2015/1137112
Guidelines:	EC 1107/2009 of the European Parliament
GLP:	no

Executive Summary

The degradation rates for reactions of ethylene urea (EU) with OH-radicals and ozone in the atmosphere were calculated using the AOPWIN program based on ATKINSON's increment method.

Based on the resulting degradation rate of $k_{OH} = 8.0290 \times 10^{-12}$ cm^3 $molecule^{-1}$ s^{-1}, the atmospheric degradation half-life of the substance via this reaction route is $t_{1/2} = 1.332$ d (12 h day). Although O_3 is likely to react with EU, the degradation rate resulting from ozone attack could not be estimated.

I. MATERIAL AND METHODS

The degradation rate resulting from attack of OH-radicals was calculated with the AOPWIN Program (Atmospheric Oxidation Program for Microsoft Windows, Syracuse Research Corp. 1997) based on ATKINSON's increment method [*Atkinson (1987)*]. The degradation rate resulting from attack of ozone was calculated according to an OECD method [*Anonymous (1992)*].

The degradation rate of EU with OH-radicals was estimated based on the structural formula. The SMILE notation used for EU in AOPWIN was:

C1NC(=O)NC1

II. RESULTS AND DISCUSSION

Assuming a pseudo-first order reaction, the degradation half-life was calculated by taking into account the diurnally and seasonally averaged concentration of hydroxyl-radicals in the troposphere. The total rate constant was estimated to be k_{OH} = 8.0290 x 10^{-12} cm^3 molecule $^{-1}$ s^{-1}.

Considering a weighted global average tropospheric hydroxyl-radical concentration of 1.5 x 10^6 mol cm^{-3}, the half-life for the degradation of EU by OH-radicals was calculated according to Equation 7.3.1-3.

Equation 7.3.1-3 Estimation of the atmospheric degradation half-life ($t_{1/2}$) of EU

$$t_{1/2} = \ln 2 / (8.0290\ 10^{-12} \times 1.5\ 10^{6})\ s$$
$$= 15.986\ h$$
$$= \underline{1.332\ d\ (12\ h\ day)}$$

Although EU contains reactive sites for an ozone attack, a reasonable approximation by AOPWIN was not possible. Therefore, although O_3 is likely to react with EU, no degradation estimation could be given.

III. CONCLUSION

Based on the results of the atmospheric degradation half-life of ethylene urea (EU) ($t_{1/2}$ = 1.332 d), it can be concluded that the substance will be degraded fast by photochemical processes in the troposphere.

CA 7.3.2 Transport via air

Metiram has a very low volatilization potential and is degraded very fast by photochemical processes.
Metabolite M222F002 (ETU) has a very low volatilization potential and is not volatilized from soil. It is degraded very fast by photochemical processes.
Metabolite M222F004 (EBIS) is potentially volatile but is degraded very fast by photochemical processes.
Metabolite M222F003 (EU) is potentially volatile but is not volatilized from soil. It is degraded fast by photochemical processes.

Consequently, there is no risk of long-range transport of metiram or its metabolites.

CA 7.3.3 Local and global effects

No effects are expected since transport via air is unlikely (for details see above).

CA 7.4 Definition of the residue

CA 7.4.1 Definition of the residue for risk assessment

According to the results presented in M-CA 7.1 – 7.3 the following compounds have to be considered for the environmental risk assessment:

Soil:
Metiram and its soil metabolites M222F002 (ETU), M222F003 (EU), M222F004 (EBIS) and M222F007 (TDIT).

For all four metabolites a risk assessment has been performed based on ecotoxicological studies with the respective metabolite. It is concluded that the risk of metabolites for soil organisms is low.

Groundwater:
Metiram and its soil metabolites M222F002 (ETU), M222F003 (EU), M222F004 (EBIS) and M222F007 (TDIT).

Due to the very fast degradation in soil and/or the strong adsorption, neither the parent compound nor one of its metabolites poses a risk of leaching to groundwater. The predicted annual leachate concentrations of metiram and all its soil metabolites were far below 0.1 µg L^{-1}.

Surface Water:
Metiram and its metabolites M222F002 (ETU), M222F003 (EU), M222F004 (EBIS) that are observed under most conditions in the aqueous environment.
Further major metabolites in water that are only observed under the highly artificial conditions of the hydrolysis and/or aqueous photolysis studies are M222F011, M222F012, M222F013, M222F015, M222F016, M222F017 and M222F018.

For the metabolites M222F002 (ETU), M222F003 (EU), M222F004 (EBIS) a risk assessment has been performed based on ecotoxicological studies with the respective metabolite or – due to the immediate formation and short half-life of the metabolite – based on studies with the parent compound. From the metabolite studies it is concluded that the risk for aquatic is low whereas for the metabolites tested within the parent studies the risk is covered by the risk of the active substance.

For the other metabolites a risk assessment is provided based on QSAR and available studies or literature data. These data indicate that the risk of these metabolites for aquatic organisms is low.

Sediment:
Metiram and its metabolite ETU.

A more detailed risk assessment for the metabolite ETU is not required due to the low aquatic toxicity of ETU.

Air:
Metiram

Metabolites M222F002 (ETU), M222F003 (EU), M222F004 (EBIS) are either not volatile and/or fast degraded by photochemical processes.

CA 7.4.2 Definition of the residue for monitoring

According to the results of the risk assessment, parent metiram should be considered for environmental monitoring. Although not required from the perspective of the risk assessment, the toxicologically relevant metabolite M222F002 (ETU) is - except for the air compartment - included in the definition of the residue for monitoring.

Soil: Metiram and M222F002 (ETU)

Ground Water: Metiram and M222F002 (ETU)

Surface Water: Metiram and M222F002 (ETU)

Sediment: Metiram and M222F002 (ETU)

Air: Metiram (parent only)

CA 7.5 Monitoring data

During the first Annex I inclusion process monitoring data on ETU have been discussed. This is reflected in the respective section of the Addendum to the Monograph (2002) for metiram which provided below (essentially summaries and discussion of von Goetz, N., Dressel, J., 2001 [CA 7.5/1, BASF DocID 2001/1020883] and von Goetz, N., 2001 [CA 7.5/2, BASF DocID 2001/1009183]).

Report:	*CA 7.5/1* *Goetz N. von,Dressel J., 2001a* *Joint position paper of Dow, Cerexagri, BASF for Mancozeb, Metiram and Maneb - Environmental fate EBDC s: Summary assessment of risk to groundwater* *2001/1020883*
Guidelines:	*none*
GLP:	*no*

Evaluation of the Joint Position Paper of Dow, Cerexagri, BASF for Mancozeb, Metiram and Maneb on risk to groundwater from exposure to EBDCs by the RMS:

The leaching potential of ETU has been assessed. The analogous assessment for carbimid, EBIS and EU is instead pending, waiting for further information on adsorption and halflives. The EDA metabolite was not considered because of its strong soil sorption.

A series of studies on groundwater findings for ETU has been considered by the notifier, their evaluation is shortly reported here; additionally, RMS has thought appropriate to add below the findings from the additional studies reported in the other two EBDC monographs, in order to reach a complete picture of the available findings on ETU in groundwater.

Boland et al (1995)
RMS considers that the evaluation of this study on groundwater monitoring in the Netherlands made by the NL as rapporteur assistant has to be taken into account, and therefore it is presented below.
On the other hand, the arguments of the notifier on the weak points of the study, and especially for using it as a proper groundwater monitoring study, are found valid (see the position paper of von Goetz, 26/1/2001, included in [*CA 7.5/2*]). These are mainly: 1) samples taken only in one year (no confirmation of results by next round, potential wrong-positive findings therefore) 2) 1993 extremely wet year 3) sampling technique not designed to sample groundwater only and therefore susceptible to cross-contamination.
The conclusive position taken by RMS is in favor of taking into account the findings of the study anyway, but considering them as only indicative of a potential for groundwater contamination.

NL review of the study

The average ETU concentration under 32 fields with potatoes was 0.11 µg/l and the 90th percentile 0.27 µg/l. The values are based on average per field for sampling round 1 and 2, which means during and after application. According to the first sampling round ETU was present in ground water before application of EBDC's. In the table average values, 90 percentile values and maximum values are given for the whole of the Netherlands and discriminated for four soil types.

Table 7.5-1: Monitoring of ETU in shallow groundwater under potato fields

Soils (number of fields)	Average concentration µg/l	90 percentile µg/l	Maximum concentration µg/l
Netherlands (32)	0.11	0.27	0.49
Sand (8)	0.13	0.25	0.28
Clay (8)	0.05	0.11	0.16
Loam (8)	0.03	0.06	0.07
Peat (8)	0.22	0.40	0.49

These figures indicate that there might be a relation between soil type and concentrations measured. Loamy soils and clay soils seem to be less sensitive to leaching compared to sandy soils and peat soils. The number of fields is too low to deduce a reliable 90 percentile for different soil types. A larger database can lead to the conclusion that loamy soils and clay soils do not exceed the threshold of 0.1 µg/l for the 90 percentile. On the other, higher concentrations after round 3 cannot be excluded.

Danish pesticide leaching assessment programme (2001)

As part of a wider leaching monitoring programme, a monitoring study has been conducted at Tylstrup (Northern Denmark), using sampling devices installed prior to the first pesticide application. The results of the first year of monitoring are presented.

The study field was 1.1 ha in size. The soil was characterised as loamy sand with 6% clay and 2 % of total organic carbon in the topsoil. The groundwater level during the study fluctuated between 3 and 4 m below soil surface. In the 1999 growing season a conservative tracer (potassium bromide), linuron, metribuzin, lambda-cyhalothrin and mancozeb were applied to potatoes. Mancozeb was applied as Dithane DG at a rate of 10 x 2 kg/ha from June to September. In the course of the study ETU was detected only in a few of the water samples from the unsaturated zone (suction cups) at levels of approximately 0.03 µg/L. No ETU was detected in the groundwater whereas for Bromide a clear concentration breakthrough was observed. The leaching risk associated with mancozeb and ETU was therefore considered to be negligible.

Groundwater monitoring at Flevoland (NL, 2000)
The groundwater of the Dutch province Flevoland was sampled and analysed for 16 pesticidal active substances. ETU was included as an important metabolite of the EBDC fungicides. 14 samples were taken from deep groundwater (>20 m below soil surface) and 51 samples were taken from shallow groundwater (<20 m). Only in two samples from wells of 4 and 6 m depth below soil surface ETU could be detected in concentrations of 0.2 and 0.17 µg/L, respectively. The land-use at both well locations was arable cropping. For these samples an analysis whether these concentrations might originate from the use of the EBDC pesticides under good agricultural practice is not available. Groundwater contamination by accidental spills or by irrigation wells must also be considered. (RMS: Evaluation of the study submitted by the notifier, it couldn't be checked because no translation was available).

US EPA National Pesticide Survey (Fisher, 1992)
Out of 1393 samples for representative agricultural areas in different regions, 1 positive finding for ETU (16 ppb) was detected. (For the description of the study see maneb monograph).
The notifier has stressed the evidence that considering that this finding, together with another one at trace level were found in states (respectively of Illinois and Iowa) which are characterized by low usage of EBDCs, whereas areas of EBDCs intensive use have not registered ETU contamination, suggesting that ETU groundwater contamination seems unlikely.

Field study in Sweden (Bergstrom, 1993)
3 Swedish potatoes sandy soil sites have been sampled for 1 year (1992-93) at 12-16.8 kg a.i/ha/year, with supplementary irrigation 175-200 mm, rainfall 511-803 mm, LOQ 0.1 µg/l, 2 pipes for each site at 2.5 and 5 m depth, no data on pH are available. (For the description of the study see maneb monograph).
ETU was always found at level < 0.1 µg/l.

Degradation of ethylenethiourea (ETU) in oxic and anoxic sandy aquifers (Jacobsen and Bossi, 1997)
In this Danish study of degradation of ETU in aquifer sediment samples drawn at different depths at the Fladerne Baek site referred to by the notifier, microbially mediated degradation in aquifers has been shown for ETU, both in oxic and anoxic conditions; temperature dependence and complete degradation in presence of microbial nitrate reduction -typical of deeper aquifers- have been found, explaining the findings of negligible amounts of ETU in groundwater in the same site notwithstanding the high application rate of EBDCs used (16 kg/ha).

Evaluation of the state of the art for ETU in groundwater

RMS can agree on the position of the notifier that considers that - taking into account the literature findings described above, the degradative potential of ETU in aquifers and the low concentration predicted by the FOCUS calculations - the groundwater contamination potential from ETU exposure should be considered low. However, the rapporteur still thinks that further investigations on the leaching potential shown by the groundwater monitoring study in the Netherlands and on the possibility of association between leaching and kind of soil should be conducted, for example by means of further modelling.

Table 7.5-2: References relied on

Author(s)	Year	Title Generated by (company or organisation), Submitted by (company(ies) or organisation), Report/file No. of submitting company For publication: reference
Bergstrom L.	1993	Occurrence of Ethylenthiourea in Swedish Groundwaters and Drinking Waters. Swedish University of Agricultural Sciences, Div. Water Quality Research.
Boland, J., S.Q. Broerse, A.A. Cornelese, A.M.A. van der Linden, R.A. Baumann, W. de Graaf, H.A.G. Heusinkveld	1995	Monitoring of ETU in the uppermost groundwater below flower bulb and potato fields in the Netherlands in 1993, RIVM report 715801003, Bilthoven.
Cornelese, A. A., P. G. H. de Jong, A. M. A. van der Linden, R. A. Baumann	1995	Behaviour of ETU in soil from two Dutch fields, RIVM reportnr. 715801002
Fisher R. L.	1992	Data Base on Occurrence of Ethylenthiourea in Groundwater. EBDC/ETU task force
Jacobsen, O.S., R. Bossi	1997	Degradation of ethylenethiourea (ETU) in oxic and anoxic sandy aquifers, FEMS Microbiology Reviews 20, 539-544
Kjaer, J., P. Olsen, P. Sjelborg, I. Fomsgard, B. Mogensen, F. Plauborg, J. O. Jorgensen, B. Lindhardt	2001	The Danish pesticide leaching assessment programme, ISBN 87-7871-095-2, Geological survey of Denmark and Greenland, Copenhagen, Denmark.
Miles, C. F., D. R. Doerge	1991	Fate of ethylenthiourea in hawaiian soil and water, J. Agric. Food Chem., 39, 214-217
Minnema, B., P. Vermeulen, P. Venema	2000	Bestrijdingsmiddelenonderzoek groundwater provicie Flevoland, TNO-rapport NITG 00-181-B, Delft, Netherlands
de Vette, H. Q. M., J. G. van Asten, A. O. Hanstveit	1999	Determination of the rate of degradation of N,N’-ethylene urea (EU; metabolite of mancozeb) in three soils, TNO Nutrition and Food Research Institute, TNO study no. IMW-98-0134-01, Delft, The Netherlands
Wright, M. C.	2000	Aerobic soil metabolism degradation rate determination for ethylenethiourea (ETU) on soil

Position paper prepared by Notifier for CTB and then submitted for the EU review on 16.05.2001:

Report:	*CA 7.5/2* *Goetz N. von, 2001a* *Metiram: Response to E-fate conclusions (Column D in SANCO 314/2001-rev. 1)* *2001/1009183*
Guidelines:	*none*
GLP:	*no*

Reasoning

On the occasion of the meeting of Elf Atochem Agri and BASF with CTB, Netherlands, CTB requested further proof on the argumentation against the use of the report „Monitoring of ETU in the uppermost groundwater below flower bulb and potato fields in the Netherlands in 1993" J. Boland et al. (1995) in the registration process for EBDC's in the Netherlands.

Statement

The study cited above was conducted 7 years ago when proper on-field-groundwater sampling techniques and designs were beginning to be developed (sampling done in year 1993, report written in 1995). It was not designed to fulfill any standard criteria for registration, but is the first study of this type. The focus of the study lies on the statistical part and therefore on the comparison between different soil types, which was done in a very thorough way by evaluating a large number of soils varying only in the soil properties.

For studies as complex as groundwater monitoring studies, the purpose of the study has a great influence on the possible use of the results. Before using a study of this type for a different purpose as the intended one it is crucial to carefully look at the premises of the study (here the study is used to determine if the use of EBDC's under good agricultural practice poses a risk to groundwater instead of using it for its original purpose of comparing the influence of different soil types on the concentration of ETU in shallow groundwater/pore water).

In the preceding year, quality criteria for groundwater sampling have been set up for the use of those data in the pesticide registration procedure (e.g. Leistra & van der Bolt (2000): Evaluation of measurements for bentazone in groundwater at about 10 m depth, Alterra, Wageningen). At present time, therefore, the report should be strictly reviewed to be sure that the quality criteria needed especially for registration purposes have been met.

In the report a wide range of soils was examined for the two crops flower bulbs and potatoes. For each crop 32 fields were tested with 8 fields each representing the four different soil types under agricultural use: sand, peat, loam and clay. This is an extraordinarily extensive study and conclusions can be drawn on the behavior of ETU in the "groundwater/pore water" near the soil surface in comparing the different soil types with one another.

However, it is difficult to draw quantitative conclusions on the groundwater concentrations of ETU from the following study mainly for the following reasons:

In this study the samples were taken **only in one year**. Thus, no confirmation of the results for a specific field was possible and wrong-positive data could not be identified. This would have been possible with a second confirmative round after the application period. In groundwater monitoring studies a large number of influences has to be taken into account, which not only enclose weather and soil conditions, but number and period of applications, cropping time, direction of groundwater flow and more. These all can contribute to wrong-positive findings and if regulation is concerned the wrong-positive findings have to be identified.

The **year 1993** was an extreme year in that the very dry spring was followed by an extremely wet summer. Therefore, some of the fields were flooded during the application period and it can be supposed that the water of the ditches was in direct contact with the shallow groundwater. Consequently, it is not sure that the concentrations detected in the study were caused by the application onto the field itself, but may have been transported there via surface water (ditches).

In the attached report (Hauck T., 2001) it is shown that with respect to the rainfall during the months June – September the year 1993 is the wettest year in 40 years for the weather station De Bilt. This weather station was used in the original report. Therefore, the FOCUS concept of referring to a 90 percentile worst case for weather for registration purposes is not followed if only results from this extreme year 1993 are taken into account.

The **sampling technique** used in this report is not designed to sample groundwater only and is susceptible to cross-contamination. The sampling technique is not described in detail in the report, instead reference is made to two SOP's which are not in our hands right now.

In the report a suction cup-like technique is used. The suction cup technique originally aims at sampling pore water. The sampled area is not well defined as inhomogeneities in the soil structure determine the sampling area. In the described study, the suction cup (a pipe with a porous ceramic head as a filter, on which a vacuum is imposed) technique is used for groundwater sampling. This is possible if the groundwater level is known and the filter of the pipe is situated in the saturated zone. In the study the groundwater level was determined in two places on each field and the average of these was extrapolated to the whole field. Then the suction cup was placed 50 cm below the thus determined groundwater level.

For most fields it is not possible to extrapolate the groundwater level. It varies over a wide range (mostly 50 cm, but variations of more than 1 m are also found in a recently conducted groundwater monitoring study: Becker-Arnold et al. (2001): „Groundwater-monitoring study on residues of kresoxim-methyl (BAS 490 F) and of the acid-metabolite BF 490-1 in BAS 490 F treated areas in the Netherlands", draft-report, raw data) which poses problems for the described sampling technique, as one can not be sure to sample groundwater and not pore water. In the study itself problems with the sampling in some fields with low porosity are indicated, which point in the same direction.

Cross contamination is more likely with this technique as compared to wells installed before the application period (which is the only alternative technique at the time being). By drilling the hole after application, treated soil from upper soil layers can be transferred to deeper soil layers or contaminate the filter and thus contaminate the obtained water sample. Even if sampling is done very thoroughly and skillfully, it is hard to fully avoid contamination.
One evidence for cross-contamination can be found in the results for the loam fields where during the application (when high concentrations of ETU are still present in the upper soil layers) findings are reported (sampling "round 2"), whereas after the application period (sampling "round 3"), nothing was found. It is not likely that this is due to dilution as the sampling of "round 3" was only about two months after the sampling "round 2" and samples were taken over the whole field.

In the Netherlands, a groundwater monitoring for ETU has been requested on the national level. Products containing EBDC fungicidal active ingredients have been commonly used in the Netherlands for decades, most prominently in the crops potato and flower bulb. The study aimed at the quantitative evaluation whether the use of EBDC fungicides has resulted in the presence of ETU in deeper groundwater.
Note: The total amounts of EBDC's that have been applied in potatoes and flower bulbs in the past are much higher than the current application rate of metiram (3 x 1.4 kg metiram/ha) that is assumed for this AIR3 dossier.

Report:	*CA 7.5/3* *Kerkdijk H., 2002a* *Monitoring of ETU in deep groundwater in the Netherlands* *2002/1011915*
Guidelines:	*none*
GLP:	*yes* *(certified by Ministerium fuer Umwelt, Raumordnung und Landwirtschaft des Landes Nordrhein-Westfalen, 40190 Duesseldorf)*

Report:	*CA 7.5/4* *Kerkdijk H., 2002b* *Monitoring of ETU in deep groundwater in the Netherlands* *2002/1012957*
Guidelines:	*none*
GLP:	*yes* *(certified by Ministerium fuer Umwelt, Raumordnung und Landwirtschaft des Landes Nordrhein-Westfalen, 40190 Duesseldorf)*

Executive Summary

A targeted monitoring study for the EBDC fungicide metabolite ETU was performed in deep groundwater wells in potato and flower bulb growing areas in the Netherlands. Those areas were identified as being the Dutch agricultural areas with the highest amount of EBDC applied. Aim of the study was to evaluate whether the use of EBDC fungicides has resulted in the presence of ETU in deep groundwater. The assessment was done by a one time sampling of wells located in EBDC use areas and subsequent analyses of the well waters.

In total a number of 170 relevant groundwater wells were selected from the main Dutch EBDC usage areas, based on filter depth as well as geographical (potato and flower bulb areas) and hydrogeological criteria. The wells were situated in the provinces Groningen, Drenthe, Overijssel, Gelderland, North Brabant, Limburg, North Holland and South Holland.

Samples were taken from 124 wells and 121 samples could be analyzed. Thereof, 2 values were removed from the assessment because the selection criteria were not met. The final set of monitored wells consisted of 119 samples.

The groundwater samples were analyzed using a method based on a combination of rotary evaporation, solid liquid partitioning and LC-MS/MS. The validation of the method was performed as part of the study.

The analysis of the samples from 119 groundwater wells showed that in 91% of all samples the ETU concentration was below 0.1 µg L^{-1}, while an ETU concentration above 0.1 µg L^{-1} was found in 11 samples. With a percentage of the groundwater samples with concentrations higher than 0.1 µg L^{-1} of 9.2% and a resulting 90th percentile concentration of 0.078µg L^{-1}, thus below 0.1 µg L^{-1}, the criterion of the Dutch decision tree for leaching of pesticides to groundwater is fulfilled.

I. MATERIALS AND METHODS

1. Reference material

Common name: Ethylene thiourea (ETU)
Code BF 222-ETU
Reg. No.: 146099
CAS-No.: 96-45-7
Chemical name (IUPAC): 2-imidazolidinethione
Chemical formula: $C_3H_6N_2S$
Molecular weight: 102.16 g mol^{-1}
Purity: 99.6%
Lot. No.: L33-99

2. Test sites

The agricultural areas, which can be regarded as major use areas of EBDC fungicides, were identified as areas with potato or flower bulb land use. From all potato and flower bulb areas identified with a GIS evaluation of digital land-use maps. Areas were then defined where ETU may be present within the selected land use areas based on hydrogeolocical criteria. The thus selected areas served as the basis for the selection of monitoring wells. Monitoring wells were located in expected water recharge areas associated with potatoes or flower bulb growing areas. Overall, the wells were selected based on the following criteria:

• EBDC historical use areas
• Depth interval of 10 ± 4 m
• Hydrologically suitable areas
• Diameter of the monitoring well ≥ 50 mm, when available

The wells were situated in the provinces Groningen, Drenthe, Overijssel, Gelderland, North Brabant, Limburg, North Holland and South Holland.

In total a number of 170 relevant groundwater wells (82 wells in potato areas plus 18 reserve wells, 52 wells in flower bulb areas, 12 wells in mixed flower bulb/potato areas and 6 extra wells, which were selected during the field phase of the study) were selected. A number of 46 wells were not sampled for several reasons e.g. because they were no longer existing. Finally, 124 wells were sampled and 121 samples could be analyzed.

3. Sampling and analysis

The experimental phase of the study was started on 5 September 2002 and was completed on 20 November 2002.

Sampling of groundwater from the well was preferably performed using a submersible pump, which was disposed of after sampling one well. If the diameter of the well was too small, a peristaltic pump was used. Water pH, water temperature, electrical conductivity (EC) and oxygen content were monitored and finally measured during pre-pumping. Sample collection started after removing groundwater at least 3 times the volume of the standing water in the well. The ground water level was watched during sampling where applicable or measured after sampling.

At each well three high-density polyethylene (HDPE) bottles of 0.5 or 1 L were filled with groundwater. 100 mL water was discarded from each bottle to prevent damages of the bottle due to expansion during freezing. Then, 20 drops of an 8 g L^{-1} solution of sodium thiosulfate preservative were added to each 900 mL sample (10 drops to 400 mL samples) to ensure the stability of the samples under frozen storage conditions for at least half a year. After sampling, the outer surface of the well tube and its cap was wiped off and the wipe was placed in Ziploc plastic bags.

Additionally, field blanks were used to verify the absence of ETU contamination from sampling equipment. For this purpose, at the field phase test facility, a glass container was filled with tap water and this water was sampled into the same bottles using the same equipment and tubing as was used for sampling in the field.

Groundwater samples and wipe samples were placed in insulated containers with dry ice and transported to the field phase test facility where they were stored at ≤ -18°C for up to two weeks. After this period for each well two bottles and the wipe samples were transported with dry ice to the analytical test facility where they were stored at ≤ -18°C until analysis.

On three occasions during the sampling phase of the study, field spikes were prepared to demonstrate adequate sample handling and storage. At the analytical test facility empty bottles with cap were weighed, filled with 900 mL of groundwater (Linschoten groundwater) and weighed again to determine the exact net mass of the water. For each field spike event, 2 bottles were supplied. In the field, 20 drops of 8 g L^{-1} solution of sodium thiosulfate were added to each bottle. To one amber bottle an amount of ETU solution was added to obtain a concentration of about 0.1 µg L^{-1}.

The analysis of samples was performed in the laboratory of TNO Nutrition and Food Research (The Netherlands). For analysis, a groundwater sample was pre-concentrated by rotary evaporation and afterwards sorbed on a column packed with Extrelut. Then, ETU was eluted from the column with dichloromethane. The organic phase extracts were concentrated by rotary evaporation until only the aqueous layer remained. The aqueous phase was brought up into 75% acetonitrile/water and adjusted to a pH of 8 to 10 with 25% ammonia. The sample was finally measured using LC-MS/MS. The limit of quantification (LOQ) was determined at 0.05 µg L^{-1}.

With each batch of samples processed on the same day, one control and one fortified control sample with a concentration of 0.1 µg L^{-1} (procedural recovery) was analyzed together with the samples.

Stability of ETU

The stability of ETU in water samples stored under identical conditions was investigated in a previous study [*Artz, S. C. (2001): ETU National Drinking Water Survey: Storage Stability and Material Validation Studies; Study Number: AACS-00-O1, Morse Laboratories*]. After 183 days a recovery of 94% ETU was obtained. As the same type and brand of bottles and preservation chemical were used in the actual sampling campaign, it was concluded that ETU remains stable during the applied storage conditions for at least 6 months.

4. Method validation

Prior to sample analysis the analytical method for determination of ETU in groundwater was validated. The validation set consisted of two unfortified control samples, five samples fortified at target LOQ (0.053 µg L^{-1}) and five samples fortified at 10 x LOQ (0.535 µg L^{-1}). The nature of the matrix effects was additionally investigated by analyzing a set of 3 standards in matrix.

Validation samples were quantified by a calibration standard curve and corrected for the average matrix effect. Average recoveries at LOQ level of 0.05 µg L^{-1} were 84% (RSD = 30%) and at 10 x LOQ 80% (RSD = 6%). The RSD of 30% for recoveries at LOQ was related to the extreme physico-chemical properties of ETU, i.e. very polar, water soluble and of low molecular weight, and was therefore considered acceptable.

II. RESULTS AND DISCUSSION

The analysis of the field blank samples resulted in a maximum ETU concentration of 0.022 µg L^{-1}. The average concentration of ETU in the control samples was 0.015 µg L^{-1}. As the results of the field blank samples and the control samples were in the same range it was concluded that no ETU originated from the sampling equipment.

The analysis of two field spike samples resulted in recoveries of 77% and 26%. The third sample was rejected due to a potential error during fortification. The storage stability study [*Artz, S. C. (2001)*] demonstrated that ETU residues in frozen groundwater samples were stable for at least 183 days. The procedural recovery was 100% at the 0.1 µg L^{-1} fortification level (RSD = 19.7%). Given the good results of the storage stability study and the procedural recovery it was assumed that the low recoveries of the field spike samples could be explained by uncertainties in the field fortification procedure.

Two values of the analytical results were removed from the assessment because the selection criteria were not met. The analytical results of the final selection of 119 groundwater samples showed that in 91% of all samples the ETU concentration was below 0.1 µg L^{-1}. In 98 samples, the ETU concentration was below the LOQ of 0.05 µg L^{-1}. In 10 samples, the ETU concentration was above LOQ but below 0.1 µg L^{-1} while an ETU concentration above 0.1 µg L^{-1} (max. 3.66 µg L^{-1}) was found in 11 samples. The results of the analysis of groundwater samples are summarized in Table 7.5-3.
To establish the 90th percentile of monitored wells, values below the LOQ were calculated with 0.5xLOQ (= 0.025 µg/L). The calculated overall 90th percentile concentration was below 0.1 µg L^{-1} (0.078 µg L^{-1}).

Table 7.5-3: Results of ETU analysis of final 119 monitored groundwater wells in the Netherlands in 2002

Sample ID	Content [µg L^{-1}]	Sample ID	Content [µg L^{-1}]	Sample ID	Content [µg L^{-1}]	Sample ID	Content [µg L^{-1}]
4777-001	< LOQ	4777-038	< LOQ	4777-084	< LOQ	4777-129	< LOQ
4777-002	< LOQ	4777-041	< LOQ	4777-085	0.077	4777-130	< LOQ
4777-003	< LOQ	4777-042	< LOQ	4777-086	< LOQ	4777-131	< LOQ
4777-004	< LOQ	4777-043	< LOQ	4777-087	< LOQ	4777-132	< LOQ
4777-005	0.065	4777-044	< LOQ	4777-088	< LOQ	4777-133	< LOQ
4777-006	< LOQ	4777-045	< LOQ	4777-089	< LOQ	4777-134	0.08
4777-007	< LOQ	4777-046	< LOQ	4777-090	< LOQ	4777-135	< LOQ
4777-008	< LOQ	4777-048	< LOQ	4777-091	< LOQ	4777-136	< LOQ
4777-010	< LOQ	4777-049	0.116	4777-092	< LOQ	4777-137	< LOQ
4777-011	< LOQ	4777-050	0.050	4777-094	0.058	4777-138	0.062
4777-012	0.386	4777-051	< LOQ	4777-097	< LOQ	4777-139	0.062
4777-013	< LOQ	4777-054	< LOQ	4777-098	< LOQ	4777-140	< LOQ
4777-014	< LOQ	4777-057	< LOQ	4777-099	< LOQ	4777-141	< LOQ
4777-015	< LOQ	4777-058	< LOQ	4777-100	< LOQ	4777-142	< LOQ
4777-017	0.340	4777-059	< LOQ	4777-101	< LOQ	4777-143	< LOQ
4777-018	< LOQ	4777-062	< LOQ	4777-102	< LOQ	4777-148	0.846
4777-019	< LOQ	4777-063	< LOQ	4777-106	< LOQ	4777-149	2.66
4777-020	< LOQ	4777-066	< LOQ	4777-107	< LOQ	4777-150	0.339
4777-021	< LOQ	4777-067	< LOQ	4777-108	< LOQ	4777-151	3.66
4777-022	< LOQ	4777-068	< LOQ	4777-109	< LOQ	4777-153	0.060
4777-024	< LOQ	4777-071	< LOQ	4777-112	< LOQ	4777-156	2.54
4777-026	< LOQ	4777-072	< LOQ	4777-113	< LOQ	4777-158	< LOQ
4777-027	< LOQ	4777-073	< LOQ	4777-116	< LOQ	4777-161	< LOQ
4777-029	< LOQ	4777-074	< LOQ	4777-118	< LOQ	4777-164	0.751
4777-030	< LOQ	4777-075	< LOQ	4777-119	< LOQ	4777-165	< LOQ
4777-031	< LOQ	4777-076	0.072	4777-121	< LOQ	4777-166	< LOQ
4777-032	< LOQ	4777-078	< LOQ	4777-123	< LOQ	4777-167	0.342
4777-033	< LOQ	4777-081	< LOQ	4777-125	0.11	4777-169	< LOQ
4777-034	< LOQ	4777-082	< LOQ	4777-127	< LOQ	4777-170	< LOQ
4777-036	< LOQ	4777-083	0.056	4777-128	< LOQ		

Limit of quantification (LOQ) = 0.05 µg L^{-1}

III. CONCLUSION

A targeted monitoring study for the EBDC fungicide metabolite ETU was performed in deep groundwater wells in potato and flower bulb growing areas in the Netherlands. Those areas were identified as being the Dutch agricultural areas with the highest amount of EBDC applied.

The analysis of the samples from 119 groundwater wells showed that in 91% of all samples the ETU concentration was below 0.1 µg L^{-1}, while an ETU concentration above 0.1 µg L^{-1} was found in 11 samples. With a percentage of the groundwater samples with concentrations higher than 0.1 µg L^{-1} of 9.2% and a resulting 90th percentile concentration of 0.078 µg L^{-1}, thus below 0.1 µg L^{-1}, the criterion of the Dutch decision tree for leaching of pesticides to groundwater is fulfilled.

Report:	*CA 7.5/5* *Hauck T., 2003a* *Statement to the study: Monitoring of ETU in deep groundwater in The Netherlands* *2003/1005451*
Guidelines:	*none*
GLP:	*no*

Executive Summary

In this evaluation the representativity of a monitoring study on ETU in deep groundwater in The Netherlands for agricultural conditions in the European Union was assessed. In the study, groundwater from 119 wells in potato and flower bulb growing areas in The Netherlands were analyzed for residues of ETU and in 11 samples or 9.2% a concentration >0.1 µg L^{-1} was detected.

The importance of potato and flower bulb growing areas and the soil characteristics of these areas in The Netherlands were analyzed and compared to the conditions in the European Union. The planting of potatoes and bulbs (flower bulbs and onions) is a dominant land use in The Netherlands (10% of total agricultural area) while for the whole European Union it is of low importance (1% of total agricultural area).

The wells where the ETU concentration was > 0.1 µg L^{-1} were spatially concentrated in two areas in the northeastern and western part of The Netherlands. They were located in areas with well-drained sandy soils originating from dune sands and eolian sands with shallow groundwater levels, a combination that can be classified as vulnerable for the leaching of substances with low adsorption affinity like ETU. The parent material dune sand and eolian sand is widespread in The Netherlands and covers almost 50% of the member state area. In contrast, in the European Union, it is very sparse and covers only 1.3% of the area.

In the European Union soils on dune sands are generally not used as agricultural soils while this is a common practice in the Netherlands.

In conclusion, the detections of ETU at a concentration > 0.1 µg L^{-1} in 11 of 119 investigated wells could be attributed to the unique agricultural conditions in the Netherlands. The groundwater wells were spatially concentrated in areas where crops with low importance on the European level are grown on highly vulnerable soils with shallow groundwater that are very sparse in the European Union and typically not used as agricultural soils. In the groundwater monitoring study, it could not be distinguished if the measured groundwater concentration of ETU originated from agricultural use of EBDC fungicides according to good agricultural practice or if they originated from other sources like accidents and spills of EBDC fungicides or even completely different sources (e.g. rubber coatings, rubber production). But even assuming that the source of the measured ETU occurrence in groundwater was solely correct agricultural use (worst-case assumption), these findings are of very low relevance for the European Union.

I. MATERIALS AND METHODS

The representativity of a of a monitoring study on ETU in deep groundwater in The Netherlands [*see CA 7.5/3 and CA 7.5/4, BASF DocID 2002/1011915 and BASF DocID 2002/1012957*] for agricultural conditions in the European Union was assessed. In the study, groundwater from 119 wells in potato and flower bulb growing areas in The Netherlands were analyzed for residues of ETU and in 11 samples or 9.2% a concentration >0.1 µg L^{-1} was detected.

An overview of the 11 samples is summarized in Table 7.5-4.

Table 7.5-4: Wells with ETU concentrations above the drinking water limit

No.	Sample ID	ETU concentration [µg L^{-1}]	Soil type [a]	Land use	Sampling location Province / Area [b]
1	4777-12	0.386	Z12	potato	Groningen / NE
2	4777-17	0.340	Z12	potato	Groningen / NE
3	4777-49	0.160	Z18	potato	Drenthe / NE
4	4777-125	0.110	Z18	flower bulb	North Holland / W
5	4777-148	0.846	Z1	flower bulb	North Holland / W
6	4777-149	2.660	Z1	flower bulb	South Holland / W
7	4777-150	0.339	Z1	flower bulb	South Holland / W
8	4777-151	3.660	Z1	flower bulb	South Holland / W
9	4777-156	2.540	Z20	flower bulb	North Holland / W
10	4777-164	0.751	Z1	flower bulb	South Holland / W
11	4777-167	0.342	Z18	flower bulb	North Holland / W

[a] Soil classification according to Dutch soil map: major soil unit Z = sandy soils
Z1 = calcareous, fine sand, fairly deep groundwater (0.25 - > 0.5 m)
Z12 = fine sand, very deep groundwater (0.8 - > 1.6 m)
Z18 = fine sand, fairly deep groundwater (0.2 - > 0.5 m)
Z20 = loamy fine sand, shallow to deep groundwater (0 - >1.6 m)
[b] NE: northeastern part of NL
W: western part of NL

A spatial analysis was performed. For this purpose, the detailed locations of the wells described in Table 7.5-4 which were provided in the monitoring study were used to determine the position of the wells on the Dutch soil map [*Steur, G.G.L. et al. (1985): "Bodemkaart van Nederland, 1:250.000", Stichting voor Bodemkartering, Wageningen*].

All groundwater wells that were sampled in the monitoring study were located in areas with potato or flower bulb cultivation. Therefore, the importance of the production of potatoes and flower bulbs on a European level was analyzed. The distribution of agricultural land used for growing potatoes and bulbs (flower bulbs and onions) in the European Union is shown in Table 7.5-5 [*EUROSTAT (2002): Area of the production of bulbs and potatoes in the EU, EUROSTAT, Berlin*].

Table 7.5-5: Agricultural area of potato and bulb production in the European Union

Country	Potatoes 2002 [1000 ha]	Potatoes 2002 [% of agricultural area]	Bulbs 2001 [1000 ha]	Bulbs 2001 [% of agricultural area]
European Union	1247	0.96	102	0.08
Belgium	62	4.43	1	0.08
Denmark	37	1.38	1	0.04
Germany	284	1.67	7	0.04
Greece	36	0.92	9	0.23
Spain	114	0.45	24	0.09
France	162	0.55	11	0.04
Ireland	14	0.31	n.s.	n.s.
Italy	81	0.54	14	0.09
Luxemburg	1	0.52	0	0.00
The Netherlands	**164**	**8.50**	**20**	**1.06**
Austria	23	0.66	2	0.07
Portugal	53	1.39	1	0.03
Finland	30	1.34	1	0.05
Sweden	32	1.03	1	0.03
United Kingdom	156	0.99	10	0.06

n.s. = not specified

For the 11 locations, the soil characteristics were analyzed based on the Dutch soil map [*Steur, G.G.L. et al., 1985*]. The representativity of the soil characteristics on a European level was assessed. For this purpose, the distribution of the parent material eolian sand and dune sand in The Netherlands and in the European Union was compared based on data from the European soil map [*European Soil Bureau (1999): "Soil Geographical Data Base of Europe, 1:1.000.000", European Soil Bureau, Space Applications Institute Joint Research Centre, Ispra, Italy*]. The data are summarized in Table 7.5-6.

Table 7.5-6: Distribution of the soil parent material eolian sand and dune sand in the European Union

Country	Eolian sand [ha]	Eolian sand [% of EU area]	Eolian sand [% of member state area]	Dune sand [ha]	Dune sand [% of EU area]	Dune sand [% of member state area]
Austria	0	0.0	0.0	0	0.0	0.0
Belgium	557451	14.1	18.2	0	0.0	0.0
Denmark	0	0.0	0.0	102332	23.8	2.4
Ireland	0	0.0	0.0	0	0.0	0.0
Finland	0	0.0	0.0	0	0.0	0.0
France	1103598	28.0	2.0	154163	35.9	0.3
Germany	0	0.0	0.0	0	0.0	0.0
Greece	0	0.0	0.0	0	0.0	0.0
Italy	0	0.0	0.0	0	0.0	0.0
Luxemburg	0	0.0	0.0	0	0.0	0.0
The Netherlands	**1608677**	**40.8**	**46.7**	**88416**	**20.6**	**2.6**
Portugal	0	0.0	0.0	84738	19.7	0.9
Spain	0485972	12.3	1.0	0	0.0	0.0
Sweden	0	0.0	0.0	0	0.0	0.0
United Kingdom	190854	4.8	0.8	0	0.0	0.0
European Union	3946551	100.0	1.2	429649	100.0	0.1

II. RESULTS AND DISCUSSION

Spatial analysis

The 11 wells were located in two areas in the northeastern and western part of The Netherlands. In the northeastern part, 3 wells were located in an area with sandy soils where potatoes dominate the land use. In the western part near the coastline, the main flower bulb region, 8 wells were spatially extremely concentrated, only some kilometers apart from each other.

Land use characteristics

All groundwater wells that were sampled in the monitoring study were located in areas with potato or flower bulb cultivation. In The Netherlands, the planting of potatoes and bulbs are dominant land uses, which cover about 10% of total agricultural area. For the whole European Union the uses are of low importance and cover only 1% of total agricultural area.

Therefore, the land use characteristics of the monitoring study were considered to be of very low relevance for the European Union.

Soil characteristics
The 11 wells were located in areas with sandy soils (see Table 7.5-4) originating from the parent material dune sand and eolian sand. The soils were characterized as young-aged with a shallow layer of developed soil where very low levels and low depths of accumulated organic carbon could be expected. Further, the soils were described as well drained with shallow groundwater levels. The agricultural use of light sandy soils on relatively coarse undergrounds with shallow groundwater, which characterizes especially the flower bulb areas in the Netherlands, was regarded as unique combination in the European Union. Groundwater beneath those soils were classified as vulnerable for the leaching of substances with low adsorption affinity like ETU.

The parent material dune sand and eolian sand is widespread in The Netherlands and covers almost 50% of the member state area (Table 7.5-6). In contrast, in the European Union, it is very sparse and covers only 1.3% of the area. In the European Union, soils on dune sand are practically not used as agricultural soils while this is a common practice in the Netherlands.

Therefore, the soil characteristics of the monitoring study were considered to be of very low relevance for the European Union.

III. CONCLUSION

The representativity of a monitoring study on ETU in deep groundwater in The Netherlands for agricultural conditions in the European Union was assessed. The assessment was based on analysis of the spatial distribution of the sampling points, land use and the soil characteristics.

All the 11 detections of ETU at concentrations $> 0.1 \mu g\ L^{-1}$ could be attributed to the unique agricultural conditions in the Netherlands. The groundwater wells were spatially concentrated in areas where crops with low importance on the European level were grown on highly vulnerable soils with shallow groundwater that are very sparse in the European Union and typically not used as agricultural soils.

In the groundwater monitoring study, it could not be distinguished if the measured groundwater concentration of ETU originated from agricultural use of EBDC fungicides according to good agricultural practice or if they originated from other sources like accidents and spills of EBDC fungicides or even completely different sources (e.g. rubber coatings, rubber production). But even assuming that the source of the measured ETU occurrence in groundwater was solely correct agricultural use (worst-case assumption), it was concluded that these findings are of very low relevance for the European Union.

Metiram

Document M-CA, Section 8

ECOTOXICOLOGICAL STUDIES ON THE ACTIVE SUBSTANCE

Compiled by:

Date: 03 November 2017

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number
29/Feb/2016	8.1.2.2 Long-term and reproductive toxicity to mammals Summary of field effect study on small herbivorous mammals added (BASF DocID 2016/104381)	BASF DocID 2016/1051894
31/Aug/2017	8.1.1.3 Sub-chronic and reproductive toxicity to birds Further evaluation of the study BASF DocID 2004/1004372 (M-CA 8.1.1.3/4) added. 8.4 Recalculation of EC_x values for the chronic H. aculeifer study CA8.4.2/2 (DocID 2010/1075822) added.	BASF DocID 2017/1161746
03/11/2017	Correction of a mistake in the introduction	

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 8 ECOTOXICOLOGICAL STUDIES ON THE ACTIVE SUBSTANCE

On request of the RMS some of the chapters have been revised. All changes concerning the request from RMS from February 2017 are highlighted in ~~blue~~ yellow and parts which are no longer valid have been crossed out.

CA 8.1 Effects on birds and other terrestrial vertebrates

Studies conducted for use in the risk assessments for birds and mammals

In addition to studies on effects on birds or terrestrial vertebrates, new studies on the residue behaviour of metiram and ETU on plants and arthropods relevant for the higher tier risk assessment of bird and terrestrial vertebrates are summarized under this chapter from M-CA 8.1/1 to 8.1/7. The studies have not been peer-reviewed on EU level, which is indicated prior each summary. A study on residues on arthropods in potatoes evaluated by RMS Italy in Addendum IV to B9 of the DAR in April 2004 and the corresponding kinetic evaluation report are summarized under M-CA 8.1/8 to 8.1/9.

For terrestrial vertebrates a field effect study on populations of small herbivorous mammals is conducted in 2015. In agreement with RMS Italy the ~~draft~~ final report will be submitted in ~~2015~~ 2016~~, hence a summary is not yet available~~. Please refer to M-CA 8.1.2 for more explanations.

Summaries on generic ecological studies and further information used in the risk assessment for birds and mammals are under M-CP 10.1.1 and M-CP 10.1.2.

Table 8.1-1: Overview of new studies / reports / documents whose summaries are given further down

Data point	Reference (BASF DocID)	Year	Title
CA 8.1/1	2013/1078062	2012	Study on the residue behaviour of metiram (BAS 222 F) on wheat (young plants) after treatment with BAS 222 28 F under field conditions in North and South Europe.
CA 8.1/2	2013/1225017	2012	Study on the residue behavior of metiram (BAS 222 F) on pea (young plants) after the application of BAS 222 28 F under field conditions in France (North), Germany, United Kingdom, Italy and Spain.
CA 8.1/3	2014/1221842	2014	Kinetic evaluation of dissipation of BAS 222 F - metiram and its metabolite ETU in wheat and peas from field trials conducted in Europe.
CA 8.1/4	2012/1017195	2013	Metiram (Polyram WG 70%) - metiram and ETU residues on arthropods after spray application in potatoes in Spain.
CA 8.1/5	2014/1221840	2014	Kinetic evaluation of dissipation of BAS 222 F - metiram and its metabolite ETU in arthropods sampled in a potato field study.
CA 8.1/6	2012/1017194	2013	Metiram (Polyram WG 70%) - metiram and ETU residues on arthropods after spray application in vineyards in France
CA 8.1/7	2014/1221841	2014	Kinetic evaluation of dissipation of BAS 222 F - metiram and its metabolite ETU in arthropods sampled in a vineyard study.
CA 8.1/8	2004/1006523	2004	Residues of metiram and ETU in potential feed items of birds and wild mammals following application of Polyram WG (BAS 222 28 F) in potatoes
CA 8.1/9	2009/1072249	2009	Estimation of DT_{50} values for metiram for soil-dwelling and leaf-dwelling arthropods
CA 8.1.1.3/5	2017/1052097	2017	Metiram (BAS 222 F) - Revised statistics for mallard reproduction study [redacted] (2004)

A study on the residue behaviour of metiram and the metabolite ETU after application of BAS 222 28 F on young wheat plants was conducted and has not been evaluated previously on EU level.

Report:	CA 8.1/1 Moreno S.,Galvez O., 2013a Study on the residue behaviour of metiram (BAS 222 F) on wheat (young plants) after treatment with BAS 222 28 F under field conditions in North and South Europe, season 2012 2013/1078062
Guidelines:	SANCO/825/00 rev. 8.1 (16 November 2010), EEC 87/18 (No. L 15/29) 1986, International guidelines for distribution and pesticides application AEPLA FAO 1985, EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, EEC 7029/VI/95 rev. 5 Appendix B, EEC 7525/VI/95 rev. 9 (March 2011)
GLP:	yes (certified by ENAC, Entidad Nacional de Acreditación, Madrid Spain)

Executive Summary

The objective of the study was to determine the residue behaviour of metiram (BAS 222 F) and the metabolite ethylene-thiourea (ETU) on young wheat plants after one application of BAS 222 28 F (Polyram). Samplings were carried out directly after and at subsequent time intervals after application. The selected application rate corresponds to the maximum single rate of 1.4 kg as/ha according the Good Agricultural Practice (critical GAP) of BAS 222 28 F.

I. MATERIAL AND METHODS

A. MATERIALS

Test Material:	BAS 222 28 F
Description:	BAS 222 28 F (metiram)
Lot/Batch #:	80449975L0; BAS 222 F: 700 g/kg (BAS 222 28 F, WG)
Purity:	not relevant
CAS#:	metiram (BAS 222 F): 9006-42-2
Crop parts(s) or processed commodity:	wheat (young plants without roots; BBCH 13-30)
Sample size:	28.33-233.80 g

B. STUDY DESIGN

Study site

During the growing season of 2012, nine independent trials were conducted in representative wheat growing areas in Germany, France, United Kingdom, Italy and Spain.

Test item and application

The trial consisted of a control plot (untreated) and one treated plot (plot 2) without replication. No product containing the test item was used on the test plots during the season.

The test item BAS 222 28 F was foliar applied on plot 2 at a nominal rate of 1.4 kg metiram/ha in a nominal spray volume of 200 L/ha at three leaves unfolded growth stage (BBCH 13) according to Good Laboratory Practice.

Sampling information

For this study treated specimens were collected as wheat whole plants without roots 1 hour after the last application as well as 1, 2, 3, 4, 5, 7, 10, 12 and 14 days thereafter. Untreated specimens were collected also as wheat whole plants without roots 1 hour after application as well as 5 and 14 days thereafter. Each specimen was collected in duplicate (“ship” and “retain”) except for the trials from BASF SE L120095 and L120439, so that reserve specimens were available in case of potential problems during sample shipment, storage or analysis.

Damaged plants were not harvested. Each specimen was collected randomly from a minimum of 12 different places within each plot. The plants were covered with a net to protect them to be eaten by birds, except for the trial L120098.

Untreated specimens were obtained prior to treated specimens when coincided at sampling timings. Each specimen was placed into an individual plastic bag and was subsequently double bagged with a second plastic bag. Specimen labels were fixed to the inner plastic bags detailing the specimen type, specimen number, trial and study number. The retain specimens were harvested at the same time of the original ones and were destroyed in the facilities of each test site or the Test Facility by agreement of the Study Director and Sponsor.

All specimens were stored at ≤ -18ºC and sent to Specimen Management in BASF SE Agricultural Center Limburgerhof.

Description of analytical methods

All specimens were analysed for metiram as carbondisulfite using BASF method no. 535/1 [L0076/01] (CS_2) and additionally using BASF method No. L0089/01 (EBDC). The limit of quantitation (LOQ) for metiram as carbondisulfide was 0.10 mg/kg and 0.05 mg/kg for the method L0089/01. The residues of ETU were determined according to the BASF method No. L0176/01 with an LOQ of 0.01 mg/kg. For further details on the analytical methods, please consult chapter M-CA, chapter 4.1.2.

The results of procedural recovery experiments averaged at about 87% for BAS 222 F (by EBDC) at fortification levels between 0.05 and 150 mg/kg, at 83% for BAS 222 F (by CS_2) at fortification levels between 0.1 and 300 mg/kg and at 88% for ETU at fortification levels between 0.01 and 1.0 mg/kg.

II. RESULTS AND DISCUSSION

The residue definition for metiram (MRL) is based on the CS_2 method (M-CA, section 6, chapter CA 6.3). Therefore, the residues based on CS_2 measurements are summarized below and will be used for DT_{50} calculations.

Metiram (by CS_2)

The metiram residues in the wheat specimens taken 0 DALA (1 HALA) ranged from 30.08 – 100.19 mg/kg. They decreased to 16.65 – 69.63 mg/kg in the specimens taken 1 DALA and further to 6.70 – 69.95 mg/kg at 2 DALA. In the specimens taken 3 DALA 11.24 – 52.79 mg/kg were determined. The residue level in the specimens taken 4 DALA was 6.61 – 65.08 mg/kg, whereas in those taken 5 DALA 2.95 – 74.04 mg/kg were found. Afterwards a continuous decline was observed in the specimens taken 7 DALA (1.67 – 52.04 mg/kg), 10 DALA (0.23 – 34.27 mg/kg) and 12 DALA (< 0.1 – 17.30 mg/kg). At the last sampling (14 DALA they remained at this level (< 0.1 – 16.42 mg/kg).

No residues of metiram above the limit of quantitation were found in any of the analysed untreated specimens, except for the specimen taken 5 DALA from trial L120097, where 0.30 mg/kg were found. Further metiram residues of 0.17 mg/kg were found in the control specimen taken 0 DALA (1 HALA) from trial L120098, as well as 0.24 mg/kg were determined in the 0 DALA (1 HALA) specimen from trial L120099. In the untreated specimens from trial L120100 metiram residues of 0.16 mg/kg were found at 0 DALA (1 HALA), at 5 DALA 0.51 mg/kg were detected. The small residue amounts found in the untreated specimens are not considered to impact the reliability of the analytical results of the study.

ETU

The ETU residues in the wheat specimens taken 0 DALA (1 HALA) ranged from 0.07 – 0.69 mg/kg. They remained at this level in the specimens taken 1 DALA (0.05 – 0.58 mg/kg) and 2 DALA (0.05 – 0.69 mg/kg). In the 3 DALA specimens 0.05 – 0.48 mg/kg were found, whereas the specimens taken 4 and 5 DALA showed a slight increase to 0.04 – 0.79 mg/kg and 0.03 – 0.82 mg/kg respectively. Afterwards the residues decreased to 0.01 – 0.42 mg/kg (7 DALA) and further to < 0.01 – 0.31 mg/kg (10 DALA). In the specimen taken 12 DALA and at the last sampling (14 DALA) they remained at this level (< 0.01 – 0.25 mg/kg and < 0.01 – 0.38 mg/kg respectively).

No residues of ETU above the limit of quantitation were found in any of the analysed untreated specimens.

Table 8.1-2: Summary of residues of metiram (by CS2) and ETU in wheat (whole plant without roots)

Trial details	Sampling timing	Sampling no.	Date	Crop growth stage (BBCH)	Residues (mg/kg)		Formation fraction (residue ETU/initial residue metiram [%]) [3]
					Metiram by CS_2 [1]	ETU by BASF method No. L0176/01	
Trial no. L120095 Study site: Palatinate, Germany	1 HALA	1	20.04.2012	13	-- [2]	0.11	--
	1 DALA	2	21.04.2012	13	-- [2]	0.21	--
	2 DALA	3	22.04.2012	13	6.70	0.09	1.34
	3 DALA	4	23.04.2012	21	-- [2]	0.13	1.94
	4 DALA	5	24.04.2012	21	-- [2]	0.08	1.19
	5 DALA	6	25.04.2012	21	2.95	0.07	1.04
	7 DALA	7	27.04.2012	23	1.87	0.04	0.60
	10 DALA	8	30.04.2012	26	0.23	< 0.01	< 0.15
	12 DALA	9	02.05.2012	31	< 0.056	< 0.01	< 0.15
	14 DALA	10	04.05.2012	31	< 0.056	< 0.01	< 0.15
Trial no. L120096 Study site: NRW, Germany	1 HALA	1	14.05.2012	13	38.61	0.09	0.23
	1 DALA	2	15.05.2012	13	26.30	0.12	0.31
	2 DALA	3	16.05.2012	14	13.25	0.08	0.21
	3 DALA	4	17.05.2012	14	13.00	0.09	0.23
	4 DALA	5	18.05.2012	14	8.98	0.09	0.23
	5 DALA	6	19.05.2012	15	6.47	0.05	0.13
	7 DALA	7	21.05.2012	17	1.67	0.01	0.01
	10 DALA	8	24.05.2012	21	0.26	< 0.01	< 0.03
	12 DALA	9	26.05.2012	23	0.12	< 0.01	< 0.03
	14 DALA	10	28.05.2012	25	< 0.1	< 0.01	< 0.03
Trial no. L120097 Study site: Hampigny, France	1 HALA	1	26.03.2012	13	100.19	0.29	0.29
	1 DALA	2	27.03.2012	13	60.21	0.58	0.58
	2 DALA	3	28.03.2012	13	51.26	0.43	0.43
	3 DALA	4	29.03.2012	21	47.86	0.48	0.48
	4 DALA	5	30.03.2012	21	30.35	0.40	0.40
	5 DALA	6	31.03.2012	21	22.92	0.29	0.29
	7 DALA	7	02.04.2012	21	22.28	0.29	0.29
	10 DALA	8	05.04.2012	22	6.95	0.08	0.08

Trial details	Sampling timing	Sampling no.	Date	Crop growth stage (BBCH)	Residues (mg/kg)		Formation fraction (residue ETU/initial residue metiram [%]) [3]
					Metiram by CS_2 [1]	ETU by BASF method No. L0176/01	
	12 DALA	9	07.04.2012	22	6.48	0.08	0.08
	14 DALA	10	09.04.2012	23	2.50	0.03	0.03
Trial no. L120098 Study site: Bicester, UK	1 HALA	1	08.08.2012	13	44.11	0.08	0.18
	1 DALA	2	09.08.2012	13	16.65	0.05	0.11
	2 DALA	3	10.08.2012	13	13.92	0.05	0.11
	3 DALA	4	11.08.2012	13	11.24	0.05	0.11
	4 DALA	5	12.08.2012	13	6.61	0.04	0.09
	5 DALA	6	13.08.2012	13	3.62	0.03	0.07
	7 DALA	7	15.08.2012	13	2.43	0.02	0.05
	10 DALA	8	18.08.2012	13	1.22	0.01	0.02
	12 DALA	9	20.08.2012	21	0.69	< 0.01	< 0.02
	14 DALA	10	22.08.2012	21	0.80	< 0.01	< 0.02
Trial no. L120099 Study site: Seville, Spain	1 HALA	1	06.03.2012	13	42.47	0.69	0.47
	1 DALA	2	07.03.2012	13	35.88	0.39	0.75
	2 DALA	3	08.03.2012	13	25.68	0.69	0.82
	3 DALA	4	09.03.2012	13-21	24.70	0.35	1.13
	4 DALA	5	10.03.2012	13-21	21.80	0.79	0.73
	5 DALA	6	11.03.2012	21	17.17	0.82	0.78
	7 DALA	7	13.03.2012	21-22	9.07	0.42	0.42
	10 DALA	8	16.03.2012	21-22	12.00	0.31	0.68
	12 DALA	9	18.03.2012	21-22	5.49	0.25	0.26
	14 DALA	10	20.03.2012	21-22	5.30	0.38	0.26
Trial no. L1200100 Study site: Seville, Spain	1 HALA	1	23.02.2012	13	71.67	0.69	0.96
	1 DALA	2	24.02.2012	13	69.63	0.39	0.54
	2 DALA	3	25.02.2012	13	69.95	0.69	0.96
	3 DALA	4	26.02.2012	13	52.79	0.35	0.49
	4 DALA	5	27.02.2012	13	65.08	0.79	1.10
	5 DALA	6	28.02.2012	13	74.05	0.82	1.14
	7 DALA	7	01.03.2012	13-14	52.04	0.42	0.59
	10 DALA	8	04.03.2012	13-14	34.27	0.31	0.43
	12 DALA	9	06.03.2012	13-14	17.30	0.25	0.35
	14 DALA	10	08.03.2012	13-14	16.42	0.38	0.53
Trial no. L1200101 Study site: Cuneo, Italy	1 HALA	1	10.03.2012	13	30.08	0.07	0.23
	1 DALA	2	11.03.2012	13	37.84	0.11	0.37
	2 DALA	3	12.03.2012	13-14	29.94	0.18	0.60
	3 DALA	4	13.03.2012	13-14	36.00	0.18	0.60
	4 DALA	5	14.03.2012	13-14	30.87	0.18	0.60
	5 DALA	6	15.03.2012	13-14	22.59	0.16	0.53
	7 DALA	7	17.03.2012	13-14	7.75	0.04	0.13
	10 DALA	8	20.03.2012	14-21	9.41	0.06	0.20
	12 DALA	9	22.03.2012	20-21	7.23	0.06	0.20
	14 DALA	10	24.03.2012	21-22	2.97	0.02	0.07
Trial no. L1200102 Study site: Cuneo, Italy	1 HALA	1	10.03.2012	13	37.20	0.08	0.22
	1 DALA	2	11.03.2012	13	49.74	0.17	0.46
	2 DALA	3	12.03.2012	13-14	31.90	0.16	0.43
	3 DALA	4	13.03.2012	13-14	43.98	0.25	0.67
	4 DALA	5	14.03.2012	13-14	40.43	0.27	0.73
	5 DALA	6	15.03.2012	13-14	27.42	0.16	0.43
	7 DALA	7	17.03.2012	13-14	14.78	0.15	0.40
	10 DALA	8	20.03.2012	14-21	12.14	0.09	0.24
	12 DALA	9	22.03.2012	20-21	4.33	0.03	0.08
	14 DALA	10	24.03.2012	21-22	8.62	0.11	0.30

Trial details	Sampling timing	Sampling no.	Date	Crop growth stage (BBCH)	Residues (mg/kg)		Formation fraction (residue ETU/initial residue metiram [%]) [3]
					Metiram by CS_2 [1]	ETU by BASF method No. L0176/01	
Trial no. L120439 Study site: Palatinate, Germany	1 HALA	1	24.05.2012	13	59.93	0.14	0.23
	1 DALA	2	25.05.2012	13	37.01	0.35	0.58
	2 DALA	3	26.05.2012	13	37.46	0.41	0.68
	3 DALA	4	27.05.2012	13	-- [2]	0.33	0.55
	4 DALA	5	28.05.2012	21	22.99	0.20	0.33
	5 DALA	6	29.05.2012	21	15.73	0.18	0.30
	7 DALA	7	31.05.2012	21	4.00	0.03	0.05
	10 DALA	8	03.06.2012	30	1.50	< 0.01	< 0.02
	12 DALA	9	05.06.2012	30	0.61	0.01	0.02
	14 DALA	10	07.06.2012	30	0.37	< 0.01	< 0.02

HALA: hours after last application; DALA: days after last application

1 The values already include the conversion factor of 1.79 from CS_2 to metiram

2 not enough specimen material for CS_2 analysis available

3 The formation fraction of ETU was not calculated in the study report BASF DocID 2013/1078062. These values are needed for the risk assessment, therefore they were calculated here based on the following equation: ETU residue / initial measured residue for metiram (by CS_2) * 100.

III. CONCLUSION

Metiram (by CS_2)
The metiram residues in the wheat specimens taken 0 DALA (1 HALA) ranged from 30.08 – 100.19 mg/kg. They continuously decreased until the last sampling (14 DALA) to a range of < 0.1 – 16.42 mg/kg.

ETU
The ETU residues in the wheat specimens taken 0 DALA (1 HALA) ranged from 0.07 – 0.69 mg/kg. Maximum observed values occurred mainly between 1 DALA and 5 DALA and ranged from 0.12 to 0.82 mg/kg. Subsequently the residues decreased to levels of < 0.01 – 0.38 mg/kg at 14 DALA.

A study on the residue behaviour of metiram and the metabolite ETU after application of BAS 222 28 F on young pea plants was conducted and has not been evaluated previously on EU level.

Report:	CA 8.1/2 Martin T., 2013a Study on the residue behavior of metiram (BAS 222 F) on pea (young plants) after the application of BAS 222 28 F under field conditions in France (North), Germany, United Kingdom, Italy and Spain, 2012 2013/1225017
Guidelines:	EC 1107/2009 of the European Parliament and of the Council of 21 Oct 2009, EEC 7029/VI/95 rev. 5, EEC 7525/VI/95 rev. 7
GLP:	yes (certified by ENAC, Entidad Nacional de Acreditación, Madrid Spain)

Executive Summary

The objective of the study was to determine the residue behaviour of metiram (BAS 222 F) and the metabolite ethylene-thiourea (ETU) on young pea plants after one application of BAS 222 28 F (Polyram). Samplings were carried out directly after and at subsequent time intervals after application. The selected application rate corresponds to the maximum single rate of the Good Agricultural Practice (critical GAP) of BAS 222 28 F.

I. MATERIAL AND METHODS

A. MATERIALS

Test Material:	BAS 222 28 F
Description:	BAS 222 28 F (metiram)
Lot/Batch #:	80449975L0; BAS 222 F: 700 g/kg (BAS 222 28 F, WG)
Purity:	not relevant
CAS#:	metiram (BAS 222 F): 9006-42-2
Crop parts(s) or processed commodity:	pea (young plants without roots; BBCH 12-23)
Sample size:	12 plants (50-100g)

B. STUDY DESIGN

Study site

During the growing season in 2012, eight independent trials were conducted in representative pea growing areas in Germany, France, United Kingdom, Italy and Spain.

Test item and application

The trial consisted of a control plot (untreated) and one treated plot (plot 2) without replication. No product containing the test item was used on the test plots during the season.

The test item BAS 222 28 F was foliar applied on plot 2 at a nominal rate of 1.4 kg metiram/ha in a nominal spray volume of 200 L/ha at two to three leaves unfolded growth stage (BBCH 12-13) according to Good Laboratory Practice.

Sampling information

For this study treated specimens were collected as pea whole plants without roots 1 hour after the last application as well as 1, 2, 3, 4, 5, 7, 10, 12 and 14 days thereafter. Untreated specimens were collected also as pea whole plants without roots 1 hour after application as well as 5 and 14 days thereafter.

All specimens were frozen within 6 hours after being taken, and remained frozen at or below -18°C, including during transportation, until analysis.

Description of analytical methods

All specimens were analysed for metiram as carbondisulfite using BASF method no. 535/1 [L0076/01] (CS_2) and additionally using BASF method No. L0089/01 (EBDC). The limit of quantitation (LOQ) for metiram as carbondisulfide was 0.10 mg/kg and 0.05 mg/kg for the method L0089/01.

The residues of ETU were determined according to the BASF method No. L0176/01 with an LOQ of 0.01 mg/kg.

For further details on the analytical methods, please consult M-CA, section 4, chapter CA 4.1.2).

The results of procedural recovery experiments averaged at about 87% for BAS 222 F (by EBDC) at fortification levels between 0.05 and 99 mg/kg, at 78% for BAS 222 F (by CS_2) at fortification levels between 0.1 and 160 mg/kg and at 88% for ETU at fortification levels between 0.01 and 0.3 mg/kg.

II RESULTS AND DISCUSSION

The residue definition for metiram (MRL) is based on the CS_2 method (M-CA, section 6, chapter CA 6.3). Therefore, the residues based on CS_2 measurements are summarized here and used for the DT_{50} calculations.

Metiram (by CS_2)

The metiram residues in the pea specimens taken at 0 DALA (1 HALA) ranged from 13.01 – 39.33 mg/kg. Subsequently a steady decline until 14 DALA was observed: 10.93 – 26.84 mg/kg (1 DALA), 6.24 – 22.36 mg/kg (2 DALA), 5.69 – 17.34 mg/kg (3 DALA), 4.26 – 13.06 mg/kg (4 DALA), 2.86 – 14.37 mg/kg (5 DALA), 0.79 – 9.81 mg/kg (7 DALA), 0.37 – 4.33 mg/kg (10 DALA), 0.17 – 2.89 mg/kg (12 DALA), resulting in a concentration of 0.12 – 2.32 mg/kg in the specimens taken at the last sampling (14 DALA).

No residues of metiram above the limit of quantitation were found in any of the analysed untreated specimens, except for the specimen taken from trial L120068, where 0.60 mg/kg were found at 0 DALA (1 HALA), 0.26 mg/kg on 5 DALA and 0.20 mg/kg on 14 DALA. Further metiram residues were detected in the control specimens taken from trial L120069, where 0.30 mg/kg were found on 5 DALA and 0.24 mg/kg on 14 DALA. In the control specimens from trial L120070 0.64 mg/kg were found at 5 DALA and 0.25 mg/kg at 14 DALA. The explanation for the contamination within the untreated plots from these three trials is that a seed treatment with Thiram was done. Nevertheless, the small residue amounts found in the untreated specimens do not affect to the residue results of the study.

ETU

The ETU residues in the pea specimens taken 0 DALA (1 HALA) ranged from 0.04 – 0.19 mg/kg and remained at this level in the specimens taken at 1 DALA (0.04 – 0.18 mg/kg), 2 DALA (0.03 – 0.25 mg/kg), 3 DALA (0.05 – 0.16 mg/kg), 4 DALA (0.04 – 0.16 mg/kg) and 5 DALA (0.04 – 0.14 mg/kg). Subsequently a steadily decline until 14 DALA was observed (0.02 – 0.12 mg/kg (7 DALA), < 0.01 – 0.06 mg/kg (10 DALA), < 0.01 – 0.04 mg/kg (12 DALA) and < 0.01 – 0.03 mg/kg (14 DALA)).

No residues of ETU above the limit of quantitation were found in any of the analysed untreated specimens.

Table 8.1-3: Summary of residues of metiram (by CS_2) and ETU in pea (whole plant without roots)

Trial details	Sampling timing	Sampling no.	Date	Crop growth stage (BBCH)	Residues (mg/kg)		Formation fraction (residue ETU/initial residue metiram [%]) [3]
					Metiram by CS_2 [1]	ETU by BASF method No. L0176/01	
Trial no. L120065 Study site: Palatinate, Germany	1 HALA	1	14.05.2012	13	-- [2]	0.04	
	1 DALA	2	15.05.2012	13	22.66	0.04	0.18
	2 DALA	3	16.05.2012	13	6.24	0.03	0.13
	3 DALA	4	17.05.2012	14	5.69	0.12	0.53
	4 DALA	5	18.05.2012	14	5.90	0.10	0.44
	5 DALA	6	19.05.2012	14	4.08	0.04	0.18
	7 DALA	7	21.05.2012	14	0.79	0.02	0.09
	10 DALA	8	24.05.2012	17	0.56	< 0.01	0.02
	12 DALA	9	26.05.2012	17	0.29	< 0.01	< 0.04
	14 DALA	10	28.05.2012	17	0.18	< 0.01	< 0.04
Trial no. L120066 Study site: Kleve, Germany	1 HALA	1	08.06.2012	13	39.33	0.06	0.15
	1 DALA	2	09.06.2012	13	18.58	0.07	0.18
	2 DALA	3	10.06.2012	13-14	15.40	0.14	0.36
	3 DALA	4	11.06.2012	14	13.63	0.13	0.33
	4 DALA	5	12.06.2012	14-15	4.26	0.04	0.10
	5 DALA	6	13.06.2012	15	2.86	0.04	0.10
	7 DALA	7	15.06.2012	17	1.08	0.02	0.05
	10 DALA	8	18.06.2012	19	0.37	< 0.01	< 0.01
	12 DALA	9	20.06.2012	21	0.17	0.01	0.03
	14 DALA	10	22.06.2012	23	0.12	< 0.01	< 0.01
Trial no. L120067 Study site: Loire-et-Cher, France	1 HALA	1	02.04.2012	12	16.73	0.04	0.24
	1 DALA	2	03.04.2012	12	10.93	0.05	0.30
	2 DALA	3	04.04.2012	12	11.34	0.07	0.42
	3 DALA	4	05.04.2012	12	9.88	0.06	0.36
	4 DALA	5	06.04.2012	13	9.10	0.07	0.42
	5 DALA	6	07.04.2012	13	11.91	0.09	0.54
	7 DALA	7	09.04.2012	13	7.83	0.08	0.48
	10 DALA	8	12.04.2012	13	3.62	0.05	0.30
	12 DALA	9	14.04.2012	14	2.08	0.03	0.18
	14 DALA	10	16.04.2012	14	1.62	0.03	0.18
Trial no. L120068 Study site: Law ford Essex, UK	1 HALA	1	12.05.2012	12-13	31.36	0.08	0.26
	1 DALA	2	13.05.2012	12-13	26.84	0.18	0.57
	2 DALA	3	14.05.2012	12-13	22.36	0.25	0.80
	3 DALA	4	15.05.2012	12-13	17.34	0.16	0.51
	4 DALA	5	16.05.2012	13	13.06	0.16	0.51
	5 DALA	6	17.05.2012	13	14.37	0.14	0.45
	7 DALA	7	19.05.2012	13-14	9.81	0.12	0.38
	10 DALA	8	22.05.2012	14-15	4.33	0.06	0.19
	12 DALA	9	24.05.2012	14-16	2.72	0.04	0.13
	14 DALA	10	26.05.2012	16	1.48	0.02	0.06
Trial no. L120069 Study site: Sevilla, Spain	1 HALA	1	13.03.2012	13	18.40	0.05	0.27
	1 DALA	2	14.03.2012	13	14.39	0.11	0.60
	2 DALA	3	15.03.2012	13	13.47	0.08	0.43
	3 DALA	4	16.03.2012	13	10.88	0.08	0.43
	4 DALA	5	17.03.2012	14	7.98	0.06	0.33
	5 DALA	6	18.03.2012	14	8.67	0.07	0.38

Trial details	Sampling timing	Sampling no.	Date	Crop growth stage (BBCH)	Residues (mg/kg)		Formation fraction (residue ETU/initial residue metiram [%]) 3)
					Metiram by CS_2 1)	ETU by BASF method No. L0176/01	
	7 DALA	7	20.03.2012	15	6.32	0.08	0.43
	10 DALA	8	23.03.2012	15	2.81	0.04	0.22
	12 DALA	9	25.03.2012	15-16	2.79	0.03	0.16
	14 DALA	10	27.03.2012	15-17	1.39	0.02	0.11
Trial no. L120070 Study site: Sevilla, Spain	1 HALA	1	19.03.2012	13	27.68	0.19	0.69
	1 DALA	2	20.03.2012	13	21.69	0.17	0.61
	2 DALA	3	21.03.2012	13	19.29	0.16	0.58
	3 DALA	4	22.03.2012	13	16.11	0.12	0.43
	4 DALA	5	23.03.2012	14	12.20	0.09	0.33
	5 DALA	6	24.03.2012	14	12.55	0.08	0.29
	7 DALA	7	26.03.2012	15	9.35	0.09	0.33
	10 DALA	8	29.03.2012	15	2.89	0.04	0.14
	12 DALA	9	30.03.2012	16	1.84	0.03	0.11
	14 DALA	10	02.04.2012	17	0.62	0.01	0.04
Trial no. L120071 Study site: Ferrara, Italy	1 HALA	1	20.03.2012	13	23.31	0.06	0.26
	1 DALA	2	21.03.2012	13-14	15.85	0.07	0.30
	2 DALA	3	22.03.2012	13-14	14.58	0.06	0.26
	3 DALA	4	23.03.2012	14-15	9.67	0.05	0.21
	4 DALA	5	24.03.2012	14-15	< 0.1	< 0.01	< 0.02
	5 DALA	6	25.03.2012	14-15	9.51	0.05	0.21
	7 DALA	7	27.03.2012	15-17	8.17	0.06	0.26
	10 DALA	8	30.03.2012	15-17	4.20	0.06	0.26
	12 DALA	9	01.04.2012	16-17	2.89	0.03	0.13
	14 DALA	10	03.04.2012	16-17	2.32	0.02	0.09
Trial no. L120072 Study site: Ferrara, Italy	1 HALA	1	29.03.2012	12-13	13.01	0.06	0.46
	1 DALA	2	30.03.2012	12-13	11.04	0.05	0.38
	2 DALA	3	31.03.2012	12-13	8.85	0.08	0.61
	3 DALA	4	01.04.2012	12-13	7.07	0.06	0.46
	4 DALA	5	02.04.2012	13-14	4.97	0.06	0.46
	5 DALA	6	03.04.2012	13-14	3.86	0.06	0.46
	7 DALA	7	05.04.2012	14-15	2.75	0.04	0.31
	10 DALA	8	08.04.2012	15-16	1.04	0.01	0.08
	12 DALA	9	10.04.2012	16-17	0.61	< 0.01	< 0.04
	14 DALA	10	12.04.2012	18-19	0.31	< 0.01	< 0.04

HALA: hours after last application; DALA: days after last application
1 The values already include the conversion factor of 1.79 from CS_2 to metiram
2 not enough specimen material for CS_2 analysis available
3 The formation fraction of ETU was not calculated in the study report BASF DocID 2013/1225017. These values are needed for the risk assessment, therefore they were calculated here based on the following equation: ETU residue / initial measured residue metiram (by CS_2) * 100.

III. CONCLUSION

Metiram (by CS_2)
The metiram residues in the pea specimens taken 0 DALA (1 HALA) ranged from 13.01 – 39.33 mg/kg. They continuously decreased until the last sampling (14 DALA) to a range of 0.12 – 2.32 mg/kg.

ETU
The ETU residues in the pea specimens taken 0 DALA (1 HALA) ranged from 0.04 – 0.19 mg/kg. Maximum observed values occurred mainly between 1 DALA and 5 DALA and ranged from 0.07 to 0.25 mg/kg. Subsequently the residues decreased to levels of < 0.01 – 0.03 mg/kg at 14 DALA.

A kinetic evaluation of dissipation of metiram and the metabolite ETU in wheat and peas was conducted. The report has not been evaluated previously on EU level.

Report:	CA 8.1/3 Sachers S., 2014a Kinetic evaluation of dissipation of BAS 222 F - metiram and its metabolite ETU in wheat and peas from field trials conducted in Europe 2014/1221842
Guidelines:	FOCUS Kinetics (2006) SANCO/10058/2005 version 1.0 of Nov. 2011
GLP:	no

This modeling report provides kinetic analysis and estimation of the dissipation times (DT_{50} values) for metiram and its metabolite ETU in wheat and peas.

MATERIAL AND METHODS

Kinetic modeling strategy

Kinetic evaluation was performed in order to derive dissipation parameters for the residues of metiram and its metabolite ETU in pea and wheat plants. Since no specific recommendation is available how to carry out the kinetic evaluation for plant residue decline studies, guidance of the FOCUS workgroup on degradation kinetics was used in order to derive degradation parameters for modeling purposes. Thus, the selected DT_{50} values are suitable input parameters for models that require single first order (SFO) DT_{50} values or conservative substitutes.

The goodness-of-fit of the kinetic models was assessed by visual inspection and statistical measures, as recommended by the FOCUS (2006) guidance on degradation kinetics. The recommended kinetic models, *i.e.* the single first order kinetics (SFO) and the Gustafson-Holden model (FOMC) were applied. For visual inspection, the recommended graphical representations of observed and modeled decline curves versus time and the residuals versus time are presented in the report. As goodness-of-fit measures, the $\chi 2$ error is provided. The kinetic model was considered appropriate if the residuals are randomly distributed around zero, the $\chi 2$ - error value ideally < 15 % and the estimated degradation parameters differed from zero at a 5% significance level (FOCUS, 2006).

According to FOCUS the χ^2 - error of 15 % should not be considered as an absolute cut-off criterion. For cases of a larger χ^2 - error (e.g. based on a large scatter in the data as it might be the case for field studies), the decision on the acceptability of the model was based on visual assessment, to evaluate if the fit still represents a reasonable description of the degradation behavior (see FOCUS 2006). If the overall pattern of decline in pesticide concentrations was represented adequately by the model and the distribution of the residuals was random (no systematic deviations), the half-life from the respective model was considered appropriate.

RESULTS

The presented DT_{50} values derived under consideration of guidance from the FOCUS kinetics working group are suitable endpoints for modeling purposes. The degradation rate constants of the different fits were estimated significantly different from zero as indicated by low P values. The visual assessment of the different fits showed that the $\chi2$ error values were acceptable as the observations were generally well described by the fitted curves and the residuals were randomly scattered around the zero line (Table 8.1-4).

Table 8.1-4: Calculated DT_{50} values for metiram in pea and wheat plants and statistical indices

Plant	Trial	Zone	Kinetic model	DT_{50} [d]	P (t-test)	$\chi2$ error[%]
peas	L120065	North	SFO	1.0	$p < 0.01$	32.7
peas	L120066	North	SFO	1.5	$p < 0.01$	18.8
peas	L120067	North	SFO	5.6	$p < 0.01$	16.0
peas	L120068	North	SFO	3.7	$p < 0.01$	6.1
peas	L120069	South	SFO	4.2	$p < 0.01$	6.3
peas	L120070	South	SFO	3.8	$p < 0.01$	7.6
peas	L120071	South	SFO [1)]	4.1	$p < 0.01$	12.1
peas	L120072	South	SFO	3.0	$p < 0.01$	4.9
wheat	L120095	North	SFO	2.4	$p < 0.01$	9.5
wheat	L120096	North	SFO	1.7	$p < 0.01$	11.0
wheat	L120097	North	SFO	2.6	$p < 0.01$	12.4
wheat	L120098	North	FOMC	1.7 [2)]	$p <0.05$	13.7
wheat	L120099	South	SFO	4.1	$p < 0.01$	9.1
wheat	L120100	South	SFO	8.5	$p < 0.01$	15.1
wheat	L120101	South	SFO	5.5	$p < 0.01$	21.4
wheat	L120102	South	SFO	5.8	$p < 0.01$	21.4
wheat	L120439	North	SFO	2.5	$p < 0.01$	14.1
Geometric mean [d]				3.2		

1 SFO model after elimination of outlier at DAT 4

2 Pseudo SFO DT_{50} derived by back-calculation from DT_{90} (DT_{90}/3.32) according to FOCUS [see Ref. 3 of the original report]

Table 8.1-5: Calculated DT_{50} values for ETU in pea and wheat plants and statistical indices

Plant	Trial	Zone	Kinetic model	DT_{50} [d]	P (t-test)	$\chi2$ error[%]
peas	L120065	North	SFO	1.6	$p < 0.05$	32.7
peas	L120066	North	SFO	1.6	$p < 0.01$	18.8
peas	L120067	North	SFO	5.2	$p < 0.01$	16.0
peas	L120068	North	SFO	4.1	$p < 0.01$	6.1
peas	L120069	South	SFO	7.0	$p < 0.01$	6.3
peas	L120070	South	SFO	4.5	$p < 0.01$	9.6
peas	L120071	South	SFO[1)]	13.0	$p < 0.05$	12.1
peas	L120072	South	SFO	3.9	$p < 0.01$	4.9
wheat	L120095	North	SFO	2.4	$p < 0.01$	9.5
wheat	L120096	North	SFO	3.1	$p < 0.01$	11.0
wheat	L120097	North	SFO	4.3	$p < 0.01$	12.4
wheat	L120098	North	SFO	3.8	$p < 0.01$	13.7
wheat	L120099	South	SFO	5.7	$p < 0.05$	9.1
wheat	L120100	South	SFO	5.1	$p < 0.05$	15.1
wheat	L120101	South	SFO	4.5	$p < 0.01$	21.4
wheat	L120102	South	SFO	4.5	$p < 0.05$	21.4
wheat	L120439	North	SFO	1.9	$p < 0.01$	14.1
Geometric mean [d]				3.9		

1 SFO model after elimination of outlier at DAT 4

CONCLUSION

The decline of metiram and its metabolite ETU residues on young plants was mostly well described by single first order kinetics. Only in field trial L120098 a pseudo SFO DT50 of metiram could be derived from a FOMC kinetic fit as conservative SFO substitute. The overall geometric mean DT_{50} of metiram in young plants is 3.2 days and is 3.9 days for the metabolite ETU.

A study on residues of metiram and the metabolite ETU on arthropods (tested as Polyram WG 70%) was conducted, which has not been evaluated on EU level.

Report:	CA 8.1/4 Henkes K.,Henkes G., 2013a Metiram (Polyram WG 70%) - metiram and ETU residues on arthropods after spray application in potatoes in Spain 2012/1017195
Guidelines:	EFSA Guidance Document: Risk assessment for birds and mammals (2009), EC 1107/2009 (14 June 2011)
GLP:	yes (certified by Landesamt fuer Umwelt, Messungen und Naturschutz Baden Wuerttemberg, Karlsruhe, Germany)

Executive Summary

The objective of the study was to determine the residue behaviour of metiram (BAS 222 F) and the metabolite ethylene thiourea (ETU) on ground- and foliage-dwelling arthropods in a potato field, after spray applications of the end-use product Polyram WG 70% (BAS 222 28 F). Samplings were carried out directly after and at subsequent time intervals after the applications. The selected application rate covers the maximum single rate of the Good Agricultural Practice (critical GAP) of BAS 222 28 F in potatoes.

I. MATERIAL AND METHODS

A. MATERIALS

Test Material:	BAS 222 28 F
Description:	BAS 222 28 F (metiram)
Lot/Batch #:	91149316K0; BAS 222 F: 700 g/kg (BAS 222 28 F, Polyram WG 70%)
Purity:	not relevant
CAS#:	metiram (BAS 222 F): 9006-42-2
Crop parts(s) or processed commodity:	ground- and foliage-dwelling arthropods in a potato field
Sample size:	ground-dwelling arthropods: 2.41-10.67 g; foliage –dwelling arthropods: 2.14-6.88g

B. STUDY DESIGN

Study site

The study was conducted 2012 in a potato field situated about 4.5 km east of the village Zazuar, in the region Castile and León in central Spain, a typical area of potato cultivation in southern Europe. The study field represented a typical potato field in the region as to both size (4.2 ha) and basic structure (i.e. distance between plant rows). Within the study field, three study plots were established with areas of 1.7 ha (study plot 1), 1.2 ha (study plot 2) and 1.3 ha (study plot 3).

Test item and application

The test item BAS 222 28 F (containing 70% of the active substance: metiram, WG formulation) was applied twice with an interval of 7 days on 05 July 2012 and 12 July 2012 in accordance with Good Agricultural Practice and Good Laboratory Practice at a nominal application rate of 1.4 kg a.s./ha (corresponding to 2.0 kg/ha BAS 222 28 F) in a spray volume of 200 L water/ha. The BBCH growth stages of the potatoes were 60 and 63 at the first and second application, respectively.

At the first application the application rate was 1445 g a.s./ha in a spray volume of 207 L water/ha at study plot 1, 1419 g a.s./ha in a spray volume of 203 L water/ha at study plot 2 and 1444 g a.s./ha in a spray volume of 206 L/ha at study plot 3. At the second application the application rate was 1419 g a.s./ha in a spray volume of 203 L water/ha at study plot 1, 1428 g a.s./ha in a spray volume of 204 L water/ha at study plot 2 and 1449 g a.s./ha in a spray volume of 207 L/ha at study plot 3.

Sampling information

Samples of natural populations of ground-dwelling arthropods were collected for residue analysis by pitfall trapping, whereas foliage-dwelling arthropods were collected by inventory spraying (using a knock-down insecticide AquaPy®: natural pyrethrum 30 g a.s./L and Piperonylbutoxid, 150 g/L). The pitfall traps were activated (opened) approx. 24 h before sampling. Over a period of 23 days, altogether 13 sampling events took place for ground-dwelling arthropods with samplings taken on each of the three study plots separately. Samples were taken 2 days before the first treatment (DAFT -2) and 1, 2, 4, 5, 6 days after the first treatment. After the second (last) treatment, samplings took place at DALT 1, 2, 3, 5, 7, 10 and 14. The overall samplings correspond to consecutive days -2, 1, 2, 4, 5, 8, 9, 10, 12, 14, 17 and 21 when related to the first application event. At each sampling event, per study plot, arthropods from all pitfall traps were pooled to provide a single sample per study plot.

A defined distance of 120 m potato row was treated with AquaPy® to obtain the minimum foliage-dwelling arthropod matrix of 4 g. All foliage-dwellers recovered from all cotton sheets (positioned between the potato plants) at each sampling event on each study plot were pooled to obtain a single sample for each study plot. Inventory spraying, hence sampling of foliage-dwelling arthropods was done 15 times. The samples were collected before the first treatment at DAFT -3, after the first treatment at DAFT 0 (approx. four hours after the first treatment) 1, 2, 4, 5, 6, and after the second, *i.e.* last treatment at DALT 0 (approx. four hours after the second application), 1, 2, 3, 5, 7, 10 and 14 corresponding to consecutive days -3, 0, 1, 2, 4, 5, 6, 7, 8, 9, 10, 12, 14, 17 and 21 when related to the first application event only.

After determination of the main taxonomic groups, the arthropods were weighed and stored at a temperature of at least -18°C until residue analysis.

Description of analytical methods

All specimens were analysed for metiram using BASF method No. L0089/01 (EBDC). The limit of quantitation (LOQ) for metiram was 0.05 mg/kg for the method L0089/01. Validation of the analytical method on arthropods is summarized in M-CA, section 4, study 4.1.2/19.

The residues of ETU were determined according to the BASF method No. L0176/01 with an LOQ of 0.01 mg/kg. The analytical method for ETU on arthropods was validated as part of the analysis in the study in vineyards (see CA 8.1/6).

The results of procedural recovery experiments averaged at about 94% for BAS 222 F (by EBDC) at fortification levels between 0.05 and 18 mg/kg and at 74% for ETU at fortification levels between 0.01 and 0.1 mg/kg.

Calculation

The initial and maximum concentrations of metiram and ETU in ground-dwelling arthropods, were calculated based on the arithmetic mean of three replicates (n=3). Additionally, the initial and maximum metiram and ETU concentration in foliage-dwelling arthropods were reported based on pooled data (the samples of all study plots of each sampling date had to be pooled to provide enough biomass for analysis).

II RESULTS AND DISCUSSION

Metiram (by EBDC)

Residues of metiram in ground-dwelling arthropods
Metiram residues in ground-dwelling arthropods collected in all study plots two days before application (DAFT -2) of the formulation BAS 222 28 F was below the LOQ of the analytical method, *i.e.* below 0.05 mg as/kg f.w..

The initial concentration of metiram (arithmetic mean, n=3) in ground-dwelling arthropods one day after the first application (DAFT 1) was measured to be 4.16 ± 0.32 mg as/kg f.w.. The maximum concentration of metiram (arithmetic mean, n=3) for the time period between the first and the second application (DAFT 1-6) was also detected on DAFT 1 with 4.16 ± 0.32 mg as/kg f.w.. Metiram levels in ground-dwelling arthropods declined to 0.20 mg as/kg f.w. (arithmetic mean, n=3) before the second application (DAFT 6).

The initial concentration of metiram (arithmetic mean, n=3) in ground-dwelling arthropods one day after the second application (DALT 1) was measured to be 0.72 ± 0.08 mg as/kg f.w (Table 8.1-6). The maximum concentration of metiram (arithmetic mean, n=3) for the time period after the second application (DALT 0-14) was detected on DALT 3 with 0.89 ± 0.33 mg as/kg f.w.. metiram levels in ground-dwelling arthropods declined to 0.16 mg as/kg f.w. (arithmetic mean, n=3) until the end of the sampling period (DALT 10).
All individual metiram residue concentrations in ground-dwelling arthropods are given in Table 8.1-6.

Table 8.1-6: Residues of metiram in ground-dwelling arthropods

DAFT (Day After First Treatment) [1)]	DALT (Day After Last Treatment) [2)]	Crop growth stage (BBCH)	Residues of metiram (mg as/kg f.w.)			
			Study plot 1	Study plot 2	Study plot 3	mean
-2	--	60	< 0.05	< 0.05	< 0.05	< 0.05
1	--	60	3.80	4.26	4.42	4.16
2	--	60	1.39	6.32	4.01	3.91
4	--	63	0.87	0.89	0.79	0.85
5	--	63	0.26	0.46	0.56	0.43
6	--	63	0.22	0.18	0.21	0.20
8	1	63	0.71	0.65	0.81	0.72
9	2	63	0.57	0.57	0.82	0.65
10	3	65	0.64	0.78	1.26	0.89
12	5	65	0.09	0.34	0.26	0.23
14	7	67	0.13	0.17	0.08	0.13
17	10	69	0.14	0.14	0.19	0.16
21	14	69	0.14	0.09	0.25	0.16

1 Date of the first treatment: 05.07.2012
2 Date of the second treatment: 12.07.2012

Residues of metiram in foliage-dwelling arthropods

Metiram residues in foliage-dwelling arthropods collected in all study plots three days before application (DAFT -3) of BAS 222 28 F was below the LOQ of the analytical method, *i.e.* below 0.05 mg as/kg f.w..

The initial concentration of metiram in foliage-dwelling arthropods on the day of the first application (DAFT 0, after application) was measured to be 17.72 mg as/kg f.w.. The maximum concentration of metiram for the time period between the first and the second application (DAFT 0-6) was also detected on DAFT 0 (after application) with 17.72 mg as/kg f.w.. Metiram levels in foliage-dwelling arthropods declined to 1.93 mg as/kg f.w. before the second application (DAFT 6).

The initial concentration of metiram in foliage-dwelling arthropods on the day of the second application (DALT 0, after application) was measured to be 9.29 mg as/kg f.w.. The maximum concentration of metiram after the second application (DALT 0-14) was also detected on DALT 0 with 9.29 mg as/kg f.w.. Metiram levels in foliage-dwelling arthropods declined to 1.02 mg as/kg f.w. until the end of the sampling period (DALT 14).

All individual metiram residue concentrations in foliage-dwelling arthropods are given in Table 8.1-7.

Table 8.1-7: Residues of metiram in foliage-dwelling arthropods

DAFT (Day After First Treatment) [1]	DALT (Day After Last Treatment) [2]	Crop growth stage (BBCH)	Residues of metiram (mg as/kg f.w.)
			Study plot 1-3 [3]
-3	--	60	< 0.05
0	--	60	17.72
1	--	60	5.66
2	--	60	6.25
4	--	63	3.41
5	--	63	3.12
6		63	1.93
7	0	63	9.29
8	1	63	7.51
9	2	63	8.69
10	3	65	2.62
12	5	65	2.66
14	7	67	2.68
17	10	69	3.43
21	14	69	1.02

1 Date of the first treatment: 05.07.2012
2 Date of the second treatment: 12.07.2012
3 Samples of all study plots were pooled prior to the analysis

ETU

Residues of ETU in ground-dwelling arthropods

The ETU residue content in ground-dwelling arthropods collected from all study plots two days before application (DAFT -2) of the formulation BAS 222 28 F was below the LOQ of the analytical method, *i.e.* below 0.01 mg as/kg f.w..

The initial concentration of ETU (arithmetic mean, n=3) in ground-dwelling arthropods one day after the first application (DAFT 1) was measured to be 0.027 ± 0.012 mg as/kg f.w.. The maximum concentration of ETU (arithmetic mean, n=3) for the time period between the first and the second application (DAFT 1-6) was detected on DAFT 2 with 0.040 ± 0.010 mg as/kg f.w.. ETU levels in ground dwelling arthropods declined to 0.013 mg as/kg f.w. (arithmetic mean, n=3) before the second application (DAFT 6).

The initial concentration of ETU (arithmetic mean, n=3) in ground-dwelling arthropods one day after the second application (DALT 1) was measured to be 0.027 ± 0.015 mg as/kg f.w.. The maximum concentration of ETU (arithmetic mean, n=3) for the time period after the second application (DALT 1-14) was detected on DALT 2 with 0.030 ± 0.000 mg as/kg f.w. and DALT 5 with 0.030 ± 0.017 mg as/kg f.w.. ETU levels in ground-dwelling arthropods declined to 0.010 mg as/kg f.w. (arithmetic mean, n=3) until the end of the sampling period (DALT 14).

All individual ETU residue concentrations in ground-dwelling arthropods are given in Table 8.1-8.

Table 8.1-8: Residues of ETU in ground-dwelling arthropods

DAFT (Day After First Treatment) [1)]	DALT (Day After Last Treatment) [2)]	Crop growth stage (BBCH)	Residues of ETU (mg as/kg f.w.)			
			Study plot 1	Study plot 2	Study plot 3	mean
-2	--	60	< 0.01	< 0.01	< 0.01	< 0.01
1	--	60	0.02	0.02	0.04	0.027
2	--	60	0.03	0.05	0.04	0.040
4	--	63	0.01	0.02	0.02	0.017
5	--	63	0.02	0.02	0.01	0.017
6	--	63	0.01	0.01	0.02	0.013
8	1	63	0.01	0.04	0.03	0.027
9	2	63	0.03	0.03	0.03	0.030
10	3	65	0.03	0.02	0.03	0.027
12	5	65	0.03	0.01	0.04	0.027
14	7	67	0.02	0.02	< 0.01	< 0.017
17	10	69	< 0.01	< 0.01	0.02	< 0.013
21	14	69	< 0.01	< 0.01	0.01	< 0.010

1 Date of the first treatment: 05.07.2012
2 Date of the second treatment: 12.07.2012

Residues of ETU in foliage-dwelling arthropods

The ETU residue content in foliage-dwelling arthropods collected from all study plots three days before application (DAFT -3) of the formulation BAS 222 28 F was below the LOQ of the analytical method, *i.e.* below 0.01 mg as/kg f.w..

The initial concentration of ETU in foliage-dwelling arthropods on the day of the first application (DAFT 0, after application) was measured to be 0.22 mg as/kg f.w.. The maximum concentration of ETU for the time period between the first and the second application (DAFT 0-6) was detected on DAFT 1 and 2 with 0.38 mg as/kg f.w., respectively. ETU levels in foliage-dwelling arthropods declined to 0.05 mg as/kg f.w. before the second application (DAFT 6).

The initial concentration of ETU in foliage-dwelling arthropods on the day of the second application (DALT 0, after application) was measured to be 0.35 mg as/kg f.w.. The maximum concentration of ETU after the second application (DALT 0-14) was detected on DALT 5 with 0.36 mg as/kg f.w.. ETU levels in foliage-dwelling arthropods declined to 0.02 mg as/kg f.w. until the end of the sampling period (DALT 14).

All individual ETU residue concentrations in foliage-dwelling arthropods are given in Table 8.1-9.

Table 8.1-9: Residues of ETU in foliage-dwelling arthropods

DAFT (Day After First Treatment) [1)]	DALT (Day After Last Treatment) [2)]	Crop growth stage (BBCH)	Residues of ETU (mg as/kg f.w.)
			Study plot 1-3 [3)]
-3	--	60	< 0.01
0	--	60	0.22
1	--	60	0.38
2	--	60	0.38
4	--	63	0.18
5	--	63	0.09
6		63	0.05
7	0	63	0.35
8	1	63	0.27
9	2	63	0.21
10	3	65	0.17
12	5	65	0.36
14	7	67	0.13
17	10	69	0.13
21	14	69	0.02

1 Date of the first treatment: 05.07.2012
2 Date of the second treatment: 12.07.2012
3 Samples of all study plots were pooled prior to the analysis, due to insufficient arthropod matrix

Conclusion

The study provides field data on the magnitude of initial residue levels and the subsequent time course of metiram and ETU residues in ground- and foliage-dwelling arthropods. These data provide a reliable basis for use in higher tier risk assessments for insectivorous or omnivorous birds and mammals.

A kinetic evaluation of dissipation of metiram and the metabolite ETU in arthropods was conducted, which has not been evaluated previously on EU level.

Report:	CA 8.1/5 Sachers S., 2014b Kinetic evaluation of dissipation of BAS 222 F - metiram and its metabolite ETU in arthropods sampled in a potato field study 2014/1221840
Guidelines:	FOCUS Kinetics (2006) SANCO/10058/2005 version 1.0 of Nov. 2011
GLP:	no

This modeling report provides kinetic analysis and estimation of the dissipation times (DT_{50} values) for metiram and its metabolite ETU.

MATERIAL AND METHODS

Calculation of DT_{50} values

Since no specific recommendation is available how to carry out the kinetic evaluation for the described experiment, guidance of the FOCUS workgroup on degradation kinetics was used in order to derive degradation parameters for modeling purposes. This means the selected DT_{50} values are suitable input parameters for models that require single first order (SFO) DT_{50} values or conservative substitutes.

The goodness-of-fit of the kinetic models was assessed by visual inspection and statistical measures, as recommended by the FOCUS (2006) guidance on degradation kinetics. The recommended kinetic models, *i.e.* the single first order kinetics (SFO) and the Gustafson-Holden model (FOMC) were applied. For visual inspection, the recommended graphical representations of observed and modeled decline curves versus time and the residuals versus time are presented in the report. As goodness-of-fit measures, the $\chi 2$ error is provided. The kinetic model was considered appropriate if the residuals are randomly distributed around zero, the $\chi 2$ - error value ideally < 15 % and the estimated degradation parameters differed from zero at a 5% significance level (FOCUS, 2006).

According to FOCUS the χ^2 - error of 15 % should not be considered as an absolute cut-off criterion. For cases of a larger χ^2 - error (e.g. based on a large scatter in the data as it might be the case for field studies), the decision on the acceptability of the model was based on visual assessment, to evaluate if the fit still represents a reasonable description of the degradation behavior (see FOCUS 2006). If the overall pattern of decline in pesticide concentrations was represented adequately by the model and the distribution of the residuals was random (no systematic deviations), the half-life from the respective model was considered appropriate.

Individual dissipation parameter estimations were conducted separately for foliage dwellers and ground dwellers in potatoes. Furthermore, two separate DT_{50} values were calculated for the residue data after the first and the second application. Afterwards, the DT_{50} values from the same trial were averaged in order to get one endpoint that is representative for the single scenario. In conservative approach (FOCUS 2006), the ETU DT_{50} values were derived by fitting SFO kinetics to the decline of the metabolite observations from the maximum onwards.

RESULTS

The presented DT_{50} values that were derived under consideration of guidance from the FOCUS kinetics working group are suitable endpoints for modeling purposes. The degradation rate constants of the different fits are significantly different from zero as indicated by low P values. The visual assessment of the different fits showed that the $\chi 2$ error values were acceptable as the observations were generally well described by the fitted curves and the residuals were randomly scattered around the zero line.

Table 8.1-10: Calculated DT_{50} values for metiram in arthropods and statistical indices

Crop	Arthropod	x^{th} application	Kinetic model 1)	DT_{50} (SFO) [d]	DT_{50} 2) (SFO) [d]	p (t-test)	$\chi 2$ error [%]
potatoes	ground dweller	1	SFO	1.5	2.4	$p < 0.01$	22.6
potatoes		2	SFO	3.8		$p < 0.01$	27.7
potatoes	foliage dweller	1	SFO	1.3	2.2	$p < 0.05$	25.7
potatoes		2	SFO	3.8		$p < 0.01$	25.0

1 SFO fit visually acceptable as the residuals are randomly scattered around zero; a bi-phasic dissipation behavior is not indicated. The elevated χ^2 error is acceptable for a field study according FOCUS 2006; k is significantly different from zero.
2 geometric mean DT_{50} of the datasets

Table 8.1-11: Calculated DT_{50} values for ETU in arthropods and statistical indices

Crop	Arthropod	x^{th} application	Kinetic model	DT_{50} (SFO) [d]	DT_{50} 1) (SFO) [d]	p (t-test)	$\chi 2$ error [%]
potatoes	ground dweller	1	SFO	2.2	4.1	$p < 0.01$	9.5
potatoes		2	SFO	7.7		$p < 0.01$	11.5
potatoes	foliage dweller	1	SFO	2.2	2.1	$p < 0.05$	16.1
potatoes		2	SFO	2.1		$p < 0.05$	18.5

1 SFO fit visually acceptable as the residuals are randomly scattered around zero; a bi-phasic dissipation behavior is not indicated. The partly elevated χ^2 error is acceptable for a field study according FOCUS 2006; k is significantly different from zero.
2 geometric mean DT_{50} of the datasets

CONCLUSION

The decline of metiram and its metabolite ETU residues on arthropods can well be described by single first order kinetics. For metiram a DT_{50} of 2.4 and 2.2 days was determined for ground-and foliage-dwelling arthropods in potatoes. For ETU the DT_{50} values correspond to 4.1 and 2.1 days in ground and foliage-dwelling arthropods.

A new study on residues of metiram and the metabolite ETU on arthropods (tested as Polyram WG 70%) was conducted and has not been evaluated previously on EU level.

Report:	CA 8.1/6 Henkes K.,Henkes G., 2013b Metiram (Polyram WG 70%) - metiram and ETU residues on arthropods after spray application in vineyards in France 2012/1017194
Guidelines:	EC 1107/2009 (14 June 2011), EFSA Guidance Document: Risk assessment for birds and mammals (2009)
GLP:	yes (certified by Landesamt fuer Umwelt, Messungen und Naturschutz Baden Wuerttemberg, Karlsruhe, Germany)

Executive Summary

The objective of the study was to determine the residue behaviour of metiram (BAS 22 F) and the metabolite ethylene-thiourea (ETU) on ground- and foliage-dwelling arthropods in a vineyard after a two-fold spray applications of the end-use product Polyram WG 70% (BAS 222 28 F). Samplings were carried out directly after and at subsequent time intervals after the applications. The selected application rate correspond to the maximum single rate of 1.4 kg as/ha according to the Good Agricultural Practice (critical GAP) of BAS 222 28 F in vineyards.

I. MATERIAL AND METHODS

A. MATERIALS

Test Material:	BAS 222 28 F
Description:	BAS 222 28 F (metiram)
Lot/Batch #:	91149316K0; BAS 222 F: 700 g/kg (BAS 222 28 F, Polyram WG 70%)
Purity:	not relevant
CAS#:	metiram (BAS 222 F): 9006-42-2
Crop parts(s) or processed commodity:	ground- and foliage-dwelling arthropods in a vineyard
Sample size:	ground-dwelling arthropods: 0.62-6.72 g; foliage –dwelling arthropods: 3.07-6.85g

B. STUDY DESIGN

Study site

The study was conducted 2012 in a vineyard situated in Amboise about 26 km east of Tours, in the region Loire Valles in central France, a typical area of vine cultivation. The study field represented a typical vineyard in the region as to both size (3 ha) and basic structure (i.e. distance between vines). Within the study field, three study plots were established with areas of 1.0 ha (study plot 1), 0.9 ha (study plot 2) and 0.9 ha (study plot 3).

Test item and application

The test item BAS 222 28 F (containing 70% of the active substance: metiram, WG formulation) was applied twice on 31 May 2012 and 07 June 2012 in accordance with Good Agricultural Practice and Good Laboratory Practice at a nominal application rate of 1.4 kg a.s./ha (corresponding to 2.0 kg/ha BAS 222 28 F) in a spray volume of 200 L water/ha. The BBCH growth stages of the vines were 57 at the first and second application, respectively.

At the first application the application rate was 1263 g a.s./ha in a spray volume of 162.39 L water/ha at study plot 1, 1404 g a.s./ha in a spray volume of 180.47 L water/ha at study plot 2 and 1468 g a.s./ha in a spray volume of 188.80 L/ha at study plot 3. At the second application the application rate was 1417 g a.s./ha in a spray volume of 166.26 L water/ha at study plot 1, 1413 g a.s./ha in a spray volume of 162.26 L water/ha at study plot 2 and 1524 g a.s./ha in a spray volume of 185.55 L/ha at study plot 3.

Sampling information

Samples of natural populations of ground-dwelling arthropods were collected for residue analysis by pitfall trapping, whereas foliage-dwelling arthropods were collected by inventory spraying (using a knock-down insecticide AquaPy®: natural pyrethrum 30 g a.s./L and Piperonylbutoxid, 150 g/L; spraying under Non-GLP). The pitfall traps were activated (opened) approx. 24 h before sampling. Over a period of 23 days, altogether 15 sampling events took place for ground-dwelling arthropods with samplings taken on each of the three study plots separately. Samples were taken before the first treatment at DAFT -2, after the first treatment at DAFT 1, 2, 4, 5, 6, and after the second, i.e. last treatment at DALT 1, 2, 3, 5, 6, 7, 11, 12 and 14 corresponding to consecutive days 8, 9, 10, 12, 14, 18, 19 and 21 when related to the first application event only. At each sampling event, per study plot, arthropods were collected from all individual pitfall traps and pooled to provide a single sample per study plot.

A defined distance of 80 m vine was treated with AquaPy® to obtain the minimum foliage-dwelling arthropod matrix of 4 g. All foliage-dwellers recovered from all cotton sheets (positioned between the vines) at each sampling event on each study plot were pooled to obtain a single sample for each study plot. Inventory spraying, hence sampling of foliage-dwelling arthropods was done 14 times. The samples were collected at DAFT 0 (approx. four hours after the first treatment) 1, 2, 4, 5, 6, and after the second, i.e. last treatment at DALT 0 (approx. four hours after the second application), 1, 2, 4, 6, 7, 10 and 13 corresponding to consecutive days 8, 9, 11, 13, 14, 17 and 20 when related to the first application event only.

After determination of the main taxonomic groups, the arthropod samples (ground-dwellers and foliage-dwellers) were weighed and stored at a temperature of at least -18°C until residue analysis.

Description of analytical methods

All specimens were analysed for metiram using BASF method No. L0089/01 (EBDC). The limit of quantitation (LOQ) for metiram was 0.05 mg/kg for the method L0089/01. Validation of the analytical method on arthropods is summarized in M-CA, section 4, study 4.1.2/19.

The residues of ETU were determined according to the BASF method No. L0176/01 with an LOQ of 0.01 mg/kg. The analytical method for ETU on arthropods was validated in the analytical part of this study.

The results of procedural recovery experiments averaged at about 93% for BAS 222 F (by EBDC) at fortification levels between 0.05 and 104 mg/kg and at 77% for ETU at fortification levels between 0.01 and 10 mg/kg.

Calculation

The initial and maximum concentrations of metiram and ETU in ground-dwelling arthropods, were calculated based on the arithmetic mean of three replicates (n=3), with exception of DAFT 5 and DALT 6, because the samples of all study plots were pooled prior to analyses for the these sampling dates.. Additionally, the initial and maximum metiram and ETU concentration in foliage-dwelling arthropods were reported based on pooled data (the samples for each sampling date of all study plots were pooled to obtain enough biomass for residue analyses).

II. RESULTS AND DISCUSSION

Metiram (by EBDC)

Residues of metiram in ground-dwelling arthropods

The metiram residue content in ground-dwelling arthropods collected from all study plots two days before application (DAFT -2) of the formulation BAS 222 28 F was below the LOQ of the analytical method, i.e. below 0.05 mg as/kg f.w..

The initial concentration of metiram (arithmetic mean, n=3) in ground-dwelling arthropods one day after the first application (DAFT 1) was measured to be 1.73 ± 0.55 mg as/kg f.w.. The maximum concentration of metiram (arithmetic mean, n=3) for the time period between the first and the second application (DAFT 1-6) was detected on DAFT 2 with 1.93 ± 0.16 mg as/kg f.w.. Metiram levels in ground-dwelling arthropods declined to 0.63 mg as/kg f.w. (arithmetic mean, n=3) before the second application (DAFT 6).

The initial concentration of metiram (arithmetic mean, n=3) in ground-dwelling arthropods one day after the second application (DALT 1) was measured to be 1.93 ± 0.53 mg as/kg f.w.. The maximum concentration of metiram (arithmetic mean, n=3) for the time period after the second application (DALT 1-14) was detected on DALT 3 with 5.59 ± 2.36 mg as/kg f.w.. Metiram levels in ground-dwelling arthropods declined to 0.20 mg as/kg f.w. (arithmetic mean, n=3) until the end of the sampling period (DALT 14).

All individual metiram residue concentrations in ground-dwelling arthropods are given in Table 8.1-12.

Table 8.1-12: Residues of metiram in ground-dwelling arthropods

DAFT (Day After First Treatment) [1]	DALT (Day After Last Treatment) [2]	Crop growth stage (BBCH)	Residues of metiram (mg as/kg f.w.)			
			Study plot 1	Study plot 2	Study plot 3	mean
-2	--	55	< 0.05	< 0.05	< 0.05	< 0.05
1	--	57	1.11	2.15	1.94	1.73
2	--	57	1.93	2.08	1.77	1.93
4	--	57	0.36	0.40	0.80	0.52
5	--	57		1.13		1.13
6	--	57	0.46	0.54	0.88	0.63
8	1	57	2.39	1.35	2.03	1.92
9	2	57	1.25	1.29	3.24	1.93
10	3	57	5.15	3.49	8.14	5.59
12	5	57-63		2.94		2.94
14	6	57-63	1.15	0.58	0.12	0.62
18	7	60-63	0.39	0.20	0.81	0.47
19	12	60-63	0.39	0.20	0.81	0.47
21	14	63	0.15	0.20	0.24	0.20

1 Date of the first treatment: 31.05.2012
2 Date of the second treatment: 07.06.2012

Residues of metiram in foliage-dwelling arthropods

The metiram residue content in foliage-dwelling arthropods collected from all study plots three days before application (DAFT -3) of the formulation BAS 222 28 F was measured to be 0.07 mg as/kg f.w. However, two days before application (DAFT -2) the metiram residue content was below the LOQ of the analytical method, i.e. below 0.05 mg as/kg f.w..

The initial concentration of metiram in foliage-dwelling arthropods on the day of the first application (DAFT 0, after application) was measured to be 20.00 mg as/kg f.w.. The maximum concentration of metiram for the time period between the first and the second application (DAFT 0-6) was also detected on DAFT 1 with 23.03 mg as/kg f.w.. Metiram levels in foliage-dwelling arthropods declined to 4.87 mg as/kg f.w. before the second application (DAFT 6).

The initial concentration of metiram in foliage-dwelling arthropods on the day of the second application (DALT 0, after application) was measured to be 36.12 mg as/kg f.w.. The maximum concentration of metiram after the second application (DALT 0- 13) was also detected on DALT 0 with 36.12 mg as/kg f.w.. Metiram levels in foliage-dwelling arthropods declined to 1.32 mg as/kg f.w. until the end of the sampling period (DALT 13).

All individual metiram residue concentrations in foliage-dwelling arthropods are given in Table 8.1-13.

Table 8.1-13: Residues of metiram in foliage-dwelling arthropods

DAFT (Day After First Treatment) [1)]	DALT (Day After Last Treatment) [2)]	Crop growth stage (BBCH)	Residues of metiram (mg as/kg f.w.)
			Study plot 1-3 [3)]
-3	--	55	0.07
-2	--	55	< 0.05
0	--	57	20.00
1	--	57	23.03
2	--	57	19.18
4	--	57	10.34
5		57	6.56
6		57	4.87
7	0	57	36.12
8	1	57	15.15
9	2	57	15.37
11	4	57	9.24
13	6	57-60	5.84
14	7	57-60	3.06
17	10	57-60	2.19
20	13	63	1.32

1 Date of the first treatment: 31.05.2012
2 Date of the second treatment: 07.06.2012
3 Samples of all study plots were pooled prior to the analysis, due to insufficient arthropod matrix

ETU

Residues of ETU in ground-dwelling arthropods

The ETU residue content in ground-dwelling arthropods collected from all study plots two days before application (DAFT -2) of the formulation BAS 222 28 F was below the LOQ of the analytical method, i.e. below 0.01 mg as/kg f.w..

The initial concentration of ETU (arithmetic mean, n=3) in ground-dwelling arthropods one day after the first application (DAFT 1) was measured to be 0.07 ± 0.04 mg as/kg f.w.. The maximum concentration of ETU (arithmetic mean, n=3) for the time period between the first and the second application (DAFT 1-6) was also detected on DAFT 1 with 0.07 ± 0.04 mg as/kg f.w.. ETU levels in ground-dwelling arthropods declined to 0.01 mg as/kg f.w. (arithmetic mean, n=3) before the second application (DAFT 6).

The initial concentration of ETU (arithmetic mean, n=3) in ground-dwelling arthropods one day after the second application (DALT 1) was measured to be 0.05 ± 0.01 mg as/kg f.w.. The maximum concentration of ETU (arithmetic mean, n=3) for the time period after the second application (DALT 1-14) was detected on DALT 3 with 0.14 ± 0.03 mg as/kg f.w.. ETU levels in ground-dwelling arthropods declined to 0.01 mg as/kg f.w. (arithmetic mean, n=3) until the end of the sampling period (DALT 14).

All individual ETU residue concentrations in ground-dwelling arthropods are given in Table 8.1-14.

Table 8.1-14: Residues of ETU in ground-dwelling arthropods

DAFT (Day After First Treatment) 1)	DALT (Day After Last Treatment) 2)	Crop growth stage (BBCH)	Residues of ETU (mg as/kg f.w.)			
			Study plot 1	Study plot 2	Study plot 3	mean
-2	--	55	< 0.01	< 0.01	< 0.01	< 0.01
1	--	57	0.05	0.11	0.05	0.070
2	--	57	0.05	0.04	0.03	0.040
4	--	57	0.01	0.02	< 0.01	0.013
5	--	57	0.04			0.04
6	--	57	< 0.01	0.01	0.02	0.013
8	1	57	0.04	0.05	0.05	0.047
9	2	57	0.06	0.04	0.13	0.077
10	3	57	0.12	0.12	0.17	0.137
13	5	57-63	0.03			0.03
14	6	57-63	0.02	0.02	0.01	0.017
19	12	60-63	0.02	0.01	0.01	0.013
21	14	63	< 0.01	< 0.01	0.01	0.01

1 Date of the first treatment: 31.05.2012
2 Date of the second treatment: 07.06.2012

Residues of ETU in foliage-dwelling arthropods

The ETU residue content in foliage-dwelling arthropods collected from all study plots three and two days before application (DAFT -3 and -2) of the formulation BAS 222 28 F was below the LOQ of the analytical method, i.e. below 0.01 mg as/kg f.w..

The initial concentration of ETU in foliage-dwelling arthropods on the day of the first application (DAFT 0, after application) was measured to be 0.19 mg as/kg f.w.. The maximum concentration of ETU for the time period between the first and the second application (DAFT 0-6) was detected on DAFT 2 with 0.42 mg as/kg f.w., respectively. ETU levels in foliage-dwelling arthropods declined to 0.05 mg as/kg f.w. before the second application (DAFT 6).

The initial concentration of ETU in foliage-dwelling arthropods on the day of the second application (DALT 0, after application) was measured to be 0.09 mg as/kg f.w.. The maximum concentration of ETU after the second application (DALT 0-13) was detected on DALT 1 with 0.21 mg as/kg f.w.. ETU levels in foliage-dwelling arthropods declined to 0.01 mg as/kg f.w. until the end of the sampling period (DALT 13).

All individual ETU residue concentrations in foliage-dwelling arthropods are given in Table 8.1-15.

Table 8.1-15: Residues of ETU in foliage-dwelling arthropods

DAFT (Day After First Treatment) [1)]	DALT (Day After Last Treatment) [2)]	Crop growth stage (BBCH)	Residues of ETU (mg as/kg f.w.)
			Study plot 1-3 [3)]
-3	--	55	< 0.01
-2	--	55	< 0.01
0	--	57	0.19
1	--	57	0.12
2	--	57	0.42
4	--	57	0.27
5		57	0.12
6		57	0.05
7	0	57	0.09
8	1	57	0.21
9	2	57	0.10
11	4	57	0.09
13	6	57-60	0.11
14	7	57-60	0.11
17	10	57-60	0.05
20	13	63	0.01

1 Date of the first treatment: 31.05.2012
2 Date of the second treatment: 07.06.2012
3 Samples of all study plots were pooled prior to the analysis, due to insufficient arthropod matrix

III. CONCLUSION

The study provides field data on the magnitude of initial residue levels and the subsequent time course of metiram and ETU residues in ground- and foliage-dwelling arthropods. These data provide a reliable basis for use in higher tier risk assessments for insectivorous or omnivorous birds and mammals.

A new study on kinetic evaluation of dissipation of metiram and the metabolite ETU in arthropods was conducted and has not been evaluated previously on EU level.

Report:	CA 8.1/7 Sachers S., 2014c Kinetic evaluation of dissipation of BAS 222 F - metiram and its metabolite ETU in arthropods sampled in a vineyard study 2014/1221841
Guidelines:	FOCUS Kinetics (2006) SANCO/10058/2005 version 1.0 of Nov. 2011
GLP:	no

This modeling report provides kinetic analysis and estimation of the dissipation times (DT_{50} values) for metiram and its metabolite ETU.

I. MATERIAL AND METHODS

Calculation of DT_{50} values

Since no specific recommendation is available how to carry out the kinetic evaluation for the described experiment, guidance of the FOCUS workgroup on degradation kinetics was used in order to derive degradation parameters for modeling purposes. This means the selected DT_{50} values are suitable input parameters for models that require single first order (SFO) DT_{50} values or conservative substitutes.

The goodness-of-fit of the kinetic models was assessed by visual inspection and statistical measures, as recommended by the FOCUS (2006) guidance on degradation kinetics. The recommended kinetic models, *i.e.* the single first order kinetics (SFO) and the Gustafson-Holden model (FOMC) were applied. For visual inspection, the recommended graphical representations of observed and modeled decline curves versus time and the residuals versus time are presented in the report. As goodness-of-fit measures, the $\chi2$ error is provided. The kinetic model was considered appropriate if the residuals are randomly distributed around zero, the $\chi2$ - error value ideally < 15 % and the estimated degradation parameters differed from zero at a 5% significance level (FOCUS, 2006).

According to FOCUS the χ^2 - error of 15 % should not be considered as an absolute cut-off criterion. For cases of a larger χ^2 - error (e.g. based on a large scatter in the data as it might be the case for field studies), the decision on the acceptability of the model was based on visual assessment, to evaluate if the fit still represents a reasonable description of the degradation behavior (see FOCUS 2006). If the overall pattern of decline in pesticide concentrations was represented adequately by the model and the distribution of the residuals was random (no systematic deviations), the half-life from the respective model was considered appropriate.

Individual dissipation parameter estimations were conducted separately for foliage dwellers and ground dwellers in potatoes. Furthermore, two separate DT_{50} values were calculated for the residue data after the first and the second application. Afterwards, the DT_{50} values from the same trial were averaged in order to get one endpoint that is representative for the single scenario. In conservative approach (FOCUS 2006), the ETU DT_{50} values were derived by fitting SFO kinetics to the decline of the metabolite observations from the maximum onwards.

II. RESULTS

The presented DT_{50} values that were derived under consideration of guidance from the FOCUS kinetics working group are suitable endpoints for modeling purposes. The degradation rate constants of the different fits were estimated significantly different from zero as indicated by low P values. The visual assessment of the different fits showed that the $\chi 2$ error values were acceptable as the observations were generally well described by the fitted curves and the residuals were randomly scattered around the zero line.

Table 8.1-16: Calculated DT_{50} values for metiram in arthropods and statistical indices

Crop	Arthropod	x^{th} application	Kinetic model	DT_{50} (SFO) [d]	DT_{50} [1] (SFO) [d]	p (t-test)	χ^2 error [%]
vine	ground dweller	1	SFO	2.9	2.9	p < 0.01	22.1
vine		2	Data set discarded [2]				
vine	foliage dweller	1	SFO	3.4	2.5	p < 0.01	16.0
vine		2	SFO	1.8		p < 0.01	22.5

1 geometric mean DT_{50} of the respective trials
2 data set discarded because of highly scattered data ($\chi^2 = 58.8\%$)

Table 8.1-17: Calculated DT_{50} values for ETU in arthropods and statistical indices

Crop	Arthropod	x^{th} application	Kinetic model	DT_{50} (SFO) [d]	DT_{50} [1] (SFO) [d]	p (t-test)	$\chi 2$ error [%]
vine	ground dweller	1	SFO	1.6	1.5	p < 0.01	30.1
vine		2	SFO	1.4		p < 0.01	11.8
vine	foliage dweller	1	SFO	1.8	3.0	p < 0.05	16.0
vine		2	SFO	5.1		p < 0.05	25.5

1 geometric mean DT_{50} of the respective trials

III. CONCLUSION

The decline of metiram and its metabolite ETU residues on arthropods was well described by single first order kinetics. For metiram a DT_{50} of 2.9 and 2.5 days was determined for ground-and foliage-dwelling arthropods in potatoes. For ETU the DT_{50} values correspond to 1.5 and 3.0 days in ground and foliage-dwelling arthropods.

The following study on arthropod residues performed with BAS 222 28 F in potatoes has been evaluated in the draft monograph of the rapporteur member state Italy of July 2000 and peer-reviewed on EU level during previous Annex I inclusion process for metiram. The study has been conducted prior to specific recommendations on the performance on arthropod residue studies given in the new EFSA guidance document (EFSA/1438/2009, Appendix N).

Report:	*CA 8.1/8* *Tilting N., 2004a* *Residues of metiram and ETU in potential feed items of birds and wild mammals following application of Polyram WG (BAS 222 28 F) in potatoes* *2004/1006523*
Guidelines:	*Draft SANCO Document 4145 (Feb. 2001)*
GLP:	*yes* *(certified by Landesamt fuer Umweltschutz und Gewerbeaufsicht, Mainz, Germany Fed.Rep.)*

Executive Summary

In this study the residues of metiram and the ETU metabolite in arthropods after spraying Polyram WG70® (BAS 222 28 F, active ingredient metiram) in potato fields were investigated.

During the year 2002 growing season, two trials (Hüttenfeld and Rossdorf) were conducted in potatoes. Polyram WP was applied three times between BBCH 37/60 and 39/69 at a rate of 1.26 kg a.s./ha at 7 day intervals with a top-down boom sprayer under common agricultural practice.

Arthropods were sampled by pitfall traps and by collecting arthropods from the leaves of the potato plants, and analyzed for metiram (BAS 222 F) using BASF method 510/0 (LOQ =f 0.05 mg/kg) and for the ETU metabolite by using BASF method 511/0 (LOQ = 0.05 mg/kg).

Residue levels of metiram (BAS 222 F) for soil-dwelling arthropods (mainly ground-dwelling beetles and spiders) and leaf dwelling arthropods (mainly Colorado beetles) can be clearly distinguished. For the two locations the mean initial residues of metiram (BAS 222 F) were 2.38 mg/kg in soil-dwelling arthropods and 15.32 mg/kg in leaf-dwelling arthropods.

Residue levels of ETU determined in leaf-dwelling arthropods from the two locations and the four sampling times after last application averaged at 0.26, 0.34, 0.28 and < 0.05 mg/kg. The small sample sizes gained in pitfall traps did not allow analyzing soil-dwelling arthropods for ETU.

I. MATERIAL AND METHODS

Test item: BAS 222 28 F, batch no. 2002-1, content of a.s. (Metiram): 70%

Test site: Two trials were conducted at two locations: Hüttenfeld and Rossdorf (both Hessia, Germany).

Test design: Each trial consisted of an untreated control subplot and a treated subplot. All applications were made as foliar sprays, using commercial ground equipment. No product containing the active substance was previously used on the test plots during the trial season. The test substance was applied three times between BBCH 37 / 60 and 39 / 69 at a rate of 1.26 kg a.s./ha at 7 day intervals with a top-down boom sprayer under common agricultural practice.

Trials were maintained with necessary crop protection measures and dripping irrigation in order to ensure a commercially acceptable crop management. To facilitate the arthropod sampling during the whole study period, no insecticides were used even under heavy infestation by Colorado beetles.

Control samples were taken at every time point and prior the collection of the treated specimens to avoid contamination. Arthropod sampling was done by beating the leaves of 3 - 4 potato plants for leaf-dwelling arthropods. From each plot two subsamples were taken. Soil-dwelling arthropods were trapped in pitfall traps. Samplings for residue analysis were made 0 days (sampling pitfall traps only), 1 days (sampling from leaves only), 3 days (pitfall and leaves), 7 days (pitfall and leaves) and 14 days (pitfall and leaves) after the 3rd application.

Endpoints: (1) Residue analysis of metiram (BAS 222 F) and the ETU metabolite in soil- and leaf-dwelling arthropods collected from treated potato fields, 0 days (for pitfall traps only), 1 day (for sampling from leaves only), 3 days (pitfall and leaves), 7 days (pitfall and leaves) and 14 days (pitfall and leaves) after the 3rd application.

(2) Taxonomic composition, biomass and number of arthropods in the samples of leaf-dwelling arthropods collected for residue analysis.

Test rates: 3 applications with 1.8 kg BAS 222 28 F/ha, corresponding to 1.26 g a.s./ha, controls without test item application.

Test conditions: Rossdorf: Mean temperature was 18°C with a maximum of 31°C and a minimum of 7 C. There were 17 days of precipitation (between 0.2 and 43 mm). The application dates were not affected by either extreme temperatures or precipitation.

Hütteneld: Mean temperature was 19°C with a maximum of 37°C and a minimum of 8°C. There were 23 days of precipitation (between 0.1 and 37 mm). The application or sampling dates were not affected by either extreme temperatures or precipitation.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

In all samples from control plots analyzed for metiram (BAS 222 F) and the ETU metabolite no or only traces of metiram (BAS 222 F) and ETU were detected.

Arthropods were sampled at two trial sites in Germany (Hüttenfeld and Rossdorf) by pitfall traps (sampling of soil-dwelling arthropods) and by collecting arthropods from the potato leaves (leaf-dwelling arthropods). The majority of the samples obtained by beating leaves consisted of Colorado beetles in different stages of development. The specimens were analyzed for metiram (BAS 222 F) by BASF method 510/0 (LOQ = 0.05 mg/kg) and for the ETU metabolite by BASF method 511/0 (LOQ = 0.05 mg/kg). Procedural recoveries were analyzed together with the analytical series and averaged to 84% for metiram (BAS 222 F) and 67% for ETU at fortification levels from 0.05 to 5.0 mg/kg.

Residue levels of metiram (BAS 222 F) for soil-dwelling arthropods (mainly ground dwelling beetles and spiders) and leaf-dwelling arthropods (mainly Colorado beetles, *Leptinotarsa decemlineata*) can be clearly distinguished. The average residues of metiram (BAS 222 F) for the two trial sites were 2.38 mg/kg for arthropods caught in pitfall traps (mainly ground-dwelling beetles and spiders). The initial average residue of metiram (BAS 222 F) for the leaf-dwelling arthropods (mainly Colorado beetles) was 15.32 mg/kg.

ETU was detected in leaf-dwelling arthropods, but at lower levels than metiram (BAS 222 F). The average residues for the two trial sites at the four sampling times after last application were 0.26, 0.34, 0.28 and < 0.05 mg/kg. Due to the small sample sizes that could be collected in pitfall traps an analysis of soil-dwelling arthropods for ETU was not possible.

The residues of metiram (BAS 222 F) in soil-dwelling and leaf-dwelling arthropods for the two locations (Hüttenfeld and Rossdorf) are presented in Table 8.1-18 and
Table 8.1-19, respectively.

Table 8.1-18: Residues of metiram (BAS 222 F) in soil-dwelling arthropods at two sampling locations

Time [DALA] [1]	Hüttenfeld	Rossdorf	Arithmetic mean of both locations
	Metiram [mg/kg]	Metiram [mg/kg]	
1	3.04	1.71	**2.38**
3	2.31	1.35	**1.83**
7	4.30 [2]	0.49	**2.40**
14	0.19	0.16	**0.18**

1) Days after the last of three successive applications.
2) Outlier according to study report.

Table 8.1-19: Residues of metiram (BAS 222 F) in leaf-dwelling arthropods at two sampling locations

Time [DALA] [1]	Hüttenfeld	Rossdorf	Arithmetic mean of both locations
	Metiram [mg/kg]	Metiram [mg/kg]	
0	16.3	18.36	**15.32**
0	16.36	10.27	
3	22.13	8.26	**12.54**
3	7.97	11.78	
7	5.41	7.44	**5.82**
7	4.63	5.8	
14	1.15	2.01	**1.96**
14	2.08	2.6	

1) Days after the last of three successive applications. Two subsamples were taken from each plot.

The overview on residues of metiram (BAS 222 F) and the metabolite ETU in soil-dwelling and leaf-dwelling arthropods for the two locations (Hüttenfeld and Rossdorf) is presented in Table 8.1-20. These data show that residues of metiram (BAS 222 F) and ETU declined rapidly within 14 days.

Table 8.1-20: Average residues of metiram (BAS 222 F) and ETU in soil dwelling arthropods and leaf dwelling arthropods

Time [DALA] [1)]	Residues in soil-dwelling arthropods (mg/kg; average of two samples)		Residues in leaf-dwelling arthropods (mg/kg; average of two samples)	
	Metiram (BAS 222 F)	ETU	Metiram (BAS 222 F)	ETU
0 / 1	2.38	-- [3)]	15.32	0.26
3	1.83	-- [3)]	12.54	0.34
7	2.40 [2)]	-- [3)]	5.82	0.28
14	0.18	-- [3)]	1.96	< 0.05

1) Days after the last of three successive applications.
2) Mean of 4.30 mg/kg and 0.49 mg/kg.
3) Due to the small amounts of soil-dwelling arthropod samples an analysis for ETU was not possible.

III. CONCLUSION

In this study residues of metiram (BAS 222 F) and the ETU metabolite in arthropods were determined. Residues of metiram (BAS 222 F) and ETU declined rapidly within 14 days in arthropods. Average residues of metiram (BAS 222 F) declined from 2.38 mg/kg (initial) to 0.18 mg/kg after 14 days for soil-dwelling arthropods. The initial average residues of metiram (BAS 222 F) for the leaf-dwelling arthropods declined from 15.32 mg/kg (initial) to 1.96 mg/kg after 14 days. Residues of ETU proved to be significantly below those of metiram (BAS 222 F). The average residues of ETU for the leaf-dwelling arthropods declined from 0.26 mg/kg to < 0.05 mg/kg after 14 days.

Evaluation of the study:
The study (BASF DocID 2004/1006523) is considered reliable. However, the sampling events within the first days after applications (conducted on day 1, 3, 7 and 14 after application), might be not detailed enough to derive precise DT_{50} values of the compound. Hence, the newly conducted arthropod residue study in potatoes (see CA 8.1/4 for residue data and CA 8.1/5 for DT_{50} calculations) is considered more appropriate to derive precise DT_{50} values for metiram and ETU on arthropods.

Results on metiram and ETU residues in arthropods in potatoes (CA 8.1/8) and resulting DT_{50} calculations (presented in CA 8.1/9) are considered supportive, but are not directly used in the risk assessment.

The following report on estimation of DT_{50} values for metiram in arthropods is based on residue data for metiram on arthropods from the study by Tilting (BASF DocID 2004/1006523 see above CA 8.1/8). The DT_{50} values from the following study (CA 8.1/9) by Horn (2009, 2009/1072249) are considered supportive data.

Report:	CA 8.1/9 Horn A., 2009a Estimation of DT50 values for metiram for soil-dwelling and leaf-dwelling arthropods 2009/1072249
Guidelines:	none
GLP:	no

Based on the residue data from the study by Tilting N., 2004 (BASF DocID 2004/1006523), DT_{50} values of metiram (BAS 222 F) for soil-dwelling arthropods and leaf-dwelling arthropods were calculated (Horn, 2009, BASF DocID 2009/1072249).

A single first-order (SFO) model was fitted to the residue data of metiram (BAS 222 F) for soil-dwelling and leaf-dwelling arthropods separately. The SFO model proved capable to describe the decline of metiram (BAS 222 F) for the different datasets. For metiram (BAS 222 F) the geometric mean DT_{50} value for soil-dwelling arthropods is 3.88 days and to 5.43 days for leaf-dwelling arthropods.

CA 8.1.1 Effect on birds

Studies with the parent molecule metiram

For the convenience of the reviewer all metiram studies on birds – peer-reviewed and evaluated in the draft monograph of the rapporteur member state Italy of July 2000, are summarized below. The endpoints relevant for the risk assessment are given in Table 8.1-1.

The avian studies were conducted with the technical active ingredient metiram (synonyms: Metiram TK 85; Metiram Premix 95 % or BAS 222 29 F) or with the formulated products Polyram DF (70 % Metiram = BAS 222 28 F, Polyram WG) and Polyram WP (71.2% purity, BAS 222 26 F) as pure metiram (pure active ingredient) is inaccessible for technical reasons. The test substance Metiram-Premix or Metiram TK, the most concentrated material, fulfils the function of a technical active ingredient.

Table 8.1.1-1: Avian toxicity endpoints of metiram (BAS 222 F) relevant for the risk assessment

Test system	Test species	Results	Test substance	Reference [BASF DocID]
Acute oral toxicity	*Colinus virginianus*	LD_{50} > 2150 mg as/kg bw	Metiram Premix BAS 222 29 F (purity 95%)	Review report 2005 [1988/5119]
Sub-chronic toxicity and reproduction	*Colinus virginianus*	NOAEC = 140.6 mg product/kg diet NOAEL = 12.7 mg product/kg bw/d NOAEC = 100 mg as/kg diet NOAEL = 9.0 mg as/kg bw/d	Polyram DF BAS 222 28 F (purity 71.12%) Calculated for pure as	DAR Vol 3, B9 [1998/10719]
	Anas platyrhynchos	NOAEC = 50 mg a.s/kg diet NOAEL = 7.9 mg as/kg bw/d	Metiram TK BAS 222 29 F (purity 95%)	Review report 2005 [1992/11151]
Higher-tier pulsed dose reproduction	*Anas platyrhynchos*	$NOAEC_{initial\ peak}$ = 150 mg as/kg diet $NOAEL_{initial\ peak}$ = 25.5 mg as/kg bw/d	Metiram TK BAS 222 29 F (purity 88.6%)	Review report 2005 [2004/1004372]

Studies with the metabolite ethylene thiourea (ETU)

For the metabolite ethylene thiourea (ETU) studies on acute and reproductive toxicity in birds are submitted. The studies have not been peer-reviewed on EU level.

Table 8.1.1-2: Avian toxicity endpoints for ETU

Test system	Test species	Results	Reference [BASF DocID]
Acute oral toxicity	*Colinus virginianus*	LD_{50} > 2250 mg/kg bw	New study [2010/7016050]
	Poephila guttata	LD_{50} = 2000 mg/kg bw	New study [2011/7003974]
Sub-chronic toxicity and reproduction	*Colinus virginianus*	NOAEC = 20 mg/kg diet NOAEL = 1.8 mg/kg bw/d	New study [2013/7000533]
	Anas platyrhynchos	NOAEC = 20 mg/kg diet NOAEL = 2.6 mg/kg bw/d	New study [2013/7000534]

The status of each study on EU level (peer-reviewed and/or EU agreed or new study) is given prior the summaries.

CA 8.1.1.1 Acute oral toxicity to birds

Studies with the parent molecule metiram

The acute toxicity study on bobwhite quail has been evaluated in the draft monograph of the rapporteur member state Italy of July 2000 and peer-reviewed on EU level during previous Annex I inclusion process for metiram.

Report:	*CA 8.1.1.1/1*
	[redacted] 1988a
	Metiram technical: 21-day acute oral LD50 study in bobwhite quail
	1988/5119
Guidelines:	*EPA 71-1*
GLP:	*yes*
	(certified by United States Environmental Protection Agency)

Executive Summary

An acute avian toxicity test with metiram was conducted in the bobwhite quail to determine the LD_{50} and the no observed effect level (NOEL). The test item was administered via a single-dose of 1470 and 2150 mg as/kg bw to 30-week old bobwhite quails. Ten birds (5 males and 5 females) were used in each group. The doses were administered in gelatin capsules. Feed was removed for 17.5 hours prior to dosing.

All groups were observed for mortality, signs of clinical toxicity, impact on food consumption and body weight for 21 consecutive days post dosing. All groups received food and water *ad libitum* throughout the test.

No mortality or signs of clinical toxicity were observed in the control and all treatment groups. No statistically significant differences in food consumption and body weights of birds in the treatment groups were observed compared to the control groups. Gross pathological examinations performed of the birds sacrificed at the termination of the test showed no evidence of toxicological effects.

In an acute toxicity test with the bobwhite quail, the LD_{50} of metiram was >2150 mg as/kg b.w.. The NOEL was 2150 mg as/kg b.w.

I. MATERIAL AND METHODS

Test item: Metiram Premix (BAS 222 F, Reg. No. 250 284), batch no. 765, purity: 95%.

Test species: Bobwhite quail (*Colinus virginianus*); indistinguishable from wild birds; age: approx. 30 weeks; source: [redacted]

Test design: Birds were administered single doses of 1470 and 2150 mg as/kg bw of metiram gelatin capsules by gavage into the crop; 5 males and 5 females per dose group were used; observation period of 21 days; assessment of mortality and signs of clinical toxicity was carried out daily; assessment of body weight was carried out on day 1, 3, 7, 14 and 21; mean food consumption (g/bird/day) was calculated from the food consumption/cage on day 3, 7, 14 and 21. Gross-mortem examinations of four arbitrarily selected birds (two male and two female) at termination of the test on day 21 after dosing.

Endpoints: LD_{50}, mortality, clinical signs, feed consumption, body weight (b.w.), gross pathological examinations.

Test concentrations: Control, 1470, 2150 mg as/kg bw (nominal).

Test conditions: Birds fasted for 17.5 h before administration of the test item; temperature 17.8 °C - 20.0 °C; relative humidity: 37% - 58%; photoperiod: 8 hours light, 16 hours dark.

Statistics: Descriptive statistics, ANOVA for body weight data.

II. RESULTS AND DISCUSSION

Biological results: No mortality occurred throughout the duration of the study. Accordingly, the highest dose causing no mortality was 2150 mg as/kg bw for males and females. No signs of toxicity were observed in the control and in all test item concentrations. The only abnormal observation noted in the treatment groups was the presence of chalky droppings under the pens at the end of day 1. Twenty-four hours later the droppings were normal in appearance. There was no treatment-related impairment of mean feed uptake. At study termination (21-d) neither the mean body weight nor the development of body weight of male and female birds was statistically significantly reduced when compared to the control group. No treatment-related macroscopic abnormalities were detected in the gross post-mortem examination. The relevant endpoints are summarized in the table below.

Table 8.1.1.1-1: Acute toxicity of metiram to the bobwhite quail (Colinus virginianus)

Mortality	Dose [mg as/kg b.w.]
Highest dose causing no treatment-related mortality	2150
LD_{50} (21 d)	> 2150
NOEL	2150

b.w. = body weight

III. CONCLUSION

In an acute toxicity test with the bobwhite quail, the LD_{50} of metiram was >2150 mg as/kg b.w.. The NOEL was 2150 mg as/kg b.w.

Studies with the metabolite ETU

The following study on acute oral toxicity study of the metabolite ETU on the bobwhite quail is new and has not been evaluated on EU level.

Report: CA 8.1.1.1/2
[redacted] 2010a
Ethylenethiourea: An acute oral toxicity study with the northern bobwhite
2010/7016050
Guidelines: EPA 850.2100, EPA 71-1
GLP: yes
(certified by United States Environmental Protection Agency)

Executive Summary

An avian acute oral toxicity test was conducted to evaluate the acute toxicity of ethylene thiourea administered to the northern bobwhite quail (*Colinus virginianus*) as a single oral dose to determine an LD_{50} and a no-observed-effect level (NOEL) for sublethal effects.

The test substance was administered via a single oral dose of 0 (control), 292, 486, 810, 1350 and 2250 mg as/kg bw to groups of 23 weeks old northern bobwhite. Ten birds (5 males and 5 females) were used in each test substance group.

A record was maintained of all mortality, signs of toxicity, and abnormal behaviour. Body weights and feed consumption was determined regularly.

No mortality occurred throughout the duration of the study in the control and in all dose groups leading to an acute oral LD value for northern bobwhite exposed to ethylene thiourea to be greater than 2250 mg as/kg bw, the highest dosage tested. The no-mortality level was 2250 mg as/kg bw. The no observed adverse effect level was 810 mg as/kg bw, based on body weight effects at the 1350 and 2250 mg as/kg dosage levels.

In an acute oral toxicity test with the northern bobwhite quail (*Colinus virginianus*), the LD_{50} of ethylene thiourea was found to be > 2250 mg as/kg bw The NOEL was 810 mg as/kg b.w.

I. MATERIAL AND METHODS

Test item:	Ethylene thiourea; 2-imidazolidinethione (Reg. No. 146099), batch no. XW7-102353-014, purity 100%
Test species:	Northern bobwhite quail (*Colinus virginianus*) phenotypically indistinguishable from wild birds; age range of birds at test initiation 23 weeks; supplier: [redacted]
Test design:	Birds were administered single doses of 292, 486, 810, 1350 and 2250 mg as/kg bw of the test substance ethylene thiourea dispersed in reverse osmose water and orally intubated directly into the crop or proventriculus of each bird using a stainless steel 14 gauge cannula; 5 males and 5 females per dose group were used. Following dosing, multiple observations of endpoints were performed on day 0 of the test, with particular attention being paid for signs of regurgitation. An observation period of 14 days followed, during which mortalities and signs of toxicity and abnormal behaviour were recorded, at least twice a day. Body weights were measured individually at the initiation of the test and on days 3, 7 and 14. Average feed consumption was determined by pen for each dosage group and the control group for days 0-3, 4-7 and 8-14. No gross-pathological examinations were conducted on all birds sacrificed at the termination of the test.
Endpoints:	Mortality, signs of toxicity, feed consumption, body weight (b.w.), Calculation of LD_{50} and NOEL.
Test concentrations:	0 (control), 292, 486, 810, 1350 and 2250 mg as/kg bw
Test conditions:	Birds were fasted for approximately 17 hours prior to dosing; temperature: 23.6 ± 0.7°C (SD), 22.9 °C (minimum) and 24.4 °C (maximum); relative humidity: 43 ± 6% (SD), 37% (minimum) and 49% (maximum); photoperiod: 8 hours light: 16 hours dark, light intensity: approx. 163 Lux.
Analytics:	No analytics were performed.
Statistics:	LD_{50}: no statistical calculation, no mortality. Food consumption: not examined. Body weight data: Dunnett's t-test using TOXSTAT® software

II. RESULTS AND DISCUSSION

Biological results:
No mortality occurred throughout the duration of the study in the control and at dose levels of 292, 486, 810, 1350 and 2250 mg as/kg bw. There were no incidences of regurgitation noted among the control birds or any treated birds. All birds in the control and the dose levels of 292, 486, 810, 1350 and 2250 mg as/kg bw were normal in appearance and behavior throughout the test with no indication of any clinical sign of toxicity.
When compared to the control group, there were no apparent treatment related effects on feed consumption among the males and females in the 292, 486, and 810 mg as/kg dosage groups or among males in the 2250 mg as/kg group. When compared to the control group and subsequent feeding intervals, there was a reduction in feeding from day 0 to 3 among the males and females in the 1350 mg as/kg dosage group and among females in the 2250 mg as/kg dosage group. For the feeding intervals of days 4 to 7 and days 8 to 14, there were no apparent treatment related effects on feed consumption at the 292, 486, 810, 1350 and 2250 mg as/kg dosage levels.

Statistical significant difference ($p<0.05$) in mean body weight were observed in males of 486 mg as/kg dosage level at the start of the test. The birds were selected by indiscriminate draw, which in this case resulted in a slight difference in mean group start weight. There was a statistically significant dose-responsive, treatment related reduction in body weight gain among the males ($p<0.05$) at the 1350 mg as/kg dosage and among the males and females ($p<0.01$) at the 2250 mg as/kg bw dosages from day 0 to 3. The mean body weights on day 3 for the males and females in the 2250 mg as/kg bw dosage level were statistically different ($p<0.05$) from the control mean body weights. From day 3 to 7 the body weight gains that were statistically greater than the control mean body weight gain was noted for the males ($p<0.05$) in the 810 mg as/kg dosage level and males ($p<0.01$) in the 2250 mg as/kg bw dosage level. These statistically significant differences in body weight reflected increased body weight compared to controls, and therefore, are considered not adverse treatment-related effects. All other body weights and body weight changes among treatment groups were not statistically significant when compared to the control group. The acute toxicity data are summarized in the table below.

Table 8.1.1.1-2: Acute toxicity of ethylene thiourea to the northern bobwhite quail (Colinus virginianus)

	Dose rate [mg as/kg bw]					
	0 (control)	292	486	810	1350	2250
Number of birds per dose group	10	10	10	10	10	10
Number of dead birds	0	0	0	0	0	0
Dead birds percentage [%]	0	0	0	0	0	0

Endpoints	Dose [mg as/kg bw]
Highest dose causing no substance-related mortality	2250
LD_{50} (14 d)	> 2250
NOEL	810

b.w. = body weight

III. CONCLUSION

In an acute oral toxicity test with the northern bobwhite (*Colinus virginianus*), the LD_{50} of ethylene thiourea was found to be > 2250 mg as/kg bw. The NOEL was 810 mg as/kg bw based on body weight effects at the 1350 and 2250 mg as/kg dosage levels.

The following study on acute oral toxicity study of the metabolite ETU with the zebrafinch is new and has not been evaluated on EU level.

Report: CA 8.1.1.1/3
[redacted] 2011a
Ethylenethiourea: An acute oral toxicity study with the zebra finch (Poephila guttata)
2011/7003974
Guidelines: EPA 850.2100, EPA 71-1
GLP: yes
(certified by United States Environmental Protection Agency)

Executive Summary

An avian acute oral toxicity test with the substance ethylene thiourea was conducted. The objective of this study was to evaluate the acute toxicity of ethylene thiourea administered to the zebra finch (*Taeniopygia* (*Poephila*) *guttata*) as a single oral dose and to determine an LD_{50} and a no-observed-effect level (NOEL) for sublethal effects.

The test substance was administered via a single oral dose of 0 (control), 259, 432, 720, 1200 and 200 mg as/kg bw to groups of 5 to 9 months old zebra finches. Ten birds (5 males and 5 females) were used in each test substance group. A record was maintained of all mortality, signs of toxicity, and abnormal behaviour. Body weights and feed consumption were recorded regularly.

There were no treatment related mortalities in the 259, 432 and 720 mg as/kg bw treatment levels. There was 10% mortality at the 1200 mg as/kg dosage level and 50% mortality at the 2000 mg as/kg dosage level that were considered to be treatment related. When compared to the control group, there were no apparent treatment related effects on body weight and feed consumption at any dosage level tested.

The acute oral LD_{50} value for zebra finch (*Taeniopygia* (*Poephila*) *guttata*) exposed to ethylene thiourea as a single oral dose was determined to be 2000 mg as/kg. The no-mortality level was 720 mg as/kg bw. The no-observed effect level (NOEL) was 259 mg as/kg bw.

I. MATERIAL AND METHODS

Test item: Ethylene thiourea; 2-imidazolidinethione, Reg. No.146099, CAS-No.: 96-45-7, batch no.XW7-102353-014, purity 100%

Test species: Zebra finch (*Taeniopygia* (*Poephila*) *guttata*) age of birds approximately 5 to 9 months at the initiation of the test; supplier: [redacted]

Test design: Birds were administered single doses of 259, 432, 720, 1200 and 2000 mg ethylene thiourea /kg bw. The test substance was orally inserted in 2 gelatine capsules into the crop of each bird. The route of administration (2 capsules) was determined from Non-GLP range finding studies in which capsule dosing had less regurgitation than gavage dosing a solution; 5 males and 5 females per dose group were used. Following dosing, multiple observations were performed on day 0 of the test, with particular attention being paid for signs of regurgitation. From test initiation until termination, all birds were observed at least twice daily. A record was maintained of all mortality, signs of toxicity, and abnormal behaviour. Body weights were measured individually on the day prior to dosing to allow time for capsule preparation (day -1) and on days 3, 7 and 14 of the test. Average estimated feed consumption was then measured from day 3 to 7, day 7 to 10 and day 10 to 14. Four of seven birds that died during the study were examined in gross necropsy.

Endpoints: Mortality, clinical signs of toxicity, feed consumption and body weight measurements were conducted on all birds, gross-pathological examinations were conducted on four birds. LD_{50} and NOEL.

Test concentrations: 0 (control), 259, 432, 720, 1200 and 2000 mg as/kg bw

Test conditions: Birds were fasted for approximately 2 – 3.5 hours prior to dosing; temperature: 22.1 ± 1.2 °C (SD); relative humidity: 22 ± 7% (SD); photoperiod: 0.25 hour dim light/eight hours light/0.25 hour dim light/16 hours dark, light intensity: approximately 172 lux.

Analytics: No analytics were performed.

Statistics: The LD_{50} value was determined to be 2000 mg as/kg, the dosage level at which 50% mortality occurred. Calculated mean values and standard deviations for body weight and feed consumption were performed using Excel in the full-precision mode.

II. RESULTS AND DISCUSSION

Biological results:
There were no incidences of regurgitation noted among the control birds or among the birds at the 259 and 1200 mg a.i/kg dosage levels. There was 10% (1 of 10) regurgitation at the 432 and 720 mg a.i./kg dosage levels and 20% (2 of 10) regurgitation at the 2000 mg a.i./kg dosage level. The regurgitating birds were not used in the study and were replaced with sufficient birds to obtain 5 males and 5 females from each level that retained the dosage given them.

There were no treatment related mortalities in the control and the 259, 432 and 720 mg as/kg bw treatment levels. There was one mortality at the 1200 mg as/kg dosage level and five mortalities at the 2000 mg as/kg dosage level that were considered to be treatment related.
When compared to the control group, there were no apparent treatment related effects on body weight and feed consumption at any dosage level tested.

All birds in the 259 mg as/kg bw dosage level were normal in appearance and behavior throughout the test. Clinical signs of toxicity were observed in the 432, 720, 1200 and 2000 mg as/kg bw treatment groups ranging from ruffled or slight ruffled appearance, slight feather loss in the lower dose groups to lethargy, loss of coordination, prostrate posture and depression at the higher dose levels.

Gross necropsy performed upon four of the seven mortalities revealed intracranial bleeding, a pasty vent, an empty crop and gizzard, and primarily empty intestines. The individual bird (in the 432 mg a.i./kg dosage level was found dead on Day 13) had an abrasion on the tip of the left wing, missing primary flight feather on the left wing and subcapsular hematomas on the left lobe of the liver. Given the results of the necropsy and the timing of the death, the mortality was not considered to be treatment related.

The acute toxicity data are summarized in the table below.

Table 8.1.1.1-3: Acute toxicity of ethylene thiourea to the zebra finch

	Dose rate [mg as/kg bw]					
	0 (control)	259	432	720	1200	2000
Number of birds per dose group	10	10	10	10	10	10
Number of dead birds	0	0	1*	0	1	5
Dead birds percentage [%]	0	0	10	0	10	50

Endpoints	Dose [mg as/kg b.w.]
Highest dose causing no substance-related mortality	2000
LD_{50} (14 d)	2000
NOEL	259

b.w. = body weight
* not considered to be treatment related due to time of mortality and lack of any signs of toxicity.

III. CONCLUSION

The acute oral LD50 value for zebra finch exposed to ethylene thiourea as a single oral dose was determined to be 2000 mg as/kg, the dosage level where 50% mortality occurred in this study. The no-mortality level was 720 mg as/kg. The no-observed effect level (NOEL) was 259 mg as/kg, based on signs of toxicity at the 432 mg as/kg dosage level.

CA 8.1.1.2 Short-term dietary toxicity to birds

Studies with the parent molecule metiram

Although Regulation 1107/2009 does not require short-term studies in birds, the following dietary toxicity study on bobwhite quail performed with a metiram containing formulation (Polyram 80 WP) is summarized below. The study has been evaluated in the draft monograph of the rapporteur member state Italy of 2000 and peer-reviewed on EU level during the last review.

Report:	*CA 8.1.1.2/1*
	[redacted] 1974a
	Eight-day dietary LC50 - Bobwhite quail - Polyram 80 WP
	1974/10255
Guidelines:	*none*
GLP:	*no*

Executive Summary

An avian dietary toxicity test was conducted to establish the dietary LC_{50} and the NOEC of Polyram 80 WP orally administered to juvenile bobwhite quail for 5 days.

The test item was administered at concentrations of 215, 464, 1000, 2150 and 4640 mg Polyram 80 WP/kg feed. Ten 14-days old Bobwhite quails were used in each treatment group. All groups were observed for mortality, signs of clinical toxicity, impact on food consumption the test-item feeding period of 5 days and for a post exposure period of three days. Body weight was recorded at the beginn of the exposure phase and at the end of the post-exposure phase. All groups received feed and water *ad libitum* throughout the test.

Mortalities occurred only in the highest test concentration; with the exception of wing droop at 1500 mg as/kg diet birds at the lower test concentrations did not reveal any signs of toxicity. Food consumption and body weight development was similar to the controls at all test concentrations except the highest.

The LC_{50} of metiram for juvenile Bobwhite quails was determined to be 3250 mg as/kg diet, the highest tested concentration.

I. MATERIAL AND METHODS

Test item: Polyram 80 WP, batch no.: M83923IR, content of a.s. metiram (BAS 222 F, Reg. No. 250 284): assumed 71.2% purity (not detailed in study)

Test species: Bobwhite quail (*Colinus virginianus*), chicks, indistinguishable from wild birds; age: approx. 14 days; source: not reported.

Test design: Birds administered different doses of the test item offered in the diet on 5 consecutive days; 10 birds per test item concentration and 50 for the control group were used; post-exposure period of 3 days; assessment for mortality and clinical signs was carried out once daily; assessment of body weight was carried out on day 0 and 8; average food consumption per bird and day was calculated from the five-day mean food consumption of each group.

Endpoints: LD_{50}, NOEC, mortality, clinical signs, feed consumption, body weight measured on day 0 and 8 .

Test concentrations: Control, 215, 464, 1000, 2150 and 4640 mg Polyram 80 WP /kg diet (nominal), corresponding to 150, 325, 700, 1500 and 3250 mg metiram/kg diet.

Reference item: Dieldrin, applied at 15.9, 25.1, 39.8, 63.1 and 100 mg as/kg diet.

Test conditions: Not reported. Non-GLP as prior implementation of GLP rules.

Analytics: Not reported.

Statistics: Descriptive statistics, probit analysis for determination of LC_{50}.

II. RESULTS AND DISCUSSION

Mortalities occurred only in the highest test concentration; with the exception of wing droop at 1500 mg as/kg diet birds at the lower test concentrations did not reveal any signs of toxicity. Food consumption was similar to the controls at all test concentrations except the highest. Body weight development during the exposure period cannot be evaluated, but seemed to be in comparable range at the end of the post-exposure period. The results are summarized in the table below.

Table 8.1.1.2-1: Avian dietary toxicity of metiram to the bobwhite quail (*Colinus virginianus*)

Group [mg as/kg diet]	Control	150	325	700	1500	3250
Mortality [%]	0.0	0.0	0.0	0.0	0.0	50.0
Feed consumption [g/5 days] 1)	519	375	390	300	400	210 (↓ 62%)
Body weight [g] 2)	28.1	26.5	27.5	29.5	27.5	25.5
Symptoms 3)	none	none	none	none	W	W, D, R
Endpoints [mg as/kg diet]						
LC_{50} (95% CL)4)	3250 (3226 - 6672)					
NOEC	700					

1) Total estimated feed consumption on days 1 - 5 of exposure period per 10 birds
2) Mean body weight on day 0 and 8 of exposure period per bird
3) Symptoms: W= wing droop; D= depression, R= labored respiration
4) Median lethal concentration calculated using probit analysis (with 95% confidence limits)

III. CONCLUSION

The LC_{50} of metiram tested as Polyram 80 WP for juvenile Bobwhite quails was determined to be 3250 mg as/kg diet. The highest tested concentration of 3250 mg as/kg diet.

Evaluation of reliability:

The EU agreed endpoint for metiram from the avian short-term study in quails (BASF DocID 1974/10255 provides an LDD_{50} 4640 of ppm (pure metiram 3250 ppm), which was calculated to correspond to a daily dose of 853 mg/kg bw/d.

The study was conducted in 1974 under non-GLP conditions. The study is considered not reliable as during the course of the study not all data were collected as foreseen by current guideline standards (OECD 405 and OCSPP 850.2200).

Due to following deficiencies, the results of the study are not considered reliable:

- Test substance purity of the tested formulation (most likely BAS 222 26 F) cannot fully be retrieved based on batch no. indicated in the report as no certificate of analysis is attached. Purity is assumed to correspond to 71.2% metiram (80% metiram complex).
- Analytical measurements of substance in the diet were not performed.
- Body weight of chicks were only measured on day 0 and 8, but not directly after the exposure period on day 5.
- Body weights were not recorded individually, but in groups of 10 chicks.
- Food consumption at the dose of 4640 mg/kg diet corresponds to 38% of average consumption in the control group with a clear indication of avoidance of food items treated with metiram at this dose.
- Daily observations for signs of toxicity or test conditions are not reported.

Conclusion: Without body weight measurements on day 5 impact of food avoidance on body weight of chicks during the exposure period cannot be retrieved. Finally, a reliable daily dose cannot be calculated. Thus, the calculated LCD_{50} based on the 5-day dietary study is not used in the risk assessment as it cannot be reliable calculated.

CA 8.1.1.3 Sub-chronic and reproductive toxicity to birds

Studies with the parent molecule metiram

All reproduction toxicity studies with the bobwhite quail and mallard duck performed with metiram have been evaluated in the draft monograph of the rapporteur member state Italy of 2000 and peer-reviewed on EU level. The pulsed dose reproduction study in the mallard was evaluated in Addendum IV to B9 chapter (April 2004). All studies were considered acceptable.

Report:	*CA 8.1.1.3/1*
	[redacted] 1989a
	Metiram premix 95%: Toxicity and reproduction study in bobwhite quail
	1989/5081
Guidelines:	*EPA 71-4*
GLP:	*yes*
	(certified by United States Environmental Protection Agency)

Executive Summary

This 1-generation study was designed to determine possible effects of administration of metiram via food on the reproduction in the bobwhite quail (*Colinus virginianus*). Effects on health, body weight development and feed consumption of adult birds as well as effects on different reproductive parameters were determined. The birds were offered the test item treated food over 21 weeks at concentrations of 0, 20, 100 and 500 mg as/kg diet. There were 15 replicates of three birds (1 male and 2 female) in each treatment group. Animals of all test groups received feed and water ad libitum throughout the test. The laid eggs were collected, incubated and the offspring was raised for 2 weeks.

Parental generation:

No treatment related adverse effects on body weight were observed at any dose. Mortalities occurred in all groups and are considered not treatment-related but rather caused by the known aggression of this species when kept in cages. However, mortality in the control group amounted to 22%, indicating that the study is of limited validity.

Effects on reproduction:

Egg quality data (number of cracked and broken eggs) were statistically significant impacted at the low dose of 20 mg as/kg diet, which was considered not treatment related as the percentage of cracked and broken eggs in the control and 20 mg as/kg diet test group fell within the historical control data. Statistically significant increases in the numbers of infertile eggs were noted for the 20 and 100 mg as/kg diet test group, but not for the dose of 500 mg as/kg diet. The percentages of infertile eggs (based on numbers of eggs set) in the control, 20 and 100 mg as/kg diet test groups all fell within the normal range. Since a dose-response relationship was not evident, these findings were not considered to be treatment related. No other statistically significant differences were noted throughout the investigation.

Overall, data related to reproductive parameters did not reveal any treatment-related adverse effects.

In a 21-week avian reproduction test with the bobwhite quail, no test item-related effects in the parent generation on mortality, birds` health, palatability, food consumption and body weight could be detected. Furthermore no test-item related adverse effect on the reproduction parameters investigated in this study could be observed in any of the treatment groups. In conclusion the NOEC of metiram was set at 500 mg as/kg diet.

I. MATERIAL AND METHODS

Test item: Metiram Premix 95% (BAS 222 F, Reg. No. 250 284), purity 93%.

Test species: Bobwhite quail *(Colinus virginianus)*, age 28 - 29 weeks, the birds were indistinguishable from wild birds; supplier: [redacted]

Test design: Bobwhite quails approaching their first breeding season were kept in groups of 1 male and 2 females per replicate; 15 replicates of three birds (1 male and 2 female) were set up. After an adaptation period of about 11 weeks to the conditions in the laboratory, birds were exposed to the test item treated diet over a period of 21 weeks (10 weeks pre-egg laying period, 11 weeks egg-laying period). Laid eggs were collected, incubated until hatching, offspring raised for 2 weeks. Gross-mortem examinations of half of the remaining parental birds at termination of the test.

Endpoints: Adult birds: mortality, clinical signs of toxicity, food consumption, palatability, development of body weight, post-mortem examination.
Reproduction parameters: egg production, egg weight, egg quality, egg shell thickness, fertility rate, early and late embryonic deaths, hatchability, 14-day survival of the chicks, body weight of survivors, post-mortem examination.

Test concentrations: Control, 20, 100 and 500 mg as/kg diet (nominal). Concentrations were adapted for content of active substance.

Test conditions: Adult bobwhite study room: temperature: 16.7 °C - 33.3°C; relative humidity: 69%; photoperiod: 8 hours fluorescent light (week 1 to week 6), 4 hours incandescent light (week 7), light cycle was increased four hours each week until a maximum of 16 hours.

Incubation of eggs: placed in a commercial incubator for 2 days before being transferred to the hatcher;
Incubator temperature: 37.1 °C - 37.7 °C.
Hatcher temperature: 33.9 °C - 40.6 °C, relative humidity: 42% - 71%.

Analytics: The test item concentrations were analyzed.

Statistics: ANOVA followed by Dunnett's-test for adult food consumption and body weight, egg weight, egg shell thickness, chicks bodyweight;
Contigency table Analysis for count data (e.g. number of eggs or hatched chicks) and proportions (e.g. number of fertile eggs of eggs initially laid) ($\alpha = 0.05$, $\alpha = 0.01$).

II. RESULTS AND DISCUSSION

Analytical measurements:

The results of the analytical verification of the test item concentration in the diet were within a range of 83.7% to 111% of the nominal concentrations. The biological results are based on the nominal values.

Parental generation:

Depressions in body weight development were seen at the highest concentration only in female birds at two weighings out of six and are therefore considered not treatment-related. Thus, no treatment related adverse effects on body weight were observed at any dose. Mortalities occurred in all groups and are considered not treatment-related but rather caused by the known aggression of this species when kept in cages.

Effects on reproduction:

The data related to egg production and chick survival did not reveal any treatment-related adverse effects. The biologically relevant number of surviving chicks per hen comparable in all groups and the body weight development was in the same range in all groups as well. The egg quality data revealed a statistically significant increase in the number of cracked and brocken eggs for the 20 mg as/kg diet test group when compared to the control group. This was considered a beneficial finding. The percentage of defective eggs in the control and 20 mg as/kg diet test group fell within the range of historical controls. Statistically significant increases in the numbers of infertile eggs were noted for the 20 and 100 mg as/kg diet test group. The percentages of infertile eggs (based on numbers of eggs set) in the control, 20 and 100 mg as/kg diet test groups all fell within the normal range. No such increase was noted for the 500 mg as/kg diet test group. Since a dose response relationship was nor evident, these findings were not considered to be treatment related. No other statistically significant differences were noted throughout the investigation. The results are summarized in the table below.

Table 8.1.1.3-1: Effects of metiram on the reproduction of the bobwhite quail (*Colinus virginianus*)

Parameter	Experimental group [mg as/kg diet]			
	Control	20	100	500
Parental generation				
Number of replicates	15	15	15	15
No. treatment-related mortality of adult birds (male/female)	0 0	0 0	0 0	0 0
Mean food consumption [g/bird/day]	21.0	22.5	21.9	21.0
Adult body weight [g] (male/female) end of egg laying period	208 230	213 220	203 230	217 225
Reproduction parameter				
No. of eggs laid / group	927	1174	1090	843
No. of cracked and broken eggs / group	170	141**	169	159
Mean egg shell thickness (mm)	0.231	0.233	0.236	0.234
No. of eggs set $^{+}$/ group	692	932	844	618
No. of fertile eggs / group	586	702	608	509
No. of infertile eggs / group	106	229**	234**	107
No. of early embryonic mortalities / group	9	13	12	8
No. of viable 8-12 day old embryos / group	577	689	596	501
No. of late embryonic mortalities / group	27	33	49	29
No. of viable 15-20-day old embryos / group	577	689	596	501
No. of total embryonic deaths / group	92	100	109	79
No. of chicks hatched / group	494	602	499	431
No. of 14-day surviving chicks / group	422	502	396	353
No. of eggs laid / female bird / week	3.0	3.6	3.3	2.9
No. of chicks hatched / female bird / week	1.6	1.8	1.5	1.5
No. of 14-day surviving chicks / female bird / week	1.4	1.5	1.2	1.2
Mean body weight of chicks at hatching (g)	7.0	7.0	7.0	6.9
Mean body weight of chicks 14 days after hatching (g)	29.5	29.0	29.0	28.7
% fertile eggs of eggs set	84.7	75.3	72.0	82.4
% viable 8-12 day old embryos of eggs set	83.4	73.9	70.6	81.1
% 14-day survivors of chicks hatched	85.4	83.4	79.4	81.9
% cracked and broken eggs of eggs laid	18.3	12.0	15.5	18.9
% early embryonic mortalities of fertile eggs	1.5	1.9	2.0	1.6
% late embryonic mortalities of fertile eggs	4.6	4.7	8.1	5.7
% "dead-in-shell" of fertile eggs	na	na	na	na
Hatchability (% chicks hatched of total eggs set)	71.4	64.6	59.1	69.7
Hatchability (% chicks hatched of fertile eggs)	84.3	85.8	82.1	84.7

+ = incubated
** = statistically significant differences compared to the control (α = 0.01)

III. CONCLUSION

In a 21-week avian reproduction test with the bobwhite quail, no test item-related effects in the parental generation on mortality, birds` health, palatability, food consumption and body weight could be detected. Furthermore no test-item related adverse effect on the reproduction parameters investigated in this study could be observed in any of the treatment groups. In conclusion the NOEC of metiram was set at 500 mg as/kg diet.

Reliability of study:

Validity criteria in quail study with BASF DocID 1989/5081 are not fulfilled according to current guideline standards of OECD 206 and OCSPP 850.2300 for avian reproduction testing based on:

- More than 10% of the control birds died or became moribund during the test
- There are greater than 13% cracked eggs in the control group.

Conclusion: Based on the high mortality and high percentage of broken eggs, the study is of very limited validity. The NOEL > 500 ppm for metiram in feed is not absolutely justified based on this study.

Report:	*CA 8.1.1.3/2*
	[redacted] 1998a
	BAS 222 28 F - 1-generation reproduction study on the bobwhite quail (Colinus virginianus) by administration in the diet
	1998/10719
Guidelines:	*EPA 71-4, OECD 206, EPA 540/9-86-139*
GLP:	*yes*
	(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)

Executive Summary

This 1-generation study was designed to determine possible effects of administration of BAS 222 28 F (Polyram WG) via food on the reproduction of the bobwhite quail (*Colinus virginianus*). Effects on health, body weight development and feed consumption of adult birds as well as effects on different reproductive parameters were determined. The birds were offered the test item treated food over 21 weeks at concentrations of 0, 100, 300 and 1000 mg as/kg diet. There were 16 replicates of two birds (1 male and 1 female) in each treatment group. Animals of all test groups received feed and water ad libitum throughout the test. The laid eggs were collected, incubated and the offspring was raised for 2 weeks.

Parental generation:

No treatment-related effects on mortality, birds` health, food consumption and body weight in the parental generation could be detected in any concentration group. Avoidance of food was not observed. At the gross-pathological evaluation at the end of the exposure period no abnormalities caused by the test item could be found.

Effects on reproduction:

No test-item related adverse effects on any reproductive parameters were observed in the group treated with 100 g a.s./kg diet. The animal treated with 300 mg/kg diet showed, when compared to control, statistically significant higher rates of cracked eggs, higher rates of early embryonic mortality and of "dead in shell". Additional to the effects at 300 mg as/kg diet the animals treated with 1000 mg/kg showed reduction (even if not statistically significant) in the number of eggs laid per female, slightly reduced mean egg weight (-5.9%) statistically significant only during the last period (weeks 11-14) of eggs-laying. As a consequence hatchability was reduced, the mean number of surviving 14-day-old chicks and of 14-days old chicks per female was significantly lower in the highest treatment group.

In a 24-week avian reproduction test with the bobwhite quail, no test item-related effects in the parent generation on mortality, birds` health, palatability, food consumption and body weight could be detected. At 300 mg as/kg diet and 1000 mg as/kg diet test-item related adverse effects on the reproduction parameters could be observed. In conclusion the NOEC of BAS 222 28 F was set at 100 mg as/kg diet, corresponding to a daily dose of 9.0 mg as/kg bw.

I. MATERIAL AND METHODS

Test item: BAS 222 28 F, batch No. 96-1, content of a.s. metiram (BAS 222 F, Reg. No. 250 284): 71.12%.

Test species: Bobwhite quail *(Colinus virginianus)*, age 4 months, the birds were indistinguishable from wild birds; supplier: [redacted]

Test design: Bobwhite quails approaching their first breeding season were kept in groups; 16 replicates of two birds (1 male and 1 female) were set up. After an adaptation period of about 2 weeks to the conditions in the laboratory, birds were exposed to the test item treated diet over a period of 24 weeks (10 weeks pre-egg laying period, 14 weeks egg-laying period). Laid eggs were collected, incubated until hatching, offspring raised for 2 weeks.

Endpoints: Adult birds: mortality, clinical signs of toxicity, food consumption, palatability, development of body weight, post-mortem examination.
Reproduction parameters: egg production, egg weight, egg quality, egg shell thickness, fertility rate, early and late embryonic deaths, hatchability, 14-day survival of the chicks, body weight of survivors, post-mortem examination.

Test concentrations: Control, 100, 300 and 1000 mg as/kg diet (nominal). The concentrations were corrected for test material purity of 71.12%.

Test conditions: Adult bobwhite study room:
temperature: 21±2 °C, relative humidity: 50% - 60%; photoperiod: 7 hours light (week 1 to week 7), 14 hours light (week 8 to week 9), 17 hours light (week 10 to end of the study); light intensity: 30 lux - 120 lux.
Incubation of eggs: placed in a commercial incubator for 21 days before being transferred to the hatcher;
Incubator temperature: 37.6 °C ± 0.5 °C, relative humidity: 60% - 70%;
Hatcher temperature: 37.7 °C - 38.2 °C, relative humidity: 80% - 90%.

Analytics: The test item concentration was analyzed by gas chromatography using a sulphur selective detector.

Statistics: Dunnett's-test for adult food consumption and body weight, egg weight, egg shell thickness, chicks bodyweight; ANOVA for body weight and food consumption, Wilcoxon-test for count data (e.g. number of eggs or hatched chicks) and proportions (e.g. number of fertile eggs of eggs initially laid) ($\alpha = 0.05$, $\alpha = 0.01$).

II. RESULTS AND DISCUSSION

Analytical measurements: The results of the analytical verification of the test item concentration in the diet were within a range of 87.3% to 104.1% of the nominal concentrations. The biological results are based on the nominal values.

Biological results:

Parental generation:

No treatment-related effects on mortality, birds` health, food consumption and body weight in the parental generation could be detected in any concentration group. Avoidance of food was not observed. At the gross-pathological evaluation at the end of the exposure period no abnormalities caused by the test item could be found.

Effects on reproduction:

- 100 mg as/kg diet:

No test-item related adverse effects on any reproductive parameters were observed in this group.

- 300 mg as/kg diet:

Treated animals showed, when compared to control, statistically significant higher rates of cracked eggs, higher rates of early embryonic mortality and of "dead in shell". As a consequence the hatchability was reduced.

- 1000 mg as/kg diet:

Treated animals showed a reduction in the number of eggs laid per female and a slightly reduced mean egg weight (-5.9%) statistically significant only during the last period (weeks 11-14) of egg-laying. Statistically significant higher rates of cracked eggs, higher rates of early embryonic mortality and of "dead in shell" lead to a reduced hatchability with a significantly lower mean number of surviving 14-day-old chicks and 14-days old chicks per female was in the highest treatment group.
The results are summarized in the table below.

Table 8.1.1.3-2: Effects of BAS 222 28 F on the reproduction of the bobwhite quail (*Colinus virginianus*)

Parameter	Experimental group [mg as/kg diet]			
	Control	**100**	**300**	**1000**
Parental generation				
Number of replicates	16	16	16	16
No. treatment-related mortality of adult birds	0	0	0	0
Mean food consumption wk 1-24 [g/bird/day]	17.7	18.5	18.8	17.8
Adult body weight [g] (male/female) end of egg laying period wk 24	213.5 247.2	212.1 248.9	214.0 245.9	214.1 237.7
Reproduction parameter				
No. of eggs laid / group	1100	1163	1071	945
No. of cracked and broken eggs / group	28	38	69	82
Mean egg weight (g)	10.1	9.9	9.8	9.5
Mean egg shell thickness (mm)	0.21	0.21	0.22	0.21
No. of eggs set $^{+}$/ group	996	1045	928	793
No. of fertile eggs / group	928	1017	883	771
No. of infertile eggs / group	68	28	45	22
No. of early embryonic mortalities / group	12	17	47	30
No. of viable 11-day old embryos / group	916	1000	836	741
No. of late embryonic mortalities / group	11	4	15	23
No. of viable 18-day old embryos / group	905	996	821	718
No. of total embryonic deaths / group	23	21	62	53
No. of "dead-in-shell" / group	144	200	202	224
No. of chicks hatched / group	761	796	619	494
No. of 14-day surviving chicks / group	655	648	515	393
No. of eggs laid / female bird / week	4.9	5.2	4.8	4.2
No. of chicks hatched / female bird / week	3.4	3.6	2.8	2.2**
No. of 14-day surviving chicks / female bird / week	2.9	2.9	2.3	1.8**
Mean body weight of chicks at hatching (g)	6.4	6.2	6.3	6.0
Mean body weight of chicks 14 days after hatching (g)	24.3	23.9	24.4	22.4
% fertile eggs of eggs set % viable 11 days old embryos of eggs set	93.2 92.0	97.3 95.7	95.2 90.1	97.2 93.4
% viable 18 days old embryos of eggs set	90.9	95.3	88.5	90.5
% viable eggs at day 18 of eggs set at day 11	98.8	99.6	98.2	96.9
% 14-day survivors of chicks hatched	86.1	81.4	83.2	79.6

Parameter	Experimental group [mg as/kg diet]			
	Control	100	300	1000
% cracked and broken eggs of eggs laid	2.6	3.3	6.4*	8.7**
% early embryonic mortalities of fertile eggs	1.3	1.7	5.3*	3.9**
% late embryonic mortalities of fertile eggs	1.2	0.4	1.7	3.0
% "dead-in-shell" of fertile eggs	15.5	19.7	22.9*	29.1**
Hatchability (% chicks hatched of total eggs set)	76.4	76.2	66.7*	62.3**
Hatchability (% chicks hatched of fertile eggs)	82.0	78.3	70.1*	64.1**

+ = incubated
* = statistically significant differences compared to the control ($\alpha = 0.05$)
** = statistically significant differences compared to the control ($\alpha = 0.01$)

III. CONCLUSION

In a 24-week avian reproduction test with the bobwhite quail, no test item-related effects in the parent generation on mortality, birds` health, palatability, food consumption and body weight could be detected. At 300 mg as/kg diet and 1000 mg as/kg diet test-item related adverse effects on the reproduction parameters could be observed. In conclusion the NOEC of BAS 222 28 F was set at 100 mg as/kg diet, corresponding to a daily dose of 9.0 mg as/kg bw.

Report:	*CA 8.1.1.3/3*
	[redacted] 1992a
	1-generation reproduction study with metiram-Premix (BAS 222 29 F) on the mallard duck (Anas platyrhynchos L.) by administration in the diet
	1992/11151
Guidelines:	*EPA 71-4, EPA 540/9-86-139, OECD 206*
GLP:	*yes*
	(certified by Ministerium fuer Umwelt und Gesundheit, Postfach 3160, 6500 Mainz)

Executive Summary

This 1-generation study was designed to determine possible effects of administration of metiram via food on the reproduction of adult mallards (*Anas platyrhynchus*). Effects on health, body weight development and food consumption of adult ducks as well as effects on different reproductive parameters were determined. Therefore birds received test item treated food for 26 weeks at concentrations of 50 and 300 mg as/kg diet. Seven birds per replicate (2 males and 5 females) were used in each group with 6 replicates each. Animals of all test groups received diet and water ad libitum throughout the test.

Parental generation:

No test-item related effects in the parental generation on mortality, birds` health, food consumption and body weight could be detected in any concentration group. Avoidance of food was not observed. At the gross-pathological evaluation at the end of the exposure period no abnormalities caused by the test item could be found.

Effects on reproduction:

No effects were observed at the 50 mg as/kg diet group. Egg production, egg weight, egg shell thickness and fertility rates were significantly reduced at 300 mg/kg diet; embryo and chick mortality were increased at this treatment level. Further, the mean number of surviving 14-day-old chicks per female was significantly lower in the highest treatment group.

In a 26-week avian reproduction test with mallards, no test-item related adverse effects on mortality, birds` health, palatability, food consumption and body weight of the parental generation could be detected. In the 300 mg as/kg diet adverse effects on reproduction parameters could be observed. In conclusion the NOEC of metiram was set at 50 mg as/kg diet, corresponding to a daily dose of 7.9 mg as/kg bw/d.

I. MATERIAL AND METHODS

Test item:	BAS 222 29 F (Metiram-Premix), batch no. 90-2, content of a.s. metiram (BAS 222 F, Reg.No. 250 284): 97%.
Test species:	Mallards (*Anas platyrhynchos*), age: 8 months; visually indistinguishable from wild birds; supplier: [redacted]
Test design:	Mallards approaching their first breeding season were kept in groups of 2 males and 5 female per replicate (6 replicates per treatment group). After an adaptation period of about 3 weeks to the conditions in the laboratory, a pre-egg production period of 10 weeks and an egg-laying period of 16 weeks followed. Birds were offered the diet with test item ad libitum over a total of 26 weeks. Laid eggs were collected, incubated until hatching, offspring raised for 2 weeks.
Endpoints:	<u>Adult birds:</u> mortality, clinical signs of toxicity, food consumption, palatability, development of body weight, post-mortem examination. <u>Reproduction parameters:</u> egg production, egg weight, egg quality, egg shell thickness, fertility rate, early and late embryonic deaths, hatchability, 14-day survival of the chicks, body weight of survivors, post-mortem examination.
Test concentrations:	Control, 50 and 300 mg as/kg diet (nominal).
Test conditions:	Adult mallard study room: temperature generally 20 C ± 2 °C; relative humidity: generally 60% - 80%; photoperiod: 7 hours light (week 1 to week 9), 10 hours light (week 10), 12 hours light (week 11), 14 hours light (week 12), 17 hours light (week 13 to end of the study); light intensity: about 170 lux. Incubation of eggs: placed in a commercial incubator for 23 days before being transferred to the hatcher; incubator temperature: 37.8 °C ± 0.5°C, relative humidity: 60% - 80%; hatcher temperature: 38 °C – 39 °C, relative humidity: 80% - 100%.
Analytics:	The test item concentrations were analyzed using gas chromatography with flame photometric detection.
Statistics:	Dunnett's-test for adult food consumption and body weight, egg weight, egg shell thickness, chicks bodyweight; Wilcoxon-test for count data (e.g. number of eggs or hatched chicks) and proportions (e.g. number of fertile eggs of eggs initially laid) ($\alpha = 0.05$, $\alpha = 0.01$).

II. RESULTS AND DISCUSSION

Analytical measurements:
The results of the analytical verification of the test item concentration in the diet were within a range of 91.7% to 98.7% of the nominal concentrations. The biological results are therefore based on the nominal values.

Biological results:

Parental generation:

No test-item related effects in the parental generation on mortality, birds` health, food consumption and body weight could be detected in any concentration group. Avoidance of food was not observed. At the gross-pathological evaluation at the end of the exposure period no abnormalities caused by the test item could be found.

Effects on reproduction:

No effects were observed at the 50 mg as/kg diet group. Egg production, egg weight, egg shell thickness and fertility rates were significantly reduced at 300 mg/kg diet; embryo and chick mortality were increased at this treatment level. Further, the mean number of surviving 14-day-old chicks per female was significantly lower in the highest treatment group. The results of the endpoints are summarized in the table below.

Table 8.1.1.3-3: Effects of metiram on the reproduction of mallards (*Anas platyrhynchos*)

Parameter	Experimental group [mg as/kg diet]		
	Control	**50**	**300**
Parental generation			
Number of replicates	6	6	6
No. of treatment-related mortality of adult birds	0	0	0
Mean food consumption [g/bird/day]	176	189	162
Adult body weight [g] (male/female) end of egg laying period	1200 1162	1200 1168	1218 1216
Reproduction parameter			
No. of eggs laid/group	1502	1635	1037*
No. of cracked eggs/group	40	34	35
Mean egg weight [g]	60.2	60.5	52.0**
Mean egg shell thickness [mm]	0.36	0.37	0.38**
Total no. of eggs set [+]/group	1330	1470	905
No. of fertile eggs per group	1273	1344	667**
No. of infertile eggs per group	57	126	238
No. of early embryonic mortalities/group	84	155	331**
No. of 14-day old embryos/group	1189	1189	336
No. of late embryonic mortalities/group	49	60	16
No. of 21-day old embryos /group	1140	1129	320
No. of total embryonic deaths/group	133	215	347
No. of "dead in shell" / group	609	677	202
No. of chicks hatched / group	531	452	118**
No. of 14-day surviving chicks / group	525	446	118**
No. of eggs laid / female bird / week	3.1	3.4	2.2*
No. of chicks hatched / female bird / week	1.1	0.9	0.2**
No. of 14-day surviving chicks / female bird	17.5	15.0	3.9**
Mean body weight of chicks at hatching [g]	34.3	33.1	34.5
Mean body weight of chicks 14 days after hatching [g]	256.2	253.5	259.5
% fertile eggs of eggs set	95.7	91.4	73.7**
% viable 14-day old embryos of eggs set	89.4	80.9	37.1
% viable 21 day embryos	85.7	76.8	35.6
% total embryonic deaths of fertile eggs	10.4	16.0	52.0

Parameter	Experimental group [mg as/kg diet]		
	Control	50	300
% 14-day survivors of chicks hatched	99.1	98.7	100.0
% cracked and broken eggs of eggs laid	2.66	2.08	3.38
% early embryonic mortalities of fertile eggs	6.6	11.5	49.6**
% late embryonic mortalities of fertile eggs	3.8	4.5	2.4
% "dead-in-shell" of fertile eggs	47.84	50.37	30.28
Hatchability (% chicks hatched of total eggs set)	39.9	30.7	13.0**
Hatchability (% chicks hatched of fertile eggs)	41.71	33.63	17.69**

+ = incubated
* = statistically significant differences compared to the control ($\alpha = 0.05$)
** = statistically significant differences compared to the control ($\alpha = 0.01$)

III. CONCLUSION

In a 26-week avian reproduction test with mallards, no test-item related adverse effects on mortality, birds` health, palatability, food consumption and body weight of the parental generation could be detected. In the 300 mg as/kg diet adverse effects on reproduction parameters could be observed. In conclusion the NOEC of metiram was set at 50 mg as/kg diet, corresponding to a daily dose of 7.9 mg as/kg bw/d.

Report:	*CA 8.1.1.3/4*
	[redacted] 2004a
	Metiram: A reproduction study with the mallard
	2004/1004372
Guidelines:	*EPA 71-4, EPA 850.2300, OECD 206*
GLP:	*yes*
	(certified by United States Environmental Protection Agency)

Report:	CA 8.1.1.3/5
	Anonymous, 2017 a
	Metiram (BAS 222 F) - revised statistics for mallard reproduction study [redacted] (2004)
	2017/1052097
Guidelines:	none
GLP:	no

Executive Summary

This 1-generation higher-tier study was designed to determine possible effects of administration of metiram via food on the reproduction of adult mallards (*Anas platyrhynchus*). The concentrations of metiram in the diets were varied over time to mimic multiple applications with a decline of residues over a period of 6 weeks during the egg laying period of the reproductive cycle. Effects on health, body weight development and food consumption of adult mallard as well as effects on different reproductive parameters were determined. Therefore birds received test item treated food for 6 weeks at initial concentrations of 100, 150 and 460 mg as/kg diet. Two birds per replicate (1 male and 1 female) were used in each group with 18 replicates initially. Animals of all test groups received diet and water ad libitum throughout the test.

Parental generation:

No test-item related effects in the parental generation on mortality, birds` health and body weight could be detected in any concentration group.

Effects on reproduction:

There were no treatment-related effects upon any of the reproductive parameters measured at either 100 or 150 mg as/kg diet. However, at the concentration of 460 mg as/kg diet, treatment related effects upon egg production, embryo viability and hatchability were observed, which were also reflected as effects on hatchlings and 14-day old survivors as percentages of eggs set.

In a 21-week avian reproduction test with mallards, no test-item related adverse effects on mortality, birds` health, palatability and body weight of the parental generation could be detected in any treatment. But treatment related effects on food consumption occurred in the 460 mg as/kg diet treatment. Furthermore no test-item related adverse effects on reproduction parameters could be observed in the 100 mg as/kg diet and 150 mg as/kg diet treatment groups, but in the 460 mg as/kg diet treatment. In conclusion the NOEC of metiram was set at 150 mg as/kg diet, corresponding to a daily dose of 25.5 mg as/kg bw/d based on the initial peak exposure.

I. MATERIAL AND METHODS

Test item: BAS 222 29 F (Metiram -Premix), lot no. 2001-1, content of a.s metiram (BAS 222 F, Reg. No. 250 284): 88.6% purity.

Test species: Mallards (*Anas platyrhynchos*), 31 weeks old, visually indistinguishable from wild birds; supplier: [redacted]

Test design: Mallards approaching their first breeding season were kept in groups of 1 male and 1 female per replicate (18 initial replicates per treatment group). After an adaptation period of about 5 weeks to the conditions in the laboratory, a pre-egg production period of 2 weeks, a pre-treatment period of 6 weeks, an egg-laying period of 6 weeks and a post-adult termination period of 6 weeks followed. Birds were offered the diet with test item ad libitum over a total of 6 weeks.

Endpoints: Adult birds: mortality, clinical signs of toxicity, food consumption, palatability, development of body weight.
Reproduction parameters: egg production, egg weight, egg quality, egg shell thickness, fertility rate, early and late embryonic deaths, hatchability, 14-day survival of the chicks, body weight of survivors.

Test concentrations: Control, 100, 150 and 460 mg as/kg diet (nominal) based on initial values adapted to 100% of a.s. from the test substance. Details of test concentrations are detailed in the table below.

Table 8.1.1.3-4: Test concentrations of metiram in the diet

Day of Study	Dose 1: 100 ppm	Dose 2: 150 ppm	Dose 3: 460 ppm
0-1	100	150	460
1-3	75.8	114	349
3-7	50.0	75	230
7-8	138	207	634
8-10	105	157	481
10-14	68.9	103	317
14-15	152	228	700
15-17	115	173	531
17-21	86.4	130	397
21-28	63.6	95.4	293
28-42	46.3	69.4	213

Test conditions: Adult mallard study room:
temperature generally 23.1 °C ± 0.9 °C; relative humidity: 62% ± 16%; photoperiod: 8 hours light (during acclimation), increasing to 17 hours light at the beginning of pre-treatment period and increasing again to 17 hours and ten minutes (until end of study). light intensity: approximately 167 lux.
Incubation of eggs: placed in a commercial incubator for 23 days before being transferred to the hatcher; incubator temperature: 37.4 °C ± 0.0 °C, relative humidity: 54% ± 0.0%; hatcher temperature: 37.4 °C ± 0.0 °C, relative humidity: 58%.

Analytics: The test item with flame photometric concentrations were analyzed using gas chromatography detection.

Statistics: ANOVA followed by Dunnett's-test for a wide range of different parental and reproductive parameters.

II. RESULTS AND DISCUSSION

Analytical measurements
The results of the analytical verification of the test item concentration in the diet were within a range of 84% to 116% of the nominal concentrations mixed in the diet, which were varied over time to mimic expected environmental concentrations following multiple applications. As the stability of the substance in the diet was sufficient, biological results are therefore based on the nominal initial values.

Biological results

Parental generation

No test-item related effects in the parental generation on mortality, birds` health and body weight could be detected in any concentration group.

Effects on reproduction and chicks

- 100 mg as/kg diet:

No biologically relevant test-item related adverse effects on any reproductive parameters were observed.

- 150 mg as/kg diet:

No biologically relevant test-item related adverse effects on any reproductive parameters were observed.

- 460 mg as/kg diet:

Treatment related effects upon egg production, embryo viability and hatchability were observed, which were also reflected as effects on hatchlings and 14-day old survivors as percentages of eggs set.

The results of the endpoints are summarized in the table below.

Table 8.1.1.3-5: Effects of metiram on the reproduction of mallards (*Anas platyrhynchos*)

Parameter	Experimental group [mg as/kg diet]			
	Control	100	150	460
Parental generation				
Number of replicates [1)]	17	16	14	18
No. of treatment-related mortality of adult birds	0	0	0	0
Mean food consumption [g/bird/day]	195.5	196.4	193.0	194.0
Adult body weight [g] (male/female) end of egg laying period	1176 1133	1193 1193	1155 1131	1190 1169
Reproduction parameter				
No. of eggs laid/group	539	501	437	412
No. of cracked eggs/group	8	1	4	3
Mean egg shell thickness [mm]	0.393	0.395	0.386	0.397
Total no. of eggs set [+]/group	478	457	390	350
No. of fertile eggs per group	436	390	238	222
No. of 21-day old embryos /group	434	387	233	219
No. of chicks hatched / group	383	346	200	165
No. of 14-day surviving chicks / group	371	342	198	163
No. of eggs laid / female bird / week	5.25	5.25	5.18	3.78
No. of 14-day surviving chicks / female bird	22	21	14	9
Mean body weight of chicks at hatching [g]	33	34	33	32
Mean body weight of chicks 14 days after hatching [g]	286	301	300	277
% hatched chicks of eggs set	78	76	56	40**
% viable embryos of eggs set	90	86	68	56**
% live 21-day embryos of viable embryos	100	99	98	98
% hatched chicks of live 21 day embryos	88	89	85	70**
% 14-day survivors of chicks hatched	96	98	99	99
% cracked and broken eggs of eggs laid	1	0	2	1
% 14-day old survivors of eggs set	75	75	55	39**
Hatchability (% chicks hatched of total eggs set)	78	76	56	40**

1) replicates with infertile pairs at start of treatment phase were excluded
\+ = incubated
* = statistically significant differences compared to the control ($\alpha = 0.05$)
** = statistically significant differences compared to the control ($\alpha = 0.01$)

III. CONCLUSION

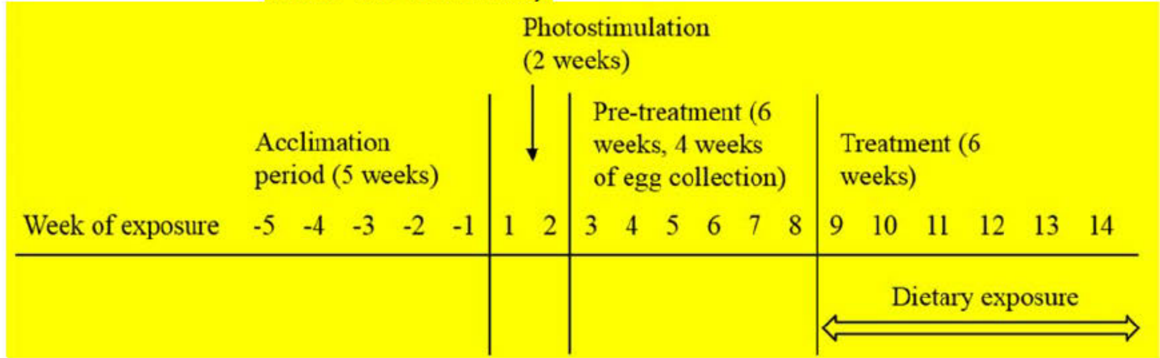
In a 21-week avian reproduction test with mallards, no test-item related adverse effects on mortality, birds` health, palatability and body weight of the parental generation could be detected in any treatment. But treatment related effects on food consumption occurred in the 460 mg as/kg diet treatment. Furthermore no test-item related adverse effects on reproduction parameters could be observed in the 100 mg as/kg diet and 150 mg as/kg treatment groups, but in the 460 mg as/kg diet treatment. In conclusion the NOEC of metiram was set at 150 mg as/kg diet, corresponding to a daily dose of 25.5 mg as/kg bw/d.

Further evaluation of the study

Exposure to metiram

A standard avian reproduction study according to OECD 206 or OPPTS 850.2300 consists of adult birds being fed treated diets with a constant dietary concentration for 8 weeks of pre-photostimulation, followed by 2-4 weeks of photostimulation and 8-10 weeks of egg collection, for a total of 20 weeks of exposure to treated diet. This study, however, was set up to mimic exposure of birds to metiram in the environment following 3 applications of a metiram-containing product. Birds in this pulsed dose exposure study were acclimated to the testing laboratory for 5 weeks, followed by a photostimulation period of 2 weeks (birds fed untreated diet). For the next 6 weeks, birds continued to be fed untreated diet and eggs were collected for the last 4 weeks of this period and hatchlings were reared to 14 days old. During the final 6 weeks of the study, birds were fed treated diets while egg collection and hatchling rearing continued (**Figure 8.1.1.3-1**).

Figure 8.1.1.3-1: Timeline of the pulse dose metiram mallard reproduction study (BASF docID 2004/1004372).



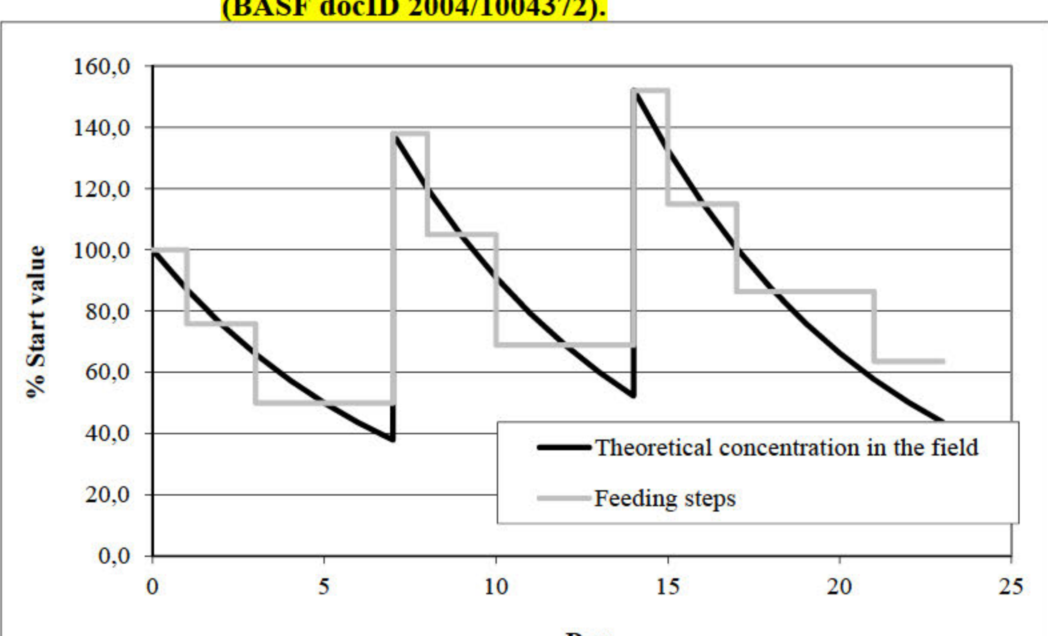
The dietary concentrations to which birds were exposed in this study were varied over time to mimic the expected environmental concentrations of metiram following 3 applications at one-week intervals, assuming a half-life of 5 days. The half-life of 5 days is well supported by results from field residue decline studies with metiram conducted with different plant species (*i.e.* wheat and peas) and with foliage and ground-dwelling arthropods sampled in potatoes and vineyards (16 plant and 3 arthropod field studies, see M-CA 8.1/1 - 8.1/9 and M-CP Table 10.1.2.2-4, 10.1.2.2-5, Table 10.1.2.2-8 and Table 10.1.2.2-9 of the dossier update).

In an avian feeding study, the concentration in the diet can be changed only in distinct intervals. Each change means an additional dose mixing and concentration control analysis. Thus a pragmatic stepwise pattern for exchanges of the diet has to be developed to have a technically feasible study design. Since the decrease after each application is assumed to follow a first order kinetic model, it was considered to be reasonable to increase the feeding intervals with time and the following exposures intervals were used:

Day 0 – 1: 100% (This assumes a longer exposure to the maximum dose than in reality, since in the field situation an immediate decline can be assumed)
Day 1 – 3: 75.8% (Theoretical value of day 2, which is the middle of the time interval)
Day 3 – 7: 50.0% (Theoretical value of day 5 as the middle of the time interval)

The use of concentration steps means necessarily, that the applied concentration in the diet will be below the theoretical concentration at the start of the feeding interval and above the theoretical concentration at the end of the feeding interval. The feeding pattern near the 3 maximum doses is presented below in comparison to the theoretical concentration of residues in the field after 3 applications in weekly intervals with an assumed half life time of 5 days (**Figure 8.1.1.3-2**).

Figure 8.1.1.3-2: Dietary exposure in the pulse dose metiram mallard reproduction study (BASF docID 2004/1004372).



Effects of exposure to metiram

While not statistically significant in the study report, it appears that there is a reduction in viable embryos/eggs set at 150 ppm. However, a comparison of the 4 weeks of pre-treatment egg data with the 6 weeks of treatment egg data shows that this apparent difference between 150 ppm and the control is not due to exposure to metiram. Pre-treatment embryo viability was 78 ± 36% for the control, but only 59 ± 47% for birds in the 150 ppm group. Of the 10 pairs of birds in the 150 ppm group that laid eggs during the pre-treatment period, 3 pairs laid no fertile eggs and a fourth pair laid only 20% fertile eggs, showing an innate difference in the fertility of the birds in the control vs. 150 ppm groups. During the treatment period, embryo viability increased compared to the pre-treatment period for both the control (78% to 90%) and 150 ppm birds (59% to 68%), showing that exposure to metiram did not decrease embryo viability for birds in the 150 ppm treatment group. In contrast, while embryo viability was similar for birds in the control and 460 ppm groups pre-treatment (78% and 73%, respectively), embryo viability at 460 ppm decreased to 56% when birds were exposed to metiram. The difference in viable embryos/eggs set from pre-treatment to treatment was on average +10%, -9%, +5%, and -19% for the control, 100 ppm, 150 ppm, and 460 ppm treatments, respectively (**Table 8.1.1.3-6**).

To account for the pre-treatment trend toward lower embryo viability in the 150 ppm treatment (59 ± 47% viable embryos/eggs set) compared to the control (78 ± 36% viable embryos/eggs set), the endpoint viable embryos/eggs set was statistically analyzed as the change from the pre-treatment to the treatment period to compare embryo viability before and during exposure to metiram. Data were not normally distributed, so a non-parametric Kruskal-Wallis test was used, which showed a statistically significant effect of metiram exposure compared to the control (p = 0.005). Using a post-hoc Dunn test, there was no difference between the control (+10% from pre-treatment to treatment) and the 100 ppm treatment (-9%, p = 0.190) or the 150 ppm treatment (+5%, p = 0.699), but a significant difference between the control and the 460 ppm treatment (-19%, p = 0.001; see **Table 8.1.1.3-6**). This shows that exposure to metiram did not affect embryo viability in the 100 or 150 ppm treatment compared to the control, but it decreased embryo viability in the 460 ppm treatment. For details of this statistical analysis, see BASF DocID 2017/1052097.

Table 8.1.1.3-6: Proportion viable embryos/egg set for mallard ducks pre-treatment and exposed to pulsed doses of metiram (BASF docID 2004/1004372). “0” for pre-treatment indicates that none of the eggs set were viable, “-“ indicates that no eggs were laid pre-treatment.

	0 ppm			100 ppm			150 ppm			460 ppm		
Rep	**Pre-treatment**	**Treatment**	**Δ[1]**	**Pre-treatment**	**Treatment**	**Δ[1]**	**Pre-treatment**	**Treatment**	**Δ[1]**	**Pre-treatment**	**Treatment**	**Δ[1]**
1	100	91	-9	100	9	-91	80	92	12	0	0	0
2	83	97	13	-	100	-	0	0	0	93	65	-28
3	100	78	-22	97	100	3	20	63	43	100	86	-14
4	100	100	0	97	100	3	-[2]	-[2]	-[2]	100	87	-13
5	0	92	92	75	100	25	88	89	0	93	64	-29
6	88	100	13	100	97	-3	-	55	-	100	83	-17
7	100	100	0	100	35	-65	100	100	0	100	97	-3
8	-	100	-	100	82	-18	-	91	-	92	77	-16
9	100	96	-4	100	97	-3	100	100	0	100	100	0
10	-	85	-	100	89	-11	0	0	0	73	8	-64
11	78	79	1	100	100	0	-	84	-	-	70	-
12	0	15	15	-	83	-	-	91	-	100	39	-61
13	96	100	4	93	100	7	97	100	3	0	0	0
14	-	100	-	67	92	25	100	86	-14	-	33	-
15	80	94	14	91	97	6	0	3	3	-	63	-
16	91	100	9	-	100	-				0	0	0
17	-	97	-							-	44	-
18										-	91	-
Mean	**78**	**90**	**10**	**94**	**86**	**-9**	**59**	**68**	**5**	**73**	**56**	**-19**
Standard deviation	**36**	**20**	**27**	**11**	**26**	**33**	**47**	**39**	**15**	**42**	**35**	**22**

[1]Change from pre-treatment to treatment
[2]The male bird in replicate 4 (pen 37) of the 150 ppm treatment died on day 8

Similarly, the absolute number of live 21 day embryos, hatchlings, and 14 day old chicks per treatment group is lower in the 150 ppm and 460 ppm treatment groups compared to the control, making it appear that there is a dose-responsive reduction in these parameters. However, when these parameters are expressed in terms of the number of viable embryos for the treatment, it can be seen that exposure to metiram did not decrease the live 21 day embryos (pre-treatment 100% vs. treatment 98%), hatchlings (pre-treatment 69% vs. treatment 84%), or 14 day old chicks (pre-treatment 69% vs. treatment 83%) in the 150 ppm treatment (**Table 8.1.1.3-7**). For each of these endpoints, the 150 ppm treatment group performed similarly or better during treatment compared to pre-treatment, showing that exposure to metiram did not have a negative effect on these parameters. In contrast, while these parameters were similar for birds in the control and 460 ppm groups pre-treatment, there was a trend toward decreased hatchlings (pre-treatment 85% vs. treatment 69%) and 14 day old survivors (pre-treatment 85% vs. treatment 68%) in the 460 ppm treatment group when birds were exposed to metiram.

The parameters live 21d embryos/viable embryos, hatchlings/viable embryos, and 14d chicks/viable embryos were also statistically analyzed as the change from the pre-treatment to the treatment period to account for pre-treatment embryo viability differences. For live 21d embryos/viable embryos, data were not normally distributed so a non-parametric Kruskal-Wallis test was used, which showed no statistically significant effect of metiram exposure compared to the control ($p = 0.117$). For hatchlings/viable embryos, data were not normally distributed so a non-parametric Kruskal-Wallis test was used, which showed no statistically significant effect of metiram exposure compared to the control ($p = 0.366$). For 14d chicks/viable embryos, data was normally distributed and variances were homogenous so an ANOVA was used ($p = 0.284$). Therefore, exposure to metiram did not affect the number of live 21 day embryos, number of hatchlings, or number of 14 day old chicks per treatment when pre-treatment differences in embryo viability were taken into account. For details of this statistical analysis, see BASF DocID 2017/1052097.

The apparent differences in the number of live 21 day embryos, hatchlings, and 14 day old chicks produced by the birds in the 150 ppm treatment compared to the control were a result of the innate difference in fertility between the birds in these groups. As discussed above, pre-treatment embryo viability was 78 ± 36% for the control, but only 59 ± 47% for birds in the 150 ppm group. The lower fertility rate of birds in the 150 ppm treatment led to a reduced number of live 21 day embryos, hatchlings, and 14 day old chicks compared to the control, independent of exposure to metiram.

Table 8.1.1.3-7: Comparison of mean reproductive parameters normalized to viable embryos for mallard ducks pre-treatment and exposed to pulsed doses of metiram (BASF docID 2004/1004372)

	0 ppm		100 ppm		150 ppm		460 ppm	
Parameter	**Pre-treatment**	**Treatment**	**Pre-treatment**	**Treatment**	**Pre-treatment**	**Treatment**	**Pre-treatment**	**Treatment**
No. Viable embryos	123	436	182	390	86	238	95	222
No. live 21 day old embryos	123	434	181	387	86	233	95	219
% live 21 day old embryos/ viable embryos	100	100	100	99	100	98	100	98
No. hatchlings	111	383	153	346	80	200	85	165
% hatchlings/ viable embryos	90	88	85	88	69	84	85	69
No. 14 day old survivors	108	371	153	342	80	198	85	163
% 14 day old survivors/viable embryos	88	84	85	87	69	83	85	68

Conclusion

This study collected data on reproduction of mallard ducks during a 6-week pre-treatment period (reproduction data collected for 4 weeks) and a 6-week treatment period. The dietary concentrations to which birds were exposed during the treatment period were varied over time to mimic the expected environmental concentrations of metiram following 3 applications at one-week intervals, assuming a half-life of 5 days as supported by field residue decline studies on plants and arthropods. While not statistically significant, it appears that there is a reduction in viable embryos, live 21 day embryos, hatchlings, and 14 day old chicks per eggs set at 150 ppm, as well as statistically significant differences at 460 ppm. However, when pre-treatment data is taken into account it can be seen that viable embryos/eggs set increased from the pre-treatment period to the treatment period for the control and 150 ppm treatment but decreased in the 460 ppm treatment, showing an effect of metiram exposure at 460 ppm but not 150 ppm. Similarly, when live 21 day embryos, hatchlings, and 14 day old chicks are expressed in terms of the number of viable embryos for the treatment, it can be seen that exposure to metiram did not decrease these parameters in the 150 ppm treatment, but did decrease them in the 460 ppm treatment. The NOEC for this study is therefore 150 ppm.

Studies with the metabolite ETU

The following reproduction studies on the bobwhite quail and the mallard duck performed with the metabolite ETU are new and have not yet been evaluated on EU level.

Report:	CA 8.1.1.3/~~5~~6 2012a Ethylenethiourea: A reproduction study with the northern bobwhite 2013/7000533
Guidelines:	EPA 850.2300
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

The objective of this study was to evaluate the effects upon the adult northern bobwhite (*Colinus virginianus*) of dietary exposure to ETU (ethylene thiourea) on adult health, body weight, and feed consumption. In addition, effects of adult exposure to ethylene thiourea on the number of eggs laid, egg shell thickness, fertility, embryo viability, hatchability, offspring body weight and survival were evaluated. Northern bobwhites were exposed to ETU at dietary concentrations of 0, 20, 100, and 400 ppm for 20 weeks.

Parental generation

There were no treatment-related mortalities, overt signs of toxicity or treatment-related effects upon body weight or feed consumption of the birds at any of the concentrations tested. At gross necropsy all but one male and four female adult birds evidenced visually enlarged thyroids at the 400 ppm test concentration, which were considered to be related to treatment.

Effects on reproduction and chicks

At the 400 ppm test concentration there was also a statistically significant reduction in live 3-week embryos as a percentage of viable embryos. While not statistically significant, there were also slight reductions in viable embryos as a percentage of the number of eggs set and hatchability, as well as corresponding reductions in hatchlings and 14-day old survivors as percentages of the eggs set for incubation that were considered to be related to treatment. There was a slight reduction in hatchling body weight at the 100 ppm test concentration and reductions in hatchling and 14-day old body weights at the 400 ppm test concentration that were statistically significant. All were considered to be related to treatment.

The no-observed-effect concentration for northern bobwhite exposed to ethylene thiourea in the diet during the study was 20 ppm (1.8 mg as/kg/day).

I. MATERIAL AND METHODS

Test item: Ethylene thiourea. (CAS No. 96457), LOT NO: XW7-102353-014, purity: 100 %

Test species: Northern bobwhite (*Colinus virginianus*), phenotypically indistinguishable from wild type; adults, age: 20 weeks of age at the initiation of the test (first day of exposure to test diet); supplier: [redacted]

Test design: Northern bobwhites approaching their first breeding season were kept in a group of 1 male and 1 female in a pen per replicate. 16 pens were allocated to the control and each treatment group. The animals were acclimatized to the laboratory conditions for 2 weeks. All adult birds and their offspring were given feed and water *ad libitum* during acclimation and testing. The study period was divided into five phases: 1. Acclimation – 2 weeks; 2. Pre-photostimulation – 7 weeks; 3. Pre-egg laying (with photostimulation) – 3 weeks; 4. Egg laying – 10 weeks; 5. Post-adult termination (final incubation, hatching and 14-day offspring rearing period – 6 weeks. All eggs were collected, artificially incubated and hatched; the young birds were maintained for 14 days. All surviving adult birds were euthanized with carbon dioxide gas, necropsied, and disposed of by incineration. During gross necropsy variation was seen between birds in length, width and shape of thyroid at the 100 ppm treatment group. Thus length and width of the thyroid was measured.

Endpoints: Adult birds: mortalities, clinical observations, gross necropsy, adult body weight, adult feed consumption

Reproductive parameters: Eggs laid, eggs cracked, eggs set, viable embryos, live 3-week embryos, hatchlings, body weight hatchlings, 14-day old survivors, body weight 14-day old survivors and egg shell thickness

Establishing of NOEC

Test concentrations: 0 (Control), 20, 100 and 400 ppm ethylene thiourea in the diet (nominal).

Test conditions: Adult bobwhite study room: temperature 24.1 ± 0.7°C; relative humidity: 57 ± 17%; ventilation: 15 times the room air /h; photoperiod: increased to 17 hours light (week 8 – to the end of study); approximately 162 lux.

Egg collection and storage: collected daily, stored in cold room: temperature: 14.2 ± 0.1°C, relative humidity: 85 ± 5%. Eggs set for incubation: temperature: 37.4 ± 0.0°C, relative humidity: 55 ± 0 %; after 21 days transferred to the hatcher: temperature: 37.3 ± 0.0°C, average relative humidity 57 ± 1 %;

Hatchlings: temperature approximately 38°C from hatching to the 14th day; average ambient room temperature 26.8 ± 1.2°C, relative humidity: 24 ± 10 %; photoperiod: 16 hours light per day.

Analytics: The test substance concentrations in the diet were analysed using HPLC

Statistics: ANOVA (for significance between groups), Dunnett's multiple comparison procedure (statistical significance of observed differences), Dunnett's method following arcsine square root transformation (examination of percentage data)

II. RESULTS AND DISCUSSION

Analytical measurements

The results of the analytical verification of the test item concentration in the diet were within a range of 88% to 100% of the nominal concentrations. Therefore, the biological results are based on the nominal values.

Biological results

Parental generation

No treatment-related effects in the parent generation on mortalities, overt signs of toxicity, body weight and feed consumption at any concentration tested. Avoidance of feed was not observed. During gross necropsy enlarged thyroids were visually observed in the 400 ppm treatment group in comparison to the control group, while thyroids in the 20 and 100 ppm group appeared similar to the control group. At the 400 ppm test concentration, all but one male and four female adult birds evidenced visually enlarged thyroids that were considered to be related to treatment (Table 8.1.1.3-8).

Table 8.1.1.3-8: Effects of ethylene thiourea on the size of the thyroids of the parental generation of Northern bobwhite (*Colinus virginianus*)

Sex	Males				Females			
Treatment Group (ppm)	0	20	100	400	0	20	100	400
Thyroids: 2x1 to 4x1 mm (Males)	16	--	--	1	--	--	--	--
Thyroids: 2x1 to 5x3 mm (females)	--	--	--	--	16	--	--	4
Thyroids: 5x4 to 6x3 mm	0	--	--	10	0	--	--	5
Thyroids: 6x4 to 7x4 mm	0	--	--	2	0	--	--	4
Thyroids: 7x5 to 8x6 mm	0	--	--	2	0	--	--	2
Thyroids: 8x7 to 9x7 mm	0	--	--	1	0	--	--	1

Effects on reproduction and chicks:

There were no treatment-related effects upon reproductive performance at the 20 or 100 ppm test concentrations, and any differences from the control group were not statistically significant for any of the reproductive parameters measured. However, at the 400 ppm test concentration there was a statistically significant reduction in live 3-week embryos as a percentage of viable embryos. While not statistically significant, there were also slight reductions in viable embryos as a percentage of the number of eggs set and hatchability, as well as corresponding reductions in hatchlings and 14-day old survivors as percentages of the eggs set for incubation that were considered to be related to treatment. Additionally there was a slight reduction in hatchling body weight at the 100 ppm test concentration (the mean body weight was decreased by 6% in comparison to the control group) that was statistically significant and reductions in hatchling and 14-day old body weights at the 400 ppm test concentration that were statistically significant. All were considered to be related to treatment.

Table 8.1.1.3-9: Effects of ethylene thiourea on the parental generation of northern bobwhite (Colinus virgianius)

Parameter	Treatment group [ppm in the diet]			
	Control	20 ppm	100 ppm	400 ppm
No. of replicates (1 male and 1 female per replicate/pen)	16	15	16	16
No. of substance-related mortalities of adult birds	0	0	0	0
Mean food consumption per treatment group [g feed/bird/day] (week 1/week 20)	14/24	14/24	14/25	15/23
Adult body weight [g] at the end of study (male / female)[1)]	233/245	218/241	213/246	200/231
Gain of adult body weight [g] at the end of study (male/female)	16/45	18/39	14/46	-1/30

1) Only surviving birds were included in the calculation for each body weight interval

Table 8.1.1.3-10: Effects of ethylene thiourea on the parental generation of northern bobwhite (*Colinus virgianius*)

Parameter	Treatment group [ppm in the diet]			
	Control	20 ppm	100 ppm	400 ppm
Total eggs laid in each dose group	818	737	865	823
Eggs laid/hen	51	49	54	51
Eggs laid/hen/day[1)]	0.56	0.54	0.59	0.57
Eggs cracked	16	32	22	24
Mean egg shell thickness (mm)	0.234 ± 0.012	0.229 ± 0.010	0.228 ± 0.015	0.234 ± 0.020
Eggs set	726	637	761	700
Viable Embryos	624	618	698	549
Live 3-week embryos	622	615	694	536**
Hatchlings	567	570	610	461
14-day old survivors	536	541	584	430
14-day old survivors/hen	34	36	37	27
Mean body weight (g) of hatchlings per group	6.2 ± 0.4	6.1 ± 0.4	5.8* ± 0.4	5.4** ± 0.5
Mean bodyweight (g) of 14-day old survivors	29 ± 2	28 ± 3	27 ± 2	26* ± 4

1) Based on 91 days of eggs production
* Significantly different from the control at $p < 0.05$
** Significantly different from the control at $p < 0.01$

Table 8.1.1.3-11: Effects of ethylene thiourea on the reproduction of northern bobwhite (*Colinus virginianus*) expressed as percentages

Parameter	Treatment group [ppm in the diet]			
	Control	20 ppm	100 ppm	400 ppm
% viable embryos of eggs set	86	97	92	77
% eggs laid/maximum laid	72	69	76	72
% viable embryos/eggs set	86	97	92	77
% live 3-week embryos/viable embryos	100	100	100	98*
%hatchlings/live 3-week old embryos	92	93	88	82
% hatchlings/eggs set	78	90	81	64
% 14-day old survivors/eggs set	74	86	78	59
% 14-day survivors of hatchlings	95	95	96	90
% cracked eggs of eggs laid	2	5	3	3
% hatchlings/maximum set	59	63	64	48
% 14-day old survivors/maximum set	56	60	61	45

* Significantly different from the control ($p < 0.01$)

III. CONCLUSION

There were no treatment-related mortalities, overt signs of toxicity or treatment-related effects upon body weight or feed consumption at any of the concentrations tested. There were no treatment-related effects upon reproductive performance in the 20 ppm treatment group. Therefore, the no-observed-effect concentration for northern bobwhite exposed to ethylene thiourea in the diet during the study was 20 ppm (1.8 mg as/kg/day).

Report:	CA 8.1.1.3/~~6~~7 2012b Ethylenethiourea: A reproduction study with the mallard 2013/7000534
Guidelines:	EPA 850.2300
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

The objective of this study was to evaluate the effects upon the adult mallard duck (*Anas platyrhynchos*) of dietary exposure to ETU (ethylene thiourea) on adult health, body weight, and feed consumption. In addition, effects of adult exposure to ethylene thiourea on the number of eggs laid, egg shell thickness, fertility, embryo viability, hatchability, offspring body weight and survival were evaluated.

Mallard ducks were exposed to ETU at dietary concentrations of 0, 4, 20, and 100 ppm for 20 weeks.

Parental generation:

There were no treatment-related mortalities, overt signs of toxicity or treatment-related effects upon body weight or feed consumption of the birds at the 4, 20 and 100 ppm concentrations tested. At the 100 ppm test concentration though, all but one of the adult birds evidenced visually enlarged thyroids during gross necropsy that were considered to be related to treatment.

Effects on reproduction:

When examined for the entire reproductive period, there were no treatment-related effects upon any of the reproductive parameters measured at the 4, 20 or 100 ppm test concentrations. However, birds in the 100 ppm treatment group exhibited enlarged thyroids at terminal necropsy that were considered to be related to treatment and slight decrease in egg production (number of eggs, number of laying hens and viable embryos of eggs set) at the begin of the egg laying phase.

The no-observed-effect concentration for mallard exposed to ethylene thiourea in the diet during the study was 20 ppm (2.6 mg as/kg/day).

I. MATERIAL AND METHODS

Test item:	Ethylene thiourea. (CAS No. 96457), LOT NO: XW7-102353-014, purity: 100 %
Test species:	Mallard duck (*Anas platyrhynchos*), phenotypically indistinguishable from wild type; adults, age: 23 weeks of age at the initiation of the test (first day of exposure to test diet); supplier:
Test design:	Mallard ducks approaching their first breeding season were kept in a group of 1 male and 1 female in a pen per replicate. 16 pens were allocated to the control and each treatment group. The animals were acclimatized to the laboratory conditions for 5 weeks. All adult birds and their offspring were given feed and water *ad libitum* during acclimation and testing. The study period was divided into five phases: 1. Acclimation – 5 weeks; 2. Pre-photostimulation – 8 weeks; 3. Pre-egg laying (with photostimulation) – 2 weeks; 4. Egg laying – 10 weeks; 5. Post-adult termination (final incubation, hatching and 14-day offspring rearing period – 6 weeks. All eggs were collected, artificially incubated and hatched; the young birds were maintained for 14 days. Adult birds were sacrificed after the egg-laying period, young birds after 14 days. Adult birds were euthanized by cervical dislocation, necropsied, and disposed of by incineration. The ducklings were euthanized with carbon dioxide and disposed of by incineration. Since variation of thyroid size in the highest test concentration was seen between birds a measurement of two diameters (dimensions) was performed for the control group and the 100 ppm treatment group.
Endpoints:	Adult birds: mortalities, clinical observations, gross necropsy, adult body weight, adult feed consumption Reproductive parameters: Eggs laid, eggs cracked, eggs set, viable embryos, live 3-week embryos, hatchlings, body weight hatchlings, 14-day old survivors, body weight 14-day old survivors and egg shell thickness Establishing of NOEC
Test concentrations:	0 (Control), 4, 20 and 100 ppm ethylene thiourea in the diet (nominal).

Test conditions: Adult bobwhite study room: temperature 22.6 ± 1.1°C; relative humidity: 64 ± 16%; ventilation: 15 times the room air /h; photoperiod: increased to 17 hours light (week 9 – to the end of study); approximately 268 lux.

Egg collection and storage: collected daily, stored in cold room: temperature: 14.2 ± 0.1°C, relative humidity: 85 ± 5%. Eggs set for incubation: temperature: 37.4 ± 0.0°C, relative humidity: 55 ± 0 %; after 21 days transferred to the hatcher: temperature: 37.3 ± 0.0°C, average relative humidity 60 ± 0 %;

Hatchlings: temperature approximately 38°C from hatching to five to seven days of age; average ambient room temperature 23.7 ± 1.0°C, relative humidity: 48 ± 13 %; photoperiod: 16 hours light per day.

Analytics: The test substance concentrations were analysed using HPLC

Statistics: ANOVA (for significance between groups), Dunnett's multiple comparison procedure (statistical significance of observed differences), Dunnett's method following arcsine square root transformation (examination of percentage data).

II. RESULTS AND DISCUSSION

Analytical measurements

The results of the analytical verification of the test item concentration in the diet were within a range of 88% to 100% of the nominal concentrations. The biological results are therefore based on the nominal values.

Biological results

Parental generation

No treatment-related effects in the parent generation on mortalities, overt signs of toxicity, body weight and feed consumption at any concentration tested. Avoidance of feed was not observed. During gross necropsy enlarged thyroids were visually observed in the 100 ppm treatment group in comparison to the control group, while thyroids in the 4 and 20 ppm group were similar to the control group. All findings observed in the 4 and 20 ppm treatment groups were considered unrelated to treatment. At the 100 ppm test concentration, all but one of the adult birds evidenced visually enlarged thyroids that were considered to be related to treatment (Table 8.1.1.3-12).

Table 8.1.1.3-12: Effects of ethylene thiourea on the size of the thyroids of the parental generation of mallard ducks (*Anas platyrhynchos*)

Sex	Males				Females			
Treatment Group (ppm)	**0**	**4**	**20**	**100**	**0**	**4**	**20**	**100**
Thyroids: 3x2 to 6x4 mm (Males)	16	--	--	1	--	--	--	--
Thyroids: 3x2 to 5x4 mm (females)	--	--	--	--	16	--	--	0
Thyroids: 8x4 to 8x6 mm	0	--	--	5	0	--	--	4
Thyroids: 10x5 to 9x7 mm	0	--	--	9	0	--	--	5
Thyroids: 10x7 to 9x8 mm	0	--	--	1	0	--	--	5
Thyroids: 10x8 mm	0	--	--	0	0	--	--	2

Reproductive results

When examined for the entire reproductive period, there were no treatment-related effects upon reproductive performance at any of the concentrations tested. When compared to the control group, there were no statistically significant differences in any of the reproductive parameters measured in the 4, 20 or 100 ppm treatment groups. However, at the 100 ppm a.i. test concentration during the first week of egg production there was a reduction in egg production (10 vs. 25), the number of hens laying (2 vs. 7) and viable embryos as a percentage of eggs set (44% vs. 73%) when compared to the control group. The differences observed were slight and transient.

Table 8.1.1.3-13: Effects of ethylene thiourea on the parental generation of mallard ducks (Anas platyrhynchos)

Parameter	Treatment group [ppm in the diet]			
	Control	**4 ppm**	**20 ppm**	**100 ppm**
No. of replicates (1 male and 1 female per replicate/pen)	16	16	16	16
No. of substance-related mortalities of adult birds	0	0	0	0
Mean food consumption per treatment group [g feed/bird/day] (week 1/week 20)	92/205	102/211	84/207	90/213
Adult body weight [g] at the end of study (male / female)	1168/1192	1156/1185	1160/1172	1134/1140
Gain of adult body weight [g] at the end of study (male/female)	40/167	15/132	26/150	2/120

2) Only surviving birds were included in the calculation for each body weight interval

Table 8.1.1.3-14: Effects of ethylene thiourea on the parental generation of mallard ducks (*Anas platyrhynchos*)

Parameter	Treatment group [ppm in the diet]			
	Control	4 ppm	20 ppm	100 ppm
Total eggs laid in each dose group	806	775	818	837
Eggs laid/hen	50	48	51	52
Eggs laid/hen/day[1)]	0.61	0.58	0.62	0.63
Eggs cracked	1	0	3	2
Mean egg shell thickness (mm)	0.398 ± 0.017	0.398 ± 0.018	0.387 ± 0.019	0.395 ± 0.017
Eggs set	722	695	739	758
Viable Embryos	666	652	670	652
Live 3-week embryos	666	647	668	643
Hatchlings	580	549	620	544
14-day old survivors	577	548	612	540
14-day old survivors/hen	36	34	38	34
Mean body weight (g) of hatchlings per group	34 ± 2	34 ± 3	36 ± 2	35 ± 3
Mean bodyweight (g) of 14-day old survivors	312 ± 16	316 ± 20	318 ± 18	321 ± 16

2) Based on 83 days of eggs production

Table 8.1.1.3-15: Effects of ethylene thiourea on the reproduction of mallard ducks (Anas platyrhynchos) expressed as percentages [1)]

Parameter	Treatment group [ppm in the diet]			
	Control	4 ppm	20 ppm	100 ppm
% viable embryos of eggs set	92	94	91	87
% eggs laid/maximum laid	73	70	74	76
% viable embryos/eggs set	92	94	91	87
% live 3-week embryos/viable embryos	100	99	100	98
%hatchlings/live 3-week old embryos	88	84	92	84
% hatchlings/eggs set	81	78	84	73
% 14-day old survivors/eggs set	80	78	83	72
% 14-day survivors of hatchlings	99	100	98	99
% cracked eggs of eggs laid	0	0	0	0
% hatchlings/maximum set	59	56	63	56
% 14-day old survivors/maximum set	59	56	63	55

1) Values represent pen means for experimental group

III. CONCLUSION

There were no treatment-related mortalities, overt signs of toxicity or treatment-related effects upon body weight, feed consumption or effects upon any of the reproductive parameters at any of the concentrations tested. However, birds in the 100 ppm treatment group exhibited enlarged thyroids at terminal necropsy that were considered to be related to treatment and a slightly reduced egg production at the begin of the egg-laying period. The no-observed-effect concentration for mallard exposed to ethylene thiourea in the diet during the study was 20 ppm (2.6 mg as/kg/day).

CA 8.1.2 Effects on terrestrial vertebrates other than birds

CA 8.1.2.1 Acute oral toxicity to mammals

Acute oral toxicity to mammals is addressed in M-CA 5.2.1. The studies previously evaluated during the Annex I inclusion process are considered to be still valid and sufficient. Therefore, no new studies were conducted with the active substance.

CA 8.1.2.2 Long-term and reproductive toxicity to mammals

Long-term and reproductive toxicity to mammals is addressed in toxicological chapter, section 5 M-CA 5.6. Studies and endpoints relevant for the reproductive risk assessment of wild mammals are discussed in M-CP 10.1.2.

In accordance with the RMS Italy a long-term field-effect study was conducted in the year 2014 to determine potential impact of metiram on small herbivorous mammal populations under field conditions. However, as communicated to RMS Italy (June 10, 2014) the field effect study had to be stopped in 2014. Despite a high trapping effort on a high number of study fields, the number of trapped small mammals was too small to run the study with metiram. It became doubtful, whether the study would bring conclusive results in the end as exposure to sufficient animals would have been difficult to demonstrate hence interpretation of any results. The status report with trapping results from 2014 are provided in a document submitted with this dossier [see KCA 8.1.2.2/1 2015/1095575].

Repeat of the field effect study was accepted by Italy and ~~will be~~ was carried out in 2015. ~~The study plan for this field effect study is included in this the original dossier [see KCA 8.1.2.2/2 2015/1123328]. In accordance with the RMS, an interim report will be provided to RMS Italy in December 2015.~~ The full final report ~~will~~ is sent end of February 2016 for inclusion in the draft registration report. The respective summary is provided below.

Report: CA 8.1.2.2/2
[redacted] 2016 a
Long-term field study to assess potential impact of Metiram on populations of the common vole in Central Europe (Germany)
2016/1043813

Guidelines: EC 1107/2009 of the European Parliament, EFSA Guidance Document: Risk assessment for birds and mammals (2009)

GLP: yes
(certified by Ministerium fuer Arbeit, Integration und Soziales des Landes Nordrhein-Westfalen, Duesseldorf)

Executive Summary

The aim of this study was to determine if there are potential long-term effects on populations of the common vole *(Microtus arvalis*) and their reproductive performance resulting from foliar spray application of the Metiram containing formulation BAS 222 28 F. To assure sufficient numbers of animals and therefore statistical validity, the application was done on commercially used grassland, a preferred habitat for the common vole. The investigation in grassland is supposed to represent a worst-case scenario of the commercial use of Metiram for other crop types such as arable crops, orchards and vineyards, which are either less preferred by common voles or only represent temporal habitats due to frequent harvest events.

I. MATERIAL AND METHODS

The study was conducted commercially used grassland fields in the vicinity of the research centre [redacted]. In total, 14 fields (7 control fields, 7 treatment fields) were selected with an average size of 1.44 ha. On treatment fields, BAS 222 28 F was applied three times with seven day intervals between the applications and 1400 g Metiram per hectare according to its use pattern.

To conduct the live trapping of small mammals 60 ‘Ugglan’ multiple-capture live traps were used at each study field. The traps were set in six rows of 10 traps each, all placed inside the grassland fields. The distance between traps was approximately ten meters. Live trapping followed a capture-mark-recapture (CMR) design, which allows identification of individually marked animals upon recapture. Each captured common vole was individually marked with a Passive Integrated Transponder (PIT), with the exception of juveniles below 10 g of body-weight which were not pitted but marked by a fur cut.

Live trapping of small mammals was conducted during eight trapping sessions. The first trapping session was started May the 27th 2015 when the vole populations were still on a low level. With three weeks between each session, trapping continued until November the 6th 2015.

A trapping session comprised four consecutive nights – one night of pre-baiting followed by three nights of live trapping. Traps were activated for trapping in the evening and checked in the morning. Traps were deactivated during the day.

In order to achieve the overall aim of determining potential long-term effects on common vole populations as a result of Metiram use, the following parameters are determined:

- Trapping efficiency and trapping success
- Population size and population development of the common vole
- Survival rates of common voles
- Body-weight and condition (signs of lesions, condition of fur, inflammations)
- Population structure (sex ratio, proportion of juveniles, proportion of reproductive active animals)

II. RESULTS

Common voles were present and captured at all 14 study sites. In total, 12853 captures of 5089 individual common voles were made.

Population densities, calculated as Minimum Number Alive (MNA), increased during the summer and reached peak densities in August/September.

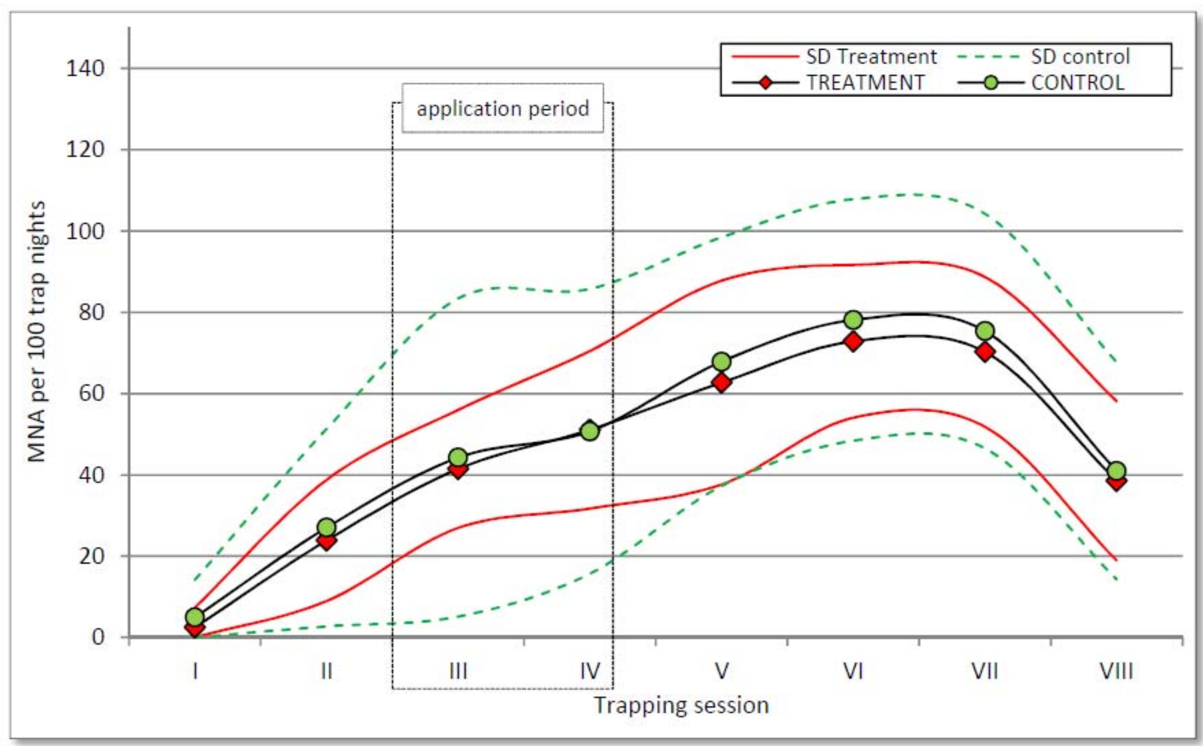


Figure 8.1.2.2-1: Development of mean MNA of common voles on treatment sites and control sites

A linear mixed model (LMM) using treatment and treatment-session interaction as explanatory variables revealed no significance neither for treatment nor the treatment-session interaction. Furthermore, the model with the treatment-session interaction was rejected by the Akaike Information Criterion (AIC) indicating that there was no time-related Metiram effect during the whole study period. Therefore, no effect of Metiram on common vole population development could be found.

Survival rates for the intervals between the trapping sessions were lower in the first and the last sessions. The pattern was the same on treatment and control fields. When analysed by a generalized linear mixed model no treatment related effects could be found.

The body weights of adult common voles remained constant around an average of 25g until trapping session V and decreased later towards 20g. The linear mixed model analysis revealed no significant treatment effect.

The sex ratio shown as the percentage of females in the population was around 50% in the beginning of the study, reached almost 70% in session IV then dropped to around 50%. In the statistical analysis no difference between treated and untreated fields could be found.

The proportion of reproductively active adults decreased during the study when more and more juveniles reached maturity but remained reproductively inactive. This could be observed for males and females but no statistical significant treatment effect was found.

The proportion of juveniles showed on both groups, treated and control fields, an increase during the course of the study until session VII. The treatment sites, however, performed slightly better, i.e. had a higher proportion of juveniles than the control sites. In session VI the difference between treated and untreated fields was found to be significant. However, a higher proportion of juveniles cannot be regarded as an adverse treatment effect.

Table 8.1.2.2-1: Summary table

	Treatment			Control		
Population dynamics (MNA)	Mean 45.4 Range 0.0 – 97.8	SD 28.2		Mean 48.6 Range 0.0 – 126.7	SD 36.1	
LMM*	$p > 0.05$		Coefficient estimate: -0.13			No significant treatment effect
Survival rates	**treatment**			**control**		
	Mean 0.46 Range 0.00 – 1.00	SD 0.20		Mean 0.45 Range 0.00 – 0.82	SD 0.20	
GLMM**	$p > 0.05$		Coefficient estimate: -0.09			No significant treatment effect
Body weight of adults [g]	**treatment (n = 3774)**			**control (n = 4214)**		
	Mean 23.6 Range 17.0 – 32.5	SD 6.6		Mean 23.6 Range 16.0 – 29.8	SD 4.5	
LMM*	$p > 0.05$		Coefficient estimate: 0.80			No significant treatment effect
Body weight of adult females [g]	**treatment (n = 2566)**			**control (n = 2784)**		
	Mean 23.9 Range 17.1 – 29.8	SD 7.4		Mean 24.1 Range 16.0 – 29.3	SD 6.0	
LMM*	$p > 0.05$		Coefficient estimate: 0.98			No significant treatment effect
Body weight of adult males [g]	**treatment (n = 1157)**			**control (n = 1395)**		
	Mean 23.3 Range 14.9 – 37.0	SD 7.8		Mean 23.2 Range 14.6 – 34.5	SD 6.7	
LMM*	$p > 0.05$		Coefficient estimate: 0.63			No significant treatment effect
Sex ratio (as % females)	**treatment**			**control**		
	Mean 56.7 Range 0.0 – 82.4	SD 19.8		Mean 57.5 Range 0.0 – 100.0	SD 17.1	
GLMM**	$p > 0.05$		Coefficient estimate: 0.03			No significant treatment effect
Reproductive activity females [%]	**treatment**			**control**		
	Mean 45.6 Range 0.0 – 100.0	SD 28.2		Mean 50.8 Range 0.0 – 100.0	SD 28.9	
GLMM**	$p > 0.05$		Coefficient estimate: -0.12			No significant treatment effect
Reproductive activity males (%)	**treatment**			**control**		
	Mean 48.0 Range 0.0 – 100.0	SD 36.7		Mean 45.4 Range 0.0 – 100.0	SD 35.0	
GLMM**	$p > 0.05$		Coefficient estimate: 0.68			No significant treatment effect
Proportion of juveniles (% of adults)	**treatment**			**control**		
	Mean 33.0 Range 0.0 – 75.0	SD 23.9		Mean 28.8 Range 0.0 – 57.6	SD 18.9	
GLMM**	**$p < 0.05$**		Coefficient estimate: 0.46			No adverse treatment effect

III. CONCLUSION

The presented study was under natural field conditions a worst-case scenario for the risk to common voles as the species occurred frequently and permanently on the treated grassland fields. The study presented here was performed on 14 fields and the trapping success was high. The data base can, therefore, be regarded as very solid. The population dynamics and general population structure observed in this study were plausible compared to the published literature, indicating an appropriate study design. For different population parameters linear mixed models were applied to test for significant effects of a Metiram treatment. No adverse effects under realistic worst-case field conditions could be found.

CA 8.1.3 Effects of active substance bioconcentration in prey of birds and mammals

The potential effects of active substance bioconcentration in prey of birds and mammals are addressed according to the guidance document EFSA/2009/1438 in the documents M-CP 10.1.1 and M-CP 10.1.2.

The partition co-efficient n-octanol/water (log Pow) of metiram is 1.76 at pH 7 (see M-CA chapter 2.7), indicating a low potential for bioaccumulation. Hence, according to EFSA/2009/1438 an assessment of bioaccumulation via the terrestrial or aquatic foodchain is not required. Consequently, no specific study is considered necessary.

CA 8.1.4 Effects on terrestrial vertebrate wildlife (birds, mammals, reptiles and amphibians)

According to the revised data requirements under regulation 1107/2009 (Commission Regulations (EU) 283/2013 and 284/2013 for the active ingredient and the plant protection products, respectively), the risk to amphibians and reptiles shall be addressed. Nevertheless, unlike birds and mammals, toxicity tests for amphibian and reptile species are not requested. In the EU there is no guidance or validated regulatory protocols yet available neither on the type of regulatory testing necessary nor how to conduct a risk assessment for amphibian and reptiles.

An extensive literature search for metiram has not revealed any relevant information with respect to the toxicity of metiram to reptiles and amphibians.

According to the new aquatic guidance document (EFSA, 2013) amphibian should be included in the aquatic and terrestrial risk assessment. In absence of GLP studies the assessment should be based on any existing relevant information (testing of amphibian is not recommended at first instance due to animal welfare reasons and the absence of standard guidelines for amphibian testing). With regard to the aquatic risk assessment several data analyses indicate that the risk assessment for aquatic organisms (and fish in particular) covers the risk assessment for aquatic phases of amphibians (Fryday and Thompson, 2012; Weltje et al., 2013). Based on these extensive data reviews it can be concluded that the acute and chronic risk to amphibians is covered by the currently requested and conducted risk assessment for aquatic organisms (CP 10.2).

Compared to aquatic studies, regulatory ecotoxicological information on amphibians based on dosing studies (LD_{50}) is rather scarce. However, in the few cases where terrestrial stages of amphibians were tested in this kind of study as birds and mammals, the general pattern is that amphibians are less sensitive than the latter two taxa (see Table 12 and 13 in Fryday and Thompson, 2012).

In the case of reptiles there is even less information available than for amphibians (see the revision by Fryday and Thompson, 2009). The risk from dietary exposure can be assumed to be lower for reptiles than for birds and mammals (Fryday and Thompson 2009). This is because reptiles are poikilotherm (*i.e.* do not maintain a constant body temperature) and as a result feeding activity will peak at warm days and will be zero during hibernation or at cold days. In contrast, birds and mammals will have to maintain a constant body temperature and, hence, will need to feed every day (Fryday & Thompson 2009). Uncertainties remain on the contribution of dermal exposure to the overall exposure to reptiles. However, in contrast to amphibians the skin of reptiles is much less permeable; its functions is in general protection and barrier and not an organ used for respiration or water/mineral exchange with the environment. Accordingly, reptiles are considered less vulnerable to dermal exposure as compared to amphibians. Nevertheless, some uncertainty with respect to the risk to reptiles, i.e. whether they are sufficiently covered by other (more standard) ecotoxicological data will remain and further research is needed.

However, metiram has been used for many years in many countries worldwide. So far, there are no publications indicating a potential risk of this compound to amphibians / reptiles and despite the long term use worldwide the applicant is not aware of a single findings or (incidence) reports that amphibians / reptiles were harmfully affected by applications of this substance.

References:

Commission Regulation (EU) No 283/2013 setting out data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. Official Journal of the European Union: 1st March 2013.

Commission Regulation (EU) No 284/2013: setting out the data requirements for plant protection products, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. Official Journal of the European Union: 1st March 2013.

Fryday S and Thompson H (2009a): Literature reviews on ecotoxicology of chemicals with a special focus on plant protection products. Lot 1. Exposure of reptiles to plant protection products. EFSA (CFT/EFSA/PPR/2008/01).

Fryday S and Thompson, H (2012): Toxicity of pesticides to aquatic and terrestrial life stages of amphibians and occurrence, habitat use and exposure of amphibian species in agricultural; Food and Environment research agency, UK.

Weltje L., Simpson P., Gross M., Crane M., Wheeler J.R. (2013): Comparative acute and chronic sensitivity of fish and amphibians: a critical review of data. Environmental Toxicology and Chemistry, Vol. 32, No. 5, pp. 984-994.

CA 8.1.5 Endocrine disrupting properties

Mammals

A full assessment of all available toxicological data with mammals on any potential of metiram to influence known endocrine mechanisms is conducted in the toxicological section under M-CA 5.8.3.

Relevance of the impact of metiram on the thyroid organ for populations of wild mammals is discussed as part of the risk assessment, specifically in the selection of the reproductive endpoint for the higher tier risk assessment in M-CP 10.1.2. A detailed evaluation is done in a separate document (BASF DocID 2015/1093577). This evaluation follows the EFSA guidance document (EFSA 2009/1438), which focus on the ecological relevance of effects relevant for reproduction and population development of wild mammals. Based on a thorough analysis of two multiple generation reproduction and two prenatal developmental toxicity studies with rats and rabbits an overall NOAEL of 32 mg as/kg bw/d as derived from the three generation reproduction toxicity study (BASF Doc ID 1981/132) is considered relevant for the reproductive risk assessment. This endpoint covers adequately potential concerns of metiram induced toxicity on the thyroid as none of the apical endpoints relevant for the reproductive or developmental of offspring were adversely affected at this dose level. For further details please refer to chapter M-CP 10.1.2 and the separate document (BASF DocID 2015/1093577).

Birds

During the preparation process for the Annex I Renewal (AIR3) dossier of the fungicidal active substance metiram, the question of potential thyroid toxicity of metiram in birds came up as a result of the analysis of the mammalian toxicological data, and available data on thyroid toxicity in birds of ethylene thiourea (ETU), a major metabolite of metiram.

This question has been addressed by the notifier in detail in a separate position paper (BASF DocID 2015/1115546). To assist the reader a full executive summary of the position paper is given below.

Report: CA 8.1.5/1
[redacted], 2015a
Metiram (BAS 222 F) - Review of reproductive toxicity endpoints for the bird risk assessment taking into account thyroid toxicity of Metiram
2015/1115546
Guidelines: none
GLP: no

EXECUTIVE SUMMARY

During the preparation process for the Annex I Renewal (AIR3) dossier of the fungicidal active substance metiram, the question of potential thyroid toxicity of metiram in birds came up as a result of the analysis of the mammalian toxicological data, and available data on thyroid toxicity in birds of ethylene thiourea (ETU), a major metabolite of metiram. In laying hens, goats and rats metiram is metabolized to a certain extend into the transient metabolite ETU. This position paper aims to demonstrate that the reproductive endpoints from the available bird reproduction studies with metiram are sufficiently protective for the avian reproductive risk assessment and are sufficient to address the question of potential thyroid toxicity of metiram in birds.
Metiram was tested in avian reproduction studies with quail and mallard. As guideline regulatory studies, the focus of these studies was on the general and reproductive toxicity of metiram to birds. Therefore, and like for the overwhelming majority of active substances, no detailed assessment was included as regard to thyroid toxicity since standard guideline studies are not designed to investigate this parameter. Still, with the available detailed assessment of fitness and reproduction parameters and gross necropsy assessment, the available studies with metiram provide adequate information on the overall effect pattern of the active substance metiram in birds. Hence, the studies allow for an evaluation of the thyroidogenic potential of metiram in birds by comparing the observed toxic effect pattern of metiram with the expected effect pattern for thyroid-toxic substances.
A conclusive overview on fitness and physiological parameters that are expected to be affected in birds under influence of thyroidogenic substances is provided in the OECD 'DETAILED REVIEW PAPER FOR AVIAN TWO-GENERATION TOXICITY TEST' (2007). In order to evaluate a potential thyroidogenic activity of metiram in birds the available avian reproduction studies with metiram are analyzed in relation to the thyroid-relevant parameters described in this OECD document. The aim of this evaluation was to evaluate if metiram shows a similar pattern typical for thyroid-mediated toxicity in birds.
Information on most parameters considered sensitive to thyroidogenicity according to OECD (2007) can be drawn from the available avian reproduction studies with metiram. No indication for thyroid toxicity of metiram was found in the studies. Consistently over the studies and species tested, thyroidogen-associated fitness endpoints were not sensitive to metiram. Instead an effect pattern of general (systemic) toxicity was observed, with effects on egg production and quality and on embryonic survival, effects which are often observed for active substances, but not specifically linked to thyroidogenic activity.

It has to be acknowledged that there are some uncertainties, since the evaluation of physiological parameters in the guideline bird reproduction studies is not highly sophisticated. Still, with gross necropsy investigation and general observation of the health status of adults and chicks some conclusions can be drawn: i.e. the gross morphological examination of birds covers to a certain extend the physiological parameter of thyroid size as an enlargement would have been visible to the observer. Such an effect was not observed in the bird reproduction studies with metiram.
In addition, with regard to the sensitivity of different thyroid-related endpoints the review paper (OECD 2007) concludes that physiological endpoints are not more sensitive than general or fitness endpoints. This is further supported by the results of an extensive public literature search conducted by the notifier on the sensitivity of thyroid effects in birds. Publications were identified that investigated both physiological parameters (e.g. thyroid size, weight, histology, hormones) as well as general fitness endpoints (e.g. body weight, food consumption, growth). Although the studies broadly differed in applied testing methodologies, test species, and endpoints investigated, a clear pattern can be derived. In the majority of cases physiological endpoints were not more sensitive than general fitness parameters and effects on physiology and general fitness occurred at the same test concentration. This clearly underlines that the available avian reproduction studies with metiram, which include a detailed assessment of fitness endpoints and cover physiological parameters within the limits of gross necropsy and general health observation, are suitable for the evaluation of the thyroidogenic potential of metiram in birds.

A direct comparison of the effect pattern of metiram and ETU in avian reproduction studies further supports the minor relevance of thyroid toxicity for the case of metiram. Metiram and ETU were both tested in bobwhite quails and mallard ducks in comparable concentration ranges, however, for both test substances the resulting effect patterns were different. For ETU the driving, sensitive endpoints are in line with the expected specific effect pattern for thyroid-active molecules, i.e. thyroid hypertrophy and reduced body weight of chicks. For metiram, in contrast, effects on egg production, egg quality and reproductive toxicity are predominant, while no indication was found for thyroid-related effects. Note that for the case of ETU the observed thyroid hypertrophy and reduced bodyweight of chicks occurred only at levels where general reproductive toxicity was observed, underlining a potential low specific sensitivity of a thyroidogenic mode of action in birds. This is in line with a conclusion of the OECD (2007) review paper on the sensitivity of thyroid effects compared to general toxicity, where it is stated that ‘the thyroid system may be ‘relatively robust and not particularly sensitive to xenobiotic effects’ (p. 108) and ‘effects will only occur at exposure concentrations that also result in general systemic toxicity’ (p. 109). This proves to be exactly the case for ETU.

In summary, based on i) the results of the avian reproduction studies with metiram, ii) the evidence from OECD (2007) and public literature on the sensitivity of physiological versus general fitness endpoints for thyroidogenic molecules, and iii) the direct comparison of the effects observed for metiram and ETU in the avian reproduction studies, it can be concluded that at the tested doses metiram does not show a typical pattern of thyroid toxicity in birds. The standard endpoints from the avian reproduction studies with metiram are sufficiently protective to address the question of potential thyroid toxicity and are suitable for use in the avian reproductive risk assessment for metiram.

CA 8.2 Effects on aquatic organisms

Since Annex I inclusion of metiram, new toxicity studies on the active substance (tested with two solo-formulations; see text below) and its major metabolites have been performed and as a result there are new endpoints which are now used in the aquatic risk assessment. Summaries of these new studies are provided below. For completeness this includes some older studies, which have not been submitted during the previous Annex I inclusion process (*e.g.* because there is no respective data requirement in the EU). In addition, summaries are provided for peer-reviewed scientific literature that was considered to be of relevance for the aquatic risk assessment of metiram.

Details on the EU agreed studies which have been already evaluated within the Annex I inclusion of metiram are provided in the EU Review documents of metiram (Monograph, Vol. 3, Annex B.9, July 2000; Addendum to the Monograph, Annex B.9, June 2002 and April 2004; EC Review Report (SANCO/4059/2001-rev 3.3), June 2005).

For better transparency and traceability of the active substance history, the results of all studies are summarized in Table 8.2-1 and Table 8.2-2. Furthermore, summaries on the EU agreed studies which are still relevant for the aquatic risk assessment are also provided below.
There are several older studies for this compound in addition to the ones presented. However, those studies are not listed in the endpoint table below and no summary is provided as these studies are partly not according to present guidelines and/or not supported by analytical measurements, whereas the respective new studies are available.

Metiram is a highly polymeric ethylen-bis-dithio carbamate (EBDC) complexed with Zinc. The compound is practically insoluble in water and subject of solvolysis in the presence of water, which means that as soon as it is dissolved in water it is not metiram any longer but a mixture of oligomers, monomers, and other degradation products. The analytical method for metiram is based on the liberation of CS_2 and therefore not specific, because every molecule able to generate CS_2 will be defined as metiram, irrespective of its chemical nature. This analytical problem is common to all polymeric EBDCs. This is the reason why the water solubility of metiram - based on CS_2-liberating material - is given as < 2 mg a.s./L (see Review Report for metiram (SANCO/4059/2001-rev 3.3, June 2005). The concurrent occurrence of undissolved metiram and its degradation products in water with varying solubility leads to inhomogeneous suspension/solution and makes the analysis of such an aqueous material extremely difficult, if not impossible.
To reduce these difficulties in aquatic ecotoxicological tests, the formulated products BAS 222 28 F and BAS 222 29 F (solo-formulations containing 70% and 85% of a.s., nominally) have been used in recent studies instead of metiram, mainly to generate a more homogenous suspension of the test material, increase the bioavailability and thus maximize exposure to the substance and its solvolysis products. In addition, several acute tests with fish were performed under flow-through conditions. When reviewing the studies in fish, *Daphnia* and algae the problem addressed above should be taken into consideration.

Full references used within the following chapters are given at the end MCA 8.2. Document N3 contains structures and synonyms for all metabolites.

Table 8.2-1: List of studies and endpoints for aquatic organisms exposed to the active metiram (BAS 222 F)

Organism	Endpoint	Value [mg/L]		Reference (BASF DocID)	EU agreed (Justification for submission of new data)
		[mg metiram/L]	**[mg product/L] (purity)**		
Fish					
Oncorhynchus mykiss [1]	96 h LC_{50}	**0.336** [2]	0.473 (71.04%)	1994/10920 + Amendment: 1995/10143	yes
Lepomis macrochirus [1]	96 h LC_{50}	> 15.9 < 31.4	> 22.3 < 44.2 (71.12%)	1996/10978	yes
Cyprinus carpio [1]	96 h LC_{50}	16.3	23.8 (68.5%)	2002/1004894	yes
Gasterosteus aculeatus [1]	96 h LC_{50}	> 57.4	> 83.8 (68.5%)	2002/1004890	yes
Leuciscus idus melanotus[1]	96 h LC_{50}	25.3	37.0 (68.5%)	2002/1004892	yes
Pimephales promelas [1]	96 h LC_{50}	8.43	12.3 (68.5%)	2002/1004893	yes
Salvelinus fontinalis [1), 4)]	96 h LC_{50}	4.50	6.12 (73.5%)	2005/1006731	no (new data generated due to authority request to refine SSD)
Pimephales promelas [3), 4)]	96 h LC_{50}	> 0.607	0.663 (91.6%)	2010/7010878	no (new data generated to address US EPA requirements)
Cyprinodon variegatus [3), 4), 5)]	96 h LC_{50}	> 0.673	0.735 (91.6%)	2010/7010879	no (new data generated to address US EPA requirements)
Oncorhynchus mykiss [1]	28 d NOEC	0.024 [6]	0.0316 (74.8%)	1990/10319 + Amendment: 1990/10320	yes
Pimephales promelas (ELS study) [3), 4)]	33 d NOEC	**0.013**	0.014 (91.6%)	2010/7012795	no (new data generated to address US EPA requirements)

Organism	Endpoint	Value [mg/L]		Reference (BASF DocID)	EU agreed (Justification for submission of new data)
		[mg metiram/L]	[mg product/L] (purity)		
Aquatic invertebrates					
Daphnia magna [1]	48 h EC_{50}	**> 0.584** [7]	> 0.821 (71.12%)	1997/10538	yes
Daphnia magna [1]	48 h EC_{50}	0.547 (nominal) 0.252 (initial measured) 0.216 (mean measured)	0.770 (nominal) 0.355 (initial measured) 0.304 (mean measured) (71.04%)	1994/10671 + Amendment: 1994/10814	yes
Daphnia magna [3), 4)]	48 h EC_{50}	0.634	0.692 (91.6%)	2010/7010877	no (new data generated to address US EPA requirements)
Americamysis bahia [3), 4), 5)]	48 h LC_{50} [8]	**> 0.147**	0.160 (91.6%)	2010/7010900	no (new data generated to address US EPA requirements)
Crassostrea virginica [3), 4), 5)]	96 h EC_{50}	**0.140**	0.153 (91.6%)	2010/7010901	no (new data generated to address US EPA requirements)
Cyclopoida [1]	48 h EC_{50}	0.660	0.960 (68.5%)	2002/1005266	yes
Chydorus sphaericus [1]	48 h EC_{50}	0.603	0.880 (68.5%)	2002/1005267	yes
Ostracoda [1]	48 h EC_{50}	> 1.77	> 2.59 (68.5%)	2002/1005268	yes
Physa acuta [1]	96 h LC_{50}	> 1.77	> 2.59 (68.5%)	2002/1005265	yes
Simocephalus vetulus [1]	48 h EC_{50}	2.09	3.05 (68.5%)	2002/1005269	yes
Daphnia magna [1]	21 d NOEC	**0.00437** [6]	0.00615 (71.12%)	1997/10521+ Amendment: 1997/10537	yes
Daphnia magna [1]	21 d EC_0	0.0062	0.0078 (80%)	1990/0310+ Amendment: 1991/10950	yes
Daphnia magna [3), 4)]	21 d NOEC	0.055	0.060 (91.6%)	2010/7014945	no (new data generated to address US EPA requirements)

Organism	Endpoint	Value [mg/L] [mg metiram/L]	[mg product/L] (purity)	Reference (BASF DocID)	EU agreed (Justification for submission of new data)
Americamysis bahia (life cycle study) 3), 4), 5)	40 d NOEC	**0.0031**	0.0034 (91.6%)	2010/7015187	no (new data generated to address US EPA requirements)
Sediment dwelling aquatic invertebrates					
Chironomus tentans (sub-chronic spiked sediment study) 3), 4), *	10 d LC_{50} 10 d NOEC	> 144 mg/kg d.s. 21.6 mg/kg d.s.	> 157 mg/kg d.s. 23.6 mg/kg d.s. (91.6%)	2010/7012893	no (new data generated to address US EPA requirements)
Leptocheirus plumulosus (sub-chronic spiked sediment study) 3), 4), 5), *	10 d LC_{50} 10 d NOEC	> 2.07 mg/kg d.s. ≥ 2.07 mg/kg d.s.	> 2.3 mg/kg d.s. ≥ 2.3 mg/kg d.s. (91.6%)	2010/7012894	no (new data generated to address US EPA requirements)
Chironomus riparius 1) (spiked water study)	28 d NOEC	0.221	0.316 (69.8%)	2001/1007679	yes
Algae 9)					
Pseudokirchneriella subcapitata 1)	72 h E_rC_{50} 72 h E_yC_{50}	0.191 0.071	0.279 0.104 (68.5%)	2002/1005284	yes
Pseudokirchneriella subcapitata 1), 4)	72 h E_rC_{50} 10) 72 h E_yC_{50} 10)	**0.021** 0.0061	0.033 0.0089 (72.5%)	2009/7000035	no (new data generated for AIR3 renewal)
Anabaena cylindrica 1)	72 h E_rC_{50} 72 h E_bC_{50}	0.360 0.264	0.526 0.385 (68.5%)	2002/1005283	yes
Anabaena flos-aquae 1)	72 h E_rC_{50} 72 h E_bC_{50}	0.189 0.052	0.276 0.076 (68.5%)	2002/1005276	yes
Anabaena flos-aquae 1), 4)	72 h E_rC_{50} 10) 72 h E_yC_{50} 10)	0.061 0.012	0.084 0.017 (72.5%)	2008/7015287	no (new data generated to address US EPA requirements)
Ankistrodesmus bibraianus 1)	72 h E_rC_{50} 72 h E_bC_{50}	1.37 0.208	2.00 0.304 (68.5%)	2002/1005272	yes
Corcontochrysis noctivaga 1)	72 h E_rC_{50} 72 h E_bC_{50}	0.232 0.131	0.339 0.191 (68.5%)	2002/1005280	yes

Organism	Endpoint	Value [mg/L]		Reference (BASF DocID)	EU agreed (Justification for submission of new data)
		[mg metiram/L]	[mg product/L] (purity)		
Cosmarium praemorsum [1)]	72 h E_rC_{50} 72 h E_bC_{50}	0.157 0.084	0.229 0.123 (68.5%)	2002/1005278	yes
Desmodesmus subspicatus [1)]	72 h E_rC_{50} 72 h E_bC_{50}	0.891 0.431	1.30 0.629 (68.5%)	2002/1005275	yes
Euglena gracilis [1)]	72 h E_rC_{50} 72 h E_bC_{50}	4.61 1.79	6.73 2.61 (68.5%)	2002/1005273	yes
Navicula pelliculosa [1)]	72 h E_rC_{50} / E_bC_{50}	> 0.206	> 0.301 (68.5%)	2002/1005277	yes
Navicula pelliculosa [1), 4)]	72 h E_rC_{50} [10)] 72 h E_yC_{50} [10)]	0.011 0.0032	0.015 0.0044 (72.5%)	2008/7015288	no (new data generated to address US EPA requirements)
Ochromonas danica [1)]	72 h E_rC_{50} 72 h E_bC_{50}	0.210 0.094	0.307 0.137 (68.5%)	2002/1005281	yes
Skeletonema costatum [1), 4), 5)]	72 h E_rC_{50} [10)] 72 h E_yC_{50} [10)]	0.025 0.020	0.035 0.028 (72.3%)	2013/7000406	no (new data generated to address US EPA requirements)
Skeletonema costatum [1), 4), 5)]	72 h E_rC_{50} [10)] 72 h E_yC_{50} [10)]	**0.0070** 0.0062	0.0097 0.0086 (72.5%)	2008/7018037	no (new data generated to address US EPA requirements)
Staurastrum gracile [1)]	72 h E_rC_{50} 72 h E_bC_{50}	0.519 0.134	0.758 0.196 (68.5%)	2002/1005282	yes
Staurastrum tetracerum [1)]	72 h E_rC_{50} 72 h E_bC_{50}	0.170 0.081	0.248 0.118 (68.5%)	2002/1005279	yes
Tetraedron caudatum [1)]	72 h E_rC_{50} 72 h E_bC_{50}	0.812 0.218	1.19 0.318 (68.5%)	2002/1005274	yes
Aquatic macrophytes [9)]					
Lemna gibba [3), 4)]	7 d E_rC_{50} / E_yC_{50}	> 0.517	> 0.564 (91.6%)	2010/7013131	no (new data generated to address US EPA requirements)

Organism	Endpoint	Value [mg/L]		Reference (BASF DocID)	EU agreed (Justification for submission of new data)
		[mg metiram/L]	[mg product/L] (purity)		
Higher-tier studies / Peer-reviewed literature studies					
Oncorhynchus mykiss (fish outdoor mesocosm) [1), 11)]	28 d NOEC	0.070	0.102 (68.5%)	2002/1005264	yes
Daphnia magna (study with unfiltered, filtered and aged solutions) [1), 4)]	48 h EC_{50}	0 h aging: 0.599 (unfiltered) > 1.69 (filtered) 4 - 6 h aging: 0.761 (unfiltered) 1.20 (filtered) 28 - 30 h aging: 0.670 (unfiltered) 1.00 (filtered)	0 h aging: 0.850 (unfiltered) > 2.4 (filtered) 4 - 6 h aging: 1.08 (unfiltered) 1.70 (filtered) 28 - 30 h aging: 0.95 (unfiltered) 1.42 (filtered) (70.5%)	2010/1059919	no (new data generated for risk refinement)
Daphnia magna (higher tier population study) [1), 12)]	28 d NOEC	0.200	0.292 (68.5%)	2002/1005270	yes
Daphnia magna (higher tier population study) [1), 4), 12)]	35 d NOAEC	0.100	0.142 (70.5%)	2006/1016038	no (new data generated due to authority request for refined risk assessment)
Outdoor microcosm (multiple applications) [1), 4), 11)]	$NOEC_{microcosm}$	0.036	0.017 - 0.051 (70.39%)	2012/1366942	no (relevant and reliable peer-reviewed scientific study; used for risk refinement) [13)]

Bold figures: Where several endpoints are available for the same group or where several endpoints are available for one study based on different effect parameters (*e.g.* for algae and macrophytes), only the relevant endpoint(s) is used in the risk assessment presented in chapter 10.2 of the MCP dossier part for Annex I renewal.

Abbreviations: ELS = early life stage; d.s. = dry sediment

* The 10-d sub-chronic study was conducted in support of the US registration (no data requirement in the EU). The results of this test are not relevant for the EU risk assessment, since a real chronic (28 day) study with *C. riparius* is available, which is considered more relevant for the risk assessment. The acute/sub-chronic risk to aquatic sediment dwelling insects is covered by the chronic spiked water study on *C. riparius*; therefore, a risk assessment based on the sub-chronic endpoints for this species is not necessary.

1) Study was conducted with the solo-formulation BAS 222 28 F (containing 70% metiram, nominally); metiram endpoints were re-calculated based on the analyzed purity of the tested formulation batch.

2) The endpoint used for risk assessment differs slightly from the endpoint reported in the EU Review for metiram due to minor differences in rounding within the LC_{50} calculations.

3) Study was conducted with the solo-formulation BAS 222 29 F (containing 85% metiram, nominally); metiram endpoints were re-calculated based on the analyzed purity of the tested formulation batch (*i.e.* 91.6%).

4) Study was not submitted during Annex I inclusion process of the active substance (study summary is provided below).

5) marine / saltwater species

[6] In the List of Endpoints (LoEP) provided in the EC Review Report (SANCO/4059/2001-rev 3.3; June 2005) the endpoints of the 28 d fish study and the 21 d study with *D. magna* re-calculated from the results of respective study with BAS 222 28 F based on the nominal content of metiram within the formulation are presented; however for all other BAS 222 28 F studies data were re-calculated based on analyzed contents of the active substance. The endpoints reported here were re-calculated based on the analyzed content of the a.s. within the formulation and thus differ slightly from the endpoints reported in the LoEP. This is considered to be a more precise calculation and thus, these endpoints are considered appropriate for risk assessment of metiram.

[7] The endpoint from the more recent acute study on *D. magna* (BASF DocID 1997/10538) is considered as relevant endpoint for the risk assessment as analytical measurements appeared more reliable; the endpoint obtained in the second study (BASF DocID 1994/10671 + Amendment 1994/10814) is not used.

[8] The 48-h LC_{50} obtained in the 96 h study is used in the risk assessment according to EU Regulation 283/2013 on the data requirements for active substances and the EFSA Aquatic Guidance (EFSA, 2013).

[9] In accordance with the new EFSA Aquatic Guidance Document (EFSA, 2013) only the EC_{50} values determined for the endpoint 'growth rate' (E_rC_{50}) are considered for the risk assessment for aquatic primary producers if both “growth rate” and “biomass” endpoints are available.

[10] In accordance to the EFSA Aquatic Guidance Document (EFSA, 2013) and OECD guideline 201 (2011) the 72 h endpoints obtained in the 96 h alga studies are considered as relevant endpoints and are presented here.

[11] Multiple applications in a static system.

[12] Population study; multiple (*i.e.* four) test substance applications

[13] Data derived from relevant peer-reviewed scientific study (for details see chapter 8.2.8 below).

Table 8.2-2: List of studies and endpoints for aquatic organisms exposed to the major metabolites of metiram (BAS 222 F)

Organism	Endpoint	Value [mg/L]	Reference (BASF DocID)	EU agreed (Justification for submission of new data)
Metabolite: Ethyleneurea (EU)				
Fish				
Oncorhynchus mykiss	96 h LC_{50}	> 122	2001/5000987	yes
Aquatic invertebrates				
Daphnia magna	48 h EC_{50}	> 985	2001/5000988	yes
Algae [1]				
Selenastrum capricornutum	72 h E_rC_{50} / E_bC_{50}	> 119 *	2001/5000986	yes
Metabolite: Ethylenethiourea (ETU)				
Fish				
Oncorhynchus mykiss	96 h LC_{50}	**> 500**	2001/1001877	yes
Lepomis macrochirus [2]	96 h LC_{50}	> 990	2008/7015434	no (new data generated to address US EPA requirements)
Cyprinodon variegates [2), 3)]	96 h LC_{50}	> 900	2008/7015440	no (new data generated to address US EPA requirements)
Other vertebrates				
Xenopus laevis	28 d NOEC	10	2002/1003402	yes
Xenopus laevis	28 d NOEC	10	2005/1043780	no (relevant and reliable peer-reviewed scientific study; used for risk refinement)

Aquatic invertebrates				
Daphnia magna	48 h EC_{50}	**21.6**	2000/1017216	yes
Americamysis bahia [2), 3)]	48 h LC_{50} [4)]	**11.0**	2008/7015438	no (new data generated to address US EPA requirements)
Crassostrea virginica [2), 3)]	96 h EC_{50}	> 110	2008/7013844	no (new data generated to address US EPA requirements)
Daphnia magna [2)]	21 d NOEC	2.0	1995/1008534	no (new data for the risk assessment of a further metabolite)
Algae [1)]				
Selenastrum capricornutum	72 h E_rC_{50} 72 h E_bC_{50}	**93.8** 23.7	2000/1017191	yes
Aquatic plants [1)]				
Lemna gibba [2)]	7 d E_rC_{50} / E_bC_{50}	> 960	2008/7015436	no (new data generated to address US EPA requirements)
Metabolite: TDIT				
Aquatic invertebrates				
Fish (QSAR) [5)]	96 h LC_{50}	991	--	no (new data for the risk assessment of a further metabolite)
Aquatic invertebrates				
Daphnia magna [2)]	48 h EC_{50}	7.12	2013/1000144	no (new data for the risk assessment of a further metabolite)
Green algae (QSAR) [5)]	96 h EC50	1.383	--	no (new data for the risk assessment of a further metabolite)

Bold figures: Where several endpoints are available for the same group or where several endpoints are available for one study based on different effect parameters (*e.g.* for algae and macrophytes), only the relevant endpoint(s) is used in the risk assessment presented in chapter 10.2 of the MCP dossier part for Annex I renewal.

* In accordance to the EFSA Aquatic Guidance Document (EFSA, 2013) and OECD guideline 201 (2011) the 72 h endpoints obtained in the 96 h alga studies are considered as relevant endpoints and are presented here.

1) In accordance with the new EFSA Aquatic Guidance Document (EFSA, 2013) only the EC_{50} values determined for the endpoint 'growth rate' (E_rC_{50}) are considered for the risk assessment for aquatic primary producers if both “growth rate” and “biomass” endpoints are available.

2) Study has not been submitted during the Annex I inclusion process of metiram. A study summary is providedd below.

3) Marine / saltwater species

4) The 48-h LC_{50} obtained in the 96 h study is used in the risk assessment according to EU Regulation 283/2013 on the data requirements for active substances and the EFSA Aquatic Guidance (EFSA, 2013).

5) Theoretical endpoint predicted by QSAR (quantitative structure-activity relationship) performed using the software ECOSAR (Version 1.11 by U.S. EPA); for details see MCP 10.2.

CA 8.2.1 Acute toxicity to fish

Data already submitted during previous Annex I inclusion process (already evaluated on EU level):

The following acute toxicity study on rainbow trout (Oncorhynchus mykiss) performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is providedd below.

Report:	*CA 8.2.1/1* [redacted] *1994a* *Acute toxicity study on the rainbow trout (Oncorhynchus mykiss WALBAUM 1792) of BAS 222 28 F in a flow-through system (96 hours)* *1994/10920*
Guidelines:	*EPA 72-1, EPA-SEP 540/9-85-006, EEC 84/449 C 1, OECD 203*
GLP:	*yes* *(certified by Ministerium fuer Arbeit, Soziales, Familie und Gesundheit, Postfach 3180, 6500 Mainz)*

Report:	*CA 8.2.1/2* [redacted] *1995a* *Amendment: Acute toxicity study on the rainbow trout (Oncorhynchus mykiss WALBAUM 1792) of BAS 222 28 F in a flow-through system (96 hours)* *1995/10143*
Guidelines:	*EPA 72-1, EPA-SEP 540/9-85-006, EEC 84/449 C 1, OECD 203*
GLP:	*yes* *(certified by Ministerium fuer Arbeit, Soziales, Familie und Gesundheit, Postfach 3180, 6500 Mainz)*

Executive Summary

Juvenile rainbow trout (approx. 7 months old) were exposed to BAS 222 28 F over a 96 h period in a flow-through system. The nominal exposure concentrations were 0, 0.01, 0.0316, 0.0464, 0.0681, 0.1, 0.146, 0.215, 0.316, 0.464, 0.681 and 1 mg BAS 222 28 F/L. Fish were observed for survival and toxic signs within 1, 4, 24, 48, 72 and 96 hours after start of exposure.

The results are based on mean measured concentrations. After 96 h BAS 222 28 F caused no mortality up to rate of 0.046 mg/L. 50% mortality was recorded at the highest test rate of 0.527 mg/L. Toxicological symptoms were observed in the two highest treatment groups of 0.244 mg/L and 0.527 mg BAS 222 28 F/L.

In a static acute toxicity study with BAS 222 28 F the LC_{50} (96 h) for rainbow trout based on mean measured concentrations was 0.473 mg/L (0.336 mg a.s./L). The NOEC has been reported as 0.046 mg/L (0.033 mg a.s./L); however, this is statistically not significant; a more realistic NOEC is 0.16 mg/L (0.114 mg a.s./L) based on the observed symptoms at the next higher concentration.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch No. 94-1, content of a.s.: metiram (BAS 222 F; Reg. No 250284): 71.04% analyzed (70% nominal).

B. STUDY DESIGN

Test species: Rainbow trout (*Oncorhynchus mykiss* WALBAUM 1792), age approximately 7 months; body weight: 2.02 (1.3 - 3.2) g, body length: 6.03 (5.2 - 7.0) cm, animal supplier: [redacted]

Test design: Flow-through (96 hours); 10 fish per treatment (1 replicate), loading 0.34 g fish/L.

Endpoints: LC_{50}, NOEC, sublethal effects.

Test concentrations: Control and 0.01, 0.0316, 0.0464, 0.0681, 0.1, 0.146, 0.215, 0.316, 0.464, 0.681 and 1 mg BAS 222 28 F/L (nominal).

Test conditions: Temperature 12 ± 1 °C; photoperiod : 16 h light : 8 h dark; pH 7.5 - 8.4; oxygen content 11.0 mg/L - 12.4 mg/L; total hardness approximately 0.5 mmol/L, conductivity: approximately 90 µhmo, no feeding, no aeration.

Analytics: The test item concentrations were analyzed with GC method (GC with FPD; 399 nm, sulfur-selective).

Statistics: Descriptive statistics, determination of LC_X and NOEC values using probit analysis

II. RESULTS AND DISCUSSION

Analytical measurements: The analytically determined concentrations of metiram were in a range of 33.6% - 60.6% at test initiation and 17.3% - 42.0% at test termination. The following biological results are based on mean measured concentrations.

Biological results: Significant mortality and sublethal symptoms were observed at the two highest concentrations. Occasional single mortalities were observed in the control and two of the lower concentrations (that is why the test report stated a (statistically not significant) NOEC of 0.046 mg/L). The results are summarized in Table 8.2.1-1.

Table 8.2.1-1: Acute toxicity of BAS 222 28 F on rainbow trout (*Oncorhynchus mykiss*)

Concentration nominal [mg/L]	Concentration mean measured [mg/L]	Mortality after 96 h [%]	Symptoms
Control	--	5#	None
0.01	n.d.	0	None
0.0316	n.d.	0	None
0.0464	n.d.	0	None
0.0681	n.d.	0	None
0.1	0.039	0	None
0.146	0.046	0	None
0.215	0.081	10	None
0.316	0.104	0	None
0.464	0.160	10	None
0.681	0.244	30	Slight discoloration, convulsions
1.0	0.527	50	Slight discoloration, mucous secretion, hyperreflexia, convulsions

	Endpoints (initially measured)	
	based on BAS 222 28 F [mg/L]	**based on BAS 222 F [mg a.s./L]**
LC_{50} (96 h)	**0.473**	**0.336**
NOEC (96 h)	0.046 / 0.160 *	0.033 / 0.114 *

n.d. = not determined
\# mean of two controls, one control with 10% and one with 0% mortality
* statistically and biologically more relevant endpoint, compared to the reported value

III. CONCLUSION

In a static acute toxicity study with BAS 222 28 F the LC_{50} (96 h) for rainbow trout based on mean measured concentrations was 0.473 mg/L (0.336 mg a.s./L). The NOEC has been reported as 0.046 mg/L (0.033 mg a.s./L); however, this is statistically not significant; a more realistic NOEC is 0.16 mg/L (0.114 mg a.s./L) based on the observed symptoms at the next higher concentration.

The following acute toxicity study on bluegill sunfish (Lepomis macrochirus) performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is providedd below.

Report:	*CA 8.2.1/3* *[redacted] 1996a* *BAS 222 28 F - Acute toxicity study on the bluegill sunfish (Lepomis macrochirus RAF.) in a flow-through system (96 hours)* *1996/10978*
Guidelines:	*EPA 72-1, EPA-SEP 540/9-85-006, EEC 84/449 C 1, OECD 203*
GLP:	*yes* *(certified by Ministerium fuer Arbeit, Soziales, Familie und Gesundheit, Postfach 3180, 6500 Mainz)*

Executive Summary

In an acute toxicity laboratory study, bluegill sunfish (*Lepomis macrochirus*) were exposed to BAS 222 28 F over a 96 h period in a flow-through system. The nominal exposure concentrations were 3.16, 4.64, 6.81, 10.0, 14.7, 21.5, 31.6, 46.4, 68.1 and 100 mg BAS 222 28 F/L. Fish were observed for survival and symptoms of toxicity within 1, 4, 24, 48, 72 and 96 hours after start of exposure.

The results are based on mean measured concentrations. After 96 h BAS 222 28 F caused no mortality up to a concentration of 4.9 mg/L. The LC_{50} (96 h) in bluegill sunfish was > 22.3 mg/L and < 44.2 mg/L. Symptoms of toxicity (apathy) were observed at concentrations of 22.3 mg BAS 222 28 F/L and higher.

BAS 222 28F was tested in an acute static laboratory study with the bluegill sunfish. The 96-hour LC_{50} was determined to be > 22.3 mg/L and < 44.2 mg/L based on mean measured concentrations, corresponding to > 15.860 and < 31.435 mg a.s./L, respectively. The NOEC was 4.9 mg/L (3.485 mg a.s./L).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch No. 96-1, content of a.s.: metiram (CAS-No.: 9006-42-2): 71.12% analyzed.

B. STUDY DESIGN

Test species: Bluegill sunfish (*Lepomis macrochirus*), juvenile animals, mean body length: 3.9 (3.3 - 5.1) cm, mean body weight: 0.82 (0.5 - 1.5) g, animal supplier: [redacted]

Test design: Flow-through system (96 hours); 10 fish per aquarium, 1 replicate per concentration, assessment of mortality and symptoms of toxicity after 1, 4, 24, 48, 72 and 96 h.

Endpoints: LC_{50}, NOEC, sublethal effects.

Test concentrations: Control and 3.16, 4.64, 6.81, 10.0, 14.7, 21.5, 31.6, 46.4, 68.1 and 100 mg BAS 222 28 F/L (nominal).

Test conditions: 60 l glass aquaria with stainless steel frame, fish loading: 0.017 g/L/day, temperature 22 - 23°C; pH approx. 7.8 - 7.9; oxygen content 8.4 - 9.3 mg/L; total hardness approx. 0.5 mmol/L, conductivity: approx. 130 µhmo, photoperiod : 16 h light : 8 h dark, no feeding, no aeration.

Analytics: The test item concentrations were analyzed using a GC method with FPD.

Statistics: Descriptive statistics, calculation of LC_x values with probit analysis.

II. RESULTS AND DISCUSSION

Analytical measurements: The analytically determined concentrations of metiram were in a range of 25.2% - 96.5% at test initiation and 63.4% - 94.0% at test termination. The following biological results are based on mean measured concentrations.

Biological results: At concentrations of 22.3 mg/L and higher symptoms and toxicity (apathy) and mortality could be observed. At concentrations of 58.3 mg/L and higher all fish were dead after 96 h. Mortalities of 20% and 10% were observed in the 6.4 and 11.2 mg/L concentrations, respectively. However, no mortality occurred at a concentration of 18.6 mg/L. The results are summarized in Table 8.2.1-2.

Table 8.2.1-2: Acute toxicity of BAS 222 28 F on bluegill sunfish (*Lepomis macrochirus*)

Concentration [mg/L] nominal	**Concentration [mg/L] mean measured**	**Mortality after 96 h [%]**	**Symptoms**
Control	--	0	None
3.16	1.90	0	None
4.64	3.60	0	None
6.81	4.90	0	None
10.00	6.40	20	None
14.70	11.20	10	None
21.50	18.60	0	None
31.60	22.30	20	Apathy
46.40	44.20	80	Apathy
68.10	58.30	100	Apathy
100.00	51.30	100	--

	Endpoints (mean measured)	
	based on BAS 222 28 F [mg/L]	**based on BAS 222 F [mg a.s./L]**
LC_{50} (96 h)	> 22.3 < 44.2 (30.2)	> 15.86 < 31.435 (21.5)
NOEC (96 h)	4.9[1)]	3.485[1)]

[1)] The NOEC has been reported as 4.9 mg/L (3.485 mg a.s./L); however, this is statistically not significant; a more realistic NOEC can be given as 18.6 mg/L (13.228 mg a.s./L) based on mortality and the observed symptoms at the next higher concentration. A statistical re-evaluation of the data using the software "ToxStat 3.5" results in an LC_{50} of 30.2 mg/L.

III. CONCLUSION

BAS 222 28F was tested in an acute static laboratory study with the bluegill sunfish. The 96-hour LC_{50} was determined to be > 22.3 mg/L and < 44.2 mg/L (30.2 mg/L) based on mean measured concentrations, corresponding to > 15.860 and < 31.435 mg a.s./L (21.5 mg a.s./L), respectively.

The following acute toxicity study on carp (Cyprinus carpio) performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.1/4* *[redacted] 2002a* *BAS 222 28 F - Acute toxicity study on the common carp (Cyprinus carpio) in a flow through system over 96 hours* *2002/1004894*
Guidelines:	*OECD 203, EPA 72-1, EEC 92/69 C 1, EPA-SEP 540/9-85-006*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In an acute toxicity laboratory study, juvenile carp (*Cyprinus carpio*) were exposed to BAS 222 28 F over a 96 h period in a flow-through system. The nominal exposure concentrations were 0, 5, 10, 22, 50 and 100 mg BAS 222 28 F/L. Fish were observed for survival and symptoms of toxicity within 1, 4, 24, 48, 72 and 96 hours after start of exposure.

The biological results are based on mean measured concentrations. No symptoms of toxicity were observed in any of the test item treatments. Mortality occurred at concentrations of 13.5 mg BAS 222 28 F/L and higher.

BAS 222 28F was tested in an acute laboratory study with carp under flow-through conditions. The 96-hour LC_{50} was determined to be 23.8 mg/L based on mean measured concentrations, corresponding to 16.303 mg a.s./L. The NOEC was 3.9 mg/L (2.672 mg a.s./L).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch No. 2000-2, content of a.s.: metiram (BAS 222 F; Reg. No 250284): 70% nominal (68.5% analyzed).

B. STUDY DESIGN

Test species: Carp (*Cyprinus carpio*), age: 5-6 weeks; mean body length: 1.0 (0.9 - 1.1) cm, mean body weight: 0.11 (0.08 - 0.14) g, , animal supplier: [redacted]

Test design: Flow-through system (96 hours); 10 fish per aquarium, 1 replicate per concentration, assessment of mortality and symptoms of toxicity after 1, 4, 24, 48, 72 and 96 h.

Endpoints: LC_{50}, NOEC, sublethal effects.

Test concentrations: Control, 5, 10, 22, 50 and 100 mg BAS 222 28 F/L (nominal).

Test conditions: Glass aquaria, fish loading 0.04 g/L, temperature: 22° C; pH 7.9 - 8.1; oxygen content 7.9 mg/L - 8.6 mg/L; total hardness approx. 2.5 mmol/L, conductivity: 550 µS/cm, photoperiod : 16 h light : 8 h dark, no feeding, no aeration.

Analytics: The test item concentrations were analyzed via GC-MS

Statistics: Descriptive statistics, calculation of LC_x values with probit analysis.

II. RESULTS AND DISCUSSION

Analytical measurements: The analytically determined concentrations of metiram were in a range of 33.2% - 66.4% at test initiation and 38.5% - 61.5% at test termination. The following biological results are based on mean measured concentrations.

Biological results: No symptoms of toxicity were observed in any of the test item treatments. Mortality occurred at concentrations of 13.5 mg BAS 222 28 F/L and higher. At the highest concentration of 51.8 mg/L all fish were dead after 4 h. The results are summarized in Table 8.2.1-3.

Table 8.2.1-3: Acute toxicity of BAS 222 28 F on carp (*Cyprinus carpio*)

Concentration [mg/L] nominal	Concentration [mg/L] mean measured	Mortality after 96 h [%]	Symptoms
Control	--	0	None
5	2.49	0	None
10	3.85	0	None
22	13.54	30	None
50	19.94	30	None
100	51.79	100	None

	Endpoints (mean measured)	
	based on BAS 222 28 F [mg/L]	**based on BAS 222 F [mg a.s./L]**
LC_{50} (96 h)	**23.8**	**16.303**
NOEC (96 h)	3.85	2.672

III. CONCLUSION

BAS 222 28F was tested in an acute laboratory study with carp under flow-through conditions. The 96-hour LC_{50} was determined to be 23.8 mg/L based on mean measured concentrations, corresponding to 16.3 mg a.s./L. The NOEC was 3.85 mg/L (2.67 mg a.s./L).

The following acute toxicity study on stickleback (Gasterosteus aculeatus) performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report: *CA 8.2.1/5*
[redacted] 2002b
BAS 222 28 F - Acute toxicity study on the stickleback (Gasterosteus aculeatus) in a flow through system over 96 hours
2002/1004890
Guidelines: *OECD 203, EEC 92/69 A V C 1, EPA 72-1, EPA-SEP 540/9-85-006*
GLP: *yes*
(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)

Executive Summary

In an acute toxicity laboratory study, juvenile stickleback (*Gasterosteus aculeatus*) were exposed to BAS 222 28 F over a 96 h period in a flow-through system. The nominal exposure concentrations were 0, 2.2, 5, 10, 22, 50, 100* mg BAS 222 28 F/L. Fish were observed for survival and symptoms of toxicity within 1, 4, 24, 48, 72 and 96 hours after start of exposure.

The results are based on mean measured concentrations. After 96 h BAS 222 28 F caused no mortality up to the highest concentration tested. Symptoms of toxicity (swimming at the bottom or surface) were observed at concentrations of 51.5 mg BAS 222 28 F/L and higher.

In a flow-through acute toxicity study with BAS 222 28 F the LC_{50} (96 h) for stickleback (*Gasterosteus aculeatus*) was determined to be > 83.8 mg/L (corresponding to 57.4 mg a.s./L) based on mean measured concentrations. The NOEC has been reported as 17.12 mg/L (11.73 mg a.s./L).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch No. 2000-2, content of a.s.: metiram (BAS 222 F; Reg. No 250284): 68.5% analyzed (70% nominal).

B. STUDY DESIGN

Test species: Stickleback (*Gasterosteus aculeatus*); juvenile fish; mean body length: 5.8 (5.4 - 6.2) cm (part 1), 5.9 (5.1 - 6.7) cm (part 2), mean body weight: 1.8 (1.4 - 2.5) g (part 1), 2.0 (1.2 - 3.2) g (part 2); animal supplier:

Test design: Flow-through system (96 hours); 10 fish per aquarium, 1 replicate per concentration, assessment of mortality and symptoms of toxicity after 1, 4, 24, 48, 72 and 96 h.

Endpoints: LC_{50}, NOEC, sublethal effects.

Test concentrations: 0 (Control), 2.2, 5, 10, 22, 50, 100 mg BAS 222 28 F/L.

Test conditions: 60 L glass aquaria with stainless steel frame; fish loading 0.3 g/L; temperature 12-13 °C; pH 8.0 - 8.1; oxygen content 8.6 mg/L - 9.4 mg/L; total hardness approx. 2.5 mmol/L, conductivity: approx. 550 µS/cm, photoperiod : 16 h light : 8 h dark ; no feeding, no aeration.

Analytics: The test item concentrations were analyzed via GC-MS.

Statistics: No statistical analysis was carried out since no lethality was observed up to the highest test concentration.

II. RESULTS AND DISCUSSION

Analytical measurements: The analytically determined concentrations of metiram were in a range of 72.7% - 92.9% at test initiation and 75.4% - 149.7% at test termination. The following biological results are based on mean measured concentrations.

Biological results: No mortality was observed up to and including the highest concentration.of 83.8 mg/L. Symptoms of toxicity were observed at concentrations of 51.5 mg BAS 222 28 F/L and higher. The results are summarized in Table 8.2.1-4.

Table 8.2.1-4: Acute toxicity of BAS 222 28 F on stickleback (*Gasterosteus aculeatus*)

Concentration [mg/L] nominal	Concentration [mg/L] mean measured	Mortality after 96 h [%]	Symptoms
Control	--	0	None
2.2	1.69	0	None
5	3.35	0	None
10	8.34	0	None
22	17.12	0	None
50	51.5	0	Swimming at the bottom/surface.
100	83.8	0	Swimming at the bottom/surface.

	Endpoints (mean measured)	
	based on BAS 222 28 F [mg/L]	**based on BAS 222 F [mg a.s./L]**
LC_{50} (96 h)	**> 83.8**	**> 57.4**
NOEC (96 h)	17.12	11.73

III. CONCLUSION

In a flow-through acute toxicity study with BAS 222 28 F the LC_{50} (96 h) for stickleback (*Gasterosteus aculeatus*) was determined to be > 83.8 mg/L (corresponding to 57.4 mg a.s./L) based on mean measured concentrations. The NOEC has been reported as 17.12 mg/L (11.73 mg a.s./L).

The following acute toxicity study on golden orfe (Leuciscus idus melanotus) performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.1/6* *[redacted] 2002c* *BAS 222 28 F - Acute toxicity study on the golden orfe (Leuciscus idus melanotus) in a flow through system over 96 hours* *2002/1004892*
Guidelines:	*EPA 72-1, OECD 203, EEC 92/69 A V C 1, EPA-SEP 540/9-85-006*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In an acute toxicity laboratory study about 10 months old golden orfe (*Leuciscus idus melanotus*) were exposed to BAS 222 28 F over a 96 h period in a flow-through system. The nominal exposure concentrations were 0, 5, 10, 22, 50 and 100 mg BAS 222 28 F/L. Fish were observed for survival and symptoms of toxicity within 1, 4, 24, 48, 72 and 96 hours after start of exposure.

The results are based on mean measured concentrations. After 96 h BAS 222 28 F caused no mortality up to and including a concentration of 23.3 mg/L. At the highest test concentration of 68.2 mg/L all fish were dead within 24 h. Symptoms of toxicity (tottering) were observed in the two highest treatment groups of 26.4 mg/L and 68.2 mg BAS 222 28 F/L.

In a flow-through acute toxicity study with BAS 222 28 F the LC_{50} (96 h) for golden orfe (*Leuciscus idus melanotus*) was determined to be 37 mg/L (corresponding to 25.3 mg a.s./L) based on mean measured concentrations. The NOEC has been reported as 23.3 mg/L (15.96 mg a.s./L).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch No. 2000-2, content of a.s.: metiram (BAS 222 F; Reg. No 250284): 68.5% analyzed (70% nominal).

B. STUDY DESIGN

Test species: Golden Orfe (*Leuciscus idus melanotus*), age: about 10 months; mean body length: 8.5 (7.5 - 9.6) cm, mean body weight: 6.57 (4.42 - 9.36) g, animal supplier: [redacted]

Test design: Flow-through system (96 hours); 10 fish per aquarium, 1 replicate per concentration, assessment of mortality and symptoms of toxicity after 1, 4, 24, 48, 72 and 96 h.

Endpoints: LC_{50}, NOEC, sublethal effects.

Test concentrations: 0 (control), 5, 10, 22, 50 and 100 mg BAS 222 28 F/L (nominal).

Test conditions: 50 L glass aquaria, fish loading: 1.3 g/L; temperature 22 °C; pH 7.9 - 8.0; oxygen content 6.7 - 7.9 mg/L; total hardness approx. 2.5 mmol/L, conductivity: approx. 550 µS/cm, photoperiod: 16 h light : 8 h dark, no feeding, no aeration.

Analytics: The test item concentrations were analyzed with a GC-MS method.

Statistics: Descriptive statistics, calculation of LC_x values with probit analysis.

II. RESULTS AND DISCUSSION

Analytical measurements: The analytically determined concentrations of metiram were in a range of 48.1 - 112.1% at test initiation and 57.6 - 99.9% at test termination. The following biological results are based on mean measured concentrations.

Biological results: BAS 222 28 F caused no mortality up to and including a concentration of 23.3 mg/L. At the highest test concentration of 68.2 mg/L all fish were dead within 24 h. Symptoms of toxicity (tottering) were observed in the two highest treatment groups of 26.4 mg/L and 68.2 mg BAS 222 28 F/L. The results are summarized in Table 8.2.1-5.

Table 8.2.1-5: Acute toxicity of BAS 222 28 F on Golden Orfe (*Leuciscus idus melanotus*)

Concentration [mg/L] nominal	Concentration [mg/L] mean measured	Mortality after 96 h [%]	Symptoms
Control	--	0	None
5	3.95	0	None
10	10.34	0	None
22	23.32	0	None
50	26.42	10	Tottering
100	68.17	100	Tottering

	Endpoints (mean measured)	
	based on BAS 222 28 F [mg/L]	**based on BAS 222 F [mg a.s./L]**
LC_{50} (96 h)	**37.0**	**25.3**
NOEC (96 h)	23.3	15.96

III. CONCLUSION

In a flow-through acute toxicity study with BAS 222 28 F the LC_{50} (96 h) for golden orfe (*Leuciscus idus melanotus*) was determined to be 37 mg/L (corresponding to 25.3 mg a.s./L) based on mean measured concentrations. The NOEC has been reported as 23.3 mg/L (15.96 mg a.s./L).

The following acute toxicity study on fathead minnow (Pimephales promelas) performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.1/7*
	[redacted] 2002d
	BAS 222 28 F - Acute toxicity study on the fathead minnow (Pimephales promelas) in a flow through system over 96 hours
	2002/1004893
Guidelines:	*EPA 72-1, OECD 203, EEC 92/69 A V C 1, EPA-SEP 540/9-85-006*
GLP:	*yes*
	(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)

Executive Summary

In an acute toxicity laboratory study, about 10 months old fathead minnow (*Pimephales promelas*) were exposed to BAS 222 28 F over a 96 h period in a flow-through system. The nominal exposure concentrations were 0, 5, 10, 22, 50 and 100 mg BAS 222 28 F/L. Fish were observed for survival and symptoms of toxicity within 1, 4, 24, 48, 72 and 96 hours after start of exposure.

The results are based on mean measured concentrations. BAS 222 28 F caused no mortality up to and including a concentration of 6.46 mg/L. 80% of the fish died in the 15.46 mg/L concentration and in the two highest treatment groups of 23.73 mg/L and 64.25 mg/L all fish had died after 96 hours.

In a flow-through acute toxicity study with BAS 222 28 F the LC_{50} (96 h) for fathead minnow (*Pimephales promelas*) was determined to be 12.3 mg/L (corresponding to 8.4 mg a.s./L) based on mean measured concentrations. The NOEC has been reported as 6.5 mg/L (4.45 mg a.s./L).

I. MATERIAL AND METHODS

A. MATERIALS

Test item:	BAS 222 28 F, batch No. 2000-2, content of a.s.: metiram (BAS 222 F; Reg. No 250284): 68.5% analyzed (70% nominal).

B. STUDY DESIGN

Test species:	Fathead minnow (*Pimephales promelas*), age about 10 months; mean body length: 4.5 (3.5 – 6.0) cm; mean body weight: 0.97 (0.34 – 1.95) g; animal supplier:
Test design:	Flow-through system (96 hours); 10 fish per aquarium, 1 replicate per concentration; assessment of mortality and symptoms of toxicity after 1, 4, 24, 48, 72 and 96 h.
Endpoints:	LC_{50}, NOEC, sublethal effects.
Test concentrations:	0 (control) 5, 10, 22, 50 and 100 mg BAS 222 28 F/L (nominal).
Test conditions:	25 L glass aquaria, fish loading: 0.4 g/L; temperature 22 °C; pH 8.0; oxygen content 7.3 - 8.3 mg/L; total hardness approx. 2.5 mmol/L, conductivity: approx. 550 µS/cm, photoperiod: 16 h light : 8 h dark no feeding, no aeration.
Analytics:	The test item concentrations were analyzed with a GC-MS method.
Statistics:	Descriptive statistics, calculation of LC_x values with probit analysis.

II. RESULTS AND DISCUSSION

Analytical measurements: The analytically determined concentrations of metiram were in a range of 47.7% - 78.5% at test initiation and 47.2% - 81.4% at test termination. The following biological results are based on mean measured concentrations.

Biological results: BAS 222 28 F caused no mortality up to and including a concentration of 6.46 mg/L. 80% of the fish died in the 15.46 mg/L concentration. Symptoms of toxicity (tottering, swimming at the surface) were observed in the two highest treatment groups of 23.73 mg/L and 64.25 mg/L, where all fish had died after 96 hours. The results are summarized in Table 8.2.1-6.

Table 8.2.1-6: Acute toxicity of BAS 222 28 F on fathead minnow (*Pimephales promelas*)

Concentration [mg/L] nominal	Concentration [mg/L] mean measured	Mortality after 96 h [%]	Symptoms
Control	--	0	None
5	3.80	0	None
10	6.46	0	None
22	15.46	80	None
50	23.73	10	Tottering
100	64.25	10	Swimming at the surface

	Endpoints (mean measured)	
	based on BAS 222 28 F [mg/L]	**based on BAS 222 F [mg a.s./L]**
LC_{50} (96 h)	**12.3**	**8.4**
NOEC (96 h)	6.5	4.45

III. CONCLUSION

In a flow-through acute toxicity study with BAS 222 28 F the LC_{50} (96 h) for fathead minnow (*Pimephales promelas*) was determined to be 12.3 mg/L (corresponding to 8.4 mg a.s./L) based on mean measured concentrations. The NOEC has been reported as 6.5 mg/L (4.45 mg a.s./L).

The following acute toxicity study on rainbow trout (Oncorhynchus mykiss) performed with the metiram metabolite ethyleneurea has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.1/8*
	[redacted] 2001a
	Ethylene urea: A 96-hour static acute toxicity test with the rainbow trout (Oncorhynchus mykiss)
	2001/5000987
Guidelines:	*OECD 203, ASTM E 729*
GLP:	*yes (certified by United States Environmental Protection Agency)*

Executive Summary

In an acute toxicity laboratory study, juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to ethyleneurea (metabolite of metiram) over a 96 h period in a static system. The nominal exposure concentrations were 0 (control), 16, 26, 43, 72 and 120 mg ethyleneurea/L (corresponding to mean measured concentrations of 16, 26, 43, 73 and 122 mg/L). Fish were observed for survival and symptoms of toxicity within 1.5, 4, 24, 48, 72 and 96 hours after start of exposure.

The results are based on nominal concentrations. After 96 h ethyleneurea caused no mortality up to the highest rate tested (120 mg/L). No symptoms of toxicity were observed in any of the treatment groups.

In a static acute toxicity study with ethyleneurea (metabolite of metiram) the LC_{50} (96 h) for rainbow trout was determined to be > 120 mg/L based on nominal concentrations. The 96 h NOEC has been reported as 120 mg/L. Based on mean measured concentrations the 96 h LC_{50} and NOEC were determined to be > 122 mg/L and 122 mg/L, respectively.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: Ethyleneurea (BF 222-EU; Reg. No 027270; metabolite of metiram); Lot No. 01743-141; purity: 90.8%.

B. STUDY DESIGN

Test species: Rainbow trout (*Oncorhynchus mykiss* WALBAUM 1792), age: about 7 weeks old; mean standard length: 3.1 (2.9 - 3.5) cm, mean total length: 3.7 (3.5-4.1) cm and mean body weight: 0.4 (0.33 - 0.59) g, animal supplier: [redacted]

Test design: Static system (96 hours); 10 fish per aquarium, 2 replicates per concentration, assessment of mortality and symptoms of toxicity after 1.5, 4, 24, 48, 72 and 96 h.

Endpoints: LC_{50}, NOEC, sublethal effects.

Test concentrations: Control and 16, 26, 43, 72 and 120 mg ethyleneurea/L (nominal), corresponding to mean measured concentrations of 16, 26, 43, 73 and 122 mg/L.

Test conditions: Glass aquaria containing 15 L of test solution, fish loading 0.27 g/L; temperature 12 - 14 °C; pH 8.1 - 8.5; oxygen content 9.2 - 9.8 mg/L; total hardness approx. 1.31 mmol/L, conductivity: approx. 315 µmhos/cm, photoperiod: 16 h light : 8 h dark , no feeding, no aeration.

Analytics: The test item concentrations were analyzed with a HPLC method.

Statistics: No statistical analysis was carried out since no lethality was observed up to the highest test concentration.

II. RESULTS AND DISCUSSION

Analytical measurements: The analytically determined concentrations of ethyleneurea were in a range of 96.7% - 104% at test initiation and 94.4% - 104% at test termination. The following biological results are based on nominal concentrations. Additionally, the results based on mean measured values are shown.

Biological results: Neither mortality nor any symptoms of toxicity were observed up to the highest concentration of 120 mg/L. The results are summarized in Table 8.2.1-7.

Table 8.2.1-7: Acute toxicity of ethyleneurea on rainbow trout (*Oncorhynchus mykiss*)

Concentration [mg/L] (nominal)	**Control**	**16**	**26**	**43**	**72**	**120**
Concentration [mg/L] (mean measured)	**--**	**16**	**26**	**43**	**73**	**122**
Mortality after 96 h [%]	0	0	0	0	0	0
Symptoms	None	None	None	None	None	None
	Endpoints [mg/L]					
	based on nominal concentrations			based on mean measured concentrations		
LC_{50} (96 h)	> 120			> 122		
NOEC (96 h)	120			122		

III. CONCLUSION

In a static acute toxicity study with ethyleneurea (metabolite of metiram) the LC_{50} (96 h) for rainbow trout was determined to be > 120 mg/L based on nominal concentrations. The 96 h NOEC has been reported as 120 mg/L. Based on mean measured concentrations the 96 h LC_{50} and NOEC were determined to be > 122 mg/L and 122 mg/L, respectively.

The following acute toxicity study on rainbow trout (Oncorhynchus mykiss) performed with the metiram metabolite ethylenethiourea (ETU) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.1/9*
	████ 2001a
	Reg.No. 146 099 - Acute toxicity study on the rainbow trout (Oncorhynchus mykiss WALBAUM 1792) in a static system (96 hours)
	2001/1001877
Guidelines:	*EPA 72-1, OECD 203, EEC 92/69 A V C 1*
GLP:	*yes*
	(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)

Executive Summary

In an acute toxicity laboratory study, juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to ethylenethiourea (ETU; metabolite of metiram) over a 96 h period in a static system. The nominal exposure concentrations were 0, 22, 50, 100, 220 and 500 mg ETU/L. Fish were observed for survival and symptoms of toxicity within 1, 4, 24, 48, 72 and 96 hours after start of exposure.

The results are based on nominal concentrations. After 96 h ethylenethiourea caused no mortality up to and including a concentration of 220 mg/L, 10% mortality occurred at the highest tested concentration of 500 mg/L. A single tumbling fish was observed in the highest concentration after 48 hours.

In a static acute toxicity study with ethylenethiourea (ETU; metabolite of metiram) the LC_{50} (96 h) for rainbow trout was determined to be > 500 mg/L based on nominal concentrations. The 96 h NOEC has been reported as 220 mg/L.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: Ethylenethiourea (ETU, Reg. No 146099; metabolite of metiram), batch-no. 01743-136, purity: 99.9%.

B. STUDY DESIGN

Test species: Rainbow trout (*Oncorhynchus mykiss* WALBAUM 1792), age: about 4 months; mean body length: 5.0 (4.7 - 5.5) cm; body weight: 1.0 (0.8 - 1.4) g, animal supplier: [redacted]

Test design: Static system (96 hours); 10 fish per aquarium, 2 replicates per concentration, assessment of mortality and symptoms of toxicity after 1, 4, 24, 48, 72 and 96 h.

Endpoints: LC_{50}, NOEC, sublethal effects.

Test concentrations: 0 (control), 22, 50, 100, 220 and 500 mg ethylenethiourea/L (nominal).

Test conditions: Glass aquaria with a stainless steel frame containing 25 L test solution, fish loading 0.4 g/L; temperature 11-12 °C; pH 8.2 - 8.6; oxygen content 8.3 - 10.3 mg/L, total hardness 2.5 mmol/L, photoperiod: 16 h light : 8 h dark; no feeding, no aeration.

Analytics: The test item concentrations were analyzed with a HPLC method.

Statistics: No statistical analysis was carried out since lethality was observed only in the highest test concentration.

II. RESULTS AND DISCUSSION

Analytical measurements: The analytically determined concentrations of ethylenethiourea (ETU) were in a range of 97.7% - 102.3% at test initiation and 99.0% - 103.1% at test termination. The following biological results are based on nominal concentrations.

Biological results: A mortality of 10% and sublethal symptoms were observed in the highest test concentration of 500 mg/L. The results are summarized in Table 8.2.1-8.

Table 8.2.1-8: Acute toxicity of ethylenethiourea on rainbow trout (*Oncorhynchus mykiss*)

Concentration [mg/L] nominal	Mortality after 96 h [%]	Symptoms
Control	0	None
22	0	None
50	0	None
100	0	None
220	0	None
500	10	Tumbling (1)
	Endpoints [mg/L] (nominal)	
LC_{50} (96 h)	> 500	
NOEC (96 h)	220	

III. CONCLUSION

In a static acute toxicity study with ethylenethiourea (ETU; metabolite of metiram) the LC_{50} (96 h) for rainbow trout was determined to be > 500 mg/L based on nominal concentrations. The 96 h NOEC has been reported as 220 mg/L.

New supplemental data (not evaluated previously on EU level):

The following acute toxicity study on the brook trout Salvelinus fontinalis performed with metiram (tested as BAS 222 28 F) has not been evaluated previously on EU level. The study was performed due to an authority request to refine a Species Sensitivity Distribution submitted in previous EU end-use product registrations.

Report: CA 8.2.1/10
[redacted] 2005a
BAS 222 28 F - Acute toxicity study on the brook trout (Salvelinus fontinalis) in a flow through system over 96 hours
2005/1006731

Guidelines: EPA 72-1, EPA 850.1075, OECD 203, EEC 92/69 A V C 1

GLP: yes
(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)

Executive Summary

In a flow-through acute toxicity laboratory study, juvenile brook trout (*Salvelinus fontinalis*) were exposed to metiram (tested with the solo-formulation BAS 222 28 F) at nominal concentrations of 0 (control), 0.22, 0.5. 1.0, 2.2 and 5.0 mg a.s./L (corresponding to mean measured concentrations of 0, 0.10, 0.456, 1.112, 2.225 and 5.098 mg a.s./L) in groups of 10 animals in glass aquaria containing 23 L water. Fish were observed for survival and symptoms of toxicity within 1 hour after start of exposure and 4, 24, 48, 72 and 96 h after start of exposure.

The biological results are based on mean measured concentrations. No mortality was observed in the control and at tested concentrations of up to and including 1.1 mg a.s./L. At the two highest tested concentrations of 2.2 mg a.s./L and 5.1 mg a.s./L, 30% and 50% mortality was observed, respectively. No symptoms of toxicity were observed in any of the test item treatment groups at test end after 96 h of exposure.

In a flow-through acute toxicity study with metiram (tested as BAS 222 28 F), the LC_{50} (96 h) for brook trout (*Salvelinus fontinalis*) was determined to be 4.5 mg a.s./L based on mean measured concentrations. The NOEC was 1.1 mg a.s./L (mean measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item:	BAS 222 28 F, batch no. 1627; content of a.s.: metiram (BAS 222 F; Reg. No 250284): 73.5% (nominal: 70%).

B. STUDY DESIGN

Test species:	Brook trout (*Salvelinus fontinalis*), approx. 3 months old; mean body length: 4.9 cm (4.4 - 5.6 cm), mean body weight: 0.94 g (0.61 - 1.43 g); supplied by [redacted]
Test design:	Flow-through system (96 hours); 10 fish per aquarium (loading 0.4 g fish/L) and per concentration, assessment of mortality and symptoms of toxicity within 1 hour after start of exposure and 4, 24, 48, 72 and 96 h after start of exposure.
Endpoints:	LC_{50}, NOEC, mortality and sub-lethal effects.
Test concentrations:	Control, 0.22, 0.5, 1.0, 2.2 and 5.0 mg a.s./L (nominal); corresponding to mean measured concentrations of 0, 0.10, 0.456, 1.112, 2.225 and 5.098 mg a.s./L.
Test conditions:	Glass aquaria with stainless steel frame (39 x 24 x 25 cm), test volume: 23 L, non-chlorinated, filtered tap water mixed with deionized water; temperature 13 °C ± 1 °C; pH 7.7 - 7.9; oxygen content 9.4 mg/L - 10.4 mg/L; total hardness: approx.100 mg $CaCO_3$/L, conductivity: approx.250 µS/cm, photoperiod : 16 h light : 8 h dark; light intensity: approx. 36 lux - 191 lux; no feeding, no aeration.
Analytics:	Analytical verification of test item concentrations was conducted using a GC-method with MS detection.
Statistics:	Descriptive statistics; Probit analysis for calculation of the LC_{50} value.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of the concentrations of the active substance was conducted in each concentration at the beginning and at the end of the test. The analytically determined concentrations of metiram ranged from 45.0% to 104.8% of nominal at test initiation and from 45.0% to 117.6% at test termination. The following biological results are based on mean measured concentrations.

Biological results: No mortality was observed in the control and at tested concentrations of up to and including 1.1 mg a.s./L. At the two highest tested concentrations of 2.2 mg a.s./L and 5.1 mg a.s./L, 30% and 50% mortality was observed, respectively. No symptoms of toxicity were observed in any of the test item treatment groups at test end after 96 h of exposure. The results are summarized in Table 8.2.1-9.

Table 8.2.1-9: Acute toxicity (96 h) of metiram (tested as BAS 222 28 F) on brook trout (*Salvelinus fontinalis*)

Concentration [mg a.s./L] (nominal)	**Control**	**0.22**	**0.5**	**1.0**	**2.2**	**5.0**
Concentration [mg a.s./L] (mean measured)	**--**	**0.10**	**0.456**	**1.112**	**2.225**	**5.098**
Mortality [%]	0	0	0	0	30	50
Symptoms	none	none	none	none	none	none
	Endpoints [mg a.s./L] (mean measured)					
LC_{50} (96 h)	**4.5**					
NOEC (96 h)	1.1					

* Calculated based on the analyzed amount of metiram in the formulation (73.5%)

III. CONCLUSION

In a flow-through acute toxicity study with metiram (tested as BAS 222 28 F), the LC_{50} (96 h) for brook trout (*Salvelinus fontinalis*) was determined to be 4.5 mg a.s./L based on mean measured concentrations. The NOEC was 1.1 mg a.s./L (mean measured).

The following acute toxicity study on the fathead minnow Pimephales promelas performed with metiram (tested as BAS 222 29 F) is not required for registration in the EU and it has not been evaluated previously on EU level. However, the study was conducted due to U.S. data requirements and it is provided for completeness.

Report: CA 8.2.1/11
[redacted] 2010a
Metiram: A 96-hour flow-through acute toxicity test with the fathead minnow (Pimephales promelas)
2010/7010878
Guidelines: EPA 850.1075, OECD 203
GLP: yes
(certified by United States Environmental Protection Agency)

Executive Summary

In a flow-through acute toxicity laboratory study, juvenile fathead minnow (*Pimephales promelas*) were exposed to a dilution water control, a solvent control and to metiram (tested with the solo-formulation BAS 222 29 F) at nominal concentrations of 0.104, 0.173, 0.288, 0.480 and 0.800 mg a.s./L (corresponding to mean measured concentrations of 0.0596, 0.114, 0.217, 0.360 and 0.607 mg a.s./L) in groups of 20 animals per concentration in stainless steel aquaria containing 15 L water. Fish were observed for survival and symptoms of toxicity 5, 24, 48, 72 and 96 h after start of exposure.

The biological results are based on mean measured concentrations. After 96 hours of exposure, no mortality occurred in the controls and in all test item concentrations over the whole study period. No symptoms of toxicity were observed in any of the test item treatment groups at test end after 96 h of exposure.

In a flow-through acute toxicity study with metiram (tested as BAS 222 29 F), the LC_{50} (96 h) for fathead minnow (*Pimephales promelas*) was determined to be > 0.607 mg a.s./L based on mean measured concentrations. The NOEC was ≥ 0.607 mg a.s./L (mean measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 29 F, batch no. 300013; content of a.s.: metiram (BAS 222 F; Reg. No 250284): 91.6% (nominal: 85%); density: 1.860 g/cm³.

B. STUDY DESIGN

Test species: Fathead minnow (*Pimephales promelas*); mean body length of dilution water control fish: 2.8 cm (2.6 - 3.0 cm), mean body weight of dilution water control fish: 0.20 g (0.16 - 0.25 g); supplied by [redacted]

Test design: Flow-through system (96 hours); 10 fish per aquarium (loading 0.13 g fish/L), 2 replicates per treatment, assessment of mortality and symptoms of toxicity 5, 24, 48, 72 and 96 h after start of exposure.

Endpoints: LC_{50}, NOEC, mortality and sub-lethal effects.

Test concentrations: Control (dilution water), solvent control (0.1 mL dimethylformamide/L), 0.104, 0.173, 0.288, 0.480 and 0.800 mg a.s./L (nominal), corresponding to mean measured concentrations of < LOQ, < LOQ, 0.0596, 0.114, 0.217, 0.360 and 0.607 mg a.s./L.

Test conditions: 25 L Teflon™ lined stainless steel aquaria, test volume: approx. 15 L, dilution water: filtered and aerated well water; flow rate: 10 volume additions per 24 hours on average in each test vessel; temperature 21.5 °C - 22.0 °C; pH 8.1 - 8.3; oxygen content 7.1 mg/L - 8.7 mg/L; total hardness: 146 mg $CaCO_3$/L, conductivity: 367 µS/cm, alkalinity: 182 mg $CaCO_3$/L, photoperiod : 16 h light : 8 h dark; light intensity: approx. 361 lux at test initiation.

Analytics: Analytical verification of test item concentrations was conducted using a GC-method with MS detection.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of the concentrations of the active substance was conducted in each concentration at the beginning, on day 2 and at the end of the test. The analytically determined concentrations of metiram ranged from 57% to 79% of nominal at test initiation and from 55% to 81% at test termination. The following biological results are based on mean measured concentrations.

Biological results: After 96 hours of exposure, no mortality occurred in the controls and in all test item concentrations. No symptoms of toxicity were observed in any of the test item treatment groups at test end after 96 h of exposure. The results are summarized in **Table 8.2.1-10**.

Table 8.2.1-10: Acute toxicity (96 h) of metiram (tested as BAS 222 29 F) on fathead minnow (*Pimephales promelas*)

Concentration [mg a.s./L] (nominal)	**Control**	**Solvent control**	**0.104**	**0.173**	**0.288**	**0.480**	**0.800**
Concentration [mg a.s./L] (mean measured)	**--**	**--**	**0.0596**	**0.114**	**0.217**	**0.360**	**0.607**
Mortality [%]	0	0	0	0	0	0	0
Symptoms	none	none	none	none	none	none	none
Endpoints [mg a.s./L] (mean measured)							
LC_{50} (96 h)	> 0.607						
NOEC (96 h)	≥ 0.607						

III. CONCLUSION

In a flow-through acute toxicity study with metiram (tested as BAS 222 29 F), the LC_{50} (96 h) for fathead minnow (*Pimephales promelas*) was determined to be > 0.607 mg a.s./L based on mean measured concentrations. The NOEC was ≥ 0.607 mg a.s./L (mean measured).

The following acute toxicity study on the sheepshead minnow Cyprinodon variegatus performed with metiram (tested as BAS 222 29 F) is not required for registration in the EU and it has not been evaluated previously on EU level. However, the study was conducted due to U.S. data requirements and it is provided for completeness.

Report: CA 8.2.1/12
[redacted] 2010b
Metiram: A 96-hour flow-through acute toxicity test with the sheepshead minnow (Cyprinodon variegatus)
2010/7010879
Guidelines: EPA 850.1075
GLP: yes
(certified by United States Environmental Protection Agency)

Executive Summary

In a flow-through acute toxicity laboratory study, juvenile sheepshead minnow (*Cyprinodon variegatus*) were exposed to a dilution water control, a solvent control and to metiram (tested with the solo-formulation BAS 222 29 F) at nominal concentrations of 0.050, 0.100, 0.200, 0.400 and 0.800 mg a.s./L (corresponding to mean measured concentrations of 0.0264, 0.0592, 0.163, 0.335 and 0.673 mg a.s./L) in groups of 20 animals per concentration in stainless steel aquaria containing 15 L water. Fish were observed for survival and symptoms of toxicity 6, 24, 48, 72 and 96 h after start of exposure.

The biological results are based on mean measured concentrations. After 96 hours of exposure, no mortality occurred in the controls and in all test item concentrations. No symptoms of toxicity were observed in any of the test item treatment groups at test end after 96 h of exposure.

In a flow-through acute toxicity study with metiram (tested as BAS 222 29 F), the LC_{50} (96 h) for sheepshead minnow (*Cyprinodon variegatus*) was determined to be > 0.673 mg a.s./L based on mean measured concentrations. The NOEC was ≥ 0.673 mg a.s./L (mean measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 29 F, batch no. 300013; content of a.s.: metiram (BAS 222 F; Reg. No 250284): 91.6% (nominal: 85%); density: 1.860 g/cm³.

B. STUDY DESIGN

Test species: Sheepshead minnow (*Cyprinodon variegatus*); mean body length of dilution water control fish: 2.4 cm (2.0 - 3.0 cm), mean body weight of dilution water control: 0.24 g (0.11 - 0.42 g); supplied by [redacted]

Test design: Flow-through system (96 hours); 10 fish per aquarium (loading 0.16 g fish/L), 2 replicates per treatment, assessment of mortality and symptoms of toxicity 6, 24, 48, 72 and 96 h after start of exposure.

Endpoints: LC_{50}, NOEC, mortality and sub-lethal effects.

Test concentrations: Control (dilution water), solvent control (0.1 mL dimethylformamide/L), 0.050, 0.100, 0.200, 0.400 and 0.800 mg a.s./L (nominal), corresponding to mean measured concentrations of < limit of quantification (LOQ), < LOQ, 0.0264, 0.0592, 0.163, 0.335 and 0.673 mg a.s./L.

Test conditions: 25 L Teflon™ lined stainless steel aquaria, test volume: approx. 15 L, dilution water: filtered and aerated natural sea water; salinity appr. 20‰; flow rate: 10 volume additions per 24 hours on average in each test vessel; temperature 21.5 °C - 22.0 °C; pH 8.0 - 8.1; oxygen content 6.7 mg/L - 7.5 mg/L; photoperiod : 16 h light : 8 h dark; light intensity: approx. 397 lux at test initiation; no feeding, no aeration.

Analytics: Analytical verification of test item concentrations was conducted using a GC-method with MS detection.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of the concentrations of the active substance was conducted in each concentration at the beginning, on day 2 and at the end of the test. The analytically determined concentrations of metiram ranged from 60% to 97% of nominal at test initiation and from 45% to 87% at test termination. The following biological results are based on mean measured concentrations.

Biological results: After 96 hours of exposure, no mortality occurred in the controls and in all test item concentrations. No symptoms of toxicity were observed in any of the test item treatment groups at test end after 96 h of exposure. The results are summarized in Table 8.2.1-11.

Table 8.2.1-11: Acute toxicity (96 h) of metiram (tested as BAS 222 29 F) on sheepshead minnow (*Cyprinodon variegatus*)

Concentration [mg a.s./L] (nominal)	**Control**	**Solvent control**	**0.050**	**0.100**	**0.200**	**0.400**	**0.800**
Concentration [mg a.s./L] (mean measured)	**--**	**--**	**0.0264**	**0.0592**	**0.163**	**0.335**	**0.673**
Mortality [%]	0	0	0	0	0	0	0
Symptoms	none	none	none	none	none	none	none
Endpoints [mg a.s./L] (mean measured)							
LC_{50} (96 h)	> 0.673						
NOEC (96 h)	≥ 0.673						

III. CONCLUSION

In a flow-through acute toxicity study with metiram (tested as BAS 222 29 F), the LC_{50} (96 h) for sheepshead minnow (*Cyprinodon variegatus*) was determined to be > 0.673 mg a.s./L based on mean measured concentrations. The NOEC was ≥ 0.673 mg a.s./L (mean measured).

***The following acute toxicity study on the bluegill sunfish Lepomis macrochirus performed with the metiram metabolite Ethylenethiourea is not required for registration in the EU and it has not been evaluated previously on EU level. However, the study was conducted due to U.S. data requirements and it is provided for completeness*.**

Report:	CA 8.2.1/13 [redacted] 2008a Ethylenethiourea - Acute toxicity to bluegill sunfish (Lepomis macrochrius) under static conditions, following OPPTS draft guideline 850.1075 2008/7015434
Guidelines:	EPA 850.1075
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

In a static acute toxicity laboratory study, bluegill sunfish were exposed to a dilution water control and to ethylenethiourea (metabolite of metiram) at nominal concentrations of 63, 130, 250, 500 and 1000 mg/L (corresponding to mean measured concentrations of 60, 120, 240, 470 and 990 mg/L) in groups of 10 animals in glass aquaria containing 15 L water, with two replicates per treatment. Fish were observed for survival and symptoms of toxicity at test initiation and 6, 24, 48, 72 and 96 hours after start of exposure.

The biological results are based on the mean measured concentrations. After 96 hours of exposure, no mortalities or adverse effects were observed in the control and at any test item concentration tested.

In a static acute toxicity study with bluegill sunfish the LC_{50} (96 h) of ethylenethiourea (metabolite of metiram) was > 990 mg/L, based on mean measured concentrations. The NOEC (96 h) was determined to be ≥ 990 mg/L (mean measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: Ethylenethiourea (BF 222-ETU, Reg. No. 146099; metabolite of metiram), ID no. TSN030606-0001; Lot. No. 04816CH; purity: 100%.

B. STUDY DESIGN

Test species: Bluegill sunfish (*Lepomis macrochirus*), SSL Lot no. 08A22; mean body length 31 mm (27 - 36 mm); mean body weight 0.43 g (0.29 - 0.69 g); supplied by [redacted]

Test design: Static system (96 hours); 10 fish per aquarium (loading 0.29 g fish/L); 2 replicates per treatment; assessment of mortality and symptoms of toxicity at test initiation and 6, 24, 48, 72 and 96 hours after start of exposure.

Endpoints: LC_{50}, NOEC, mortality and sub-lethal effects.

Test concentrations: Control, 63, 130, 250, 500 and 1000 mg ethylenethiourea/L (nominal), corresponding to 0, 60, 120, 240, 470 and 990 mg/L (mean measured).

Test conditions: Glass aquaria (39 x 20 x 25 cm), test volume: 15 L, non-chlorinated, well water; temperature: 21 - 22 °C; pH 6.6 - 7.9; oxygen content: 6.6 mg/L - 9.4 mg/L; total hardness: 38 - 44 mg $CaCO_3$/L; specific conductivity: 170 µmhos/cm; photoperiod 16 h light : 8 h dark; light intensity: 360 lux - 540 lux; no aeration, no feeding.

Analytics: Analytical verification of test item concentrations was conducted using a HPLC-method with UV detection.

Statistics: Descriptive statistics; Kruskal-Wallis'Test for determination of the NOEC.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of the test item was conducted in each concentration at the beginning of the test, 48 h after start of exposure and at the end of the test. Mean measured concentrations of ethylenethiourea ranged from 94% to 99% of nominal concentrations. The following biological results are based on mean measured concentrations.

Biological results: After 96 hours of exposure, no mortalities or adverse effects were observed in the control and at any test item concentration tested. The results are summarized in Table 8.2.1-12.

Table 8.2.1-12: Acute toxicity (96 h) of ethylenethiourea (metabolite of metiram) on bluegill sunfish (*Lepomis macrochirus*)

Concentration [mg/L] (nominal)	**Control**	**63**	**130**	**250**	**500**	**1000**
Concentration [mg/L] (mean measured)	**--**	**60**	**120**	**240**	**470**	**990**
Mortality [%]	0	0	0	0	0	0
Symptoms	none	none	none	none	none	none
	Endpoints [mg ethylenethiourea/L] (mean measured)					
LC_{50} (96 h)	> 990					
NOEC (96 h)	≥ 990					

III. CONCLUSION

In a static acute toxicity study with bluegill sunfish the LC_{50} (96 h) of ethylenethiourea (metabolite of metiram) was > 990 mg/L, based on mean measured concentrations. The NOEC (96 h) was determined to be ≥ 990 mg/L (mean measured).

The following acute toxicity study on the sheepshead minnow Cyprinodon variegatus performed with the metiram metabolite ethylenethiourea is not required for registration in the EU and it has not been evaluated previously on EU level. However, the study was conducted due to U.S. data requirements and it is provided for completeness.

Report: CA 8.2.1/14
[redacted] 2008b
Ethylenethiourea - acute toxicity to sheepshead minnow (cyprinodon variegatus) under static conditions, following OPPTS guideline 850.1075
2008/7015440
Guidelines: EPA 850.1075
GLP: yes
(certified by United States Environmental Protection Agency)

Executive Summary

In a static acute toxicity laboratory study, sheepshead minnow (*Cyprinodon variegatus*) were exposed to a dilution water control and to ethylenethiourea (ETU, metabolite of metiram) at nominal concentrations of 63, 130, 250, 500 and 1000 mg/L (corresponding to mean measured concentrations of 55, 110, 230, 470 and 900 mg/L) in groups of 10 animals in glass aquaria containing 15 L water, with two replicates per treatment. Fish were observed for survival and symptoms of toxicity at test initiation and 6, 24, 48, 72 and 96 hours after start of exposure.

The biological results are based on mean measured concentrations. After 96 hours of exposure, no mortality occurred in the controls and in all test item concentrations. No symptoms of toxicity were observed in any of the test item treatment groups at test end after 96 h of exposure.

In a static acute toxicity study with sheepshead minnow (*Cyprinodon variegatus*) the LC_{50} (96 h) of ETU (metabolite of metiram) was > 900 mg/L, based on mean measured concentrations. The NOEC (96 h) was determined to be ≥ 900 mg/L (mean measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: Ethylenethiourea (BF 222-ETU, Reg. No. 146099; metabolite of metiram), Lot. No. 04816CH; purity: 100%.

B. STUDY DESIGN

Test species: Sheepshead minnow (*Cyprinodon variegatus*); SSL Lot no. 08A05; mean body length: 2.4 cm (1.6 - 3.0 cm), mean body weight: 0.28 g (0.08 - 0.55 g); supplied by [redacted]

Test design: Static system (96 hours); 10 fish per aquarium (loading 0.19 g fish/L); 2 replicates per treatment; assessment of mortality and symptoms of toxicity at test initiation and 6, 24, 48, 72 and 96 hours after start of exposure.

Endpoints: LC_{50}, NOEC, mortality and sub-lethal effects.

Test concentrations: Control (dilution water), 63, 130, 250, 500 and 1000 mg ETU/L (nominal), corresponding to mean measured concentrations of < LOQ, 55, 110, 230, 470 and 900 mg/L.

Test conditions: Glass aquaria (39 x 20 x 25 cm), test volume: 15 L, dilution water: natural filtered sea water (salinity: 20 - 22‰); temperature 21 - 22 °C; pH 7.4 - 7.8; oxygen content 4.8 mg/L - 7.7 mg/L; photoperiod: 16 h light : 8 h dark; light intensity: approx. 440 - 570 lux; no feeding, slight aeration from 72 h onwards.

Analytics: Analytical verification of test item concentrations was conducted using a HPLC-method with UV detection.

Statistics: Descriptive statistics; Kruskal-Wallis'Test for determination of the NOEC.

II. RESULTS AND DISCUSSION

Analytical verification of the test item concentration was conducted in each concentration at the beginning of the test, 48 h after start of exposure and at the end of the test. Mean measured concentrations of ETU ranged from 85% to 92% of nominal at test initiation, from 83% to 90% after 48 h and from 85% to 98% at test termination. The following biological results are based on mean measured concentrations.

Biological results: After 96 hours of exposure, no mortality occurred in the controls and in all test item concentrations. No symptoms of toxicity were observed in any of the test item treatment groups at test end after 96 h of exposure. The results are summarized in Table 8.2.1-13.

Table 8.2.1-13: Acute toxicity (96 h) of ETU (metabolite of metiram) on sheepshead minnow (*Cyprinodon variegatus*)

Concentration [mg/L] (nominal)	**Control**	**63**	**130**	**250**	**500**	**1000**
Concentration [mg/L] (mean measured)	**--**	**55**	**110**	**230**	**470**	**900**
Mortality [%]	0	0	0	0	0	0
Symptoms	none	none	none	none	none	none
	Endpoints [mg ETU/L] (mean measured)					
LC_{50} (96 h)	> 900					
NOEC (96 h)	≥ 900					

III. CONCLUSION

In a static acute toxicity study with sheepshead minnow (*Cyprinodon variegatus*) the LC_{50} (96 h) of ETU (metabolite of metiram) was > 900 mg/L, based on mean measured concentrations. The NOEC (96 h) was determined to be ≥ 900 mg/L (mean measured).

CA 8.2.2 Long-term and chronic toxicity to fish

Data already submitted during previous Annex I inclusion process (already evaluated on EU level):

The following chronic toxicity study on rainbow trout (Oncorhynchus mykiss) performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.2/1* *[redacted] 1990a* *Sublethal toxic effects of Polyram WG (= BAS 222 28 F) on rainbow trout (Oncorhynchus mykiss WALBAUM 1792) in a flow-through system (28 days); OECD 204* *1990/10319*
Guidelines:	*OECD 204*
GLP:	*yes* *(certified by Ministerium fuer Umwelt und Gesundheit, Postfach 3160, 6500 Mainz)*

Report:	*CA 8.2.2/2* *[redacted] 1990a* *Amendment to the report of May 28, 1990 on the study: Sublethal toxic effects of Polyram WG (= BAS 222 28 F) on rainbow trout (Oncorhynchus mykiss WALBAUM 1792) in a flow-through system (28 days); OECD 204* *1990/10320*
Guidelines:	*OECD 204*
GLP:	*yes* *(certified by Ministerium fuer Umwelt und Gesundheit, Postfach 3160, 6500 Mainz)*

Executive Summary

The chronic toxicity of BAS 222 28 F to rainbow trout (*Oncorhynchus mykiss*) was evaluated in a 28-day juvenile growth test under flow-through conditions. Fish were exposed to a control and 0.001, 0.010, 0.0316 and 0.100 mg BAS 222 28 F/L (nominal). Mortality was assessed daily and symptoms of toxicity daily during the week. Body weight and body length were determined at the end of the study.

The results are based on nominal concentrations. No mortality occurred up to and including 0.0316 mg BAS 222 28 F/L. One fish (5%) died at the highest concentration of 0.100 mg/L. Slight discoloration was observed at 0.100 mg/L, but only at the beginning of the study. No statistically significant effects on body weight and body length were observed up to and including 0.100 mg/L.

In a juvenile growth test conducted under flow-through conditions, rainbow trout were exposed to BAS 222 28 F over a 28-day period. The overall NOEC (survival, toxic signs, growth parameters) was 0.032 mg/L (0.024 mg a.s./L) based on nominal concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch No. 90-1, content of a.s.: metiram (BAS 222 F; Reg. No 250284): 74.8% analyzed (70% nominal).

B. STUDY DESIGN

Test species: Rainbow trout (*Oncorhynchus mykiss* WALBAUM 1792), age approx. 5 months; body weight: 7.9 (7.5 - 8.5) g, body length: 8.7 (8.0 - 9.2) cm, animal supplier: [redacted]

Test design: Flow-through system (28 d); 20 fish per concentration and control (1 replicate). Daily assessment of mortality; assessment of toxic symptoms daily during the week; determination of weight and length of fish at test termination.

Endpoints: NOEC values based on mortality, toxic signs, body weight and body length.

Test concentrations: Control, 0.001, 0.010, 0.0316 and 0.100 mg BAS 222 28 F/L (nominal).

Test conditions: Aquaria (water volume 60 L), dilution water: purified drinking water, total hardness: about 2.4 mmol/L, temperature 15 - 18 °C; pH 7.6 - 7.9; oxygen content: 8.2 - 10.5 mg/L; flow rate: 10 L/hour/test aquarium; photoperiod: 16 hours light: 8 hours darkness, feeding with commercial fish diet (Ssniff trout starter); continuous aeration with oil-free air.

Analytics: The test item concentrations were analyzed with a GC method (GC with FPD; 399 nm)

Statistics: Descriptive statistics; ANOVA followed by Dunnett´s test for evaluation of body weight and body length.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical measurements were conducted for the 0.100 mg/L test concentration. Mean concentration of the test substance were within 70 – 200% of nominal concentration. Mean recovery in the fortified sample of 0.100 mg/L nominal concentration was 88.4 - 97.0%. The biological results based on nominal concentrations.

Biological results: No mortality occurred up to and including 0.0316 mg BAS 222 28 F/L. One fish (5%) died in the highest treatment of 0.100 mg/L. Slight discoloration was observed at 0.100 mg/L only at the beginning of the study. No statistically significant effects on body weight and body length were observed up to and including 0.100 mg/L (Dunnett's test). The results are summarized in Table 8.2.2-1.

Table 8.2.2-1: Chronic toxicity of BAS 222 28 F on rainbow trout (*Oncorhynchus mykiss*)

Concentration [mg/L] nominal	**Control**	**0.001**	**0.010**	**0.0316**	**0.100**
Mortality (28 d) [%]	0	0	0	0	5
Toxic signs	none	none	none	none	Slight discoloration
Mean body weight (28 d) [g]	22.10	22.32	22.95	22.24	21.96
Mean body length (28 d) [cm]	12.53	12.70	12.63	12.71	12.23

	Endpoints (nominal)	
	based on BAS 222 28 F [mg/L]	**based on BAS 222 F [mg a.s./L]**
NOEC (survival)	0.0316	0.0236
NOEC (growth)	0.100	0.0748
NOEC (toxic signs)	0.0316	0.0236
NOEC (overall)	**0.0316**	**0.0236**

III. CONCLUSION

In a juvenile growth test conducted under flow-through conditions, rainbow trout were exposed to BAS 222 28 F over a 28-day period. The overall NOEC (survival, toxic signs, growth parameters) was 0.032 mg/L (0.024 mg a.s./L) based on nominal concentrations.

The following 28 d fish outdoor mesocosm study on rainbow trout (Oncorhynchus mykiss) performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.2/3*
	█████, 2002a
	Effect of multiple applications of BAS 222 28 F on the rainbow trout (Oncorhynchus mykiss) in a chronic, juvenile growth test
	2002/1005264
Guidelines:	*OECD 215*
GLP:	*yes*
	(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)

Executive Summary

The effect of multiple applications of BAS 222 28 F on rainbow trout (*Oncorhynchus mykiss*) was investigated in a chronic, juvenile growth test under more realistic conditions over 28 days. The test was performed in small mesocosms with 4 applications of BAS 222 28 F at a 7 day spraying interval. Fish were exposed to a control and 0.032, 0.100, 0.316, 1.0 and 3.16 mg BAS 222 28 F/L (nominal). Mortality and visual symptoms were assessed daily. Body weight and body length were determined at the end of the study.

The results are based on nominal concentrations. BAS 222 28 F caused 8.3% mortality at a concentration of 0.032 mg/L. At a rate of 0.316 mg/L fish mortality was 12.5%. At treatment rates of 1 mg/L and higher all fish had died until study termination. A statistically significant effect on the mean body length was observed in the group exposed to 0.316 mg/L when compared to the control (Dunnett's test, $p \leq 0.05$). No statistically significant differences on the body weight were observed in any treatment group compared to the control. Reduced food consumption was observed in one replicate of the 0.316 mg/L treatment from DAT 8 - DAT 13. No other symptoms were observed in any of the treatments.

In a juvenile growth test with multiple applications conducted in small mesocosms, rainbow trout were exposed to BAS 222 28 F over a 28-day period. The overall NOEC (survival, toxic signs, growth parameters) was 0.10 mg/L (corresponding to 0.07 mg a.s./L) based on nominal concentrations. The LC_{50} was 0.40 mg/L (0.27 mg a.s./L).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch No. 2000-2, content of a.s.: metiram (BAS 222 F; Reg. No 250284): 68.5% analyzed (70% nominal).

B. STUDY DESIGN

Test species: Rainbow trout (*Oncorhynchus mykiss*), age: approx. 3 months; body weight: 1.66 (1.25 - 2.04) g, body length: 5.4 (4.7 - 6.0) cm, animal supplier: [redacted]

Test design: Small outdoor mesocosms with c. 460 L water, test duration: 28 d; 4 applications with a 7-day spraying interval; 12 fish per replicate, 2 replicates/concentration, 4 replicates for the control. Daily assessment of mortality and visual symptoms, determination of weight and length of fish at test termination.

Endpoints: NOEC, LOEC, LC_{50}, based on mortality, toxic signs, body weight and body length.

Test concentrations: Control, 0.032, 0.100, 0.316, 1.0 and 3.16 mg BAS 222 28 F/L, corresponding to 0, 0.022, 0.069, 0.216, 0.685 and 2.165 mg a.s./L.

Test conditions: Quadratic stainless steel basins (0.96 m x 0.96 m x 0.6 m), approx. 2 cm natural sediment layer, about 50 cm natural water layer; temperature 4.7 - 19.8 °C; pH 8.27 - 9.56; oxygen content: 68.2% - 111.9%; conductivity: 427 - 547 µS/cm; total hardness: 1.77 - 2.35 mmol/L; feeding with commercial fish diet (Forellenfutter, Zeigler); from day 2 ventilation of basins.

Analytics: The test item concentrations were analyzed with GC/MS method CP 402 and HPLC method 373/2 with UV detection.

Statistics: Standard procedures; ANOVA, Dunnett test for determination of NOEC and LOEC (body weight, body length); probit analysis for determination of LC_{50}; Fisher´s exact test for determination of NOEC (mortality).

II. RESULTS AND DISCUSSION

Analytical measurements: The measured concentration of metiram in the stock solutions ranged from 93.7% - 135.8%, thus confirmed the nominal data. Measured concentrations of the analyzed water samples shortly after application ranged from 73.1% - 112.7% of nominal. Measured concentrations shortly before the new application ranged from 1.6% - 35.8% of the initial concentration, demonstrating the rapid degradation of metiram. The concentration of the metabolite ETU did not increase with the applications and remained at a level of 0.16 mg/L. As the amounts of test compound introduced into the basins were well documented and confirmed the correct application of test substance, the biological results are based on nominal initial peak concentrations.

Biological results: At a rate of 0.316 mg/L fish mortality was 12.5%. At treatment rates of 1 mg/L and higher all fish had died until study termination. A statistically significant effect on the mean body length was observed in the group exposed to 0.316 mg/L when compared to the control (Dunnett's test, $p \leq 0.05$). No statistically significant differences on the body weight were observed in any treatment group compared to the control. Reduced food consumption was observed in one replicate of the 0.316 mg/L treatment from DAT 8 - DAT 13. No other symptoms were observed in any of the treatments. The results are summarized in Table 8.2.2-2.

Table 8.2.2-2: Chronic toxicity of BAS 222 28 F on rainbow trout (*Oncorhynchus mykiss*)

Concentration (nominal) [mg BAS 222 28 F/L]	**Control**	**0.032**	**0.100**	**0.316**	**1.000**	**3.16**
Concentration [mg a.s./L] nominal	**Control**	**0.022**	**0.069**	**0.216**	**0.685**	**2.165**
Mortality (28 d) [%]	0	8.3	0	12.5*	100**	100**
Toxic signs (during the study)	none	none	none	Reduced food consumption	--	--
Mean body weight (28 d) [g]	3.98	4.17	4.09	3.55	--	--
Mean body length (28 d) [cm]	7.13	7.29	7.22	6.79*	--	--

	Endpoints (nominal)	
	based on BAS 222 28 F [mg/L]	**based on BAS 222 F [mg a.s./L]**
NOEC (28 d)	**0.10**	**0.07**
LOEC (28 d)	**0.32**	**0.22**
LC_{50} (28 d)	**0.40**	**0.27**

* Statistically significant compared to control (Fisher´s exact test (mortality), Dunnett test (length), $p \leq 0.05$)
** Statistically significant compared to control (Fisher´s exact test (mortality), $p \leq 0.01$)

III. CONCLUSION

In a fish juvenile growth test conducted in small outdoor mesocosms, rainbow trout were exposed to multiple (4) applications of BAS 222 28 F over a 28-day period. The overall NOEC (survival, toxic signs, growth parameters) was 0.10 mg/L (corresponding to 0.07 mg a.s./L) based on nominal concentrations. The LC_{50} was 0.40 mg/L (0.27 mg a.s./L).

CA 8.2.2.1 Fish early life stage toxicity test

New supplemental data (not evaluated previously on EU level):

The following early life-stage test on fathead minnow (Pimephales promelas) performed with metiram (tested as BAS 222 29 F) is not required for registration in the EU and it has not been evaluated previously on EU level. The study was conducted due to U.S. data requirements and is provided for completeness.

Report:	CA 8.2.2.1/1
	[redacted] 2010c
	Metiram: An early life-stage toxicity test with the fathead minnow (Pimephales promelas)
	2010/7012795
Guidelines:	OECD 210, EPA 850.1400
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

The chronic toxicity of metiram to fathead minnow (*Pimephales promelas*) was evaluated in a 33-day early life-stage test under flow-through conditions. Embryos were exposed to a dilution water control, a solvent control and to metiram (tested with the solo-formulation BAS 222 29 F) at nominal concentrations of 0.0031, 0.0063, 0.013, 0.025 and 0.050 mg a.s./L (corresponding to mean measured concentrations of 0.0016, 0.0032, 0.0063, 0.013 and 0.021 mg a.s./L). Hatchability, survival rate and behavior of fathead minnow embryos and fry were assessed throughout the study. Individual fish lengths and weights were measured at test termination.

The results are based on mean measured concentrations. Egg hatch was complete on day 5 in the control groups and all test item treatments. There was no statistically significant decrease in hatching success in any treatment group compared to the pooled controls. Post-hatch survival of larvae was statistically significantly reduced compared to the pooled control at the highest test item concentration of 0.021 mg a.s./L. The statistically significant reductions in growth in the 0.0016 and 0.0063 mg a.s./L treatments were not considered to be treatment-related as they were not dose-responsive. Sublethal effects, observed as fish exhibiting morphological deformations (crooked spine) or smaller appearance, were noted at all tested concentrations of metiram with the highest frequency in the 0.021 mg a.s./L treatment.

In an early life-stage study with fathead minnow (*Pimephales promelas*) the overall NOEC (33 d) for metiram (tested as BAS 222 29 F) was determined to be 0.013 mg a.s./L, based on mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 29 F, batch no. 300013; content of a.s.: metiram (BAS 222 F; Reg. No 250284): 91.6% (nominal: 85%); density: 1.860 g/cm³.

B. STUDY DESIGN

Test species: Fathead minnow (*Pimephales promelas*), eggs less than 24 hours at test initiation, source: [redacted]

Test design: Flow-through system (33 d); 5 test item concentrations plus a dilution water control and a solvent control; 4 replicate test chambers per treatment with 20 fertilized eggs in each; a proportional diluter system was used for continuous introduction of the solutions to the test chambers. During the embryo stage, the developing embryos were incubated in glass cups. At the end of hatch (day 5), fish were released into the test chamber. Daily assessment of hatch, survival, signs of toxicity and abnormal behavior. At test termination surviving animals were sacrificed and measured for length and weight.

Endpoints: NOEC values based on hatchability, survival, toxic signs and growth.

Test concentrations: Control (dilution water); solvent control (0.1 mL dimethylformamide/L); 0.0031, 0.0063, 0.013, 0.025 and 0.050 mg a.s./L (nominal), corresponding to mean measured concentrations of 0.0016, 0.0032, 0.0063, 0.013 and 0.021 mg a.s./L.

Test conditions: Test vessels: 9 L glass aquaria with a test volume of approx. 7 L; 4 replicate test chambers; glass incubation cups (used during embryo stage, approximately 50 mm diameter) closed on one end with nylon screen mesh; one incubation cup per test chamber; filtered and aerated well water; temperature: 25.0°C - 25.6°C; pH 7.9 - 8.2; oxygen content: 7.2 mg/L - 8.2 mg/L; total hardness: 136 - 148 mg $CaCO_3$/L; alkalinity: 176 - 186 mg $CaCO_3$/L; conductivity: 333 - 381 µmhos/cm; light intensity: 169 lux at test initiation; photoperiod: 16 hours light : 8 hours dark; flow rate: from test initiation to day 12: approx. 8 volume additions per 24 hours per vessel, from day 12 onwards: 15 volume additions per 24 hours per vessel; feeding: newly-hatched larvae were fed 2-3 times daily *ad libitum* live brine shrimp nauplii (*Artemia sp.*) until 2 days before study termination.

Analytics: Analytical verification of test item concentrations was conducted using a GC-method with MS detection.

Statistics: Descriptive statistics; t-test for comparison of the dilution water control and solvent control data ($p \leq 0.05$); Fisher's Exact test with Bonferroni-Holmes correction ($p \leq 0.05$) to calculate NOEC values for hatching success and survival data; ANOVA followed by Dunnett's test ($p \leq 0.05$) to calculate NOEC values for growth data.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of the test item concentrations was conducted in all concentrations at test initiation, at regular intervals during the study (day 7, 14, 21 and 28) and at test end. Additionally, samples from the 0.0031 and 0.050 mg a.s./L nominal treatments were collected to determine the potential impact of centrifugation on measured concentrations of metiram. Because the measured concentrations of the centrifuged and uncentrifuged samples were approximately the same, the mean results of the centrifuged samples were reported. Recoveries of metiram ranged from 47% to 76% of nominal concentrations in all treatments at test initiation and from 28% to 45% of nominal at test termination. Since the 0.0031 mg a.s./L nominal treatment level was below the limitation of the method, the analytical results for this treatment level were not reliable. However, it was well below the no-observed-effect concentration (NOEC) of the study and had no impact on the interpretation of the study results. The following biological results are based on mean measured concentrations.

Biological results: No statistically significant differences were determined between the control and the solvent control except for mean body length and dry weight data (t-test, $p \leq 0.05$). As the difference in mean total lengths between the two controls was slight and not biologically meaningful, the data from both control groups were pooled and the pooled data set was used for statistical evaluation of treatment related effects. However, regarding the dry weight data treatment groups were compared to the data from both the negative and solvent control groups. Egg hatch was complete on day 5 in the control groups and all test item treatments. There was no statistically significant decrease in hatching success in any treatment group compared to the pooled controls (Fisher's Exact test, $p \leq 0.05$). Post-hatch survival of larvae was statistically significantly reduced compared to the pooled control at the highest test item concentration of 0.021 mg a.s./L (Fisher's Exact Test with Bonferroni-Holmes correction, $p \leq 0.05$). The statistically significant reductions in growth in the 0.0016 and 0.0063 mg a.s./L treatments (Dunnett's test, $p \leq 0.05$) were not considered to be treatment-related as they were not dose-responsive. Sublethal effects, observed as fish exhibiting morphological deformations (crooked spine) or smaller appearance, were noted at all tested concentrations of metiram with the highest frequency in the 0.021 mg a.s./L treatment. The results are summarized in Table 8.2.2.1-1.

Table 8.2.2.1-1: Chronic toxicity of metiram (tested as BAS 222 29 F) to fathead minnow (*Pimephales promelas*) in an fish early life-stage test (33 d)

Concentration [mg a.s./L] (nominal)	**Control**	**Solvent control**	**0.0031**	**0.0063**	**0.013**	**0.025**	**0.050**
Concentration [mg a.s./L] (mean measured)	**--**	**--**	**0.0016**	**0.0032**	**0.0063**	**0.013**	**0.021**
Embryo survival at hatch on day 5 [%]	98	99	98	99	100	100	100
Survival of larvae 28 days post hatch [%]	92	92	88	95	97	94	65 *
Mean total length ± Std. Dev. (28 d post hatch) [mm]	24.1 ± 0.42	23.6 ± 0.17	23.1 ± 0.34 +, 2)	23.6 ± 0.13	22.9 ± 0.52 +, 2)	23.7 ± 0.13	22.8 ± 0.32 1)
Mean wet weight ± Std. Dev. (28 d post hatch) [mg]	105.2 ± 3.9	100.6 ± 2.7	95.2 ± 2.1 +, 2)	102.0 ± 1.6	96.1 ± 1.7 +, 2)	106.2 ± 4.3	95.9 ± 4.8 1)
Mean dry weight ± Std. Dev. (28 d post hatch) [mg]	19.9 ± 0.80	18.6 ± 0.41	18.0 ± 0.48 #, 2)	19.0 ± 0.33	17.9 ± 0.67 #, 2)	19.7 ± 0.62	17.8 ± 0.45 1)
	Endpoints [mg metiram/L] (mean measured)						
$NOEC_{overall}$ (33 d)	0.013						

* Statistically significant differences compared to the pooled control (Fisher's Exact Test with Bonferroni-Holmes correction, $p \leq 0.05$).
+ Statistically significant differences compared to the pooled control (Dunnett's test, $p \leq 0.05$).
# Statistically significant differences compared to the dilution water control, (Dunnett's test, $p \leq 0.05$).
1) The 0.021 mg a.s./L treatment group was excluded from analyses of growth due to a significant effect on survival.
2) Statistically significant reductions in growth detected in the 0.0016 and 0.0063 mg a.s./L treatment groups in comparison to the control groups were not dose-responsive since no significant reductions were detected in the 0.013 mg a.s./L treatment group in comparison to the control groups.

III. CONCLUSION

In an early life-stage study with fathead minnow (*Pimephales promelas*) the overall NOEC (33 d) for metiram (tested as BAS 222 29 F) was determined to be 0.013 mg a.s./L, based on mean measured concentrations.

CA 8.2.2.2 Fish full life cycle test

No study required; thus, this point is not addressed *via* (new) toxicity studies.

CA 8.2.2.3 Bioconcentration in fish

The log P_{ow} of the active substance metiram was determined to be < 3 (see EC Review Report for metiram; SANCO/4059/2001-rev 3.3, June 2005). Hence, the accumulation potential of metiram in aquatic non-target organisms is considered to be low and no bioconcentration studies are required; thus, this point is not addressed *via* (new) toxicity studies.

CA 8.2.3 Endocrine disrupting properties

Currently there are no final criteria from the European Comission which are defining endocrine disrupting properties for ecotoxicology. The active substance metiram is not fulfilling the interim criteria for toxicology (see MCA-8.1.5) of the EU Regulation 1107/2009 and therefore no further studies are triggered at this stage. Furthermore based on the physical, chemical and structural characteristics of the active substance metiram, as well as based on results of available long-term fish studies there is no indication of endocrine disrupting properties of this active substance. Further details are discussed for the aquatic part in a separate document (DocID 2015/1161953).

CA 8.2.4 Acute toxicity to aquatic invertebrates

CA 8.2.4.1 Acute toxicity to Daphnia magna

Data already submitted during previous Annex I inclusion process (already evaluated on EU level):

The following chronic toxicity study on Daphnia magna performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Furthermore the endpoint from the more recent acute study on D. magna (BASF DocID 1997/10538) is considered as relevant endpoint for the risk assessment as analytical measurements appeared more reliable; the endpoint obtained in this second study below is not used. Therefore no summary is provided

Report:	*CA 8.2.4.1/1* *Jatzek H.-J., 1994a* *Determination of the acute toxicity of BAS 222 28 F to the water flea Daphnia magna STRAUS* *1994/10671*
Guidelines:	*EEC 79/831 A V C 2*
GLP:	*yes* *(certified by Ministerium fuer Arbeit, Soziales, Familie und Gesundheit, Postfach 3180, 6500 Mainz)*

Report:	*CA 8.2.4.1/2* *Jatzek H.-J., 1994b* *Determination of the acute toxicity of BAS 222 28 F to the water flea Daphnia magna STRAUS* *1994/10814*
Guidelines:	*EEC 79/831 A V C 2*
GLP:	*yes* *(certified by Ministerium fuer Arbeit, Soziales, Familie und Gesundheit, Postfach 3180, 6500 Mainz)*

The following acute toxicity study on Daphnia magna performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.4.1/3* *Maisch R., 1997a* *Determination of the acute effect of BAS 222 28 F on the swimming ability of the water flea Daphnia magna STRAUS* *1997/10538*
Guidelines:	*EEC 79/831 A V C 2*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a semi-static acute toxicity laboratory study, water flea neonates (less than 24 h old) were exposed to BAS 222 28 F at concentrations of 0, 0.100, 0.178, 0.316, 0.563 and 1.00 mg/L in an EDTA free test medium. Daphnids were observed for immobility 0, 3, 6, 24 and 48 hours after start of exposure.

The results are based on initially measured concentrations. 5% of daphnids were immobile at the nominal concentration of 0.485 mg/L after 48 hours of exposure. In the highest treatment group of 0.821 mg/L 25% immobility was observed.

In a 48 hours semi-static acute toxicity study with *Daphnia magna* the EC_{50} of BAS 222 28 F based on initially measured concentrations was > 0.821 mg/L (> 0.584 mg a.s./L), the NOEC was 0.485 mg/L (0.345 mg a.s./L).

I. MATERIAL AND METHODS

A. MATERIALS

Test item:	BAS 222 28 F, batch No. 96-1, content of a.s.: metiram (BAS 222 F; Reg. No 250284): 71.12% analyzed (70% nominal).

B. STUDY DESIGN

Test species:	Water flea (*Daphnia magna* STRAUS 1820), neonates at test initiation less than 24 hours old; from in-house culture, clone obtained from Institute National de Recherche Chimique Appliquee, France.
Test design:	48 hours semi-static test (renewal after 24 h), 6 treatment groups (5 test concentrations, control), 4 replicates with 5 daphnids in each; assessment of immobility after 0, 3, 6, 24 and 48 hours.
Endpoints:	EC_0, EC_{50} based on immobility of daphnids.
Test concentrations:	Control, 0.100, 0.178, 0.316, 0.563 and 1.00 mg BAS 222 28 F/L (nominal).
Test conditions:	Glass vessels, dilution water "M4" Elendt medium, however, modified to be free of EDTA or other complex building substances, test volume 100 mL; pH 8.0 - 8.1; oxygen content: 8.4 mg/L - 8.8 mg/L; conductivity: 638 µS/cm; light intensity: about 5 - 6 µE/(m²*s); temperature 19.9 °C - 21.6 °C; total hardness: 2.68 mmol/L, photoperiod: 16 h light : 8 h dark; no feeding, no ventilation.
Analytics:	The test item concentrations were analyzed with GC method CP067.
Statistics:	Descriptive statistics.

II. RESULTS AND DISCUSSION

Analytical measurements: The analytically determined concentrations of metiram were in a range of 79.2% - 235.7% at test initiation and 27.5% - 82.9% at test termination. The biological results are based on initially measured concentrations.

Biological results: 5% of daphnids were immobile at the initially measured concentration of 0.485 mg/L after 48 hours of exposure. In the highest treatment group of 0.821 mg/L 25% immobility was observed. The results are summarized in Table 8.2.4.1-1.

Table 8.2.4.1-1: Effect (48 h) of BAS 222 28 F on *Daphnia magna* mobility

Concentration [mg/L] nominal	**Control**	**0.100**	**0.178**	**0.316**	**0.563**	**1.00**
Concentration [mg/L] initially measured	**-**	**0.099**	**0.141**	**0.745**	**0.485**	**0.821**
Immobility (24 h) [%]	0	0	0	0	5	0
Immobility (48 h) [%]	0	0	0	0	5	25

	Endpoints (based on initially measured concentrations[#])	
	based on BAS 222 28 F [mg/L]	**based on BAS 222 F [mg a.s./L]**
EC_{50} (48 h)	> 0.**821** (nominal: > 1.00) > 0.511*	**> 0.584**
EC_0 (48 h)	0.485 (nominal: 0.563) 0.288*	0.345

[#] Endpoints based on initially measured concentrations are re-calculated from the data of the original report

* The endpoints of EC_{50} > 0.511 mg/L and EC_0 = 0.288 mg/L as stated within the respective report are based on the median analytical recovery rate of all tested concentrations after 48 h (i.e. 51.1% of nominal).

III. CONCLUSION

In a 48 hours semi-static acute toxicity study with *Daphnia magna* the EC_{50} of BAS 222 28 F based on initially measured concentrations was > 0.821 mg/L (> 0.584 mg a.s./L), the NOEC was 0.485 mg/L (0.345 mg a.s./L).

The following acute toxicity study on Daphnia magna performed with the metiram metabolite ethyleneurea (EU) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.4.1/4* *Palmer S.J. et al., 2001b* *Ethylene urea: A 48-hour static acute toxicity test with the cladoceran (Daphnia magna)* *2001/5000988*
Guidelines:	*OECD 202*
GLP:	*yes* *(certified by United States Environmental Protection Agency)*

Executive Summary

In a static acute toxicity laboratory study (48 h), water flea neonates (less than 24 h old) were exposed to ethyleneurea (metabolite of metiram) at nominal concentrations of 0, 130, 216, 360, 600 and 1000 mg/L (corresponding to mean measured concentrations of 126, 214, 354, 589 and 985 mg/L). Daphnids were observed for immobility 20, 24 and 48 hours after start of exposure.

The results are based on nominal concentrations. No mortalities, no immobility and no overt signs of toxicity occurred during the test period up to a test concentration of 600 mg ethyleneurea/L. 10% mortality were observed after 48 h of exposure at 1000 mg ethyleneurea/L.

In a 48 hours static acute toxicity study with *Daphnia magna* the EC_{50} of ethyleneurea (metabolite of metiram) based on nominal concentrations was > 1000 mg/L, the NOEC was 600 mg/L. Based on mean measured concentrations the 48 h EC_{50} and NOEC were determined to be > 985 mg/L and 589 mg/L, respectively.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: Ethyleneurea (BF 222-EU; Reg. No 027270; metabolite of metiram), Lot No. 01743-141; purity: 90.8%.

B. STUDY DESIGN

Test species: Water flea (*Daphnia magna*), neonates at test initiation less than 24 hours old; from cultures maintained by Wildlife International Ltd., Easton, Maryland, USA.

Test design: 48 hours static test, 6 treatment groups (5 test concentrations, control), 4 replicates with 5 daphnids in each; assessment of mortality, immobility, signs of toxicity, after approx. 20, 24 and 48 hours.

Endpoints: NOEC, EC_{50} based on mortality/immobility of daphnids.

Test concentrations: Control, 130, 216, 360, 600 and 1000 mg ethyleneurea/L (nominal); (corresponding to mean measured concentrations of 126, 214, 354, 589 and 985 mg/L)

Test conditions: Glass beakers, filtered well water, test volume 250 mL; pH 8.2 - 8.6; oxygen content: 8.5 - 9.2 mg/L; temperature 20.1 - 20.8 °C; conductivity: 300 µmhos/cm; total hardness: 136 mg/L ($CaCO_3$); alkalinity: 182 mg/L ($CaCO_3$); light intensity: approx. 304 lux; photoperiod: 16 h light : 8 h dark; no feeding, no ventilation.

Analytics: The test item concentrations were analyzed with HPLC.

Statistics: Visual interpretation.

II. RESULTS AND DISCUSSION

Analytical measurements: The analytically determined concentrations of ethyleneurea were in the range of 97.1% - 99.4% at test initiation and between 96.3% and 100% at test termination. The biological results are based on nominal concentrations. Additionally, the results based on mean measured values are shown.

Biological results: No mortalities, no immobility and no overt signs of toxicity occurred during the test period up to 600 mg ethyleneurea/L. 10% mortality were observed after 48 h of exposure at 1000 mg ethyleneurea/L. The results are summarized in Table 8.2.4.1-2.

Table 8.2.4.1-2: Effect (48 h) of ethyleneurea on *Daphnia magna* mobility

Concentration [mg/L] (nominal)	**Control**	**130**	**216**	**360**	**600**	**1000**
Concentration [mg/L] (mean measured)	**--**	**126**	**214**	**354**	**589**	**985**
Immobility (24 h) [%]	0	0	0	0	0	0
Immobility (48 h) [%]	0	0	0	0	0	10

	Endpoints [mg/L]	
	based on nominal concentrations	based on mean measured concentrations
EC_{50} (48 h)	> 1000	> 985
NOEC (48 h)	600	589

III. CONCLUSION

In a 48 hours static acute toxicity study with *Daphnia magna* the EC_{50} of ethyleneurea (metabolite of metiram) based on nominal concentrations was > 1000 mg/L, the NOEC was 600 mg/L. Based on mean measured concentrations the EC_{50} and NOEC were determined to be > 985 mg/L and 589 mg/L, respectively.

The following acute toxicity study on Daphnia magna performed with the metiram metabolite ethylenethiourea (ETU) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.4.1/5* *Hisgen H., 2000a* *Reg.No. 146 099 - Determination of the acute effect on the swimming ability of the water flea Daphnia magna STRAUS* *2000/1017216*
Guidelines:	*EEC 92/32 A V C 2, OECD 202 Part I (1984), EPA 850.1010, ISO 6341, ISO/DIS 10706*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a static acute toxicity laboratory study (48 h), water flea neonates (less than 24 h old) were exposed to ethylenethiourea (ETU; metabolite of metiram) at concentrations of 0, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 mg/L (nominal). Daphnids were observed for immobility and other signs of toxicity 24 and 48 hours after start of exposure.

The results are based on nominal concentrations. No immobility occurred at concentrations up to and including 3.13 mg ETU/L. 10% to 80% of the animals were immobilized at higher test concentrations.

In a 48 hours static acute toxicity study with *Daphnia magna* the EC_{50} of ETU (metabolite of metiram) was 21.6 mg/L, based on nominal concentrations. No significant mortality was observed at 6.25 mg/L (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: Ethylenethiourea (BF 222-ETU, Reg. No. 146099; metabolite of metiram), batch no. L33-99, purity: 99.6%.

B. STUDY DESIGN

Test species: Water flea (*Daphnia magna* STRAUS), neonates older than 2 but less than 24 hours old at test initiation, source: in-house culture.

Test design: 48 hours static test, 8 treatment groups (7 test concentrations, control), 4 replicates with 5 daphnids in each; assessment of mortality, immobility, signs of toxicity, after approx. 24 and 48 hours.

Endpoints: NOEC and EC_{50} were based on immobility of daphnids.

Test concentrations: Control, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 mg ETU/L (nominal).

Test conditions: Glass vessel 20 mL, test volume 10 mL, dilution water “M4” (Elendt medium); total hardness: 2.42 mmol/L, conductivity: 625 µS/cm, alkalinity: 0.86 mmol/L at test initiation; pH 7.9 - 8.1; oxygen content: 8.4 - 8.9 mg/L; temperature: 20.1 - 21.8 °C; photoperiod 16 hours light: 8 hours dark; no feeding and no aeration.

Analytics: The test item concentrations were quantified by external calibration using HPLC (method CF-A 446).

Statistics: For statistical calculation of the EC_{50} the moving average method was used.

II. RESULTS AND DISCUSSION

Analytical measurements: The test concentrations were confirmed by analytical measurements (93.7% - 109%). Therefore, biological results are based on mean measured concentrations.

Biological results: No immobility occurred at concentrations up to and including 3.13 mg ETU/L. 10% to 80% of the animals were immobilized at higher test concentrations. The results are summarized in Table 8.2.4.1-3

Table 8.2.4.1-3: Effects (48 h) of ETU (metabolite of metiram) on *Daphnia magna*

Concentration [mg/L] (nominal)	**control**	**1.56**	**3.13**	**6.25**	**12.5**	**25**	**50**	**100**
Immobility (24 h) [%]	0	0	0	0	5	25	55	35
Immobility (48 h) [%]	0	0	0	10	40	50	75	80
	Endpoints [mg/L] (nominal)							
EC_{50} (48 h)	21.6 (95% confidence limit: 16.0 – 29.1)							
EC_0 *	6.25							

* The EC0 is the highest concentration tested, at which ≤10% of the animal were immobile.

III. CONCLUSION

In a 48 hours static acute toxicity study with *Daphnia magna* the EC_{50} of ETU (metabolite of metiram) was 21.6 mg/L, based on nominal concentrations. No significant mortality was observed at 6.25 mg/L (nominal).

<u>New supplemental data (not evaluated previously on EU level):</u>

The following acute toxicity study on Daphnia magna performed with metiram (tested as BAS 222 29 F) is provided in support of the aquatic risk assessment and has not been evaluated previously on EU level. The study was conducted due to U.S. data requirements and is provided for completeness.

Report:	CA 8.2.4.1/6 Minderhout T. et al., 2010d Metiram: A 48-hour flow-through acute toxicity test with the Cladoceran (Daphnia magna) 2010/7010877
Guidelines:	EPA 850.1010, OECD 202
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

In a 48-hour flow-through acute toxicity laboratory study, water flea neonates were exposed to metiram (tested as BAS 222 29 F) at nominal concentration of 0.104, 0.173, 0.288, 0.480 and 0.800 mg a.s./L, (corresponding to mean measured concentrations of 0.0559, 0.110, 0.201, 0.373 and 0.670 mg a.s./L). Additionally, a dilution water control and a solvent control were set up. The test was conducted with 2 replicates per concentration, containing 10 daphnids each.
The daphnids were observed for immobility and symptoms of toxicity 4.5, 24 and 48 hours after start of exposure.

The biological results are based on mean measured concentrations. After 48 hours of exposure, no immobility of daphnids was observed in the control groups, and at the test item concentrations of 0.110 and 0.201 mg a.s./L. The single immobile daphnid observed in the lowest treatment group was not considered dose-responsive and was therefore not included in the calculation of the 48-hour EC_{50} value. At the second highest and the highest test item concentration, 10% and 55% immobility were noted, respectively, after 48 hours of exposure. After 48 hours of exposure, sub-lethal effects (i.e. lethargy, discoloration and captivity at water surface either with normal or lethargic behavior after gentle submersion) were found in all test item concentrations.

In a 48-hour flow-through acute toxicity study with *Daphnia magna*, the EC_{50} of metiram (tested as BAS 222 29 F) was 0.634 mg a.s./L based on mean measured concentrations. The NOEC was determined to be 0.201 mg a.s./L (mean measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 29 F, lot no. 300013; content of a.s.: metiram (BAS 222 F; Reg. No 250284): 91.6% (nominal: 85%); density: 1.860 g/cm³.

B. STUDY DESIGN

Test species: Water flea (*Daphnia magna* STRAUS), neonates collected from in house culture; < 24 h old at test initiation.

Test design: Flow-through system (48 hours), 5 test item concentrations plus dilution water control and solvent control, 2 replicates with 10 daphnids in each; assessment of immobility after 4.5, 24 and 48 hours.

Endpoints: NOEC and EC_{50} based on immobility of daphnids.

Test concentrations: Control (dilution water); solvent control (0.1 mL dimethylformamide/L); 0.104, 0.173, 0.288, 0.480 and 0.800 mg a.s./L (nominal), corresponding to mean measured concentrations of 0.0559, 0.110, 0.201, 0.373 and 0.670.

Test conditions: Test chambers: 25 L stainless steel aquaria; test volume: 22 L; test compartments consisting of glass beakers (6.5 cm diameter, 12 cm height) with nylon screen attached to two holes on the sides of the beaker; dilution water: filtered, aerated and sterilized well water; flow rate: 5 volume additions per 24 hours on average in each test vessel; pH: 8.1 - 8.2; oxygen concentration: 7.8 mg/L - 9.0 mg/L; temperature: 19.9 °C - 20.1 °C; total hardness: 148 mg $CaCO_3$/L at test initiation; conductivity: 364 µS/cm at test initiation; alkalinity: 186 mg $CaCO_3$/L; photoperiod: 16 h light : 8 h dark; no feeding.

Analytics: Analytical verification of test item concentrations was conducted using a GC-method with MS detection.

Statistics: Descriptive statistics; probit analysis for determination of the EC_{50}.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of the concentrations of the active substance was conducted at the beginning and at the end of the test. The analytically determined concentrations of metiram ranged from 46% to 83% of nominal at test initiation and from 61% to 85% at test termination. The following biological results are based on mean measured concentrations.

Biological results: After 48 hours of exposure, no immobility of daphnids was observed in the control groups, whereas 5% immobility were observed at the lowest test item concentration of 0.0559 mg a.s./L. Since no immobility occurred at the test item concentrations of 0.110 and 0.201 mg a.s./L, the single immobile daphnid observed in the lowest treatment group was not considered dose-responsive and was therefore not included in the calculation of the 48-hour EC_{50} value. At the second highest and the highest test item concentration, 10% and 55% immobility were noted, respectively, after 48 hours of exposure. After 48 hours of exposure, sub-lethal effects (*i.e.* lethargy, discoloration and captivity at water surface either with normal or lethargic behavior after gentle submersion) were found in all test item concentrations. For results see Table 8.2.4.1-4.

Table 8.2.4.1-4: Effects of metiram on *Daphnia magna* mobility

Concentration [mg a.s./L] (nominal)	**Control**	**Solvent control**	**0.104**	**0.173**	**0.288**	**0.480**	**0.800**
Concentration [mg a.s./L] (mean measured)	**--**	**--**	**0.0559**	**0.110**	**0.201**	**0.373**	**0.670**
Immobility (24 h) [%]	0	0	0	0	0	0	0
Immobility (48 h) [%]	0	0	5	0	0	10	55
Endpoints [mg metiram/L] (mean measured)							
EC_{50} (48 h)	0.634 (95% confidence limits: 0.531 - 0.890)						
NOEC (48 h)	0.201						

III. CONCLUSION

In a 48-hour flow-through acute toxicity study with *Daphnia magna*, the EC_{50} of metiram (tested as BAS 222 29 F) was 0.634 mg a.s./L based on mean measured concentrations. The NOEC was determined to be 0.201 mg a.s./L (mean measured).

In addition to the standard acute toxicity studies on Daphnia magna which have already been evaluated during the Annex I inclusion process a new study with differentially filtered stock solutions of metiram (tested as BAS 222 28 F) was conducted. The study was performed to generate further data for a risk refinement and has not been evaluated previously on EU level.

Report:	CA 8.2.4.1/7 Weltje L.,Janson G.-M., 2010a Acute toxicity of the Metiram formulation BAS 222 28 F to Daphnia magna STRAUS using unfiltered, filtered and aged solutions 2010/1059919
Guidelines:	OECD 202, EPA 850.1010, EEC 79/831 A V C 2 update 1990
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

In a series of seven 48-hour static acute toxicity laboratory tests, water flea neonates were exposed to BAS 222 28 F at nominal concentrations of 0 (control), 0.15, 0.3, 0.6, 1.2 and 2.4 mg/L in 4 replicates per concentration, containing 5 daphnids each. Three tests were conducted using either unfiltered, 0.2 µm filtered or 1.2 µm filtered fresh test item solutions. After 4 - 6 hours of ageing of the stock solution, two tests were started with either unfiltered or 0.2 µm filtered test media. Finally, two further tests were initiated after the stock solution had aged for 28 - 30 h using either unfiltered or 0.2 µm filtered test solutions. Daphnids were observed for immobility 24 hours and 48 hours after start of exposure.

The biological results are based on nominal concentrations. After 48 hours of exposure, filtration significantly reduced toxicity of the test item, since all EC_{50} values were higher after filtration compared to EC_{50} values obtained with unfiltered test solutions. The unfiltered test medium remained approximately equally toxic during a maximum ageing time of 28 - 30 h, while the 0.2 µm filtered test medium slightly increased in its toxicity (*i.e.* less than a factor of two).

In a 48-hour static acute toxicity study with *Daphnia magna*, the EC_{50} values based on nominal concentrations of BAS 222 28 F using fresh test item solutions were 0.85 mg/L for the unfiltered test media and > 2.4 mg/L for both the 1.2 µm filtered and the 0.2 µm filtered test media. In the tests conducted with aged test media (stock solution aged for 4 -6 hours), the EC_{50} values were determined to be 1.08 mg/L and 1.70 mg/L (nominal) for the unfiltered test media and the 0.2 µm filtered test media, respectively. After 28 - 30 h of ageing the resulting EC_{50} values were 0.95 mg/L and 1.42 mg/L (nominal) for the unfiltered test media and for the 0.2 µm filtered test media, respectively. The NOEC was determined to be 0.6 mg/L (nominal) for the unfiltered, fresh solutions.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2002-1; content of a.s.: metiram (BAS 222 F, Reg. no. 250284): 70.5% (nominal: 70.0%); density: 1.740 g/cm^3.

B. STUDY DESIGN

Test species: Water flea (*Daphnia magna* STRAUS), neonates collected from in-house culture (originally obtained from Institute National de Recherché Chimique Appliquée, France), > 2 < 24 hours old at test initiation.

Test design: Static system (48 hours), total of 7 tests; 5 test concentrations plus control, 4 replicates with 5 daphnids in each; assessment of immobility after 24 and 48 hours. A series of 7 tests was conducted using combinations of ageing of the stock solutions and filtration: at t = 0 h, test media were unfiltered, filtered over 1.2 µm or filtered over 0.2 µm. At t = 4 - 6 h, test media were either unfiltered or filtered over 0.2 µm. At t = 28 - 30 h, test media were either unfiltered or filtered over 0.2 µm.

Endpoints: NOEC, EC_{50} based on immobility of daphnids.

Test concentrations: Control, 0.15, 0.3, 0.6, 1.2 and 2.4 mg BAS 222 28 F/L (nominal).

Test conditions: Glass vessels, test volume 50 mL, dilution water "M4" (Elendt medium); pH 7.78 - 7.99; oxygen content: 7.8 mg/L - 9.2 mg/L; total hardness: 2.43 mmol/L - 2.46 mmol/L at test initiation; conductivity: 664 µS/cm - 681 µS/cm at test initiation; temperature: 20.5 °C - 22.0 °C; light intensity 500 lux - 1000 lux; photoperiod: 16 h light : 8 h dark, no feeding, no aeration.

Analytics: Analytical verification of test item concentrations was conducted using a GC-method with MS detection.

Statistics: Descriptive statistics; probit analysis using linear maximum likelihood regression for EC_{50} calculation and Fisher’s test for NOEC calculation.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at the beginning and at the end of the test. Mean measured concentrations of BAS 222 28 F in the unfiltered test media ranged from 79.0% to 119.4% of the nominal values at test initiation and from 30.0% to 37.3% of nominal at test termination. After 4 - 6 h of ageing of the stock solution, mean recoveries in unfiltered test media were between 90.4% and 117.1% of the nominal concentrations at test initiation and between 31.9% and 69.8% of nominal at test end. After 28 - 30 h of ageing of the stock solution, mean measured concentrations of BAS 222 28 F in the unfiltered test media were in the range of 49.8% to 55.3% of nominal at test initiation and between 21.5% and 32.6% of nominal at test termination. Filtration caused a significant reduction of the recovered concentrations of the test item, whereas the effect of ageing was less pronounced and only clearly visible after 28 - 30 h of ageing of the stock solution in the unfiltered media.

As the measured concentrations confirmed correct application of the test item, the following biological results are based on nominal test item concentrations. Moreover, this allows for optimal comparison of the 7 tests. A summary of all recoveries at test initiation and test end is provided in Table 8.2.4.1-5.

Table 8.2.4.1-5: Minimum and maximum analytical recoveries of BAS 222 28 F in the different test media at test start (t = 0 h) and test end (t = 48 h)

Mean recovery [% of nominal concentration]		**t = 0 h**	**t = 48 h**
unfiltered	fresh solutions	93.4 - 119.4 [1)] 79.0 - 107.0 [2)]	30.0 - 37.3 *
	4 - 6 h aged solutions	90.4 - 117.1	31.9 - 69.8
	28 - 30 h aged solutions	49.8 - 55.3 *	21.5 - 32.6 *
filtered over 1.2 µm	fresh solutions	1.5 - 15.4 * 5.4 - 30.4 *	5.59 - 10.72 *
	4 - 6 h aged solutions	n.d.	n.d.
	28 - 30 h aged solutions	n.d.	n.d.
filtered over 0.2 µm	fresh solutions	7.4 - 8.2 * 20.4 - 25.5 *	-- #
	4 - 6 h aged solutions	55.7 - 66.0	17.6 - 31.4*
	28 - 30 h aged solutions	32.8 - 73.9 *	11.8 - 15.3*

n.d. = not determined
1) Samples received on 12.07.2005
2) Samples received on 13.07.2005
* one or more values were not reliable since they were below the Limit of Quantitation (LoQ)
# analytical concentrations could not be determined, because all values were below the Limit of Quantitation (LoQ)

Biological results: After 48 hours of exposure, filtration significantly reduced toxicity of the test item, since all EC_{50} values were higher after filtration compared to EC_{50} values obtained with unfiltered test solutions. The unfiltered test medium remained approximately equally toxic during a maximum ageing time of 28 - 30 h, while the 0.2 µm filtered test medium slightly increased in its toxicity (*i.e.* less than a factor of two). For results see
Table **8.2.4.1-6**.

Table 8.2.4.1-6: Effects of BAS 222 28 F on Daphnia magna immobility

Concentration [mg/L] (nominal)			Control	0.15	0.3	0.6	1.2	2.4
fresh solutions [1]	unfiltered	Immobility (24 h) [%]	0	0	0	0	0	55
		Immobility (48 h) [%]	0	0	0	10	90	100
	filtered over 1.2 µm	Immobility (24 h) [%]	0	0	0	0	0	0
		Immobility (48 h) [%]	0	0	0	0	0	10
	filtered over 0.2 µm	Immobility (24 h) [%]	0	0	0	0	0	0
		Immobility (48 h) [%]	0	0	0	0	0	10
4 - 6 h aged solutions [2]	unfiltered	Immobility (24 h) [%]	0	0	0	0	25	65
		Immobility (48 h) [%]	0	0	0	5	60	100
	filtered over 0.2 µm	Immobility (24 h) [%]	0	0	0	0	10	40
		Immobility (48 h) [%]	0	0	0	0	15	85
28 - 30 h aged solutions [2]	unfiltered	Immobility (24 h) [%]	0	0	0	0	15	80
		Immobility (48 h) [%]	0	0	0	5	80	100
	filtered over 0.2 µm	Immobility (24 h) [%]	0	0	0	0	15	50
		Immobility (48 h) [%]	0	0	0	0	30	95
			Endpoints [mg BAS 222 28 F/L] (nominal)					
EC_{50} (48 h)	fresh solutions	unfiltered	0.85 (confidence limits: 0.73 - 0.99)					
		filtered over 1.2 µm	> 2.4					
		filtered over 0.2 µm	> 2.4					
	4 - 6 h aged solutions	unfiltered	1.08 (confidence limits: 0.90 - 1.28)					
		filtered over 0.2 µm	1.70 (confidence limits: 1.43 - 2.03)					
	28 - 30 h aged solutions	unfiltered	0.95 (confidence limits: 0.80 - 1.11)					
		filtered over 0.2 µm	1.42 (confidence limits: 1.21 - 1.69)					
NOEC (48 h) (fresh solutions, unfiltered) [$]			0.6					

[1] The same control was used for unfiltered, 1.2 µm and 0.2 µm filtered solutions
[2] The same control was used for unfiltered and 0.2 µm filtered solutions started at 4 - 6 h and at 28 - 30 h, respectively
[$] The NOEC value was only determined for this combination

III. CONCLUSION

In a 48-hour static acute toxicity study with *Daphnia magna*, the EC_{50} values based on nominal concentrations of BAS 222 28 F using fresh test item solutions were 0.85 mg/L for the unfiltered test media and > 2.4 mg/L for both the 1.2 µm filtered and the 0.2 µm filtered test media. In the tests conducted with aged test media (stock solution aged for 4 -6 hours), the EC_{50} values were determined to be 1.08 mg/L and 1.70 mg/L (nominal) for the unfiltered test media and the 0.2 µm filtered test media, respectively. After 28 - 30 h of ageing the resulting EC_{50} values were 0.95 mg/L and 1.42 mg/L (nominal) for the unfiltered test media and for the 0.2 µm filtered test media, respectively. The NOEC was determined to be 0.6 mg/L (nominal) for the unfiltered, fresh solutions.

The following acute toxicity study on Daphnia magna performed with the metiram metabolite TDIT is provided in support of the aquatic risk assessment has not been evaluated previously on EU level.

Report:	CA 8.2.4.1/8 Nierzedska E., 2013a Reg.No. 4670450 (metabolite of BAS 222 F, Metiram, TDIT) - Daphnia magna, acute immobilization test 2013/1000144
Guidelines:	OECD 202 (2004)
GLP:	yes (certified by Bureau for Chemical Substances and Preparations, Lodz, Poland)

Executive Summary

In a static acute toxicity laboratory study, water flea neonates were exposed to TDIT (metabolite of metiram) at nominal concentrations of 0 (control), 0.95, 3.05, 9.77, 31.25 and 100 mg/L in 4 replicates per concentration containing 5 daphnids each. Daphnids were observed for immobility 24 hours and 48 hours after start of exposure.

The biological results are based on nominal concentrations of the test item. After 48 h of exposure, no immobility of daphnids was observed in the control, whereas 5%, 50%, 55% 80% and 85% immobility were observed at the test item concentrations of 0.95, 3.05, 9.77, 31.25 and 100 mg/L, respectively. Statistically significant effects on mobility of daphnids were determined at the four highest test item concentrations.

In a 48-hour static acute toxicity study with *Daphnia magna* the EC_{50} of TDIT (metabolite of metiram) was determined to be 7.12 mg/L based on nominal concentrations. The NOEC was 0.95 mg/L (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: TDIT (metabolite of metiram, Reg. No. 4 670 450), batch no. L83-138, content: 2,3,7,8-tetrahydrodiimidazo[2,1-b:1'2'-e][1,3,5]thiadiazine-5-thione (purity is 91.4%, tolerance ± 1.0%) and 0.3% dichloromethane (Reg.No. 4 108 316).

B. STUDY DESIGN

Test species: Water flea (*Daphnia magna* STRAUS), neonates from in-house culture, < 24 hours old at test initiation.

Test design: Static system (48 hours), 5 test concentrations plus control, 4 replicates with 5 daphnids in each; assessment of immobility after 24 and 48 hours.

Endpoints: EC_{50} and NOEC based on immobility of daphnids.

Test concentrations: Control, 0.95, 3.05, 9.77, 31.25 and 100 mg TDIT/L (nominal).

Test conditions: 150 mL glass beakers, test volume 100 mL, dilution water: "M7" (Elendt medium); temperature: 21.3 °C - 22.7 °C; pH 7.49 - 7.54 at test initiation and pH 7.49 - 7.66 at test termination; oxygen content: 8.6 mg/L - 8.7 mg/L at test initiation and 8.4 mg/L - 9.2 mg/L at test termination; photoperiod: 16 hours light : 8 hours dark; no feeding and no aeration.

Analytics: Analytical verification of test item concentrations was conducted using a LC-method with DAD detection.

Statistics: Descriptive statistics. Fisher`s Exact Binomial Test with Bonferroni Correction for determination of the NOEC ($\alpha = 0.05$); probit analysis for determination of the EC_{50} value.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentration was conducted in each concentration at the beginning and at the end of the test. Mean measured values for TDIT ranged from 94.1% to 100.9% of nominal at test initiation and from 94.0% to 102.3% of nominal at test termination. As analytical data confirmed correct application of the test item, the following biological results are based on nominal concentrations.

Biological results: After 48 h of exposure, no immobility of daphnids was observed in the control, whereas 5%, 50%, 55% 80% and 85% immobility were observed at the test item concentrations of 0.95, 3.05, 9.77, 31.25 and 100 mg/L, respectively. Statistically significant effects on mobility of daphnids were determined at the four highest test item concentrations (Fisher's Exact Binomial Test with Bonferroni Correction, $\alpha = 0.05$). For results see Table 8.2.4.1-7.

Table 8.2.4.1-7: Effect of TDIT (metabolite of metiram) on *Daphnia magna* mobility

Concentration [mg/L] (nominal)	**Control**	**0.95**	**3.05**	**9.77**	**31.25**	**100**
Immobility (24 h) [%]	0	0	0	30	25	50
Immobility (48 h) [%]	0	5	50 *	55 *	80 *	85 *
	Endpoints [mg TDIT/L] (nominal)					
EC_{50} (48 h)	7.12 (95% confidence limits: 3.75 - 12.75)					
NOEC (48 h)	0.95					

* Statistically significantly different compared to the control (Fisher's Exact Binomial Test with Bonferroni Correction, $\alpha = 0.05$)

III. CONCLUSION

In a 48-hour static acute toxicity study with *Daphnia magna* the EC_{50} of TDIT (metabolite of metiram) was determined to be 7.12 mg/L based on nominal concentrations. The NOEC was 0.95 mg/L (nominal).

CA 8.2.4.2 Acute toxicity to an additional aquatic invertebrate species

Data already submitted during previous Annex I inclusion process (already evaluated on EU level):

The following acute toxicity study on the zooplankton species Chydorus sphaericus performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.4.2/1* *Junker M., 2002b* *Effect of BAS 222 28 F on the immobility of Chydorus sphaericus in a 48 hours static, acute toxicity test* *2002/1005267*
Guidelines:	*OECD 202*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a static acute toxicity laboratory study a mixed age population of the zooplankton species *Chydorus sphaericus* was exposed to BAS 222 28 F at nominal concentrations of 0, 0.10, 0.20, 0.40, 0.80 and 1.60 mg product/L (corresponding to initially measured concentrations of 0, 0.080, 0.18, 0.32, 0.71 and 1.41 mg/L. Animals were observed for immobility 24 and 48 hours after start of exposure.

The results are based on initially measured concentrations. No immobility occurred at concentrations up to and including 0.180 mg BAS 222 28 F/L. At 1.41 mg/L, 90% of the animals were immobile after 48 hours. At 0.71 and 1.41 mg/L young individuals of *Chydorus sphaericus* did not survive after 48 hours.

In an 48 hours static acute toxicity study with *Chydorus sphaericus* the EC_{50} of BAS 222 28 F based on initially measured concentrations was 0.880 mg/L (corresponding to 0.603 mg a.s./L). The NOEC was determined to be 0.180 mg BAS 222 28 F/L (corresponding to 0.123 mg a.s./L; initially measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch 2000-2, content of a.s.: metiram (BAS 222 F; Reg. No 250284): 68.5% (70% nominal).

B. STUDY DESIGN

Test species: *Chydorus sphaericus*, Crustacea, Cladocera, mixed age population, approx. same size; individuals collected from mesocosm facility, prior adapted to test conditions.

Test design: 48 hours static test, 6 treatment groups (5 test concentrations, control), 4 replicates with 5 individuals each; assessment of immobility after 24 and 48 hours.

Endpoints: NOEC, EC_{50} based on immobility.

Test concentrations: Control, 0.10, 0.20, 0.40, 0.80 and 1.60 mg BAS 222 28 F/L (nominal); corresponding to initially measured concentrations of 0, 0.080, 0.18, 0.32, 0.71 and 1.41 mg/L.

Test conditions: Multiwell tissue culture plate, test volume 3 mL, dilution water "M4" Elendt medium; conductivity: 696 µS/cm; total hardness: 2.53 mmol/L; pH 8.05 - 8.08; oxygen content: 9.0 mg/L - 9.1 mg/L; temperature 20 °C, photoperiod: 16 h light : 8 h dark; light intensity: < 1500 lux; no feeding, no ventilation.

Analytics: Analytical verification of test substance concentrations was conducted using HPLC with UV-detection.

Statistics: Descriptive statistics, log-log analysis.

II. RESULTS AND DISCUSSION

Analytical measurements: Measured values for metiram at test initiation ranged from 79.4% to 89.3% (average 85.7%) of nominal. The biological results are based on initially measured concentrations.

Biological results: No immobility occurred at concentrations up to and including 0.180 mg BAS 222 28 F/L. At 1.41 mg/L, 90% of the animals were immobile after 48 hours. At 0.71 and 1.41 mg/L young individuals of *Chydorus sphaericus* did not survive after 48 hours. The results are summarized in Table 8.2.4.2-1.

Table 8.2.4.2-1: Effect (48 h) of BAS 222 28 F on *Chydorus sphaericus* mobility

Concentration [mg/L] (nominal)	**Control**	**0.10**	**0.20**	**0.40**	**0.80**	**1.6**
Concentration [mg/L] (initially measured)	**-**	**0.080**	**0.18**	**0.32**	**0.71**	**1.41**
Immobility (24 h) [%]	0	0	0	0	15	50
Immobility (48 h) [%]	0	0	0	10	30	90

	Endpoints (initially measured)	
	based on BAS 222 28 F [mg/L]	**based on BAS 222 F [mg a.s./L]**
EC_{50} (48 h)	0.880 (95% confidence limits: 0.730 - 1.05)	0.603 (95% confidence limits: 0.500 - 0.720)
NOEC (48 h)	0.180	0.123

III. CONCLUSION

In an 48 hours static acute toxicity study with *Chydorus sphaericus* the EC_{50} of BAS 222 28 F based on initially measured concentrations was 0.880 mg/L (corresponding to 0.603 mg a.s./L). The NOEC was determined to be 0.180 mg BAS 222 28 F/L (corresponding to 0.123 mg a.s./L; initially measured).

The following acute toxicity study on the zooplankton species Simocephalus vetulus performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.4.2/2* *Junker M., 2002c* *Effect of BAS 222 28 F on Simocephalus vetulus in a 48 hours static, acute toxicity test* *2002/1005269*
Guidelines:	*OECD 202*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a static acute toxicity laboratory study a mixed age population of the zooplankton species *Simocephalus vetulus* was exposed to BAS 222 28 F at nominal concentrations of 0, 0.20, 0.40, 0.80, 1.60 and 3.20 mg/L (corresponding to initially measured concentrations of 0, 0.18, 0.31, 0.67, 1.46 and 3.05 mg BAS 222 28 F/L). *Simocephalus vetulus* were observed for immobility 24 and 48 hours after start of exposure.

The results are based on initially measured concentrations. No immobility occurred at 0.18 mg BAS 222 28 F/L. At 3.05 mg/L, 50% of *Simocephalus vetulus* were immobile after 48 h.

In a 48 hours static acute toxicity study with *Simocephalus vetulus* the EC_{50} of BAS 222 28 F based on initially measured concentrations was 3.05 mg/L (corresponding to 2.09 mg a.s./L). The NOEC was determined to be 0.18 mg/L (corresponding to 0.123 mg a.s./L; initially measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch 2000-2, content of a.s.: metiram (BAS 222 F; Reg. No 250284): 68.5% (70% nominal).

B. STUDY DESIGN

Test species: *Simocephalus vetulus*, Crustacea, Cladocera, mixed age population, approx. same size; individuals collected from mesocosm facility, prior adapted to test conditions.

Test design: 48 hours static test, 6 treatment groups (5 test concentrations, control), 4 replicates with 5 individuals each; assessment of immobility after 24 and 48 hours.

Endpoints: NOEC, EC_{50} based on immobility.

Test concentrations: Control, 0.20, 0.40, 0.80, 1.60 and 3.20 mg BAS 222 28 F/L (nominal); corresponding to initially measured concentrations of 0, 0.18, 0.31, 0.67, 1.46 and 3.05 mg/L.

Test conditions: Glass vessels, test volume 50 mL, dilution water "M4" Elendt medium; conductivity: 670 µS/cm; total hardness: 2.44 mmol/L, pH 7.96 - 7.97; oxygen content: 8.9 mg/L - 9.0 mg/L; temperature 20 °C - 21° C; light intensity: < 1500 lux; photoperiod: 16 h light : 8 h dark; no feeding, no ventilation.

Analytics: Analytical verification of test substance concentrations was conducted using HPLC with UV-detection.

Statistics: None, NOEC, EC_{50} and EC_{100} were determined by visual analysis.

II. RESULTS AND DISCUSSION

Analytical measurements: Measured values for metiram at test initiation ranged from 78.7% to 95.3% (average 87.4%) of nominal. The biological results are based on initially measured concentrations.

Biological results: No immobility occurred at 0.18 mg BAS 222 28 F/L. At 3.05 mg/L, 50% of *Simocephalus vetulus* were immobile after 48 h. The results are summarized in Table 8.2.4.2-2

Table 8.2.4.2-2: Effect (48 h) of BAS 222 28 F on *Simocephalus vetulus* mobility

Concentration [mg/L] nominal	**Control**	**0.20**	**0.40**	**0.80**	**1.60**	**3.20**
Concentration [mg/L] initially measured	**-**	**0.18**	**0.31**	**0.67**	**1.46**	**3.05**
Immobility (24 h) [%]	0	0	0	5	10	25
Immobility (48 h) [%]	0	0	5	15	40	50

	Endpoints (initially measured)	
	based on BAS 222 28 F [mg/L]	**based on BAS 222 F [mg a.s./L]**
EC_{50} (48 h)	**3.05**	**2.09**
NOEC (48 h)	0.18	0.12

III. CONCLUSION

In a 48 hours static acute toxicity study with *Simocephalus vetulus* the EC_{50} of BAS 222 28 F based on initially measured concentrations was 3.05 mg/L (corresponding to 2.09 mg a.s./L). The NOEC was determined to be 0.18 mg/L (corresponding to 0.123 mg a.s./L; initially measured).

***The following acute toxicity study on a mixed age population of zooplankton of the order Cyclopoida performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below*.**

Report:	*CA 8.2.4.2/3 Junker M., 2002d Effect of BAS 222 28 F on Cyclopoida in a 48 hours static, acute toxicity test 2002/1005266*
Guidelines:	*OECD 202*
GLP:	*yes (certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a static acute toxicity laboratory a mixed age population of zooplankton of the order Cyclopoida was exposed to BAS 222 28 F at nominal concentrations of 0, 0.20, 0.40, 0.80, 1.60 and 3.20 mg/L (corresponding to initially measured concentrations of 0, 0.16, 0.30, 0.63, 1.47 and 2.96 mg/L). Observations for mortality were conducted 24 and 48 hours after start of exposure.

The results are based on initially measured concentrations. No effects were observed at concentrations up to and including 0.63 mg BAS 222 28 F/. At 1.47 mg/L all Cyclopoida were dead after 48 hours (one dead animal was found in the control).

In a 48 hours static acute toxicity study with Cyclopoida the EC_{50} of BAS 222 28 F based on initially measured concentrations was 0.96 mg/L (corresponding to 0.66 mg a.s./L). The NOEC was determined to be 0.63 mg BAS 222 28 F/L (corresponding to 0.43 mg a.s./L; initially measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch 2000-2, content of a.s.: metiram (BAS 222 F; Reg. No 250284): 68.5% (70% nominal).

B. STUDY DESIGN

Test species: Cyclopoida (Crustacea, Copepoda), mixed age population, approx. same size; individuals collected from mesocosm facility, prior adapted to test conditions.

Test design: 48 hours static test, 6 treatment groups (5 test concentrations, control), 4 replicates with 3 individuals each; assessment of immobility after 24 and 48 hours.

Endpoints: NOEC, EC_{50} based on mortality.

Test concentrations: Control, 0, 0.20, 0.40, 0.80, 1.60 and 3.20 mg BAS 222 28 F/L (nominal); corresponding to initially measured concentrations of 0, 0.16, 0.30, 0.63, 1.47 and 2.96 mg/L.

Test conditions: Petri dishes, test volume 6 mL, dilution water "M4" Elendt medium; ; conductivity: 696 µS/cm; total hardness: 2.54 mmol/L, pH 8.20 - 8.26; oxygen content: 8.7 mg/L - 8.9 mg/L, temperature 20 °C - 21° C; light intensity: < 1500 lux; photoperiod: 16 h light : 8 h dark; no feeding, no ventilation.

Analytics: Analytical verification of test substance concentrations was conducted using HPLC with UV-detection.

Statistics: Descriptive statistics

II. RESULTS AND DISCUSSION

Analytical measurements: Measured values for metiram at test initiation ranged from 73.9% to 92.4% (average 82.9%) of nominal. The biological results are based on initially measured concentrations.

Biological results: No effects were observed at concentrations up to and including 0.63 mg BAS 222 28 F/. At 1.47 mg/L all Cyclopoida were dead after 48 hours (one dead animal was found in the control). The results are summarized in Table 8.2.4.2-3.

Table 8.2.4.2-3: Effect (48 h) of BAS 222 28 F on Cyclopoida mortality

Concentration [mg/L] (nominal)	**Control**	**0.20**	**0.40**	**0.80**	**1.60**	**3.20**
Concentration [mg/L] (initially measured)	**-**	**0.16**	**0.30**	**0.63**	**1.47**	**2.96**
Mortality (24 h) [%]	0	0	0	0	8.3	83.3
Mortality (48 h) [%]	8.3	0	0	0	100	100
	Endpoints (initially measured)					
	based on BAS 222 28 F [mg/L]			**based on BAS 222 F [mg a.s./L]**		
EC_{50} (48 h)	**0.96** (95% confid. limits: > 0.63 < 1.47)			**0.66** (95% confid. limits: > 0.43 < 1.01)		
NOEC (48 h)	0.63			0.43		

III. CONCLUSION

In a 48 hours static acute toxicity study with Cyclopoida the EC_{50} of BAS 222 28 F based on initially measured concentrations was 0.96 mg/L (corresponding to 0.66 mg a.s./L). The NOEC was determined to be 0.63 mg BAS 222 28 F/L (corresponding to 0.43 mg a.s./L; initially measured).

The following acute toxicity study on a mixed age population of Ostracoda performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.4.2/4* *Junker M., 2002e* *Effect of BAS 222 28 F on Ostracoda in a 48 hours static, acute toxicity test* *2002/1005268*
Guidelines:	*OECD 202*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a static acute toxicity laboratory a mixed age population of Ostracoda, genus *Heterocypris* was exposed to BAS 222 28 F at nominal concentrations of 0, 0.20, 0.40, 0.80, 1.60 and 3.20 mg/L (corresponding to initially measured concentrations of 0, 0.15, 0.28, 0.54, 1.23 and 2.59 mg BAS 222 28 F/L). Observations for mortality were conducted 24 and 48 hours after start of exposure.

The results are based on initially measured concentrations. No significant effects on *Heterocypris* sp. were observed. No other unusual observations were made.

In a 48 hours static acute toxicity study with Ostracoda the EC_{50} of BAS 222 28 F based on initially measured concentrations was > 2.59 mg/L (corresponding to > 1.77 mg a.s./L). The NOEC was determined to be ≥ 2.59 mg/L (corresponding to ≥ 1.77 mg a.s./L; initially measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch 2000-2, content of a.s.: metiram (BAS 222 F; Reg. No 250284): 68.5% (70% nominal).

B. STUDY DESIGN

Test species: Ostracoda (Crustacea), genus *Heterocypris*, mixed age population, approx. same size; individuals collected from mesocosm facility, prior adapted to test conditions.

Test design: 48 hours static test, 6 treatment groups (5 test concentrations, control), 3 replicates with 3 individuals each; assessment of mortality after 24 and 48 hours.

Endpoints: NOEC, EC_{50} based on mortality.

Test concentrations: Control, 0.20, 0.40, 0.80, 1.60 and 3.20 mg BAS 222 28 F/L (nominal); corresponding to initially measured concentrations of 0, 0.15, 0.28, 0.54, 1.23 and 2.59 mg/L.

Test conditions: Multiwell tissue culture plate, test volume 3 mL, dilution water "M4" Elendt medium; conductivity: 680 µS/cm; total hardness: 2.43 mmol/L, pH 8.0 - 8.1; oxygen content: 8.9 mg/L - 9.0 mg/L; temperature 20 °C; light intensity: < 1500 lux; photoperiod: 16 h light : 8 h dark; no feeding, no ventilation.

Analytics: Analytical verification of test substance concentrations was conducted using HPLC with UV-detection.

Statistics: None applicable.

II. RESULTS AND DISCUSSION

Analytical measurements: Measured values for metiram at test initiation ranged from 67.6% to 80.9% (average 74.4%) of nominal. The biological results are based on initially measured concentrations.

Biological results: No significant effects on *Heterocypris* were observed. No other unusual observations were made. The results are summarized in Table 8.2.4.2-4.

Table 8.2.4.2-4: Effect (48 h) of BAS 222 28 F on Ostracoda mortality

Concentration [mg/L] (nominal)	**Control**	**0.20**	**0.40**	**0.80**	**1.60**	**3.20**
Concentration [mg/L] (initially measured)	**-**	**0.15**	**0.28**	**0.54**	**1.23**	**2.59**
Mortality (24 h) [%]	0	0	0	0	0	0
Mortality (48 h) [%]	0	0	0	0	0	0
	Endpoints (initially measured)					
	based on BAS 222 28 F [mg/L]			**based on BAS 222 F [mg a.s./L]**		
EC_{50} (48 h)	**> 2.59**			**> 1.77**		
NOEC (48 h)	≥ 2.59			≥ 1.77		

III. CONCLUSION

In a 48 hours static acute toxicity study with Ostracoda the EC_{50} of BAS 222 28 F based on initially measured concentrations was > 2.59 mg/L (corresponding to > 1.77 mg a.s./L). The NOEC was determined to be ≥ 2.59 mg/L (corresponding to ≥ 1.77 mg a.s./L; initially measured).

The following acute toxicity study on aquatic snail Physa acuta performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.4.2/5* *Junker M., 2002f* *Effect of BAS 222 28 F on Physa acuta in a 96 hours static, acute toxicity test* *2002/1005265*
Guidelines:	*OECD 202*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 96 h static acute toxicity laboratory a mixed age population of the aquatic snail *Physa acuta* was exposed to BAS 222 28 F at nominal concentrations of 0, 0.20, 0.40, 0.80, 1.60 and 3.20 mg/L (corresponding to initially measured concentrations of 0, 0.15, 0.28, 0.54, 1.23 and 2.59 mg BAS 222 28 F/L). Observations on mortality and other signs of toxicity were conducted 24, 48, 72 and 96 hours after start of exposure.

The results are based on initially measured concentrations. No significant effects on survival of *Physa acuta* were observed at any concentration level. No other unusual observations were made.

In a 96 hours static acute toxicity study with *Physa acuta* the LC_{50} of BAS 222 28 F based on initially measured concentrations was > 2.59 mg/L (corresponding to > 1.77 mg a.s./L). The NOEC (96 h) was determined to be ≥ 2.59 mg/L (corresponding to ≥1.77 mg a.s./L; initially measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch 2000-2, content of a.s.: metiram (BAS 222 F; Reg. No 250284): 68.5% (70% nominal).

B. STUDY DESIGN

Test species: *Physa acuta* (Gastropoda, Physidae), mixed age population, two larger and one medium-sized snails per replicate; individuals collected from mesocosm facility, prior adapted to test conditions.

Test design: 96 hours static test, 6 treatment groups (5 test concentrations, control), 3 replicates with 3 individuals each; assessment of mortality and other signs of toxicity after 24, 48, 72 and 96 hours.

Endpoints: NOEC, LC_{50} based on mortality.

Test concentrations: Control, 0.20, 0.40, 0.80, 1.60 and 3.20 mg BAS 222 28 F/L (nominal); corresponding to initially measured concentrations of 0, 0.15, 0.28, 0.54, 1.23 and 2.59 mg/L)

Test conditions: Glass vessels, test volume 50 mL, dilution water "M4" Elendt medium; conductivity: 680 µS/cm; total hardness: 2.43 mmol/L, pH 8.04 - 8.06; oxygen content: 8.9 mg/L - 9.0 mg/L; temperature 20 °C - 21° C; light intensity: < 1500 lux; photoperiod: 16 h light : 8 h dark; no feeding, no ventilation.

Analytics: Analytical verification of test substance concentrations was conducted using HPLC with UV-detection.

Statistics: None applicable.

II. RESULTS AND DISCUSSION

Analytical measurements: Measured values for metiram at test initiation ranged from 67.6% to 80.9% (average 74.4%) of nominal. The biological results are based on initially measured concentrations.

Biological results: No significant effects on survival of *Physa acuta* were observed at any concentration level. No other unusual observations were made. The results are summarized in Table 8.2.4.2-5.

Table 8.2.4.2-5: Acute toxicity (96 h) of BAS 222 28 F on *Physa acuta*

Concentration [mg/L] (nominal)	**Control**	**0.20**	**0.40**	**0.80**	**1.60**	**3.20**
Concentration [mg/L] (initially measured)	**-**	**0.15**	**0.28**	**0.54**	**1.23**	**2.59**
Mortality (24 h) [%]	0	0	0	0	0	0
Mortality (48 h) [%]	0	0	0	0	0	0
Mortality (72 h) [%]	0	0	0	0	0	0
Mortality (96 h) [%]	0	0	0	0	0	0
	Endpoints (initially measured)					
	based on BAS 222 28 F [mg/L]			**based on BAS 222 F [mg a.s./L]**		
LC_{50} (96 h)	> **2.59**			> **1.77**		
NOEC (96 h)	≥ 2.59			≥ 1.77		

III. CONCLUSION

In a 96 hours static acute toxicity study with *Physa acuta* the LC_{50} of BAS 222 28 F based on initially measured concentrations was > 2.59 mg/L (corresponding to > 1.77 mg a.s./L). The NOEC (96 h) was determined to be ≥ 2.59 mg/L (corresponding to ≥1.77 mg a.s./L; initially measured).

<u>New supplemental data (not evaluated previously on EU level):</u>

The following acute toxicity study on the saltwater mysid Americamysis bahia performed with metiram (tested as BAS 222 29 F) is not required for registration in the EU and it has not been evaluated previously on EU level. However, the study was conducted due to U.S. data requirements and it is provided for completeness.
The 48-h LC_{50} obtained in the 96 h study on A. bahia is used as relevant endpoint for the risk assessment in accordance with the EU Regulation 283/2013 (European Commission, 2013) which describes the data requirements for active substances. Also the recent EFSA Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters (EFSA, 2013) advises to use the 48-h endpoint, which allows an easy comparison to the other standard acute invertebrate tests. Therefore only the 48-h results are shown below.

Report:	CA 8.2.4.2/6 Minderhout T. et al., 2010e Metiram: A 96-hour flow-through acute toxicity test with the saltwater mysid (Americamysis bahia) 2010/7010900
Guidelines:	EPA 850.1035
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

In a 96-hour flow-through acute toxicity laboratory study, saltwater mysids were exposed to nominal concentrations of 0.013, 0.025, 0.050, 0.100 and 0.200 mg metiram/L (corresponding to mean measured concentrations of 0.0071, 0.015, 0.033, 0.068 and 0.147 mg a.s./L) in groups of 10 animals in test chambers containing 15 L water with 2 replicates per concentration. Additionally, a dilution water control and a solvent control were set up. Saltwater mysids were observed for survival and symptoms of toxicity after 3.5, 24, 48, 72 and 96 hours of exposure.

The biological results are based on mean measured concentrations (measured over the 96 h study period). After 48 hours of exposure, no mortality was observed in the control and at the two lowest test item concentrations of 0.0071 and 0.015 mg a.s./L, whereas 5% mortality was observed in the solvent control and at test item concentrations of 0.033 and 0.068 mg a.s./L. At the highest test item concentration of 0.147 mg a.s./L, 50% of the mysids were dead after 48 h of exposure. No sub-lethal effects were observed after 48 h of exposure at any test item concentration tested.

In a flow-through acute toxicity study with saltwater mysids (*Americamysis bahia*), the LC_{50} (48 h) for metiram (tested as BAS 222 29 F) was determined to be > 0.147 mg a.s./L based on mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item:	BAS 222 29 F, batch no. 300013; content of a.s.: metiram (BAS 222 F; Reg. No 250284): 91.6% (nominal: 85%); density: 1.860 g/cm³.

B. STUDY DESIGN

Test species:	Saltwater mysid (*Americamysis bahia*), juveniles, age: less than 24 hours; source: in-house cultures.
Test design:	Flow-through system (96 hours); 10 mysids per test chamber, 2 replicates per concentration; assessment of mortality and symptoms of toxicity at 3.5, 24, 48, 72 and 96 hours after start of exposure.
Endpoints:	LC_{50} (48 h), mortality and sub-lethal effects.
Test concentrations:	Control (dilution water), solvent control (0.1 mL dimethylformamide/L), 0.013, 0.025, 0.050, 0.100 and 0.200 mg metiram/L (nominal), corresponding to mean measured concentrations of < limit of quantification (LOQ), < LOQ, 0.0071, 0.015, 0.033, 0.068 and 0.147 mg a.s./L.
Test conditions:	25 L Teflon-lined stainless steel aquaria, test volume approx. 15 L, test compartments: glass container (12 cm diameter, 19 cm height) with nylon screen attached to two holes on the sides of the container; filtered, aerated and sterilized natural seawater mixed with well water, salinity: approx. 20‰; temperature: 24.7 °C - 24.8 °C; pH 8.0 - 8.1; oxygen content: 6.1 mg/L - 7.4 mg/L; flow rate: approx. 10 volume additions/24 h/test vessel; photoperiod 16 h light : 8 h; light intensity 314 lux; mysids were fed daily with live brine shrimps (*Artemia* sp.).
Analytics:	Analytical verification of test item concentrations was conducted using a GC-method with MS-detection.
Statistics:	Descriptive statistics, probit analysis for calculation of the LC_{50} value.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of the active substance was conducted in each concentration at test initiation, 48 h after start of exposure and at test termination after 96 h. Measured concentrations of metiram ranged from 54% to 68% of nominal concentrations at test initiation, from 51% to 71% 48 h after start of exposure and from 53% to 89% of nominal at test termination. The following biological results are based on mean measured concentrations (measured over the 96 h study period).

Biological results: After 48 hours of exposure, no mortality was observed in the control and at the two lowest test item concentrations of 0.0071 and 0.015 mg a.s./L, whereas 5% mortality was observed in the solvent control and at test item concentrations of 0.033 and 0.068 mg a.s./L. At the highest test item concentration of 0.147 mg a.s./L, 50% of the mysids were dead after 48 h of exposure. No sub-lethal effects were observed after 48 h of exposure at any test item concentration tested. The results are summarized in Table 8.2.4.2-6.

Table 8.2.4.2-6: Acute toxicity (48 h) of metiram (tested as BAS 222 29 F) to saltwater mysids (*Americamysis bahia*)

Concentration [mg a.s./L] (nominal)	**Control**	**Solvent control**	**0.013**	**0.025**	**0.050**	**0.100**	**0.200**
Concentration [mg a.s./L] (mean measured)	**--**	**--**	**0.0071**	**0.015**	**0.033**	**0.068**	**0.147**
Mortality (48 h) [%]	0	5	0	0	5	5	50
Symptoms (48 h)	none	none	none	none	none	none	none
Endpoints [mg metiram/L] (mean measured)							
LC_{50} (48 h)	> 0.147						

III. CONCLUSION

In a flow-through acute toxicity study with saltwater mysids (*Americamysis bahia*), the LC_{50} (48 h) for metiram (tested as BAS 222 29 F) was determined to be > 0.147 mg a.s./L based on mean measured concentrations.

The following acute toxicity study on the eastern oyster Crassostrea virginica performed with metiram (tested as BAS 222 29 F) is not required for registration in the EU and it has not been evaluated previously on EU level. However, the study was conducted due to U.S. data requirements and it is provided for completeness.

Report:	CA 8.2.4.2/7 Minderhout T. et al., 2010f Metiram: A 96-hour shell deposition test with the eastern oyster (Crassostrea virginica) 2010/7010901
Guidelines:	EPA 850.1025
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

In a 96-hour acute toxicity laboratory study the effect of metiram on shell deposition of eastern oysters was investigated under flow-through conditions. The eastern oysters were exposed to metiram (tested as solo-formulation BAS 222 29 F) at nominal concentrations of 0.0065, 0.022, 0.072, 0.240 and 0.800 mg a.s./L (corresponding to mean measured concentrations of 0.0042, 0.014, 0.056, 0.211 and 0.729 mg a.s./L) in groups of 20 oysters per replicate with two replicates per treatment. Additionally, a dilution water control and a solvent control were set up. Eastern oysters were observed for survival and symptoms of toxicity 5, 24, 48, 72 and 96 h after start of exposure. Measurements of shell deposition for each oyster were made after 96 hours.

The biological results are based on mean measured concentrations of the test item. After 96 hours of exposure, survival of oysters was 100% in the control and the solvent control. No mortality occurred at test item concentrations of up to and including the highest test item concentration. No sub-lethal effects were noted during the exposure period in the controls and the test item treatments. Statistically significant inhibition of shell growth compared to the pooled control was observed at the four highest test item concentrations.

In a flow-through acute toxicity study with eastern oysters (*Crassostrea virginica*), the LC_{50} (96 h) and the EC_{50} (96 h) for metiram (tested as BAS 222 29 F) was > 0.729 mg a.s./L and 0.140 mg a.s./L, respectively based on mean measured concentrations. The NOEC (96 h) was determined to be 0.0042 mg a.s./L (mean measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 29 F, lot no. 300013; content of a.s.: metiram (BAS 222 F; Reg. No 250284): 91.6% (nominal: 85%); density: 1.860 g/cm³.

B. STUDY DESIGN

Test species: Eastern oyster (*Crassostrea virginica*), length: 30.0 - 35.4 mm; source: "Circle C Oyster Ranch", Ridge, Maryland, USA.

Test design: Flow-through system (96 hours); 5 test item concentrations plus a control and a solvent control, 2 replicates for each test item concentration and the controls with 20 oysters per test chamber; assessment of mortality and symptoms of toxicity 5, 24, 48, 72 and 96 h after start of exposure; measurements of shell deposition 96 hours after start of exposure.

Endpoints: EC_{50} and NOEC for shell growth inhibition, LC_{50} for mortality.

Test concentrations: Control (dilution water), solvent control (0.1 mL dimethylformamide/L), 0.0065, 0.022, 0.072, 0.240 and 0.800 mg metiram/L (nominal), corresponding to mean measured concentrations of < limit of quantification (LOQ), < LOQ, 0.0042, 0.014, 0.056, 0.211 and 0.729 mg a.s./L.

Test conditions: 54 L glass aquaria, test volume 27 L, dilution water: natural filtered seawater, aerated and diluted with freshwater, flow rate: approx. 19 volume additions per 24 hours in each test vessel; salinity: 20‰ - 21‰; temperature: 18.3°C - 19.4°C; pH 8.1 - 8.3; oxygen content: 6.7 mg/L - 7.3 mg/L; photoperiod 16 h light : 8 h dark; light intensity: 424 lux; feeding: suspension of marine microalgae at a nominal rate of 5.8×10^9 cells/oyster/day.

Analytics: Analytical verification of test item concentrations was conducted using a GC-method with MS-detection.

Statistics: Descriptive statistics; linear interpolation for calculation of EC_{50}; comparison of shell deposition data from the control groups using an appropriate statistical test ($\alpha = 0.05$), Bonferroni t-test for calculation of the NOEC value for shell deposition data ($\alpha = 0.05$).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of the active substance was conducted in each concentration at test initiation, 48 h after start of exposure and at test termination. Measured concentrations of metiram ranged from 67% to 102% of nominal concentrations at test initiation, from 48% to 93% 48 h after start of exposure and from 61% to 79% of nominal at test termination. The following biological results are based on mean measured concentrations.

Biological results: After 96 hours of exposure, survival of oysters was 100% in the control and the solvent control. No mortality occurred at test item concentrations of up to and including the highest test item concentration. No sub-lethal effects were noted during the exposure period in the controls and the test item treatments. Control and solvent control oysters deposited an average of 5.3 and 4.7 mm of new shell during the test, respectively. No statistically significant difference in shell deposition was observed between the control groups ($\alpha = 0.05$). Subsequent statistical analyses were performed by comparing the pooled control data to the treatment data. Statistically significant inhibition of shell growth compared to the pooled control was observed at the four highest test item concentrations (Bonferroni t-test, $\alpha = 0.05$). The results are summarized in Table 8.2.4.2-7.

Table 8.2.4.2-7: Acute toxicity (96 h) of metiram (tested as BAS 222 29 F) to eastern oysters (*Crassostrea virginica*)

Concentration [mg a.s./L] (nominal)	**Control**	**Solvent control**	**0.0065**	**0.022**	**0.072**	**0.240**	**0.800**
Concentration [mg a.s./L] (mean measured)	**--**	**--**	**0.0042**	**0.014**	**0.056**	**0.211**	**0.729**
Mortality after 96 h [%]	0	0	0	0	0	0	0
Shell growth inhibition after 96 h [% of pooled control]	--	--	10	28 *	38 *	60 *	78 *
	Endpoints [mg metiram/L] (mean measured)						
LC_{50} (96 h)	> 0.729						
EC_{50} (96 h)	0.140 (95% confidence limits: 0.048 - 0.265)						
NOEC (96 h)	0.0042						

* Statistically significantly different compared to the pooled control (Bonferroni t-test, $\alpha = 0.05$).

III. CONCLUSION

In a flow-through acute toxicity study with eastern oysters (*Crassostrea virginica*), the LC_{50} (96 h) and the EC_{50} (96 h) for metiram (tested as BAS 222 29 F) was > 0.729 mg a.s./L and 0.140 mg a.s./L, respectively based on mean measured concentrations. The NOEC (96 h) was determined to be 0.0042 mg a.s./L (mean measured).

The following acute toxicity study on the saltwater mysid Americamysis bahia performed with the metiram metabolite Ethylenethiourea is not required for registration in the EU and it has not been evaluated previously on EU level. However, the study was conducted due to U.S. data requirements and it is provided for completeness.
The 48-h LC_{50} obtained in the 96 h study on A. bahia is used as relevant endpoint for the risk assessment in accordance with the EU Regulation 283/2013 (European Commission, 2013) which describes the data requirements for active substances. Also the recent EFSA Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters (EFSA, 2013) advises to use the 48-h endpoint, which allows an easy comparison to the other standard acute invertebrate tests. Therefore only the 48-h results are shown below.

Report: CA 8.2.4.2/8
Soucy K., 2008c
Ethylenethiourea - Ethylenethiourea - acute toxicity to mysids (Americamysis bahia) under static conditions, following OPPTS guideline 850.1035
2008/7015438
Guidelines: EPA 850.1035
GLP: yes
(certified by United States Environmental Protection Agency)

Executive Summary

In a 96-hour static acute toxicity laboratory study, saltwater mysids were exposed to ethylenethiourea (ETU, metabolite of metiram) at nominal concentrations of 1.6, 3.1, 6.3, 13, 25 and 50 mg ETU/L (corresponding to mean measured concentrations of 1.5, 3.0, 6.4, 14, 26 and 53 mg/L) in groups of 10 animals in glass beakers containing 0.9 L water with 2 replicates per concentration. Additionally, a dilution water control was set up. Saltwater mysids were observed for survival and symptoms of toxicity after 24, 48, 72 and 96 hours of exposure.

The biological results are based on mean measured concentrations (measured over the 96 h study period). After 48 hours of exposure, no mortality was observed in the control and the test item concentration of 3.0 mg ETU/L, whereas 5% mortality occurred at the lowest test item concentration of 1.5 mg/L. At the test item concentrations of 6.4 and 14 mg/L, 5% and 70% mortality was observed, respectively. At the two highest test item concentrations of 26 and 53 mg/L, all mysids were dead after 48 h of exposure. No sub-lethal effects were found at any test item concentration after 48 hours.

In a static acute toxicity study with saltwater mysids (*Americamysis bahia*), the LC_{50} (48 h) of ETU (metabolite of metiram) was determined to be 11 mg/L based on mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: Ethylenethiourea (BF 222-ETU; Reg. no. 146 099; metabolite of metiram); lot. no.: 04816CH; purity: 100%.

B. STUDY DESIGN

Test species: Saltwater mysid (*Americamysis bahia*), juveniles, age: less than 24 hours; source: in-house cultures; originally obtained from Aquatic BioSystems, Inc., Fort Collins, Colorado, USA.

Test design: Static system (96 hours); 10 mysids per test vessel, 2 replicates per concentration; assessment of mortality and symptoms of toxicity at 24, 48, 72 and 96 hours after start of exposure.

Endpoints: LC_{50} (48 h), mortality and sub-lethal effects.

Test concentrations: Control (dilution water), 1.6, 3.1, 6.3, 13, 25 and 50 mg ETU/L (nominal), corresponding to mean measured concentrations of < limit of quantification (LOQ), 1.5, 3.0, 6.4, 14, 26 and 53 mg/L.

Test conditions: 1.0 Lglass beakers, test volume 0.9 L; filtered natural seawater diluted with well water, salinity: 20‰; temperature: 23 °C - 25 °C; pH 7.3 - 7.9; oxygen content: 4.0 mg/L - 8.4 mg/L; photoperiod 16 h light : 8 h; light intensity 47 - 77 footcandles (510 - 830 lux); gentle aeration from 48 h after test initiation on; mysids were fed daily with live brine shrimp nauplii (*Artemia salina*).

Analytics: Analytical verification of test item concentrations was conducted using an HPLC-method with UV-detection.

Statistics: Descriptive statistics, binomial probability analysis for calculation of the LC_{50} value.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at test initiation and at test termination. Measured concentrations of ethylenethiourea ranged from 87.5% to 100.0% of nominal concentrations at test initiation and from 93.8% to 115.4% of nominal at test termination. The following biological results are based on mean measured concentrations (measured over the 96 h study period).

Biological results: After 48 hours of exposure, no mortality was observed in the control and the test item concentration of 3.0 mg ETU/L, whereas 5% mortality occurred at the lowest test item concentration of 1.5 mg/L. At the test item concentrations of 6.4 and 14 mg/L, 5% and 70% mortality was observed, respectively. At the two highest test item concentrations of 26 and 53 mg/L, all mysids were dead after 48 h of exposure. No sub-lethal effects were found at any test item concentration after 48 hours. The results are summarized in Table 8.2.4.2-8.

Table 8.2.4.2-8: Acute toxicity 48 h) of ETU (metabolite of metiram) to saltwater mysids (*Americamysis bahia*)

Concentration [mg/L] (nominal)	**Control**	**1.6**	**3.1**	**6.3**	**13**	**25**	**50**
Concentration [mg/L] (mean measured)	**--**	**1.5**	**3.0**	**6.4**	**14**	**26**	**53**
Mortality (48 h) [%]	0	5	0	5	70	100	100
Symptoms (48 h)	none	none	none	none	none	n.d.	n.d.
Endpoints [mg ETU/L] (mean measured)							
LC_{50} (48 h)	11 (95% confidence limits: 6.4 - 26)						

n.d. = not determined; all animals dead

III. CONCLUSION

In a static acute toxicity study with saltwater mysids (*Americamysis bahia*), the LC_{50} (48 h) of ETU (metabolite of metiram) was determined to be 11 mg/L based on mean measured concentrations.

The following acute toxicity study on the eastern oyster Crassostrea virginica performed with the metiram metabolite Ethylenethiourea is not required for registration in the EU and it has not been evaluated previously on EU level. However, the study was conducted due to U.S. data requirements and it is provided for completeness.

Report:	CA 8.2.4.2/9 York D.O., 2008a Ethylenethiourea - Acute toxicity to eastern oyster (Crassostrea virginica) under flow through conditions, following OPPTS guideline (draft) 850.1025 2008/7013844
Guidelines:	EPA 850.1025
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

In a 96-hour acute toxicity laboratory study the effect of ethylenethiourea (ETU, metabolite of metiram) on shell deposition of eastern oysters was investigated under flow-through conditions. The eastern oysters were exposed to nominal concentrations of 9.3, 16, 26, 43, 72 and 120 mg ETU/L (corresponding to mean measured concentrations of 15, 22, 31, 42, 68 and 110 mg/L) in groups of 20 oysters per aquarium with two replicates per treatment. Additionally, a dilution water control was set up. Eastern oysters were observed for survival and symptoms of toxicity at test initiation, 6 h after start of exposure and at each subsequent 24-hour interval until termination of the test. Measurements of shell deposition for each oyster were made after 96 hours.

The biological results are based on mean measured concentrations of the test item. After 96 hours of exposure, no mortality or sub-lethal effects were observed in the control and up to and including the highest test item concentration. Statistically significant inhibition of shell growth compared to the control occurred at the two highest test item concentrations.

In a flow-through acute toxicity study with eastern oysters (*Crassostrea virginica*) the LC_{50} (96 h) and the EC_{50} (96 h) for ETU (metabolite of metiram) were both > 110 mg/L based on mean measured concentrations. The NOEC (96 h) was determined to be 42 mg/L (mean measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: Ethylenethiourea (BF 222-ETU; Reg. no. 146 099; metabolite of metiram); lot. no.: 04816CH; purity: 100%.

B. STUDY DESIGN

Test species: Eastern oyster (*Crassostrea virginica*), valve height: 44 ± 2.8 mm; source: "Circle C Oysters", Ridge, Maryland, USA.

Test design: Flow-through system (96 hours); 6 test item concentrations plus a control, 2 replicates for each test item concentration and the control with 20 oysters per aquarium; assessment of mortality and symptoms of toxicity at test initiation, 6 h after start of exposure and at each subsequent 24-hour interval until termination of the test; measurements of shell deposition 96 hours after start of exposure.

Endpoints: EC_{50} and NOEC for shell growth inhibition, mortality and symptoms of toxicity.

Test concentrations: Control (dilution water), 9.3, 16, 26, 43, 72 and 120 mg ETU/L (nominal), corresponding to mean measured concentrations of < limit of quantification (LOQ), 15, 22, 31, 42, 68 and 110 mg/L.

Test conditions: Glass aquaria (49.5 x 25.5 x 29 cm); test volume 18 L, dilution water: natural filtered seawater, diluted with freshwater, flow rate: 75 mL/minute (approx. 6.0 volume additions per 24 hours in each test vessel; salinity: 19‰ - 20‰; temperature: 21°C - 22°C; pH 7.6 - 7.9; oxygen content: 7.0 mg/L - 7.7 mg/L; photoperiod 16 h light : 8 h dark; feeding: algae (*Tetraselmus maculata*) at a rate of 10^7 cells/mL three times daily.

Analytics: Analytical verification of test item concentrations was conducted using an HPLC-method with UV-detection.

Statistics: Descriptive statistics; Williams' Test for calculation of the NOEC value for shell deposition data.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at test initiation and at test termination. Measured concentrations of ETU ranged from 98.6% to 161.3% of nominal concentrations at test initiation and from 91.7% to 150.5% of nominal at test termination. The following biological results are based on mean measured concentrations.

Biological results: After 96 hours of exposure, no mortality or sub-lethal effects were observed in the control and at up to and including the highest test item concentration. Control oysters deposited an average of 2.0 mm of new shell during the test. Statistically significant inhibition of shell growth compared to the control occurred at the two highest test item concentrations (Williams' Test). The results are summarized in Table 8.2.4.2-9.

Table 8.2.4.2-9: Acute toxicity (96 h) of ETU (metabolite of metiram) to eastern oysters (*Crassostrea virginica*)

Concentration [mg/L] (nominal)	**Control**	**9.3**	**16**	**26**	**43**	**72**	**120**
Concentration [mg/L] (mean measured)	**--**	**15**	**22**	**31**	**42**	**68**	**110**
Mortality after 96 h [%]	0	0	0	0	0	0	0
Shell growth inhibition after 96 h [% of control]	--	11	11	0	12	29 *	25 *
	Endpoints [mg ETU/L] (mean measured)						
LC_{50} (96 h)	> 110						
EC_{50} (96 h) #	> 110						
NOEC (96 h)	42						

* Statistically significantly different compared to the control (Williams' Test).
\# Empiric estimation of EC_{50} value.

III. CONCLUSION

In a flow-through acute toxicity study with eastern oysters (*Crassostrea virginica*) the LC_{50} (96 h) and the EC_{50} (96 h) for ETU (metabolite of metiram) were both > 110 mg/L based on mean measured concentrations. The NOEC (96 h) was determined to be 42 mg/L (mean measured).

CA 8.2.5 Long-term and chronic toxicity to aquatic invertebrates

CA 8.2.5.1 Reproductive and development toxicity to Daphnia magna

Data already submitted during previous Annex I inclusion process (already evaluated on EU level):

The following chronic toxicity study on Daphnia magna performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.5.1/1* *Maisch R., 1997b* *Determination of the chronic effect of BAS 222 28 F on the reproduction of the water flea Daphnia magna STRAUS in a flow-through test system* *1997/10521*
Guidelines:	*EPA 850.1300*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Report:	*CA 8.2.5.1/2* *Maisch R., 1997c* *Determination of the chronic effect of BAS 222 28 F on the reproduction of the water flea Daphnia magna STRAUS in a flow-through test system* *1997/10537*
Guidelines:	*EPA 850.1300*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 21-day flow-through toxicity test, effects of BAS 222 28 F on water fleas (*Daphnia magna*) were examined. Neonates less than 24 hours old were exposed to nominal concentrations of 0.00313, 0.00625, 0.0125, 0.0250, 0.0500 and 0.100 mg/L (corresponding to measured concentrations of 0.000770, 0.00154, 0.00308, 0.00615, 0.0123 and 0.0246 mg/L based on the median analytical recovery rate over all test concentrations and all sampling days of 24.6%), and a control. All treatment groups and the control consisted of 4 replicates, each containing 5 parent daphnids. After 21 days parent mortality, body length and reproductive performance were assessed.

The results are based on the median analytical recovery rate over all test concentrations and all sampling days (24.6%). After 21 days of exposure, one parent daphnid died in the control group. Survival was significantly reduced compared to control in the 0.0123 mg/L and 0.0246 mg/L treatments. First brood was observed after 8 days in the control and all treatments. The number of offspring varied between 89.7 and 131.6, with significant reductions compared to control at 0.0123 mg/L and higher. At test end the parent body length ranged from 3.9 mm to 4.0 mm and no statistically significant reduction occurred.

In a 21-day flow-through toxicity study with *Daphnia magna* the NOEC of BAS 222 28 F was 0.00615 mg/L (corresponding to 0.00437 mg a.s./L) based on measured concentrations (*i.e.* a median recovery rate of 24.6%).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch 96-1, content of a.s.: metiram (BAS 222 F; Reg. No 250284): 71.12% analyzed (70% nominal).

B. STUDY DESIGN

Test species: Water flea (*Daphnia magna* STRAUS), neonates, age at test initiation > 2 < 24 hours, source: in-house culture, originally supplied by Institut National de Recherché Chimique Appliquée, France, 1978.

Test design: Flow-through test (21 days); 6 test concentrations plus control, four replicates per concentration and controls, five parent daphnids per replicate and treatment; check of the study and recording: daily, assessment of parent mortality, body length and reproduction after 21 days.

Endpoints: NOEC, parent mortality, parent length and reproduction.

Test concentrations: Control, 0.00313, 0.00625, 0.0125, 0.0250, 0.0500 and 0.100 mg BAS 222 28 F/L (nominal); corresponding to measured concentrations of 0.000770, 0.00154, 0.00308, 0.00615, 0.0123 and 0.0246 mg/L based on the median analytical recovery rate over all test concentrations and all sampling days of 24.6%.

Test conditions: Test chambers (volume 250 mL), flow-through rate approx. 1250 mL/day, dilution water “M4” (Elendt medium); pH 7.8 - 8.0; oxygen content: 7.7 mg/L - 9.6 mg/L; water temperature: 19.1 °C - 21.4 °C; photoperiod: 16 hours light : 8 hours dark; light intensity; 5 - 6 µE/(m² * s); feeding with algae (*Scenedesmus subspicatus*).

Analytics: The test item concentrations were analyzed using GC-method CP067/1.

Statistics: Descriptive statistics; ANOVA via Duncan´s multiple range test for evaluation of LOEC and NOEC.

II. RESULTS AND DISCUSSION

Analytical results: Mean recoveries of BAS 222 28 F were in the range of 19.2% - 106.1% of nominal test item concentrations at study initiation. At study termination analyzed concentrations of BAS 222 28 F were between 6.4% and 36.9% of nominal concentrations. As the analytical measurements varied significantly, the theoretical concentrations of the test samples could not be confirmed, therefore the biological results are based on the median analytical recovery rate over all test concentrations and all sampling days of 24.6%.

Biological results: After 21 days of exposure, one parent daphnid died in the control group. Survival was significantly reduced compared to control (Duncan´s multiple range test) in the 0.0123 mg/L and 0.0246 mg/L treatments. First brood was observed after 8 days in the control and all treatments. The number of offspring varied between 89.7 and 131.6, with significant reductions compared to control (Duncan´s multiple range test) at 0.0123 mg/L and higher. At test end the parent body length ranged from 3.9 mm to 4.0 mm and no statistically significant reduction occurred. The results are summarized in Table 8.2.5.1-1.

Table 8.2.5.1-1: Effects of BAS 222 28 F (21 d) on *Daphnia magna* reproduction and parent mortality and body length

Concentration [mg/L] (nominal)	**Control**	**0.00312**	**0.00625**	**0.0125**	**0.0250**	**0.0500**	**0.100**
Concentration [mg/L] (measured)#	**Control**	**0.000770**	**0.00154**	**0.00308**	**0.00615**	**0.0123**	**0.0246**
Parent mortality [%]	5	10	5	0	15	30*	30*
av. offspring/parent	131.6	128.0	120.0	114.0	130.6	114.0*	89.7*
day of first brood	8	8	8	8	8	8	8
av. body length [mm]	3.9	3.9	3.9	3.9	4.0	4.0	3.9
	Endpoints (based on the median recovery rate)						
	based on BAS 222 28 F [mg/L]			**based on BAS 222 F [mg a.s./L]**			
NOEC (21 d)	**0.00615**			**0.00437**			

* Statistically significant differences compared to the control (Duncan`s multiple range test)
\# Measured values calculated with median analytical recovery rate over all test concentrations and all sampling days of 24.6%

III. CONCLUSION

In a 21-day flow-through toxicity study with *Daphnia magna* the NOEC of BAS 222 28 F was 0.00615 mg/L (corresponding to 0.00437 mg a.s./L) based on measured concentrations (*i.e.* a median recovery rate of 24.6%).

The following chronic toxicity study on Daphnia magna performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Furthermore the endpoint from the more recent study on D. magna (BASF DocID 1997/10521) is considered as relevant endpoint for the risk assessment as analytical measurements appeared more reliable; the endpoint obtained in this second study below is not used. Therefore no summary is provided.

Report:	*CA 8.2.5.1/3* *Jatzek H.-J., 1990a* *Determination of the longterm effects of Polyram WG BAS 222 28 F Prod.-Nr. 81708 on the parthenogenetic reproduction rate of the waterflea Daphnia magna STRAUS* *1990/0310*
Guidelines:	*EEC XI/681/86*
GLP:	*yes* *(certified by Ministerium fuer Umwelt und Gesundheit, Postfach 3160, 6500 Mainz)*

Report:	CA 8.2.5.1/4 Jatzek H.-J., 1991a Determination of the longterm effects of Polyram WG BAS 222 28 F on the parthenogenetic reproduction rate of the waterflea Daphnia magna STRAUS 1991/10950
Guidelines:	EEC XI/681/86
GLP:	yes (certified by Ministerium fuer Umwelt und Gesundheit, Postfach 3160, 6500 Mainz)

The following chronic population development study on Daphnia magna performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.5.1/5* *Dohmen G.P., 2002a* *Effect of BAS 222 28 F on the population development of Daphnia magna STRAUS in an extended laboratory test* *2002/1005270*
Guidelines:	*EPA 72-2, EPA 850.1300, HARAP, CLASSIC, OECD 211*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 28-day static population experiment effects of metiram (tested as BAS 222 28 F) on water fleas (*Daphnia magna*) of mixed age classes were examined. The test substance was applied four times with 7 day intervals at nominal concentrations of 0.025, 0.050, 0.10, 0.20, 0.40, 0.80 mg a.s./L. For all treatment groups 2 replicates were tested. The control consisted of 4 replicates. The assessment of *Daphnia* population development was done over the entire experimental period. In addition, the fate of the test substance was monitored during the study.

Biological results were based on nominal concentrations. Daphnia populations showed typical exponential growth during the first two weeks of the experiment. Starting with 10 daphnids per aquarium, after 14 days about 300 daphnids per aquarium were found. Thereafter, population growth slowed down. Only the two highest concentrations had a negative impact on population development of *Daphnia* even after several applications. The initial population increase in the first two weeks (following 2-3 treatments) was nearly identical in all treatment groups < 0.40 mg/L. Variability between treatments started to increase slightly after about 14 to 19 days, however, a statistically significant reduction was only found at the two highest concentrations of 0.40 mg a.s./L and 0.80 mg a.s./L. But even at 0.80 mg a.s./L, there was still significant population growth, indicating rapid recovery after the treatment period.

In a 28-day population study with *Daphnia magna* the NOEC of metiram (applied as formulated product BAS 222 28 F) was 0.20 mg a.s./L and the corresponding LOEC was determined to be 0.40 mg a.s./L.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch 2000-2, content of a.s.: metiram (BAS 222 F; Reg. No 250284): 68.5% (70% nominal).

B. STUDY DESIGN

Test species: Water flea (*Daphnia magna* STRAUS) from in-house culture, originally supplied by Institut National de Recherche Chimique Appliquée, France, 1978; mixed age classes (one adult, 2 daphnids approximately < 2 days old, 2 juvenile daphnids ≥ 2 < 5 days old and 2 daphnids approx. 5-8 days old).

Test design: Population experiment, static test (28 days); 6 test concentrations with two replicates plus control with four replicates, four test substance applications at 7 day intervals (i.e. at DAT 0, 7, 14 and 21), 10 daphnids intially per replicate and treatment; assessment of daphnia population development over experimental period.

Endpoints: Population development over time, NOEC, LOEC;.

Test concentrations: Control, 0.025, 0.050, 0.10, 0.20, 0.40 and 0.80 mg a.s./L (nominal).

Test conditions: Small aquaria filled with 1.5 L, dilution water “M4” (Elendt medium); pH 7.8 - 8.5; oxygen content: 8.78 mg/L - 10.18 mg/L; water temperature: 20 ± 1 °C; photoperiod: 16 hours light: 8 hours dark; light intensity; 5.5 - 7.2 klux; feeding with algae, no ventilation.

Analytics: Analytical verification of test substance concentrations was conducted using GC/MS method CP 402 and using HPLC with UV-detection.

Statistics: Descriptive statistics; ANOVA via Dunnett´s and Bonferroni t-test for determination of NOEC and LOEC.

II. RESULTS AND DISCUSSION

Analytical results: The measured metiram concentrations in all application solutions ranged from 73.8% to 117.2% of nominal and thus confirmed the nominal data. metiram degraded rapidly, thus two days after the applications measured concentrations were around 20%. Seven days after the first and second application metiram concentrations were close to zero. Seven days after the last treatment 18% of nominal concentration was found in the highest treatment group. In addition to metiram data, concentrations of the main degradation product, ETU, were determined within the aquaria of the high treatment group. ETU degraded less fast within this system and thus showed some build up during the application intervals up to 0.4 - 0.5 mg/L after the third and fourth application. Biological results are based on nominal concentrations.

Biological results: *Daphnia* populations showed typical exponential growth during the first two weeks of the experiment (with a short lack phase during the first days). Starting with 10 daphnids per aquarium, after 14 days about 300 daphnids per aquarium were found. Thereafter, population growth slowed down. Therefore - to allow further population growth and thus observations of respective effects - the populations were reduced on day 21 and day 26. For this, about 300 mL samples of the aquaria were taken and poured over a sieve to retain the *Daphnia* within the samples; the filtered water was returned into the aquaria (this caused the observed reductions in all treatments on day 21 and 26). Only the two highest concentrations had a negative impact on population development of daphnia even after several applications. The initial increase in the first two weeks (following 2-3 treatments) was nearly identical in all treatment groups < 0.40 mg/L. Variability between treatments started to increase slightly after about 14 to 19 days, however, a statistically significant reduction was only found at the two highest concentrations of 0.40 mg a.s./L and 0.80 mg a.s./L. But even at 0.80 mg a.s./L there was still significant population growth, indicating rapid recovery after the treatment period.

The results are summarized in Table 8.2.5.1-2.

Table 8.2.5.1-2: Effects of BAS 222 28 F on *Daphnia magna* population density

Concentration [mg a.s./L] (nominal)	**Control**	**0.025**	**0.050**	**0.10**	**0.20**	**0.40**	**0.80**
Population density (12 d)	253	251.5	233.5	272.0	208.0	132.5*	41.5*
Population density (14 d)	282.0	283.0	281.0	311.0	287.5	207.5*	86.0*
Population density (21 d)	371.25	367.5	363.0	414.5	375.5	268.0*	110.0*
Population density (23 d)	390.0	386.5	378.5	352.0	389.0	250.5*	153.0*
Population density (28 d)	370.5	363.0	388.5	382.5	343.0	235.5*	175.5*
	Endpoints [mg a.s./L] (nominal)						
NOEC (28 d)	**0.20**						
LOEC (28 d)	0.40						

* Statistically significant as compared to the control (Bonferroni t-test, α = 0.05)

III. CONCLUSION

In a 28-day population study with *Daphnia magna* the NOEC of metiram (applied as formulated product BAS 222 28 F) was 0.20 mg a.s./L and the corresponding LOEC was determined to be 0.40 mg a.s./L.

New supplemental data (not evaluated previously on EU level):

The following life-cycle test on the cladoceran Daphnia magna performed with metiram (tested as BAS 222 29 F) is not required for registration in the EU and it has not been evaluated previously on EU level. The study was conducted due to U.S. data requirements and it is provided for completeness.

Report:	CA 8.2.5.1/6 Minderhout T. et al., 2010g Metiram: A flow-through life-cycle toxicity test with the Cladoceran (Daphnia magna) 2010/7014945
Guidelines:	EPA 850.1300, OECD 211
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

In a 21-day flow-through toxicity test, effects of metiram (tested as solo-formulation BAS 222 29 F) to water fleas (*Daphnia magna*) were examined. Neonates less than 24 hours old were exposed to nominal concentrations of 0.0085, 0.019, 0.041, 0.091 and 0.200 mg metiram/L (corresponding to mean measured concentrations of 0.0039, 0.0094, 0.023, 0.055 and 0.144 mg a.s./L). Additionally, a dilution water control and a solvent control were set up. All treatment groups and the controls consisted of 2 replicates containing two test compartments with five daphnids in each compartment. Parent mortality and sub-lethal effects were assessed daily, assessment of reproduction was conducted on days 8, 10, 13, 15, 17 and 21 and measurement of growth were made at test termination.

The biological results are based on mean measured concentrations. After 21 days of exposure, statistically significant differences in parent mortality were observed at the highest test item concentration in comparison to the pooled control. Daphnids in the test item treatment groups that survived until test termination generally appeared normal, with the exception of one daphnid in the 0.023 mg a.s./L treatment and two daphnids in the 0.144 mg a.s./L treatment which showed discoloration and lethargy. The first day of brood production in the water control and solvent control replicates and in all metiram treatment replicates was day 8 of the test, indicating that there was no apparent delay in the onset of reproduction at any metiram concentration tested. On Day 21 of the test, 7, 4, 2 and 8 immobile neonates were noted in the water control and the 0.0094, 0.023 and 0.055 mg a.s./L treatment groups, respectively. On Day 15 of the test, three aborted or shed eggs were present in two of the 0.0094 and 0.023 mg a.s./L test compartments. There was no statistically significant decrease in mean offspring production in comparison to the pooled control at up to and including the highest test item concentration.

In a 21-day flow-through toxicity study with *Daphnia magna* the NOEC of metiram (tested as BAS 222 29 F) was determined to be 0.055 mg a.s./L based on mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item:	BAS 222 29 F, batch no. 300013; content of a.s.: metiram (BAS 222 F; Reg. No 250284): 91.6% (nominal: 85%); density: 1.860 g/cm³.

B. STUDY DESIGN

Test species:	Water flea (*Daphnia magna* STRAUS), neonates from in-house culture; < 24 hours old at test initiation.
Test design:	Flow-through system (21 days), 5 test concentrations plus control and solvent control, two replicates containing two test compartments; five daphnids per test compartment (20 daphnids per test item concentration); daily assessment of parent mortality and sub-lethal effects; assessment of reproduction (number of live neonates per day) on days 8, 10, 13, 15, 17 and 21; measurement of body length and dry weight at test termination.
Endpoints:	NOEC, parent mortality, reproduction, parent length and dry weight.
Test concentrations:	Control (dilution water); solvent control (0.1 mL dimethylformamide/L); 0.0085, 0.019, 0.041, 0.091 and 0.200 mg metiram/L (nominal); corresponding to mean measured concentrations of 0.0039, 0.0094, 0.023, 0.055 and 0.144 mg a.s./L.
Test conditions:	25 L Teflon®-lined stainless steel aquaria, test volume approx. 22 L, test compartments: 300 mL glass beakers (6.5 cm diameter and 12 cm height) with nylon screen covering two holes on opposite sides of each test compartment; dilution water: filtered, aerated and sterilized well water; temperature: 19.8 °C - 20.0 °C; pH 8.0 - 8.1; oxygen content: 5.9 mg/L - 8.8 mg/L; total hardness: 138 - 146 mg $CaCO_3$/L; alkalinity: 180 - 184 mg $CaCO_3$/L; conductivity: 309 - 398 µmhos/cm; flow rate: approx. 5 volume additions/24 h/test vessel; photoperiod 16 hours light : 8 hours dark; light intensity: 440 lux; feeding: mixture of yeast, cereal grass media, and trout chow plus suspension of freshwater green alga (*Pseudokirchneriella subcapitata),* 3 x daily until day 8, 4 x daily until day 20, 1 x on day 21.
Analytics:	Analytical verification of test item concentrations was conducted using a GC-method with MS-detection.
Statistics:	Descriptive statistics; comparison of data of the control and solvent control groups using an appropriate statistical test ($p \leq 0.05$); Fisher's Exact test with Bonferroni-Holmes correction ($p \leq 0.05$), Dunnett's one-tailed test ($p \leq 0.05$) and Bonferroni's one-tailed test ($p \leq 0.05$) for determination of the NOEC values.

II. RESULTS AND DISCUSSION

Analytical results: Analytical verification of the active substance concentrations was conducted in all treatments at day 0, 7, 14 and 21. Mean recoveries of metiram were in the range of 34% to 74% of nominal concentrations during the course of the study. Measured concentrations of metiram ranged from 45% to 70% at test initiation and from 34% to 74% at test termination. The following biological results are based on mean measured concentrations.

Biological results: No statistically significant differences between the control groups were found for any parameter tested (appropriate statistical test, $p > 0.05$). Therefore, subsequent statistical analyses were performed by comparing the pooled control data to the treatment data for the respective parameters.
After 21 days of exposure, statistically significant differences in parent mortality were observed at the highest test item concentration in comparison to the pooled control (Fisher's Exact test with Bonferroni-Holmes correction; $p \leq 0.05$). Because of the significant effect on survival, the highest test item treatment group was excluded from the statistical analysis of reproduction and growth data.
Daphnids in the test item treatment groups that survived until test termination generally appeared normal, with the exception of one daphnid in the 0.023 mg a.s./L treatment and two daphnids in the 0.144 mg a.s./L treatment which showed discoloration and lethargy.
The first day of brood production in the water and solvent control replicates and in all metiram treatment replicates was day 8 of the test, indicating that there was no apparent delay in the onset of reproduction at any metiram concentration tested. On Day 21 of the test, 7, 4, 2 and 8 immobile neonates were noted in the water control and the 0.0094, 0.023 and 0.055 mg a.s./L treatment groups, respectively. On Day 15 of the test, three aborted or shed eggs were present in two of the 0.0094 and 0.023 mg a.s./L test compartments. There was no statistically significant decrease in mean offspring production in comparison to the pooled control up to and including the highest test item concentration (Dunnett's one-tailed test, $p \leq 0.05$).
Bonferroni's one-tailed test indicated that there was a statistically significant decrease in mean length in the second highest test item concentration in comparison to the pooled control ($p \leq 0.05$). However, the difference in length of 0.1 mm was not considered biologically meaningful.
Dunnett's one-tailed test indicated a statistically significant decrease in mean dry weight in the 0.0094 mg a.s./L treatment group in comparison to the pooled control ($p \leq 0.05$). However, it did not appear to be dose-responsive since no significant effects were noted in the 0.023 and 0.055 mg a.s./L treatment groups when compared to the pooled controls.

The results are summarized in Table 8.2.5.1-3.

Table 8.2.5.1-3: Effects of metiram (tested as BAS 222 29 F) on *Daphnia magna* reproduction, growth and parent mortality after 21 days of exposure

Concentration [mg a.s./L] (nominal)	**Control**	**Solvent control**	**Pooled control**	**0.0085**	**0.019**	**0.041**	**0.091**	**0.200**
Concentration [mg a.s./L] (mean measured)	**--**	**--**	**--**	**0.0039**	**0.0094**	**0.023**	**0.055**	**0.144**
Parent mortality [%]	0	5	2	5	0	5	5	20 *
Mean no. of offspring/reproductive Day (± Std. Dev.)	11.1 ± 1.9	11.1 ± 0.38	11.1 ± 1.3	12.4 ± 2.5	12.5 ± 0.56	11.3 ± 0.99	11.7 ± 1.2	8.9 ± 1.8 a)
Mean length (± Std. Dev.) [mm]	4.8 ± 0.10	4.8 ± 0.00	4.8 ± 0.07	4.9 ± 0.00	4.8 ± 0.06	4.7 ± 0.08	4.7 ± 0.05 #	4.4 ± 0.09 a)
Mean dry weight (± Std. Dev.) [mg]	0.90 ± 0.08	0.92 ± 0.05	0.91 ± 0.06	0.92 ± 0.11	0.82 ± 0.04 +	0.84 ± 0.04	0.86 ± 0.03	0.68 ± 0.07 a)
Endpoints [mg metiram/L] (mean measured)								
$NOEC_{overall}$ (21 d)	0.055							

* Statistically significant effects compared to the pooled control (Fisher's Exact test with Bonferroni-Holmes correction, p ≤ 0.05)

\# Statistically significant decrease in comparison to the pooled control (Bonferroni's one-tailed test, p ≤ 0.05), but not considered biologically meaningful.

\+ Statistically significant decrease in comparison to the pooled control (Dunnett's one-tailed test, p ≤ 0.05), but not dose-responsive.

a) The highest test item treatment group was excluded from the statistical analysis of reproduction and growth data due to a significant effect on survival.

III. CONCLUSION

In a 21-day flow-through toxicity study with *Daphnia magna* the NOEC of metiram (tested as BAS 222 29 F) was determined to be 0.055 mg a.s./L based on mean measured concentrations.

***The following population study on the cladoceran Daphnia magna performed with metiram (tested as BAS 222 28 F) was performed to generate further data for a risk refinement and has not been evaluated previously on EU level*.**

Report: CA 8.2.5.1/7
Dohmen G.P.,Bergtold M., 2006a
Effect of Metiram - Applied as BAS 222 28 F - On the population development of Daphnia magna
2006/1016038

Guidelines: OECD 211

GLP: yes
(certified by Landesamt fuer Umweltschutz und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

In a 35-day semi-static extended laboratory study, effects of metiram (tested as BAS 222 28 F) on water fleas (*Daphnia magna*) mixed age population were examined. The test substance was applied four times with 7 day intervals at nominal concentrations of 0.050, 0.100, 0.200, 0.400, 0.800 mg a.s./L. For all test item treatment groups 2 replicates were tested with 10 daphnids each. The control consisted of 4 replicates. The development of *Daphnia* populations and test item concentrations were monitored in regular intervals throughout the experiment.

The biological results are based on nominal concentrations. Adverse effects on daphnid population parameters were observed at test concentrations higher than 0.100 mg a.s./L.

In a 35-day population study with *Daphnia magna* the NOAEC of metiram (applied as formulated product BAS 222 28 F) was 0.100 mg a.s./L, based on nominal concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2000-2; content of a.s.: metiram (BAS 222 F; Reg. No 250284): 68.5% (nominal: 70%).

B. STUDY DESIGN

Test species: Water flea (*Daphnia magna* STRAUS) from in-house culture, originally supplied by Institut National de Recherché Chimique Appliquée, France, 1978; mixed age classes (2 adult daphnids > 12 days old; 3 sub-adults 3 - 8 days old and 5 neonates < 24 h old).

Test design: Population experiment, semi-static test (35 days); 5 test concentrations with two replicates plus control with four replicates, four test substance applications at 7 day intervals, 10 daphnids per replicate and treatment; assessment of *Daphnia* population development over the experimental period.

Endpoints: NOAEC, LOAEC; population development over time.

Test concentrations: Control, 0.0709, 0.142, 0.284, 0.567 and 1.130 mg BAS 222 28 F/L, corresponding to 0, 0.050, 0.100, 0.200, 0.400, 0.800 mg a.s./L (nominal);

Test conditions: Small aquaria filled with 1.5 L dilution water "M4" (Elendt medium); water replacement latest before 10% decrease of water level; pH 7.32 - 8.34; oxygen content: 7.62 mg/L - 10.49 mg/L; water temperature: 19 °C ± 1 °C; photoperiod: 16 hours light: 8 hours dark; light intensity; < 2 klux; feeding with algae, no ventilation.

Analytics: Analytical verification of test substance concentrations was conducted using GC-method with MS detection.

Statistics: Descriptive statistics; ANOVA via Bonferroni t-test for determination of NOAEC and LOAEC.

II. RESULTS AND DISCUSSION

Analytical measurements: At day 0, 7, 14 and 21, when freshly prepared test item was applied, recoveries of metiram ranged between about 60% and 130% at the first three treatments and were up to 190% at the last treatment. Metiram degrades rapidly; accordingly mean recoveries in the treated aquaria decreased to about 10% - 20% two days after the initial treatment. Six days after treatment, metiram residues could only be detected in the two highest treatments at a max. of 5.1% of nominal concentrations; similarly after the second application. Residues following the third and fourth application were slightly higher. At day 35 recoveries were 0 - 7% of the nominal test concentration, except for the 0.400 mg a.s./L treatment group with about 30%. ETU was found at concentrations of up to 0.180 mg/L at the highest treatment. Initially there was a buildup of ETU levels, but towards the end of the study no or only minor ETU residues were detected. The following biological results are based on nominal concentrations.

Biological results: *Daphnia* population development was rapid with high initial growth rates, but growth slowed down after day 12 - 14. Population maxima were reached at day 23 in the control and the three lowest treatment levels; thereafter population densities leveled off to densities around 670 - 770 daphnids per vessel in the control and the two lowest concentrations. The population growth at the two lowest test concentrations were slightly higher than in the controls. At 0.200 mg a.s./L population development was slightly reduced after 7 days following the second application. The developments of populations in the 0.400 mg a.s./L and 0.800 mg a.s./L treatments were strongly affected, but recovery was observed even after multiple treatments with 0.400 mg a.s./L. Statistically significant adverse effects were observed at concentrations higher than 0.100 mg a.s./L. The results are summarized in Table 8.2.5.1-4.

Table 8.2.5.1-4: Effects of metiram (tested as BAS 222 28 F) on *Daphnia magna* population density

Concentration [mg a.s./L] nominal	**Control**	**0.050**	**0.100**	**0.200**	**0.400**	**0.800**
Population density (35 d) (mean numbers of daphnids)	668.3	711.0	771.5	445.5*	320.5*	0
	Endpoints [mg metiram/L] (nominal)					
NOAEC (35 d)	**0.100**					
LOAEC (35 d)	0.200					

* Statistically significant difference compared to the control (Bonferroni t-test, $\alpha = 0.05$)

III. CONCLUSION

In a 35-day population study with *Daphnia magna* the NOAEC of metiram (applied as formulated product BAS 222 28 F) was 0.100 mg a.s./L, based on nominal concentrations.

The following chronic toxicity study on Daphnia magna performed with the metiram metabolite ethylenethiourea (ETU) has not been evaluated previously on EU level during previous Annex I inclusion process for metiram. The obtained endpoint is considered relevant for the risk assessment of metiram and thus, relevant information and the results for ethylenethiourea of this study are described in the following summary.

Report:	CA 8.2.5.1/8 Graves W.C., 1995a Ethylene thiourea (ETU): A flow-through life-cycle toxicity test with the Cladoceran (daphnia magna) 1995/1008534
Guidelines:	OECD 202
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

In a 21-day flow-through toxicity test, effects of ethylenethiourea to water fleas (*Daphnia magna*) were examined. Parent daphnids were exposed to nominal concentrations of 0.25, 0.50, 1.0, 2.0 and 4.0 mg/L (corresponding to mean measured concentrations of 0.28, 0.54, 1.0, 2.0 and 4.1 mg/L). Additionally, a dilution water control was set up. All treatment groups and the control consisted of 4 replicates containing 10 parent daphnids each. Parent mortality and sub-lethal effects were assessed daily, assessment of reproduction was conducted on days 10, 12, 14, 17, 19 and 21.

The biological results are based on mean measured concentrations. After 21 days of exposure no parent mortality was observed in the control and at up to and including the second highest test item concentration of 2.0 mg/L, whereas statistically significant differences in parent mortality were observed at the highest test item concentration of 4.1 mg/L in comparison to the control. Because of the significant effect on survival, the highest test item treatment group was excluded from the statistical analysis of reproduction and growth data. No adverse effects on growth or reproduction were observed in the control and at up to and including the second highest test item concentration of 2.0 mg/L.

In a 21-day semi-static toxicity study with *Daphnia magna* the overall NOEC of ethylenethiourea was determined to be 2.0 mg/L based on mean measured concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: Ethylenethiourea (BF 222-ETU, Reg. No. 146099; metabolite of metiram), TD no. 94-001; Lot. No. 02506 EV; purity: 96.2%.

B. STUDY DESIGN

Test species: Water flea (*Daphnia magna*), neonates at test initiation less than 24 hours old; from cultures maintained by Wildlife International Ltd., Easton, Maryland, USA.

Test design: Flow-through test (21 days); 5 test concentrations plus a dilution water control, four replicates per concentration and control, ten parent daphnids per replicate and treatment; daily assessment of parent mortality and presence of brood pouch, assessment of reproduction three times per week and at test termination, measurement of dry weight and body length of surviving first-generation daphnids at test termination.

Endpoints: NOEC, parent mortality, parent growth and reproduction.

Test concentrations: Control, 0.25, 0.50, 1.0, 2.0 and 4.0 mg ethylenethiourea/L (nominal); corresponding to mean measured concentrations of 0, 0.28, 0.54, 1.0, 2.0 and 4.1 mg/L.

Test conditions: Glass beakers (500 mL, about 7 cm Ø x 13 cm), test volume: approx. 6.5 L; dilution water: filtered and UV-sterilized well water; temperature: 19.9 – 20.3 °C; pH 8.1 - 8.3; oxygen content: 7.9 mg/L – 8.8 mg/L, (oxygen saturation > 60%); total hardness of test water: 140 mg $CaCO_3$/L; conductivity of test water: 310 - 320 µmhos/cm; photoperiod 16 hours light : 8 hours dark; light intensity: approx. 464 lux; flow rate: approx.. 14 volume additions every 24 h; feeding: 0.3 mL YCT (mixture of yeast Cerophyll® and trout chow) and 3.0 mL living cells of *Scenedesmus subspicatus* three times a day.

Analytics: Analytical verification of test item concentrations was conducted using a LC-method with Diode Array Detector.

Statistics: Descriptive statistics; 2 X 2 contingency tables were used for evaluation of survival, analysis of reproduction and growth was performed by ANOVA via Bonferroni t-test.

II. RESULTS AND DISCUSSION

Analytical results: Analytical verification of the test substance concentrations was conducted at test initiation, at day 7 and 14 and at the end of the test. Analysed contents of ethylenethiourea were in the range of 92% - 108% of nominal test item concentrations at study initiation. At study termination analyzed concentrations of ethylenethiourea were between 98% and 142% of nominal concentrations. The following biological results are based on the mean measured test concentrations.

Biological results: After 21 days of exposure no parent mortality was observed in the control and at up to and including the second highest test item concentration of 2.0 mg/L, whereas statistically significant differences in parent mortality were observed at the highest test item concentration of 4.1 mg/L in comparison to the control (2 X 2 contingency tables; $p > 0.05$). Because of the significant effect on survival, the highest test item treatment group was excluded from the statistical analysis of reproduction and growth data. No adverse effects on growth or reproduction were observed in the control and at up to and including the second highest test item concentration of 2.0 mg/L. The results are summarized in Table 8.2.5.1-5.

Table 8.2.5.1-5: Effects of ethylenethiourea on *Daphnia magna* reproduction, growth and parent mortality after 21 days of exposure

Concentration [mg/L] (nominal)	**Control**	**0.25**	**0.50**	**1.0**	**2.0**	**4.0**
Concentration [mg/L] (mean measured)	**--**	**0.28**	**0.54**	**1.0**	**2.0**	**4.1**
Parent mortality [%]	8	10	5	8	5	50 *
Mean no. of offspring/reproductive day	3.3	3.4	3.3	3.1	3.1	1.7
Mean length (± Std. Dev.) [mm]	3.8 (± 0.09)	3.8 (± 0.09)	3.8 (± 0.08)	3.7 (± 0.11)	3.7 (± 0.09)	3.5 (± 0.10) +
Mean dry weight (± Std. Dev.) [mg]	0.52 (± 0.09)	0.50 (± 0.07)	0.51 (± 0.07)	0.48 (± 0.07)	0.51 (± 0.07)	0.31 (± 0.07) +
Endpoints [mg ethylenthiourea/L] (mean measured)						
$NOEC_{overall}$ (21 d)	2.0					

* Statistically significant decrease in comparison to the control (2 X 2 contingency tables, $p \leq 0.05$)
+ Reproduction and growth of daphnids in the highest test item treatment group was reduced when compared to the control group. The highest test item group was excluded from the statistical analysis of reproduction and growth data due to a significant effect on survival.

III. CONCLUSION

In a 21-day semi-static toxicity study with *Daphnia magna* the overall NOEC of ethylenethiourea was determined to be 2.0 mg/L based on mean measured concentrations.

CA 8.2.5.2 Reproductive and development toxicity to an additional aquatic invertebrate species

New supplemental data (not evaluated previously on EU level):

The following chronic study on the saltwater mysid Americamysis bahia performed with metiram (tested as BAS 222 29 F) is not required for registration in the EU and it has not been evaluated previously on EU level. The study was conducted due to U.S. data requirements and it is provided for completeness.

Report:	CA 8.2.5.2/1 Minderhout T. et al., 2010h Metiram: A flow-through life-cycle toxicity test with the saltwater mysid (Americamysis bahia) 2010/7015187
Guidelines:	EPA 850.1350
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

The chronic toxicity of metiram (tested as BAS 222 29 F) to saltwater mysids (*Americamysis bahia*) was evaluated in a 40-day full-life cycle test under flow-through conditions. Mysids were exposed to a dilution water control, a solvent control and to nominal test item concentrations of 0.0016, 0.0031, 0.0063, 0.012 and 0.025 mg metiram/L. Survival, reproductive success and symptoms of toxicity were assessed throughout the study. Length, dry weight and wet weight of surviving mysids were determined at test termination.

The biological results are based on nominal concentrations. The first day of brood production in the controls and in all treatment groups was day 8 of the test, indicating that there was no apparent delay in the onset of reproduction at any concentration tested. After 15 days of exposure, statistically significant differences in survival of juvenile mysids was observed at the highest test item concentration, when compared to the solvent control, while survival in all treatment groups was significantly different compared to the dilution water control. Results are not considered dose-responsive except for the highest test treatment group of 0.025 mg a.s./L since survival in all treatment groups up to and including 0.013 mg a.s./L was above/equal to 70%. During exposure from test initiation to pairing on day 15 sub-lethal effects, *i.e.* lethargy, loss of equilibrium, surfacing and erratic swimming, were observed in the two highest treatment groups as well as lethargy of isolated individuals in the controls and the lowest test item concentration. Sub-lethal effects from paring to test termination (*i.e.* few mysids exhibited lethargy and/or were impinged on the screen of the test compartment above the waterline) were infrequent and comparable to those observed in the control groups. Therefore, they were not considered to be treatment related.

At test termination, no statistically significant differences in survival were observed at any tested concentration, when compared to the pooled control. However, there was a statistically significant reduction in the mean number of young produced per female at the two highest test item concentrations compared to the pooled control. Although reproduction in the 0.0063 mg a.s./L treatment group is not statistically significant, the observed decrease of 44% compared to the pooled control is considered treatment-related. No statistically significant, treatment-related decreases in mean total length for males and females as well as mean dry weight for males and females were found in any of the treatment groups compared to data from either control groups or the pooled control.

In a 40-day flow-through toxicity study with saltwater mysids (*Americamysis bahia*), the overall NOEC of metiram (tested as BAS 222 29 F) was determined to be 0.0031 mg a.s./L based on nominal concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 29 F, batch no. 300013; content of a.s.: metiram (BAS 222 F; Reg. No 250 284): 91.6% (nominal: 85%); density: 1.860 g/cm³.

B. STUDY DESIGN

Test species: Test species: Saltwater mysid (*Americamysis bahia*), juveniles, age: less than 24 hours; source: in-house cultures.

Test design: Flow-through system (40 days), 5 test concentrations plus control and solvent control, with four replicates each; 15 mysids per test chamber (60 mysids per treatment group); on day 15 when the sex of mysids could be determined, up to five male/female pairs were segregated from the population in each glass aquarium and assigned to reproductive compartments in each replicate test chamber, with one pair per compartment, unpaired mysids were sexually differentiated and placed in separate test chambers; daily assessment of parent mortality and sub-lethal effects; daily assessment of reproduction (number of live neonates per day); measurement of body length and dry weight at test termination.

Endpoints: NOEC, mortality and sub-lethal effects for juvenile and adult mysids, reproduction, parent length and dry weight.

Test concentrations: Control (dilution water), solvent control (0.1 mL dimethylformamide/L), 0.0016, 0.0031, 0.0063, 0.012 and 0.025 mg metiram/L (nominal).

Test conditions:	Test chambers: from test initiation until day 15: 9 L glass aquaria, test volume approx. 2.5 L, containing four test compartments: 2 L glass beakers (12 cm in diameter and 19 cm height) with nylon screen covering two holes on opposite sides of each test compartment. From day 15 on: 19 L glass aquaria, test volume approx. 14.5 L, containing up to five reproduction compartments: petri dishes of 10 cm diameter with sides of nylon mesh screen; dilution water: filtered, aerated, sterilized and diluted seawater; flow rates: juvenile test chamber: at least 36 volume additions per 24 hours, adult test chamber: at least 6 volume additions per 24 hours; salinity: 19 - 21‰; temperature: 24.2°C - 26.3°C; pH 7.9 - 8.2; oxygen content: 4.7 - 7.8 mg/L; photoperiod 14 h light : 10 h dark with a 120 minute transition period between dark and light; light intensity: 258 lux; feeding: live *Artemia sp.* nauplii up to four times per day, no feeding during the final 24 hours; continuous aeration.
Analytics:	Analytical verification of test item concentrations was conducted using a GC-method with MS-detection. Stock solution samples were analyzed by ICP-AES.
Statistics:	Descriptive statistics; comparison of data of the control and solvent control groups using a t-test ($p \leq 0.05$); Fisher's Exact test ($p \leq 0.05$) and Dunnett's test ($p \leq 0.05$) for determination of the NOEC values.

II. RESULTS AND DISCUSSION

Analytical results: Analytical verification of the active substance concentrations was conducted in all treatments at days 0, 7, 14, 21, 28, 35 and 40, except for the two highest test item concentrations of 0.012 and 0.025 mg metiram/L where no data is available. Regarding the two lowest test item concentrations of 0.0016 and 0.0031 mg a.s./L the analytical results were below the limit of quantification (LoQ = 0.002 mg a.s./L) and therefore could not be analyzed reliably. Mean recovery in the test item concentration of 0.0063 mg metiram/L was 55% of nominal at test initiation and 9.0% of nominal at test termination. Additionally, analytical verification of stock solution samples was conducted for all test item concentrations. Calculated metiram concentrations ranged from 79.3% to 123% of nominal. Due to the low solubility and the high hydrolysis rate (DT50 < 1 day) of the test substance, metiram recoveries in the test solutions were low in all treatment groups. However, since the nominal concentrations of the stock solutions delivered to these levels were confirmed, the following biological results are based on nominal concentrations.

Biological results: The first day of brood production in the dilution water - and solvent control replicates and in all treatment replicates was day 8 of the test, indicating that there was no apparent delay in the onset of reproduction at any test item concentration tested. After 15 days of exposure prior to pairing, survival of juvenile mysids in the control and the solvent control group was 98% and 83%, respectively. Since there were statistically significant differences in survival between the control and solvent control group (t-test, $p > 0.05$), the treatment groups were compared individually to the control and solvent control. The surviving mysids in the control groups appeared normal, with exception of two lethargic mysids in the solvent control group. After 15 days of exposure, statistically significant differences in survival were solely observed at the highest test item concentration of 0.025 mg a.s./L , when compared to the solvent control (Fisher's Exact test, $p \leq 0.05$), while survival in all treatment groups was significantly different from the dilution water control group (Fisher's Exact test, $p \leq 0.05$). However, results are not considered dose-responsive except for the highest test treatment group of 0.025 mg a.s./L since survival in all treatment groups up to and including 0.013 mg a.s./L was above/equal to 70%. During exposure from test initiation to pairing on day 15 sub-lethal effects, i.e. lethargy, loss of equilibrium, surfacing and erratic swimming, were observed in the two highest treatment groups as well as lethargy of isolated individuals in the controls and the lowest test item concentration. Sub-lethal effects from pairing to test termination (i.e. few mysids exhibited lethargy and/or were impinged on the screen of the test compartment above the waterline) were infrequent and comparable to those observed in the control groups. Therefore, they were not considered to be treatment related. After 40 days of exposure, survival of adult mysids in the control and the solvent control groups was 72% and 70%, respectively. Since no statistically significant difference in survival between the control groups was found (t- test, $p > 0.05$), subsequent statistical analysis was performed by comparing the pooled control data to the treatment data. At test termination, no statistically significant differences in survival were observed at any tested concentration, when compared to the pooled control (Fisher's Exact test, $p \leq 0.05$). There was a statistically significant reduction in the mean number of young produced per female at the two highest test item concentrations compared to the pooled control (Dunnett's test, $p \leq 0.05$). Although reproduction in the 0.0063 mg a.s./L treatment group is not statistically significant, the observed decrease of 44% compared to the pooled control is considered treatment-related. Dunnett's test ($p > 0.05$) indicated that there were no statistically significant differences in mean total length for males and females as well as for mean dry weight for males in any of the treatment groups compared to data from either control group or the pooled control Regarding mean dry weight for female mysids, a significant decrease was observed at the lowest test item concentration of 0.0016 mg a.s./L (Dunnett's test, $p \leq 0.05$). However, the significant reduction detected in this group was not dose-responsive, and therefore not considered to be treatment related. The results are summarized in Table 8.2.5.2-1.

Table 8.2.5.2-1: Chronic toxicity (40 d) of metiram (tested as BAS 222 29 F) to saltwater mysids (*Americamysis bahia*)

Concentration [mg a.s./L] (nominal)	Control	Solvent control	Pooled control	0.0016	0.0031	0.0063	0.012	0.025
Survival after 15 days [%] (pre-pairing)	98	83	91	80 +	70 +	85 +	77 +	43 + #
Survival after 40 days [%] (post-pairing)	72	70	71	76	71	57	76	54
Mean no. of offspring/reproductive day (± SD)	0.246 ± 0.20	0.236 ± 0.055	0.241 ± 0.087	0.213 ± 0.072	0.232 ± 0.17	0.137 ± 0.071 [1)]	0.0769 ± 0.0039 * [2)]	0.0867 ± 0.075 *
Mean length males (± SD) [mm]	8.41 ± 0.16	8.09 ± 0.15	8.25 ± 0.23	8.20 ± 0.24	8.23 ± 0.32	8.22 ± 0.29	8.02 ± 0.24	8.11 ± 0.28
Mean length females (± SD) [mm]	8.67 ± 0.085	8.64 ± 0.23	8.66 ± 0.16	8.56 ± 0.26	8.82 ± 0.21	8.75 ± 0.092	8.65 ± 0.27	8.53 ± 0.20
Mean dry weight males (± SD) [mg]	1.06 ± 0.078	0.96 ± 0.070	1.01 ± 0.088	0.97 ± 0.047	0.98 ± 0.079	1.03 ± 0.043	1.03 ± 0.20	1.10 ± 0.20
Mean dry weight females (± SD) [mg]	1.46 ± 0.085	1.28 ± 0.073	1.37 ± 0.12	1.24 ± 0.13 * [3)]	1.35 ± 0.059	1.37 ± 0.065	1.27 ± 0.078	1.33 ± 0.20
	Endpoints [mg metiram/L] (nominal)							
$NOEC_{overall}$ (40 d)	0.0031							

n.d. = not determined; SD = standard deviation
* Statistically significant decrease compared to the pooled control (Dunnett's test, p ≤ 0.05)
+ Statistically significant decrease compared to the dilution water control (Fisher's Exact test, p ≤ 0.05).
\# Statistically significant decrease compared to the solvent control (Fisher's Exact test, p ≤ 0.05).
1) While the reproduction was not statistically significant compared to the pooled control, the difference of 44% was considered treatment-related.
2) Mysid pair was apparently not transferred into the new test compartment on Day 25 of the test; results were therefore calculated with the exclusion of Replicate C test compartment 4.
3) While the decrease in weight was statistically significant compared to the dilution water control, it was not considered to be treatment-related since the difference was slight and was not dose-responsive.

III. CONCLUSION

In a 40-day flow-through toxicity study with saltwater mysids (*Americamysis bahia*), the overall NOEC of metiram (tested as BAS 222 29 F) was determined to be 0.0031 mg a.s./L based on nominal concentrations.

CA 8.2.5.3 Development and emergence in Chironomus riparius

Data already submitted during previous Annex I inclusion process (already evaluated on EU level):

The following 28 day chronic, spiked water toxicity study on Chironomus riparius performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.5.3/1* *Dohmen G.P., 2001a* *Effect of Metiram - Applied as formulated product BAS 222 28 F - On the development of sediment dwelling larvae of Chironomus riparius in a water-sediment system* *2001/1007679*
Guidelines:	*BBA proposal: Effects of plant protection products on the development of sediment dwelling larvae of Chironomus riparius in water-sediment system 1995*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 28-day static sediment test, midge larvae (*Chironomus riparius*) were exposed to metiram (tested as BAS 222 28 F). Nominal test concentrations were 0.01, 0.032, 0.1, 0.316, 1.0 and 3 mg BAS 222 28 F/L (corresponding to 0.007, 0.022, 0.07, 0.221, 0.7 and 2.1 mg a.s./L). Additionally an untreated control was tested. All test item concentrations had 3 replicates, whereas 5 replicates were made for the control. 25 larvae were added to each test vessel.

Biological results are based on nominal concentrations. More than 80% of the chironomids emerged in all but the two highest test concentrations. Statistically significant effects were obtained at the test concentration of 3 mg/L (41.9% reduction in emergence compared to control), respectively at 1 mg/L depending on the statistical method used (22% reduction of emergence compared to control). Statistically significant differences between the overall development rates of treatments and control were found only at the test concentration of 3 mg/L.

In a 28-day sediment test with *Chironomus riparius* the NOEC for development rate was determined to be 1.0 mg BAS 222 28 F/L (corresponding to 0.7 mg metiram/L), the NOEC for emergence rate was 0.316 mg BAS 222 28 F/L (0.221 mg a.s./L) and the EC_{50} for emergence and development rate was > 3.0 mg BAS 222 28 F/L (> 2.1 mg a.s./L).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch No. 98-1, content of a.s. metiram (BAS 222 F; Reg. No 250284): 69.8% analyzed (70% nominal).

B. STUDY DESIGN

Test species: *Chironomus riparius*, up to 3 days old larvae at test initiation; source: in-house.

Test design: Static system; test duration 28 days; 6 concentrations plus a control; 3 replicates per concentration, 5 replicates for the control; 25 larvae were added to each vessel; assessment of emergence rate and development.

Endpoints: NOEC and EC_{50} (regarding emergence rate and development).

Test concentrations: Control, 0.01, 0.032, 0.1, 0.316, 1.0 and 3 mg BAS 222 28 F/L (nominal); corresponding to 0.007, 0.022, 0.07, 0.221, 0.7 and 2.1 mg a.s./L.

Test conditions: Glass vessels with 2 cm sediment layer (soil according to OECD 207), 1.8 L M4 water (Elendt medium) according to a 16.5 cm water layer; pH 7.71 - 8.01; oxygen content: 7.4 mg/L – 8.4 mg/L; total hardness: 2.58 mmol/L; conductivity: 692 µS/cm; feeding with TetraMin, slight ventilation; water temperature: 19.5 °C – 20.7 °C; light intensity: 780 - 1030 lux; photoperiod: 16 h light : 8 h darkness.

Analytics: The test item concentrations were analyzed using GC (method CP 067).

Statistics: Descriptive statistics, ANOVA followed by Dunnett´s, Bonferroni-, or Willam´s-test; probit-, logit- or log-log-analysis was used for determination of EC-values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical measurements of test substance were performed in water, pore water and sediment. Due to the insolubility of the active substance and its rapid degradation, the analytical results for the water phase were rather erratic. This behavior is well known for metiram. Significant quantities were recovered from the sediment (up to 0.47 mg/kg in the high treatment group). As the amounts of test compound introduced into vessels were well documented and confirmed the correct application of test substance, the results are based on nominal concentrations.

Biological results: Biological results are based on nominal concentrations. More than 80% of the chironomids emerged in all but the two highest test concentrations. Statistically significant effects were obtained at the test concentration of 3 mg/L (41.9% reduction in emergence compared to control), respectively at 1 mg/L depending on the statistical method used (22% reduction of emergence compared to control). Statistically significant differences between the overall development rates of treatments and control were found only at the test concentration of 3 mg/L. The results are summarized in Table 8.2.5.3-1.

Table 8.2.5.3-1: Effects of metiram (tested as BAS 222 28 F) on emergence and development of *Chironomus riparius*

Concentration (nominal) [mg BAS 222 28 F/L]	**Control**	**0.01**	**0.032**	**0.1**	**0.316**	**1.0**	**3.0**
Concentration (nominal) [mg a.s./L]	**Control**	**0.007**	**0.022**	**0.07**	**0.221**	**0.7**	**2.1**
Emergence rate (ER)	0.872	0.880	0.800	0.813	0.840	0.680*	0.507**
Development rate per day (DR)	0.0641	0.0686	0.0681	0.0681	0.0672	0.0655	0.0581**

	Endpoints (nominal)	
	based on BAS 222 28 F [mg/L]	**based on metiram [mg a.s./L]**
$NOEC_{emergence}$	0.316	0.221
$NOEC_{development}$	1.0	0.7
$EC_{50\ development\ /\ emergence}$	> 3.0	> 2.1

Statistically significant difference compared to control (**Dunnett´s and William´s-test, *only according to William´s-test, $p < 0.05$)

III. CONCLUSION

In a 28-day sediment test with *Chironomus riparius* the NOEC for development rate was determined to be 1.0 mg BAS 222 28 F/L (corresponding to 0.7 mg metiram/L), the NOEC for emergence rate was 0.316 mg BAS 222 28 F/L (0.221 mg a.s./L) and the EC_{50} for emergence and development rate was > 3.0 mg BAS 222 28 F/L (> 2.1 mg a.s./L).

New supplemental data (not evaluated previously on EU level):

The following sub-chronic spiked sediment study on Chironomus tentans larvae with metiram (tested as BAS 222 29 F) is not required for registration in the EU and it has not been evaluated previously on EU level. The study was conducted due to U.S. data requirements and it is provided for completeness.

Report:	CA 8.2.5.3/2 Thomas S. et al., 2010a Metiram: A 10-day flow-through survival and growth sediment toxicity test with Chironomus tentans using spiked sediment 2010/7012893
Guidelines:	EPA 850.1735, ASTM E 1706-05
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

In a 10-day flow-through sub-chronic spiked sediment study, midge larvae (Chironomus tentans) were exposed to metiram (tested as BAS 222 29 F) at nominal concentrations of 4.7, 9.4, 19, 38, 75 and 150 mg a.s./kg dry sediment (corresponding to initial measured concentrations of 2.75, 5.17, 12.2, 21.6, 77.6 and 144 mg a.s./kg dry sediment). Additionally, a dilution water control was set up. All test item concentrations and the control had 8 replicates containing 10 larvae each. In addition to the organisms placed in the test compartments at the beginning of the test, an additional 20 organisms were impartially selected at the beginning of the test and measured for dry weight. Assessment of survival and growth of surviving midges (measured as ash-free dry weight) was conducted at the end of the test. Sub-lethal effects were assessed daily.

The biological results are based on nominal sediment concentrations. Few midges showed abnormal behavior during the test (i.e. midges on the surface of the sediment, midges climbing the walls of the test compartment) in the control and at all tested concentrations. Survival was statistically significantly reduced compared to the control at the highest test item concentration. Statistically significant effects compared to the control on dry weight of the midges were observed at the two highest test item concentrations.

In a sub-chronic 10-day flow-through sediment test with *Chironomus tentans* the overall NOEC of metiram (tested as BAS 222 29 F) was determined to be 21.6 mg a.s./kg dry sediment based on initial measured sediment concentrations. Both the LC_{50} and the EC_{50} for growth were > 144 mg a.s./kg dry sediment (initial measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 29 F, batch no. 300013; content of a.s.: metiram (BAS 222 F; Reg. No 250284): 91.6% (nominal: 85%); density: 1.860 g/cm³.

B. STUDY DESIGN

Test species: Midge (*Chironomus tentans*), larvae, 10 days old at test initiation; source: "Environmental Consulting and Testing", Superior, Wisconsin, USA.

Test design: Flow-through system (10 days); 6 test concentrations plus dilution water control, 8 replicates per test item concentration and for the control; 10 larvae per replicate; in addition to the organisms placed in the test compartments at the beginning of the test, an additional 20 organisms were impartially selected at the beginning of the test and measured for dry weight; assessment of survival and growth (ash-free dry weight) at the end of the test; sub-lethal effects were assessed daily.

Endpoints: LC_{50}, EC_{50} (regarding growth) and NOEC.

Test concentrations: Control (dilution water), 4.7, 9.4, 19, 38, 75 and 150 mg a.s./kg dry sediment (nominal), corresponding to initial measured concentrations of 2.75, 5.17, 12.2, 21.6, 77.6 and 144 mg a.s./kg dry sediment.

Test conditions: Test compartments: 300 mL glass beakers with stainless steel mesh covered holes on opposite sides of the beaker; approx. 100 mL spiked sediment (artificial sediment similar to OECD 218 (< 1% humic acid and dolomite, 5% alpha-cellulose, 14% silt and clay (kaolin clay) and 80% industrial quartz sand), pH 6.8); approx. 150 - 175 mL overlying water (depth 6.7 cm); dilution water: filtered and aerated well water; flow rate: approx. 2 volume additions/24 h/test compartment; pH: 8.1 - 8.3; oxygen content: 5.6 mg/L - 8.1 mg/L; total hardness: 116 - 140 mg $CaCO_3$/L; alkalinity: 174 - 194 mg $CaCO_3$/L; conductivity: 355 - 378 µS/cm; ammonia: < 0.17 mg NH_3/L; water temperature: 22.5 °C - 23.4 °C; light intensity: 507 lux; photoperiod: 16 h light : 8 h dark; food: 1.5 mL of a 4 g/L flake food suspension on days 0 - 9.

Analytics: Analytical verification of test item concentrations was conducted using a GC-method with MS detection.

Statistics: Descriptive statistics; Kruskal-Wallis test ($p < 0.05$) and Dunnett's test ($p < 0.05$) for determination of the NOEC values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of active substance concentrations in the sediment, the overlaying water and the pore water was conducted in each concentration at the beginning and the end of the test. Recoveries in the sediment were in a range between 55% - 103% of the nominal concentrations at test initiation and between 13% and 30% of nominal at test termination. Overlaying water concentrations ranged from below the limit of quantification (LOQ) to 0.659 µg a.s./L on day 0 and from below the LOQ to 0.261 µg a.s./L on day 10. The pore water concentrations ranged from 0.579 to 17.2 µg a.s./L at test initiation and from below the LOQ to 13.4 µg a.s./L at test termination. The following biological results are based on the nominal sediment concentrations.

Biological results: Few midges showed abnormal behavior during the test (i.e. midges on the surface of the sediment, midges climbing the walls of the test compartment) in the control and at all tested concentrations. Survival was statistically significantly reduced compared to the control at the highest test item concentration (Kruskal-Wallis test, $p < 0.05$). Statistically significant effects compared to the control on dry weight of the midges were observed at the two highest test item concentrations (Dunnett's test, $p < 0.05$). The results are summarized in Table 8.2.5.3-2.

Table 8.2.5.3-2: Effects of metiram (tested as BAS 222 29 F) on survival and growth of *Chironomus tentans* after 10 days of exposure

Concentration [mg a.s./kg dry sediment] (nominal)	**Control**	**4.7**	**9.4**	**19**	**38**	**75**	**150**
Concentration [mg a.s./kg dry sediment] (nominal)	**--**	**2.75**	**5.17**	**12.2**	**21.6**	**77.6**	**144**
Mean survival [%]	100	98	99	99	84	91	68 *
Mean dry weight [mg]	1.37	1.29	1.23	1.22	1.21	1.01 +	1.13 +
	Endpoints [mg a.s./kg dry sediment] (initial measured)						
LC_{50} / $EC_{50\ (growth)}$	> 144						
$NOEC_{overall}$	21.6						

* Statistically significant differences compared to the control (Kruskal-Wallis test, $p < 0.05$).
+ Statistically significant differences compared to the control (Dunnett's test, $p < 0.05$).

III. CONCLUSION

In a sub-chronic 10-day flow-through sediment test with *Chironomus tentans* the overall NOEC of metiram (tested as BAS 222 29 F) was determined to be 21.6 mg a.s./kg dry sediment based on initial measured sediment concentrations. Both the LC_{50} and the EC_{50} for growth were > 144 mg a.s./kg dry sediment (initial measured).

CA 8.2.5.4 Sediment dwelling organisms

New supplemental data (not evaluated previously on EU level):

The following sub-chronic spiked sediment study on the marine amphipod Leptocheirus plumulosus with metiram (tested as BAS 222 29 F) is not required for registration in the EU and it has not been evaluated previously on EU level. The study was conducted due to U.S. data requirements and it is provided for completeness.

Report:	CA 8.2.5.4/1 Thomas S. et al., 2010b Metiram: A 10-day flow-through whole sediment toxicity test with Leptocheirus plumulosus using spiked sediment 2010/7012894
Guidelines:	EPA 850.1740
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

In a 10-day static sub-chronic spiked sediment study, marine amphipods (*Leptocheirus plumulosus*) were exposed to metiram (tested as BAS 222 29 F) at nominal concentrations of 0 (control), 0.31, 0.63, 1.3, 2.5 and 5.0 mg a.s./kg dry sediment (corresponding to initial measured concentrations of 0.150, 0.366, 0.659, 1.13 and 2.07 mg a.s./kg dry sediment). All test item concentrations and the control group had 5 replicates consisting of 20 amphipods per replicate. Assessment of survival and behavior of the amphipods was performed daily.

The biological results are based on nominal sediment concentrations. After 10 days of exposure, no statistically significant effects on survival compared to the control were observed at test item concentrations up to and including the highest tested concentration. Abnormal behavior (i.e. organism leaving the sediment or on the surface of the sediment) was observed in the control and at the test item concentrations of 1.3, 2.5 and 5.0 mg a.s./kg dry sediment after 10 days of exposure.

In a 10-day static acute sediment test with *Leptocheirus plumulosus*, the LC_{50} of metiram (tested as BAS 222 29 F) was determined to be > 2.07 mg a.s./kg dry sediment based on initial measured concentrations. The NOEC was ≥ 2.07 mg a.s./kg dry sediment (initial measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 29 F, batch no. 300013; content of a.s.: metiram (BAS 222 F; Reg. No 250284): 91.6% (nominal: 85%); density: 1.860 g/cm³.

B. STUDY DESIGN

Test species: Marine amphipod (*Leptocheirus plumulosus*), 2 - 4 mm length; source: "Chesapeake Cultures", Hayes, Virginia, USA.

Test design: Static system (10 days); 5 test concentrations plus a control, 5 replicates per test item concentration and for the control, 20 amphipods per replicate; assessment of survival and signs of toxicity or abnormal behavior after 10 days.

Endpoints: NOEC and LC_{50}.

Test concentrations: Control (dilution water), 0.31, 0.63, 1.3, 2.5 and 5.0 mg a.s./kg dry sediment (nominal), corresponding to initial measured concentrations of 0.150, 0.366, 0.659, 1.13 and 2.07 mg a.s./kg dry sediment.

Test conditions: 1 L glass beakers; 175 mL sediment (artificial sediment similar to OECD 218 (<1% humic acid and dolomite, 5% alpha-cellulose, 14% silt and clay (kaolin clay) and 80% industrial quartz sand), pH 6.8); 775 mL overlying water (filtered natural seawater mixed with well water); salinity: 19.0 - 21.0‰; pH 8.0 - 8.1; oxygen content: 6.9 mg/L - 7.7 mg/L; water temperature: 25.0°C - 25.6°C; ammonia: < 0.17 ppm; light intensity: 541 lux; photoperiod: 24 h light; light intensity: 541 lux; gentle aeration; no feeding.

Analytics: Analytical verification of test item concentrations was conducted using a GC-method with MS detection.

Statistics: Descriptive statistics, Dunnett's t-test ($p < 0.05$) for determination of the NOEC value.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of active substance concentrations in the sediment, the overlaying water and the pore water was conducted in each concentration at the beginning and the end of the test. Recoveries in the sediment were in a range between 41% - 58% of the nominal concentrations at test initiation and between 39% and 62% of nominal at test termination. Overlaying water concentrations were below the limit of quantification (LOQ) and 0.013 mg a.s./L on day 0 and were below the LOQ on day 10. The pore water concentrations ranged from 0.013 to 0.621 mg a.s./L at test initiation and were below the LOQ at test termination. The following biological results are based on the nominal sediment concentrations.

Biological results: After 10 days of exposure, no statistically significant effects on survival compared to the control were observed at test item concentrations up to and including the highest tested concentration (Dunnett's t-test, $p < 0.05$). Abnormal behavior (i.e. organism leaving the sediment or on the surface of the sediment) was observed in the control and at the test item concentrations of 1.3, 2.5 and 5.0 mg a.s./kg dry sediment after 10 days of exposure. The results are summarized in Table 8.2.5.4-1.

Table 8.2.5.4-1: Effect of metiram (tested as BAS 222 29 F) on survival of *Leptocheirus plumulosus*

Concentration [mg a.s./kg dry sediment] (nominal)	**Control**	**0.31**	**0.63**	**1.3**	**2.5**	**5.0**
Concentration [mg a.s./kg dry sediment] (initial measured)	**--**	**0.150**	**0.366**	**0.659**	**1.13**	**2.07**
Mean survival (10 d) [%]	94	88	90	91	93	84
	Endpoints [mg a.s./kg dry sediment] (initial measured)					
LC_{50} (10 d)	> 2.07					
NOEC (10 d)	≥ 2.07					

III. CONCLUSION

In a 10-day static acute sediment test with *Leptocheirus plumulosus*, the LC_{50} of metiram (tested as BAS 222 29 F) was determined to be > 2.07 mg a.s./kg dry sediment based on initial measured concentrations. The NOEC was ≥ 2.07 mg a.s./kg dry sediment (initial measured).

CA 8.2.6 Effects on algal growth

CA 8.2.6.1 Effects on growth of green algae

Data already submitted during previous Annex I inclusion process (already evaluated on EU level):

The following algae toxicity study on the freshwater green alga Pseudokirchneriella subcapitata performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.6.1/1* *Kubitza J., 2002a* *Effect of Metiram (applied as formulated product BAS 222 28 F) on the growth of Pseudokirchneriella subcapitata* *2002/1005284*
Guidelines:	*OECD 201*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 72-hours static toxicity study, the growth of the green alga *Pseudokirchneriella subcapitata* was investigated. The following nominal concentrations of metiram (tested as BAS 222 28 F) were applied: 0 (control), 0.002, 0.007, 0.021, 0.069, 0.206, 0.685 mg a.s./L. Assessment of growth was conducted daily. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and biomass based on number of cells.

The biological results are based on nominal concentrations. No morphological effects on algae could be observed up to 0.206 mg a.s./L. At 0.685 mg a.s./L the cells appeared slightly bigger than those in the control or at lower test concentrations.

In a 72-hour algae test with *Pseudokirchneriella subcapitata* the E_rC_{50} of metiram (applied as formulation BAS 222 28 F) was determined to be 0.191 mg a.s./L and the E_bC_{50} was 0.071 mg a.s./L (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2000-2; content of a.s.: metiram (BAS 222 F, Reg. No. 250284): 68.5% analyzed (nominal: 70%).

B. STUDY DESIGN

Test species: Unicellular fresh water green alga, *Pseudokirchneriella subcapitata* (Reinsch) Korshikov (syn. *Selenastrum capricornutum* Prinz) SAG 61.81, Chlorophyta, Chlorophyceae, Ankistrodesmaceae; stock obtained from “Sammlung von Algenkulturen” Göttingen, Germany.

Test design: Static system; test duration 72 hours; 6 test concentrations, each with 5 replicates plus a control with 10 replicates; daily assessment of growth.

Endpoints: Effects on growth rate and biomass.

Test concentrations: Control, 0.002, 0.007, 0.021, 0.069, 0.206, 0.685 mg metiram/L (nominal); corresponding to 0, 0.003, 0.01, 0.03, 0.1, 0.3 and 1 mg BAS 222 28 F/L

Test conditions: Sterile synthetic test medium, pH 8.0 at test initiation; glass Erlenmeyer flasks; continuous shaking; initial cell densities 1 x 10^4 cells/mL; temperature 22 °C ± 1 °C; continuous light at about 8000 lux.

Analytics: Analytical verification of test item concentrations was conducted using a GC/MS method.

Statistics: Descriptive statistics, probit-analysis for determination of the EC-values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in the stock solution at test initiation. The average measured value for metiram was 84.9% of nominal at test initiation. The following biological results are based on nominal concentrations of the test item.

Biological results: No morphological effects on algae could be observed up to 0.206 mg a.s./L. At 0.685 mg a.s./L the cells appeared slightly bigger than those in the control or at lower test concentrations. The effects on algal growth are summarized in Table 8.2.6.1-1.

Table 8.2.6.1-1: Effects of metiram (tested as BAS 222 28 F) on the growth of the green alga *Pseudokirchneriella subcapitata*

Concentration BAS 222 28 F (nominal) [mg/L]	**Control**	**0.003**	**0.01**	**0.03**	**0.1**	**0.3**	**1**
Concentration metiram (nominal) [mg a.s./L]	**Control**	**0.002**	**0.007**	**0.021**	**0.069**	**0.206**	**0.685**
Inhibition in 72 h (growth rate) [%]	--	0.9	-0.5	1.7	9.3	58.0	89.7
Inhibition in 72 h (biomass) [%]	--	4.1	-2.5	8.6	38.9	93.1	97.6
	Endpoints [mg a.s./L] (nominal)						
E_rC_{50} (0-72 h)	0.191 (95% confidence limits: 0.181 – 0.201)						
E_rC_{10} (0-72 h)	0.049 (95% confidence limits: 0.046 - 0.053)						
E_bC_{50} (0-72 h)	0.071 (95% confidence limits: 0.067 – 0.075)						
E_bC_{10} (0-72 h)	0.016 (95% confidence limits: 0.015 - 0.017)						

III. CONCLUSION

In a 72-hour algae test with *Pseudokirchneriella subcapitata* the E_rC_{50} of metiram (applied as formulation BAS 222 28 F) was determined to be 0.191 mg a.s./L and the E_bC_{50} was 0.071 mg a.s./L (nominal).

The following algae toxicity study on the green alga Ankistrodesmus bibraianus performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.6.1/2* *Kubitza J., 2002b* *Effect of Metiram (applied as formulated product BAS 222 28 F) on the growth of the green alga Ankistrodesmus bibraianus* *2002/1005272*
Guidelines:	*OECD 201*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 72-hours static toxicity study, the growth of green alga *Ankistrodesmus bibraianus* was investigated. The following nominal concentrations of metiram (tested as BAS 222 28 F) were applied: 0 (control), 0.007, 0.022, 0.069, 0.219, 0.685, 2.165 and 6.850 mg a.s./L. Assessment of growth was conducted daily. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and biomass based on number of cells.

The biological results are based on nominal concentrations. No morphological effects on algae could be observed up to and including 0.685 mg a.s./L. At 2.165 mg a.s./L and 6.850 mg a.s./L the cells appeared smaller than those in the control or at lower test concentrations.

In a 72-hour algae test with *Ankistrodesmus bibraianus* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 1.365 mg a.s./L and the E_bC_{50} was 0.208 mg a.s./L (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2000-2; content of a.s. metiram (BAS 222 F, Reg. No. 250 284): 68.5% analyzed (nominal: 70%).

B. STUDY DESIGN

Test species: Unicellular fresh water green alga, *Ankistrodesmus bibraianus* (Reinsch) Korshikov (syn. *Selenastrum bibraianum*), SAG 278-1, Chlorophyta, Chlorophyceae, Ankistrodesmaceae; stock obtained from “Sammlung von Algenkulturen” Göttingen, Germany.

Test design: Static system; test duration 72 hours; 7 test concentrations, each with 5 replicates plus a control with 10 replicates; daily assessment of growth.

Endpoints: Effects on growth rate and biomass.

Test concentrations: Control, 0.007, 0.022, 0.069, 0.219, 0.685, 2.165 and 6.850 mg metiram/L (nominal); corresponding to 0, 0.01, 0.032, 0.1, 0.32, 1.0, 3.16 and 10 mg BAS 222 28 F/L.

Test conditions: Glass Erlenmeyer flasks; nutrient solution according to OECD 201, pH 8.0 at test initiation; initial cell densities 1 x 10^4 cells/mL; temperature 22 °C ± 1 °C; continuous light at about 8000 lux; continuous shaking.

Analytics: Analytical verification of test item concentrations was conducted using GC/MS method CP 402.

Statistics: Descriptive statistics, probit-analysis for determination of the EC-values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in the stock solution used for the preparation of the test solutions. Test substance recovery was determined to be 101.9% of the nominal concentration. Thus, biological results are based on nominal concentrations of the test item.

Biological results: No morphological effects on algae could be observed up to 0.685 mg a.s./L. At 2.16 mg a.s./L the cells appeared smaller than those in the control or at lower test concentrations. The effects on algal growth are summarized in Table 8.2.6.1-2.

Table 8.2.6.1-2: Effects of metiram (tested as BAS 222 28 F) on the growth of the green alga *Ankistrodesmus bibraianus*

Concentration BAS 222 28 F (nominal) [mg/L]	**Control**	**0.010**	**0.030**	**0.10**	**0.30**	**1.00**	**3.16**	**10**
Concentration metiram (nominal) [mg a.s./L]	**Control**	**0.007**	**0.022**	**0.069**	**0.219**	**0.685**	**2.165**	**6.850**
Inhibition in 72 h (growth rate) [%]	--	0.9	2.7	9.6	19.4	37.3	62.5	74.0
Inhibition in 72 h (biomass) [%]	--	2.8	14.0	27.3	58.1	75.4	84.9	93.2
	Endpoints [mg a.s./L] (nominal)							
E_rC_{50} (0-72 h)	1.365 (95% confidence limits: 1.248 – 1.492)							
E_rC_{10} (0-72 h)	0.079 (95% confidence limits: 0.070 - 0.090)							
E_bC_{50} (0-72 h)	0.208 (95% confidence limits: 0.193 – 0.224)							
E_bC_{10} (0-72 h)	0.015 (95% confidence limits: 0.013 - 0.017)							

III. CONCLUSION

In a 72-hour algae test with *Ankistrodesmus bibraianus* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 1.365 mg a.s./L and the E_bC_{50} was 0.208 mg a.s./L (nominal).

The following algae toxicity study on the green alga Desmodesmus subspicatus performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.6.1/3* *Kubitza J., 2002c* *Effect of Metiram (applied as formulated product BAS 222 28 F) on the growth of the green alga Desmodesmus subspicatus* *2002/1005275*
Guidelines:	*OECD 201*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 72-hours static toxicity study, the growth of green alga *Desmodesmus subspicatus* was investigated. The following nominal concentrations of metiram (tested as BAS 222 28 F) were applied: 0 (control), 0.021, 0.055, 0.137, 0.377, 0.986, 2.603 and 6.850 mg a.s./L. Assessment of growth was conducted daily. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and biomass based on number of cells.

The biological results are based on nominal concentrations. No morphological effects on algae could be observed up to and including 0.137 mg a.s./L. At 0.377 mg a.s. /L a few cells, at 0.986 mg a.s. /L about 30%, at 2.603 mg a.s. /L about 80% and at 6.850 mg a.s./L more or less all cells appeared translucent and deformed.

In a 72-hour algae test with *Desmodesmus subspicatus* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.891 mg a.s./L and the E_bC_{50} was 0.431 mg a.s./L (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2000-2; content of a.s. metiram (BAS 222 F, Reg. No. 250 284): 68.5% analyzed (nominal: 70%).

B. STUDY DESIGN

Test species: Unicellular fresh water green alga, *Desmodesmus subspicatus* (Hegewald & Schmidt; formerly *Scenedesmus subspicatus*) SAG 86.81, Chlorophyta, Chlorophyceae, Scenedesmaceae; stock obtained from "Sammlung von Algenkulturen" Göttingen, Germany.

Test design: Static system; test duration 72 hours; 7 test concentrations, each with 5 replicates plus a control with 10 replicates; daily assessment of growth.

Endpoints: Effects on growth rate and biomass.

Test concentrations: Control, 0.021, 0.055, 0.137, 0.377, 0.986, 2.603 and 6.850 mg metiram/L (nominal); corresponding to 0.03, 0.08, 0.2, 0.55, 1.44, 3.8 and 10 mg BAS 222 28 F/L.

Test conditions: Glass Erlenmeyer flasks; nutrient solution according to OECD 201, pH 8.0 at test initiation; initial cell densities 1 x 10^4 cells/mL; temperature 22 °C ± 2 °C; continuous light at about 8000 lux; continuous shaking.

Analytics: Analytical verification of test item concentrations was conducted using GC/MS method CP 402.

Statistics: Descriptive statistics, probit-analysis for determination of the EC-values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test substance concentrations was conducted in the stock solution at test initiation. The measured value of metiram was 97.2%, confirming the nominal data. Therefore, biological results are based on nominal concentrations of the test item.

Biological results: No morphological effects on algae could be observed up to 0.137 mg a.s./L. At 0.377 mg a.s. /L a few cells, at 0.986 mg a.s. /L about 30%, at 2.603 mg a.s. /L about 80% and at 6.850 mg a.s./L more or less all cells appeared translucent and deformed. The effects on algal growth are summarized in Table 8.2.6.1-3.

Table 8.2.6.1-3: Effects of metiram (tested as BAS 222 28 F) on the growth of the green alga *Desmodesmus subspicatus*

Concentration BAS 222 28 F (nominal) [mg/L]	**Control**	**0.030**	**0.080**	**0.200**	**0.550**	**1.440**	**3.8**	**10**
Concentration metiram (nominal) [mg a.s./L]	**Control**	**0.021**	**0.055**	**0.137**	**0.377**	**0.986**	**2.603**	**6.850**
Inhibition in 72 h (growth rate) [%]	--	2.8	-0.8	6.2	19.9	44.3	90.1	95.2
Inhibition in 72 h (biomass) [%]	--	-1.0	-7.1	17.9	40.6	80.4	99.2	97.8
	Endpoints [mg a.s./L] (nominal)							
E_rC_{50} (0-72 h)	0.891 (95% confidence limits: 0.846 – 0.938)							
E_rC_{10} (0-72 h)	0.183 (95% confidence limits: 0.169 - 0.197)							
E_bC_{50} (0-72 h)	0.431 (95% confidence limits: 0.412 – 0.451)							
E_bC_{10} (0-72 h)	0.118 (95% confidence limits: 0.110 - 0.126)							

III. CONCLUSION

In a 72-hour algae test with *Desmodesmus subspicatus* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.891 mg a.s./L and the E_bC_{50} was 0.431 mg a.s./L (nominal).

The following algae toxicity study on the green alga Tetraedron caudatum performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.6.1/4* *Kubitza J., 2002d* *Effect of Metiram (applied as formulated product BAS 222 28 F) on the growth of the alga Tetraedron caudatum* *2002/1005274*
Guidelines:	*OECD 201*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 72 hour static toxicity study, the growth of the green alga *Tetraedron caudatum* was investigated. The following nominal concentrations of metiram (tested as BAS 222 28 F) were applied: 0 (control), 0.014, 0.034, 0.075, 0.164, 0.384, 0.891 and 2.055 mg a.s./L. Assessment of growth was conducted daily. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and biomass based on number of cells.

The biological results are based on nominal concentrations. No morphological effects on algae could be observed up to and including 0.384 mg a.s./L. At 0.891 mg a.s. /L a third and at 2.055 mg a.s. /L about two thirds of cells appeared rounder than those in the control.

In a 72-hour algae test *Tetraedron caudatum* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.812 mg a.s./L and the E_bC_{50} was 0.218 mg a.s./L (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2000-2; content of a.s. metiram (BAS 222 F, Reg. No. 250 284): 68.5% analyzed (nominal: 70%).

B. STUDY DESIGN

Test species: Unicellular fresh water green alga, *Tetraedron caudatum* (Corda) Hansgirg SAG 23.81, Chlorophyta, Chlorophyceae, Oocystaceae; stock obtained from "Sammlung von Algenkulturen" Göttingen, Germany.

Test design: Static system; test duration 72 hours; 7 test concentrations, each with 5 replicates plus a control with 10 replicates; daily assessment of growth.

Endpoints: Effects on growth rate and biomass.

Test concentrations: Control, 0.014, 0.034, 0.075, 0.164, 0.384, 0.891, 2.055 mg metiram/L (nominal; corresponding to 0, 0.02, 0.05, 0.11, 0.24, 0.56, 1.3 and 3 mg BAS 222 28 F/L.

Test conditions: Glass Erlenmeyer flasks; nutrient solution according to OECD 201, pH 8.0 at test initiation; initial cell densities 1 x 10^4 cells/mL; temperature 22 °C ± 1 °C; continuous light at about 8000 lux; continuous shaking.

Analytics: Analytical verification of test item concentrations was conducted using GC/MS method CP 402.

Statistics: Descriptive statistics, probit-analysis for determination of the EC-values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in the stock solution at test initiation. The measured value of metiram was 96.1%, confirming the nominal data. Therefore, biological results are based on nominal concentrations of the test item.

Biological results: No morphological effects on algae could be observed up to and including 0.384 mg a.s./L. At 0.891 mg a.s. /L a third and at 2.055 mg a.s. /L about two thirds of cells appeared rounder than those in the control did. The effects on algal growth are summarized in Table 8.2.6.1-4.

Table 8.2.6.1-4: Effects of metiram (tested as BAS 222 28 F) on the growth of the green alga *Tetraedron caudatum*

Concentration BAS 222 28 F (nominal) [mg/L]	**Control**	**0.02**	**0.05**	**0.11**	**0.24**	**0.56**	**1.3**	**3**
Concentration metiram (nominal) [mg a.s./L]	**Control**	**0.014**	**0.034**	**0.075**	**0.164**	**0.384**	**0.891**	**2.055**
Inhibition in 72 h (growth rate) [%]	--	1.5	9.4	12.0	23.1	40.3	53.5	62.8
Inhibition in 72 h (biomass) [%]	--	2.6	20.1	26.4	45.5	63.9	75.8	89.2
	Endpoints [mg a.s./L] (nominal)							
E_rC_{50} (0-72 h)	0.812 (95% confidence limits: 0.741 – 0.890)							
E_rC_{10} (0-72 h)	0.050 (95% confidence limits: 0.044 - 0.056)							
E_bC_{50} (0-72 h)	0.218 (95% confidence limits: 0.205 – 0.232)							
E_bC_{10} (0-72 h)	0.022 (95% confidence limits: 0.020 - 0.024)							

III. CONCLUSION

In a 72-hour algae test *Tetraedron caudatum* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.812 mg a.s./L and the E_bC_{50} was 0.218 mg a.s./L (nominal).

The following algae toxicity study on the green alga Staurastrum tetracerum performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.6.1/5* *Kubitza J., 2002e* *Effect of Metiram (applied as formulated product BAS 222 28 F) on the growth of Staurastrum tetracerum* *2002/1005279*
Guidelines:	*OECD 201*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 72-hours static toxicity laboratory study, the growth of the green alga *Staurastrum tetracerum* was investigated. The following nominal concentrations of metiram (teste as BAS 222 28 F) were applied: 0 (control), 0.007, 0.014, 0.041, 0.096, 0.233, 0.562 and 1.37 mg a.s./L. Assessment of growth was conducted daily. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and biomass based on number of cells.

The biological results are based on nominal concentrations. No morphological effects on algae could be observed up to and including 0.562 mg a.s./L. At 1.370 mg a.s. /L some cells appeared deformed.

In a 72-hour algae test with *Staurastrum tetracerum* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.170 mg a.s./L and the E_bC_{50} was 0.081 mg a.s./L (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2000-2; content of a.s. metiram (BAS 222 F, Reg. No. 250 284): 68.5% analyzed (nominal: 70%).

B. STUDY DESIGN

Test species: Unicellular fresh water green alga, *Staurastrum tetracerum* (Kützing) Ralfs SAG B7.94, Chlorophyta, Conjugatophyceae, Desmidiaceae; stock obtained from "Sammlung von Algenkulturen" Göttingen, Germany.

Test design: Static system; test duration 72 hours; 7 test concentrations, each with 5 replicates plus a control with 10 replicates; daily assessment of growth.

Endpoints: Effects on growth rate and biomass.

Test concentrations: Control, 0.007, 0.014, 0.041, 0.096, 0.233, 0.562 and 1.37 mg metiram/L (nominal); corresponding to 0, 0.01, 0.02, 0.06, 0.14, 0.34, 0.82 and 2 mg BAS 222 28 F/L.

Test conditions: Glass Erlenmeyer flasks; nutrient solution according to OECD 201, pH 8.0 at test initiation; initial cell densities 1 x 10^4 cells/mL; temperature 22 °C ± 1 °C; continuous light at about 8000 lux; continuous shaking.

Analytics: Analytical verification of test item concentrations was conducted using GC/MS method CP 402.

Statistics: Descriptive statistics, probit-analysis for determination of the EC-values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in the stock solution at test initiation. The measured value of metiram was 88.6%, confirming nominal data. Therefore, biological results are based on nominal concentrations of the test item.

Biological results: No morphological effects on algae could be observed up to and including 0.562 mg a.s./L. At 1.370 mg a.s. /L some cells appeared deformed. The effects on algal growth are summarized in Table 8.2.6.1-5.

Table 8.2.6.1-5: Effects of metiram (tested as BAS 222 28 F) on the growth of the green alga *Staurastrum tetracerum*

Concentration BAS 222 28 F (nominal) [mg/L]	**Control**	**0.01**	**0.02**	**0.06**	**0.14**	**0.34**	**0.82**	**2.0**
Concentration metiram (nominal) [mg a.s./L]	**Control**	**0.007**	**0.014**	**0.041**	**0.096**	**0.233**	**0.562**	**1.370**
Inhibition in 72 h (growth rate) [%]	--	3.2	13.9	61.8	57.2	64.5	56.5	56.0
Inhibition in 72 h (biomass) [%]	--	4.6	29.4	65.1	65.7	64.3	62.7	71.9
	Endpoints [mg a.s./L] (nominal)							
E_rC_{50} (0-72 h)	0.170 (95% confidence limits: 0.151 – 0.190)							
E_rC_{10} (0-72 h)	0.002 (95% confidence limits: 0.0012 - 0.0021)							
E_bC_{50} (0-72 h)	0.081 (95% confidence limits: 0.073 – 0.090)							
E_bC_{10} (0-72 h)	0.001 (95% confidence limits: 0.0007 - 0.0013)							

III. CONCLUSION

In a 72-hour algae test with *Staurastrum tetracerum* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.170 mg a.s./L and the E_bC_{50} was 0.081 mg a.s./L (nominal).

The following algae toxicity study on the green alga Staurastrum gracile performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.6.1/6* *Kubitza J., 2002f* *Effect of Metiram (applied as formulated product BAS 222 28 F) on the growth of Staurastrum gracile* *2002/1005282*
Guidelines:	*OECD 201*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 72 hour static toxicity laboratory study, the growth of green alga *Staurastrum gracile* was investigated. The following nominal concentrations of metiram (tested as BAS 222 28 F) were applied: 0 (control), 0.007, 0.021, 0.041, 0.096, 0.233, 0.569 and 1.370 mg a.s./L. Assessment of growth was conducted daily. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and biomass based on number of cells.

The biological results are based on nominal concentrations. No morphological effects on algae could be observed up to and including 0.096 mg a.s./L. At 0.233 mg a.s. /L and higher the cells appeared roundish and translucent.

In a 72-hour algae test with *Staurastrum gracile* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.519 mg a.s./L and the E_bC_{50} was 0.134 mg a.s./L (nominal)

I. MATERIAL AND METHODS

A. MATERIALS

Test item:	BAS 222 28 F, batch no. 2000-2; content of a.s. metiram (BAS 222 F, Reg. No. 250 284): 68.5% analyzed (nominal: 70%).

B. STUDY DESIGN

Test species:	Unicellular fresh water green alga, *Staurastrum gracile* Ralfs, UTEX LB 562, Chlorophyta, Conjugatophyceae, Desmidiaceae; stock obtained from "The Culture Collection of Algae" at the University of Texas, Austin, USA.
Test design:	Static system; test duration 72 hours; 7 test concentrations, each with 5 replicates plus a control with 10 replicates; daily assessment of growth.
Endpoints:	Effects on growth rate and biomass.
Test concentrations:	Control, 0.007, 0.021, 0.041, 0.096, 0.233, 0.569, 1.37 mg metiram/L (nominal); corresponding to 0.01, 0.03, 0.06, 0.14, 0.34, 0.83 and 2 mg BAS 222 28 F/L.
Test conditions:	Glass Erlenmeyer flasks; nutrient solution according to OECD 201, pH 8.0 at test initiation initial cell densities 1 x 10^4 cells/mL; temperature 22 °C ± 1 °C; continuous light at about 8000 lux; continuous shaking;.
Analytics:	Analytical verification of test item concentrations was conducted using GC/MS method CP 402.
Statistics:	Descriptive statistics, probit-analysis for determination of the EC-values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in the stock solution at test initiation. The average value for metiram was 97.1%, confirming nominal data. Therefore, biological results are based on nominal concentrations of the test item.

Biological results: No morphological effects on algae could be observed up to and including 0.096 mg a.s./L. At 0.233 mg a.s. /L and higher the cells appeared roundish and translucent. The effects on algal growth are summarized in Table 8.2.6.1-6.

Table 8.2.6.1-6: Effects of metiram (tested as BAS 222 28 F) on the growth of the green alga *Staurastrum gracile*

Concentration BAS 222 28 F (nominal) [mg/L]	**Control**	**0.01**	**0.03**	**0.06**	**0,14**	**0.34**	**0.83**	**2**
Concentration metiram (nominal) [mg a.s./L]	**Control**	**0.007**	**0.021**	**0.0041**	**0.096**	**0.233**	**0.569**	**1.370**
Inhibition in 72 h (growth rate) [%]	--	0.3	12.2	11.3	33.1	48.7	49.3	56.6
Inhibition in 72 h (biomass) [%]	--	3.6	21.8	28.1	58.9	67.7	68.1	77.4
	Endpoints [mg a.s./L] (nominal)							
E_rC_{50} (0-72 h)	0.519 (95% confidence limits: 0.467 – 0.576)							
E_rC_{10} (0-72 h)	0.020 (95% confidence limits: 0.017 - 0.023)							
E_bC_{50} (0-72 h)	0.134 (95% confidence limits: 0.124 – 0.145)							
E_bC_{10} (0-72 h)	0.007 (95% confidence limits: 0.006 - 0.008)							

III. CONCLUSION

In a 72-hour algae test with *Staurastrum gracile* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.519 mg a.s./L and the E_bC_{50} was 0.134 mg a.s./L (nominal)

The following algae toxicity study on the green alga Cosmarium praemorsum performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.6.1/7* *Kubitza J., 2002g* *Effect of Metiram (applied as formulated product BAS 222 28 F) on the growth of Cosmarium praemorsum* *2002/1005278*
Guidelines:	*OECD 201*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 72-hour static toxicity laboratory study, the growth of green alga *Cosmarium praemorsum* was investigated. The following nominal concentrations of metiram (tested as BAS 222 28 F) were applied: 0 (control), 0.021, 0.041, 0.096, 0.206, 0.445, 0.959 and 2.055 mg a.s./L. Assessment of growth was conducted daily. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and biomass based on number of cells.

The biological results are based on nominal concentrations. No morphological effects on the algae could be observed at concentrations up to and including 0.041 mg metiram/L. At 0.096, 0.206 and 0.445 mg a.s./L about 25% and at higher test concentrations more or less all cells appeared pale and deformed.

In a 72-hour algae test with *Cosmarium praemorsum,* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.157 mg a.s./L and the E_bC_{50} was 0.084 mg a.s./L (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2000-2; content of a.s. metiram (BAS 222 F, Reg. No. 250 284): 68.5% analyzed (nominal: 70%).

B. STUDY DESIGN

Test species: Unicellular fresh water green alga, *Cosmarium praemorsum,* de Brebisson, SAG B 612-13, Chlorophyta, Conjugatophyceae, Desmidiaceae; stock obtained from "Sammlung von Algenkulturen" Göttingen, Germany.

Test design: Static system; test duration 72 hours; 7 test concentrations, each with 5 replicates plus a control with 10 replicates; daily assessment of growth.

Endpoints: Effects on growth rate and biomass.

Test concentrations: Control, 0.021, 0.041, 0.096, 0.206, 0.445, 0.959 and 2.055 mg metiram/L (nominal); corresponding to0, 0.03, 0.06, 0.14, 0.3, 0.65, 1.4 and 3 mg BAS 222 28 F/L.

Test conditions: Glass Erlenmeyer flasks; nutrient solution according to OECD 201, pH 8.0 at test initiation; initial cell densities 1 x 10^4 cells/mL; temperature 22 °C ± 2 °C; continuous light at about 8000 lux; continuous shaking.

Analytics: Analytical verification of test item concentrations was conducted using GC/MS method CP 402.

Statistics: Descriptive statistics, probit-analysis for determination of the EC-values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in the stock solution at test initiation. The measured value for metiram was 105.6%, confirming nominal values. Therefore, biological results are based on nominal concentrations of the test item.

Biological results: No morphological effects on the algae could be observed at concentrations up to and including 0.041 mg metiram/L. At 0.096, 0.206 and 0.445 mg a.s./L about 25% and at higher test concentrations more or less all cells appeared pale and deformed. The effects on algal growth are summarized in Table 8.2.6.1-7.

Table 8.2.6.1-7: Effects of metiram (tested as BAS 222 28 F) on the growth of the green alga *Cosmarium praemorsum*

Concentration BAS 222 28 F (nominal) [mg/L]	**Control**	**0.03**	**0.06**	**0.14**	**0.3**	**0.65**	**1.4**	**3**
Concentration metiram (nominal) [mg a.s./L]	**Control**	**0.021**	**0.041**	**0.096**	**0.206**	**0.445**	**0.959**	**2.055**
Inhibition in 72 h (growth rate) [%]	--	2.7	32.8	47.3	55.3	75.5	79.8	87.4
Inhibition in 72 h (biomass) [%]	--	6.0	46.9	64.3	72.3	82.4	83.9	87.7
	Endpoints [mg a.s./L] (nominal)							
E_rC_{50} (0-72 h)	0.157 (95% confidence limits: 0.148 – 0.168)							
E_rC_{10} (0-72 h)	0.013 (95% confidence limits: 0.012 - 0.015)							
E_bC_{50} (0-72 h)	0.084 (95% confidence limits: 0.078 – 0.091)							
E_bC_{10} (0-72 h)	0.006 (95% confidence limits: 0.005 - 0.007)							

III. CONCLUSION

In a 72-hour algae test with *Cosmarium praemorsum,* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.157 mg a.s./L and the E_bC_{50} was 0.084 mg a.s./L (nominal).

The following algae toxicity study on the green alga Selenastrum capricornutum performed with the metiram metabolite ethyleneurea (EU) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.
In accordance to the EFSA Aquatic Guidance Document (EFSA, 2013) and OECD guideline 201 (2011) the 72 h endpoints obtained in the 96 h study are considered as relevant endpoint for the aquatic risk assessment, therefore both the 72 h and 96 h endpoints are reported in the study summary below.

Report:	*CA 8.2.6.1/8* *Palmer S.J. et al., 2001c* *Ethylene urea: A 96-hour toxicity test with the freshwater alga (Selenastrum capricornutum)* *2001/5000986*
Guidelines:	*EPA 850.5400, OECD 201, EEC 92/69 C 3*
GLP:	*yes* *(certified by United States Environmental Protection Agency)*

Executive Summary

In a 96-hours static toxicity laboratory study, the growth of green alga *Selenastrum capricornutum* was investigated. The following nominal concentrations of ethyleneurea (EU; metabolite of metiram) were applied: 0 (control), 7.5, 15, 30, 60, 120 mg/L (corresponding to mean measured concentrations of 7.5, 15, 29, 58 and 119 mg/L). Assessment of growth was conducted daily. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and biomass based on number of cells.

Biological results are based on nominal concentrations. After 72 and 96 hours of exposure there were no noticeable changes in cell morphology in any treatment group when compared to the control. Inhibition of biomass and growth rate was < 10% compared to control in all treatment groups.

In a 96-hour algae test with *Selenastrum capricornutum,* the 72 h and 96 h E_rC_{50} and E_bC_{50} values of ethyleneurea (metabolite of metiram) were all determined to be > 120 mg/L based on nominal concentrations. Based on mean measured concentrations the the 72 h and 96 h E_rC_{50} and E_bC_{50} values were both determined to be > 119 mg/L.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: Ethyleneurea (BF 222-EU; Reg. No 027270; metabolite of metiram); Lot No. 01743-141; purity: 90.8%

B. STUDY DESIGN

Test species: Unicellular green alga *Selenastrum capricornutum,* obtained from the University of Toronto Culture Collection and maintained in culture at Wildlife International, Ltd., Easton, Maryland.

Test design: Static system; test duration 96 hours; 5 test concentrations plus a control, each with 3 replicates; daily assessment of growth.

Endpoints: Effects on growth rate and biomass.

Test concentrations: Control, 7.5, 15, 30, 60, 120 mg/L, (nominal); corresponding to mean measured concentrations of 7.5, 15, 29, 58 and 119 mg/L.

Test conditions: Glass Erlenmeyer flasks (volume: 250 mL);100 mL sterilized nutrient solution; initial cell densities 1 x 10^4 cells/mL; pH 7.3 – 8.7; temperature 22.4 °C – 23.6 °C; continuous light at about 3880 – 4690 lux.

Analytics: Analytical verification of test item concentrations was conducted using HPLC method KP-017-00.

Statistics: Descriptive statistics, normality and homogeneity of variance using the Shapiro-Wilk´s and Levene´s tests, Dunnett´s test, EC values and corresponding 95% confidence limits were calculated statistically by linear interpolation versus concentration.

II. RESULTS AND DISCUSSION

Analytical measurements: The analytically determined concentrations of ethyleneurea were in a range of 96.5% - 99.4% at test initiation and 98.2% - 99.7% at test termination, confirming nominal data. Therefore, biological results are based on nominal concentrations. Additionally, the results based on mean measured values are shown.

Biological results: After 72 and 96 hours of exposure there were no noticeable changes in cell morphology in any treatment group when compared to the control. The effects on algal growth are summarized in Table 8.2.6.1-8.

Table 8.2.6.1-8: Effects of ethyleneurea (metabolite of metiram) on the green alga *Selenastrum capricornutum*

Concentration [mg/L] (nominal)	**Control**	**7.5**	**15**	**30**	**60**	**120**
Concentration [mg/L] (mean measured)	**--**	**7.5**	**15**	**29**	**58**	**119**
Inhibition in 72 h (growth rate) [%] #	--	0.76	-0.24	-0.32	1.9	-1.4
Inhibition in 96 h (growth rate) [%] #	--	0.27	-0.57	-0.23	0.04	-0.93
Inhibition in 72 h (biomass) [%] #	--	3.2	-1.4	-1.5	8.2	-6.2
Inhibition in 96 h (biomass) [%] #	--	1.5	-3.3	-1.3	0.076	-5.3
	Endpoints [mg//L] (nominal)					
	based on nominal concentrations			based on mean measured concentrations		
E_rC_{50} / E_bC_{50} (0-72 h)	> 120			> 119		
E_rC_{50} / E_bC_{50} (0 - 96 h)	> 120			> 119		

Negative values indicate stimulated growth compared to the control.

III. CONCLUSION

In a 96-hour algae test with *Selenastrum capricornutum,* the 72 h and 96 h E_rC_{50} and E_bC_{50} values of ethyleneurea (metabolite of metiram) were all determined to be > 120 mg/L based on nominal concentrations. Based on mean measured concentrations the the 72 h and 96 h E_rC_{50} and E_bC_{50} values were both determined to be > 119 mg/L.

The following algae toxicity study on the green alga Pseudokirchneriella subcapitata performed with the metiram metabolite ethylenethiourea (ETU) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.6.1/9* *Reuschenbach P., 2000a* *Reg.No. 146 099 - Determination of the inhibitory effect on the cell multiplication of unicellular green algae* *2000/1017191*
Guidelines:	*EEC 92/69 A V C 3, OECD 201*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 72-hours static toxicity laboratory study, the effect of ethylenethiourea (ETU; metabolite of metiram) on the growth of the green alga *Pseudokirchneriella subcapitata* was investigated. The following nominal concentrations were applied: 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 mg ETU/L. Assessment of growth was conducted daily. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and biomass based on number of cells.

Biological results are based on nominal concentrations. Growth rate and biomass of *Pseudokirchneriella subcapitata* were inhibited at concentrations of 25 mg ETU/L and higher.

In a 72-hour algae test with *Pseudokirchneriella subcapitata,* the E_rC_{50} of ethylenethiourea (ETU; metabolite of metiram) was determined to be 93.8 mg/L, the E_bC_{50} was 23.7 mg/L based on nominal concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

Test item:	Ethylenethiourea (BF 222-ETU, Reg. No. 146099; metabolite of metiram), batch no. L33-99, purity: 99.6%

B. STUDY DESIGN

Test species:	Unicellular green alga *Pseudokirchneriella subcapitata,* (Chodat), SAG 61.81, stock obtained from “Collection of algal cultures” Göttingen, Germany.
Test design:	Static system; test duration 72 hours; 9 test concentrations, each with 3 replicates plus a control with 5 replicates; daily assessments of growth.
Endpoints:	Effects on growth rate and biomass.
Test concentrations:	Control, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 mg ETU/L (nominal).
Test conditions:	Glass Erlenmeyer flasks (volume: 250 mL); 100 mL test medium prepared according to test guidelines, pH 7.9 - 8.2; initial cell densities 1 x 10^4 cells/mL; temperature 23 °C ± 2 °C; continuous light at about 60-120 µE/(m² s) at a wave length of 400-700 nm.
Analytics:	Analytical verification of test item concentrations was conducted using HPLC method CF-A 446.
Statistics:	Descriptive statistics; determination of EC values by linear regression analysis.

II. RESULTS AND DISCUSSION

Analytical measurements: The analytically determined concentrations of ETU were in a range of 85.0% - 107.4% at test initiation and between 97.2% and 118.0% at test termination, confirming nominal data. Therefore, biological results are based on nominal concentrations.

Biological results: The effects on algal growth are summarized in Table 8.2.6.1-9.

Table 8.2.6.1-9: Effects of ethylenethiourea (ETU; metabolite of metiram) on the green alga *Pseudokirchneriella subcapitata*

Concentration [mg/L] (nominal)	Control	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100
Inhibition in 72 h (growth rate) [%] #	--	-0.9	-1.9	-0.2	2.1	-1.6	-0.4	24.7	34.7	50.6
Inhibition in 72 h (biomass) [%] #	--	-5.0	-5.0	-1.1	3.8	-8.7	-1.6	54.5	74.0	77.8
	Endpoints [mg ETU/L] (nominal)									
E_rC_{50} (0-72 h)	93.8									
E_rC_{10} (0-72 h)	16.7									
E_bC_{50} (0-72 h)	23.7									
E_bC_{10} (0-72 h)	14.4									

Negative values indicate stimulated growth compared to the control.

III. CONCLUSION

In a 72-hour algae test with *Pseudokirchneriella subcapitata,* the E_rC_{50} of ethylenethiourea (ETU; metabolite of metiram) was determined to be 93.8 mg/L, the E_bC_{50} was 23.7 mg/L based on nominal concentrations.

New supplemental data (not evaluated previously on EU level):

The following study on the freshwater green alga Pseudokirchneriella subcapitata performed with metiram (tested as BAS 222 28 F) is provided in support of the aquatic risk assessment and has not been evaluated previously on EU level.
In accordance to the EFSA Aquatic Guidance Document (EFSA, 2013) and OECD guideline 201 (2011) the 72 h endpoints obtained in the 96 h study are considered as relevant endpoint for the aquatic risk assessment, therefore both the 72 h and 96 h endpoints are reported in the study summary below.

Report:	CA 8.2.6.1/10 Minderhout T. et al., 2009a Polyram (BAS 222 28 F): A 96-hour toxicity test with the freshwater alga (Pseudokirchneriella subcapitata) 2009/7000035
Guidelines:	OECD 201, EEC 92/69 C 3, EPA 850.5400, ASTM E 1218-90
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

In a 96-hour static toxicity laboratory study, the effect of metiram (tested as BAS 222 28 F) on the growth of the green alga *Pseudokirchneriella subcapitata* was investigated. The following nominal concentrations were applied: 0 (control), 0.0047, 0.0094, 0.019, 0.038, 0.075 and 0.150 mg a.s./L (corresponding to geometric mean measured concentrations of 0.0016, 0.0036, 0.0044, 0.0092, 0.030 and 0.055 mg a.s./L). Assessment of growth was conducted 0 h, 24 h, 48 h and 96 h after test initiation.

The biological results are based on geometric mean measured concentrations over the 96 h exposure period. No morphological effects on algae were observed in the control and at any of the test item concentrations tested over the 96 h study period. Statistically significant effects on all measured parameters compared to the control were observed at three highest test item concentrations of 0.0092, 0.030 and 0.055 mg a.s./L after 72 h and 96 h of exposure.

In a 96-hour algae test with *Pseudokirchneriella subcapitata*, the 96 h E_rC_{50} and E_yC_{50} values for metiram (tested as BAS 222 28 F) were determined to be 0.033 mg a.s./L and 0.0089 mg a.s./L, respectively, based on geometric mean measured concentrations. The 72 h E_rC_{50} and E_yC_{50} values were 0.021 mg a.s./L and 0.0061 mg a.s./L, respectively.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 1653; content of a.s.: metiram (BAS 222 F, Reg. no. 250284); 72.5% (nominal: 70.0%); density: 1.740 g/cm^3.

B. STUDY DESIGN

Test species: Unicellular fresh water green alga, *Pseudokirchneriella subcapitata* (Reinsch) Korshikov (syn. *Selenastrum capricornutum* Prinz); stock obtained from the "University of Toronto Culture Collection”.

Test design: Static system; test duration 96 hours; 6 test concentrations plus a control, each with 3 replicates per treatment; daily assessment of growth.

Endpoints: EC_{50} with respect to growth rate and yield after exposure over 72 and 96 hours.

Test concentrations: Control; 0.0047, 0.0094, 0.019, 0.038, 0.075 and 0.150 mg a.s./L (nominal), corresponding to geometric mean measured concentrations of 0.0016, 0.0036, 0.0044, 0.0092, 0.030 and 0.055 mg a.s./L.

Test conditions: 250 mL Erlenmeyer flasks; test volume 100 mL; freshwater algal medium according to OECD 201; pH 7.9 at test initiation, pH 7.8 - 8.1 at test termination; temperature: 25.1 - 25.7 °C; initial cell densities 1 x 10^4 cells/mL; continuous light at about 4410 - 4600 lux; continuous shaking at 100 rpm.

Analytics: Analytical verification of test item concentrations was conducted using a GC-method with MS detection.

Statistics: Descriptive statistics; non-linear regression or linear interpolation for determination of EC_{50} values for growth rate and yield, Dunnett’s test ($\alpha = 0.05$) for determination of NOEC value.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at the beginning and at the end of the test. The analyzed contents of metiram ranged from 86% to 102% of nominal at test initiation and from 6% to 18% of nominal at test termination. The decrease in concentrations of metiram in the treatment groups over the 96-hour exposure period was attributed to breakdown of the test substance by hydrolysis. Since the measured concentrations of metiram varied by more than 20% of the nominal values, the following biological results are based on geometric mean measured concentrations over the 96 h exposure period.

Biological results: No morphological effects on algae were observed in the control and at any of the test item concentrations tested over the 96 h study period. Statistically significant effects on all measured parameters compared to the control were observed at three highest test item concentrations of 0.0092, 0.030 and 0.055 mg a.s./L after 72 h and 96 h of exposure (Dunnett's test, α = 0.05). The effects on algal growth rate and yield are summarized in Table 8.2.6.1-10.

Table 8.2.6.1-10: Effect of metiram (tested as BAS 222 28 F) on the growth of green alga *Pseudokirchneriella subcapitata*

Concentration [mg a.s./L] (nominal)	**Control**	**0.0047**	**0.0094**	**0.019**	**0.038**	**0.075**	**0.150**
Concentration [mg a.s./L] (geometric mean measured)	**--**	**0.0016**	**0.0036**	**0.0044**	**0.0092**	**0.030**	**0.055**
Inhibition in 72 h (cell density) [%] #	--	-31	-0-68	26	71 *	95 *	99 *
Inhibition in 72 h (area under the growth curve) [%] #	--	-19	3.8	27	71 *	94 *	98 *
Inhibition in 72 h (growth rate) [%] #	--	-4.0	0.48	5.9	24 *	59 *	83 *
Inhibition in 72 h (yield) [%] #	--	-31	-0.68	26	72 *	96 *	99 *
Inhibition in 96 h (cell density) [%] #	--	-4.9	-3.5	7.5	55 *	93 *	100 *
Inhibition in 96 h (area under the growth curve) [%] #	--	-12	-1.5	15	61 *	94 *	99 *
Inhibition in 96 h (growth rate) [%] #	--	-0.74	-0.30	1.1	13 *	43 *	87 *
Inhibition in 96 h (yield) [%] #	--	-4.9	-3.5	7.5	55 *	93 *	100 *
Endpoints [mg metiram/L] (geometric mean measured)							
E_rC_{50} (72 h)	0.021 (95% confidence limits: 0.018 - 0.025)						
E_yC_{50} (72 h)	0.0061 (95% confidence limits: 0.0041 - 0.0091)						
E_rC_{50} (96 h)	0.033 (95% confidence limits: 0.031 - 0.035)						
E_yC_{50} (96 h)	0.0089 (95% confidence limits: 0.0070 - 0.011)						

\# Negative values indicate stimulated growth compared to the control.
* Statistically significant differences compared to the control (Dunnett's test, α = 0.05).

III. CONCLUSION

In a 96-hour algae test with *Pseudokirchneriella subcapitata*, the 96 h E_rC_{50} and E_yC_{50} values for metiram (tested as BAS 222 28 F) were determined to be 0.033 mg a.s./L and 0.0089 mg a.s./L, respectively, based on geometric mean measured concentrations. The 72 h E_rC_{50} and E_yC_{50} values were 0.021 mg a.s./L and 0.0061 mg a.s./L, respectively.

CA 8.2.6.2 Effects on growth of an additional algal species

Data already submitted during previous Annex I inclusion process (already evaluated on EU level):

The following algae toxicity study on the yellow-brown alga Corcontochrysis noctivaga performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.6.2/1* *Kubitza J., 2002h* *Effect of Metiram (applied as formulated product BAS 222 28 F) on the growth of Corcontochrysis noctivaga* *2002/1005280*
Guidelines:	*OECD 201*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 72 hour static toxicity laboratory study, the growth of yellow-brown alga *Corcontochrysis noctivaga* was investigated. The following nominal concentrations of metiram (tested as BAS 222 28 F) were applied: 0 (control), 0.002, 0.007, 0.021, 0.069, 0.206, 0.685 and 2.055 mg a.s./L, nominal. Assessment of growth was conducted daily. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and biomass based on number of cells.

The biological results are based on nominal concentrations. No morphological effects on algae could be observed up to and including 0.206 mg a.s./L. At 0.685 mg a.s. /L and 2.055 mg a.s. /L the cells appeared deformed and more pale than those in the control and lower test concentrations.

In a 72-hour algae test with *Corcontochrysis noctivaga,* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.232 mg a.s./L and the E_bC_{50} was 0.131 mg a.s./L (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2000-2; content of a.s. metiram (BAS 222 F, Reg. No. 250 284): 68.5% analyzed (nominal: 70%).

B. STUDY DESIGN

Test species: Unicellular fresh water alga, *Corcontochrysis noctivaga* (Kalina), SAG 5.83, Chrysophyta, Haptophyceae, Prymnesiaceae; stock obtained from "Sammlung von Algenkulturen" Göttingen, Germany.

Test design: Static system; test duration 72 hours; 7 test concentrations, each with 5 replicates plus a control with 10 replicates; daily assessments of growth.

Endpoints: Effects on growth rate and biomass.

Test concentrations: Control, 0.002, 0.007, 0.021, 0.069, 0.206, 0.685 and 2.055 mg metiram/L (nominal); corresponding to 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1 and 3 mg BAS 222 28 F/L.

Test conditions: Glass Erlenmeyer flasks; nutrient solution according to OECD 201, pH 8.0 at test initiation; initial cell densities 3 x 10^4 cells/mL; temperature 22 °C ± 2 °C; continuous light at about 8000 lux; continuous shaking.

Analytics: Analytical verification of test item concentrations was conducted using GC/MS method CP 402.

Statistics: Descriptive statistics, probit-analysis for determination of the EC-values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in the stock solution at test initiation. The measured value for metiram was 98.9%, confirming nominal data. Therefore, biological results are based on nominal concentrations of the test item.

Biological results: No morphological effects on algae could be observed up to and including 0.206 mg a.s./L. At 0.685 mg a.s. /L and 2.055 mg a.s. /L the cells appeared deformed and more pale than those in the control and lower test concentrations. The effects on algal growth are summarized in Table 8.2.6.2-1.

Table 8.2.6.2-1: Effects of metiram (tested as BAS 222 28 F) on the growth of *Corcontochrysis noctivaga*

Concentration BAS 222 28 F (nominal) [mg/L]	**Control**	**0.003**	**0.01**	**0.03**	**0.1**	**0.3**	**1**	**3**
Concentration metiram (nominal) [mg a.s./L]	**Control**	**0.002**	**0.007**	**0.021**	**0.069**	**0.206**	**0.685**	**2.055**
Inhibition in 72 h (growth rate) [%]	--	-1.8	-0.5	4.5	24.8	55.7	70.0	90.8
Inhibition in 72 h (biomass) [%]	--	-4.2	0.7	13.5	46.6	70.0	78.3	88.5
	Endpoints [mg a.s./L] (nominal)							
E_rC_{50} (0-72 h)	0.232 (95% confidence limits: 0.218 – 0.247)							
E_rC_{10} (0-72 h)	0.031 (95% confidence limits: 0.028 - 0.035)							
E_bC_{50} (0-72 h)	0.131 (95% confidence limits: 0.123 – 0.141)							
E_bC_{10} (0-72 h)	0.013 (95% confidence limits: 0.012 - 0.015)							

III. CONCLUSION

In a 72-hour algae test with *Corcontochrysis noctivaga,* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.232 mg a.s./L and the E_bC_{50} was 0.131 mg a.s./L (nominal).

The following algae toxicity study on the yellow-brown alga Ochromonas danica performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.6.2/2* *Kubitza J., 2002i* *Effect of Metiram (applied as formulated product BAS 222 28 F) on the growth of Ochromonas danica* *2002/1005281*
Guidelines:	*OECD 201*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 72-hour static toxicity laboratory study, the growth of yellow brown alga *Ochromonas danica* was investigated. The following nominal concentrations of metiram (tested as BAS 222 28 F) were applied: 0 (control), 0.002, 0.007, 0.021, 0.069, 0.206, 0.685 and 2.055 mg a.s./L. Assessment of growth was conducted daily. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and biomass based on number of cells.

The biological results are based on nominal concentrations. No morphological effects on algae could be observed at concentrations up to and including 0.069 mg a.s./L. At 0.206 mg a.s. /L about 50% and at the higher concentrations all cells appeared smaller and frayed.

In a 72-hour algae test with *Ochromonas danica,* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.210 mg a.s./L and the E_bC_{50} was 0.094 mg a.s./L (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2000-2; content of a.s. metiram (BAS 222 F, Reg. No. 250 284): 68.5% analyzed (nominal: 70%).

B. STUDY DESIGN

Test species: Unicellular fresh water alga, *Ochromonas danica* (Wyssotzki) Pringsheim, SAG 933-7, Chrysophyta, Chrysophyceae, Ochromonadaceae; stock obtained from "Sammlung von Algenkulturen" Göttingen, Germany.

Test design: Static system; test duration 72 hours; 7 test concentrations, each with 5 replicates plus a control with 10 replicates; daily assessment of growth.

Endpoints: Effects on growth rate and biomass.

Test concentrations: Control, 0.002, 0.007, 0.021, 0.069, 0.206, 0.685 and 2.055 mg metiram/L (nominal); corresponding to 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1 and 3 mg BAS 222 28 F/L.

Test conditions: Glass Erlenmeyer flasks; nutrient solution according to OECD 201, pH 8.0 at test initiation; initial cell densities 3 x 10^4 cells/mL; temperature 22 °C ± 1 °C; continuous light at about 8000 lux; continuous shaking.

Analytics: Analytical verification of test item concentrations was conducted using GC/MS method CP 402.

Statistics: Descriptive statistics, probit-analysis for determination of the EC-values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in the stock solution at test initiation. The measured value for metiram was 89.1%, confirming nominal data. Therefore, biological results are based on nominal concentrations of the test item.

Biological results: No morphological effects on algae could be observed at concentrations up to and including 0.069 mg a.s./L. At 0.206 mg a.s. /L about 50% and at higher concentrations all cells appeared smaller and frayed. The effects on algal growth are summarized in Table 8.2.6.2-2.

Table 8.2.6.2-2: Effects of metiram (tested as BAS 222 28 F) on the growth of the Chrysophyta alga *Ochromonas danica*

Concentration BAS 222 28 F (nominal) [mg/L]	**Control**	**0.003**	**0.01**	**0.03**	**0.1**	**0.3**	**1**	**3**
Concentration metiram (nominal) [mg a.s./L]	**Control**	**0.002**	**0.007**	**0.021**	**0.069**	**0.206**	**0.685**	**2.055**
Inhibition in 72 h (growth rate) [%]	--	-1.9	-1.8	1.1	10.8	45.2	92.0	100.0
Inhibition in 72 h (biomass) [%]	--	-5.5	-0.8	10.6	30.9	79.0	99.1	100.0
	Endpoints [mg a.s./L] (nominal)							
E_rC_{50} (0-72 h)	0.210 (95% confidence limits: 0.201 – 0.220)							
E_rC_{10} (0-72 h)	0.068 (95% confidence limits: 0.064 - 0.073							
E_bC_{50} (0-72 h)	0.094 (95% confidence limits: 0.089 – 0.098)							
E_bC_{10} (0-72 h)	0.027 (95% confidence limits: 0.025 - 0.029)							

III. CONCLUSION

In a 72-hour algae test with *Ochromonas danica,* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.210 mg a.s./L and the E_bC_{50} was 0.094 mg a.s./L (nominal).

The following toxicity study on the single-celled flagellate protist Euglena gracilis performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.6.2/3* *Kubitza J., 2002j* *Effect of Metiram (applied as formulated product BAS 222 28 F) on the growth of the alga Euglena gracilis* *2002/1005273*
Guidelines:	*OECD 201*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 72-hour static toxicity laboratory study, the growth of the single-celled flagellate protist *Euglena gracilis* was investigated. The following nominal concentrations of metiram (tested as BAS 222 28 F) were applied: 0 (control), 0.069, 0.206, 0.685, 2.055, 6.85, 20.55 and 68.50 mg a.s./L. Assessment of growth was conducted daily. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and biomass based on number of cells.

The biological results are based on nominal concentrations. No morphological effects on the *E. gracilis* could be observed at concentrations up to and including 0.206 mg a.s./L. At 0.685 mg a.s./L and 2.055 mg a.s./L a few, at 6.850 mg a.s./L and 20.55 mg a.s./L about 75% and at 68.5 mg a.s./L all cells appeared deformed

In a 72-hour toxicity test with *Euglena gracilis,* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 4.612 mg a.s./L and the E_bC_{50} was 1.793 mg a.s./L (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2000-2; content of a.s. metiram (BAS 222 F, Reg. No. 250 284): 68.5% analyzed (nominal: 70%).

B. STUDY DESIGN

Test species: Single-celled flagellate protist, *Euglena gracilis,* UTEX LB 884, Euglenophyta, Euglenophyceae, Euglenaceae; stock obtained from "The Culture Collection of Algae" at the University of Texas, Austin, USA.

Test design: Static system; test duration 72 hours; 7 test concentrations, each with 5 replicates plus a control with 10 replicates; daily assessment of growth.

Endpoints: Effects on growth rate and biomass.

Test concentrations: Control, 0.069, 0.206, 0.685, 2.055, 6.85, 20.55 and 68.50 mg metiram/L (nominal), corresponding to 0, 0.1, 0.3, 1, 3, 10, 30 and 100 mg BAS 222 28 F/L.

Test conditions: Glass Erlenmeyer flasks; nutrient solution according to OECD 201, pH 8.0 at test initiation; initial cell densities 3 x 10^3 cells/mL; temperature 22 °C ± 1 °C; continuous light at about 8000 lux; continuous shaking.

Analytics: Analytical verification of test item concentrations was conducted using GC/MS method CP 402.

Statistics: Descriptive statistics, probit-analysis for determination of the EC-values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in the stock solution at test initiation. The measured value for metiram was 104.9%, confirming the nominal data. Therefore, biological results are based on nominal concentrations of the test item.

Biological results: No morphological effects on the *E. gracilis* could be observed at concentrations up to and including 0.206 mg a.s./L. At 0.685 mg a.s./L and 2.055 mg a.s./L a few, at 6.850 mg a.s./L and 20.55 mg a.s./L about 75% and at 68.5 mg a.s./L all cells appeared deformed. The effects on growth of *E. gracilis* are summarized in Table 8.2.6.2-3.

Table 8.2.6.2-3: Effects of metiram (tested as BAS 222 28 F) on the growth of *Euglena gracilis*

Concentration BAS 222 28 F (nominal) [mg/L]	**Control**	**0.1**	**0.3**	**1**	**3**	**10**	**30**	**100**
Concentration metiram (nominal) [mg a.s./L]	**Control**	**0.069**	**0.206**	**0.685**	**2.055**	**6.85**	**20.55**	**68.50**
Inhibition in 72 h (growth rate) [%]	--	0.1	7.4	16.7	35.6	49.5	72.8	100.0
Inhibition in 72 h (biomass) [%]	--	5.6	17.8	30.0	54.0	72.4	82.9	100.0
	Endpoints [mg a.s./L] (nominal)							
E_rC_{50} (0-72 h)	4.612 (95% confidence limits: 4.301 – 4.945)							
E_rC_{10} (0-72 h)	0.436 (95% confidence limits: 0.391 – 0.486)							
E_bC_{50} (0-72 h)	1.793 (95% confidence limits: 1.665 – 1.931)							
E_bC_{10} (0-72 h)	0.125 (95% confidence limits: 0.110 - 0.142)							

III. CONCLUSION

In a 72-hour toxicity test with *Euglena gracilis,* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 4.612 mg a.s./L and the E_bC_{50} was 1.793 mg a.s./L (nominal).

The following algae toxicity study on the diatom Navicula pelliculosa performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.6.2/4* *Kubitza J., 2002k* *Effect of Metiram (applied as formulated product BAS 222 28 F) on the growth of Navicula pelliculosa* *2002/1005277*
Guidelines:	*OECD 201*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 72-hours static toxicity laboratory study, the growth of diatom *Navicula pelliculosa* was investigated. The following nominal concentrations of metiram (tested as BAS 222 28 F) were applied: 0 (control), 0.007, 0.014, 0.034, 0.089 and 0.206 mg a.s./L. Assessment of growth was conducted daily. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and biomass based on number of cells.

The biological results are based on nominal concentrations. No morphological effects on the algae could be observed at concentrations up to and including 0.089 mg metiram/L. At 0.206mg a.s./L, the cells had a tendency to be round.

In a 72-hour algae test with *Navicula pelliculosa,* the E_rC_{50} and the E_bC_{50} of metiram (applied as BAS 222 28 F) were both determined to be > 0.206 mg a.s./L (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2000-2; content of a.s. metiram (BAS 222 F, Reg. No. 250 284): 68.5% analyzed (nominal: 70%).

B. STUDY DESIGN

Test species: Freshwater algae (diatoms), *Navicula pelliculosa* (Breb.) Hilse, UTEX 661, Chrysophyta, Bacillariophyceae, Naviculeae; stock obtained from "The Culture Collection of Algae" at the University of Texas at Austin.

Test design: Static system; test duration 72 hours; 5 test concentrations, each with 5 replicates plus a control with 10 replicates; daily assessment of growth.

Endpoints: Effects on growth rate and biomass.

Test concentrations: Control, 0.007, 0.014, 0.034, 0.089 and 0.206 mg metiram/L (nominal); corresponding to 0, 0.01, 0.02, 0.05, 0.13 and 0.3 mg BAS 222 28 F/L.

Test conditions: Glass Erlenmeyer flasks; nutrient solution according to OECD 201; pH 8.0 at test initiation; initial cell densities 1 x 10^4 cells/mL; temperature 22 °C ± 1 °C; continuous light at about 8000 lux; continuous shaking.

Analytics: Analytical verification of test item concentrations was conducted using GC/MS method CP 402.

Statistics: Descriptive statistics, probit-analysis for determination of the EC-values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentration was conducted in the stock solution at test initiation. The measured value for metiram was 85.0%, confirming the nominal data. Therefore, biological results are based on nominal concentrations of the test item.

Biological results: No morphological effects on the algae could be observed at concentrations up to and including 0.089 mg metiram/L. At 0.206 mg a.s./L, the cells had a tendency to be round. The effects on algal growth are summarized in Table 8.2.6.2-4.

Table 8.2.6.2-4: Effects of metiram (tested as BAS 222 28 F) on the diatom alga *Navicula pelliculosa*

Concentration BAS 222 28 F (nominal) [mg/L]	**Control**	**0.01**	**0.02**	**0.05**	**0.13**	**0.3**
Concentration metiram (nominal) [mg a.s./L]	**Control**	**0.007**	**0.014**	**0.034**	**0.089**	**0.206**
Inhibition in 72 h (growth rate) [%]	--	2.3	7.5	10.3	15.2	18.9
Inhibition in 72 h (biomass) [%]	--	1.2	10.1	17.2	25.8	34.7
	Endpoints [mg a.s./L] (nominal)					
E_rC_{50} (0-72 h)	> 0.206 (extrapolated 4.413 (95% confidence limits: 2.213 – 8.800))					
E_rC_{10} (0-72 h)	0.039 (95% confidence limits: 0.032 – 0.047)					
E_bC_{50} (0-72 h)	> 0.206 (extrapolated 0.453 (95% confidence limits: 0.364 – 0.565))					
E_bC_{10} (0-72 h)	0.020 (95% confidence limits: 0.017 - 0.023)					

III. CONCLUSION

In a 72-hour algae test with *Navicula pelliculosa,* the E_rC_{50} and the E_bC_{50} of metiram (applied as BAS 222 28 F) were both determined to be > 0.206 mg a.s./L (nominal).

The following algae toxicity study on the blue-green alga Anabaena flos-aquae performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.6.2/5* *Kubitza J., 2002l* *Effect of Metiram (applied as formulated product BAS 222 28 F) on the growth of the blue-green alga Anabaena flos-aquae* *2002/1005276*
Guidelines:	*OECD 201*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 72-hours static toxicity laboratory study, the growth of blue-green alga *Anabaena flos-aquae* was investigated. The following nominal concentrations of metiram (tested as BAS 222 28 F) were applied: 0 (control), 0.002, 0.005, 0.014, 0.034, 0.096, 0.260 and 0.685 mg a.s./L. Assessment of growth was conducted daily. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and biomass based on number of cells.

The biological results are based on nominal concentrations. No morphological effects on the algae could be observed at concentrations up to and including 0.260 mg metiram/L. At 0.685 mg a.s./L, the cells appeared slightly smaller than those in the control did.

In a 72-hour algae test with *Anabaena flos-aquae,* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.189 mg a.s./L and the E_bC_{50} was 0.052 mg a.s./L (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item:	BAS 222 28 F, batch no. 2000-2; content of a.s. metiram (BAS 222 F, Reg. No. 250 284): 68.5% analyzed (nominal: 70%).

B. STUDY DESIGN

Test species:	Filamentous blue-green alga *Anabaena flos-aquae,* (Lyngbye) de Brèbisson, UTEX 1444, Cyanophyta, Cyanophyceae, Nostoceae; stock obtained from "The Culture Collection of Algae" at the University of Texas, Austin, USA.
Test design:	Static system; test duration 72 hours; 7 test concentrations, each with 5 replicates plus a control with 10 replicates; daily assessment of growth.
Endpoints:	Effects on growth rate and biomass.
Test concentrations:	Control, 0.002, 0.005, 0.014, 0.034, 0.096, 0.260 and 0.685 mg metiram/L (nominal); corresponding to 0, 0.003, 0.008, 0.02, 0.05, 0.14, 0.38 and 1 mg BAS 222 28 F/L.
Test conditions:	Glass Erlenmeyer flasks; nutrient solution according to OECD 201; pH 8.0 at test initiation; initial cell densities 1 x 10^4 cells/mL; temperature 24 °C ± 1 °C; continuous light at about 2300 lux; continuous shaking.
Analytics:	Analytical verification of test item concentrations was conducted using GC/MS method CP 402.
Statistics:	Descriptive statistics, probit-analysis for determination of the EC-values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentration was conducted in the stock solution at test initiation. The measured value for metiram was 89.3%, confirming the nominal data. Therefore, biological results are based on nominal concentrations of the test item.

Biological results: No morphological effects on the algae could be observed at concentrations up to and including 0.260 mg metiram/L. At 0.685 mg a.s./L, the cells appeared slightly smaller than those in the control did. The effects on algal growth are summarized in Table 8.2.6.2-5.

Table 8.2.6.2-5: Effects of metiram (tested as BAS 222 28 F) on the blue-green alga *Anabaena flos-aquae*

Concentration BAS 222 28 F (nominal) [mg/L]	**Control**	**0.003**	**0.008**	**0.02**	**0.05**	**0.14**	**0.38**	**1**
Concentration metiram (nominal) [mg a.s./L]	**Control**	**0.002**	**0.005**	**0.014**	**0.034**	**0.096**	**0.260**	**0.685**
Inhibition in 72 h (growth rate) [%]	--	-0.9	1.0	2.6	6.2	20.9	77.1	77.9
Inhibition in 72 h (biomass) [%]	--	-0.7	7.7	14.1	28.9	64.8	95.1	98.3
	Endpoints [mg a.s./L] (nominal)							
E_rC_{50} (0-72 h)	0.189 (95% confidence limits: 0.179 – 0.200)							
E_rC_{10} (0-72 h)	0.037 (95% confidence limits: 0.034 – 0.041)							
E_bC_{50} (0-72 h)	0.052 (95% confidence limits: 0.050 – 0.055)							
E_bC_{10} (0-72 h)	0.011 (95% confidence limits: 0.010 - 0.012)							

III. CONCLUSION

In a 72-hour algae test with *Anabaena flos-aquae,* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.189 mg a.s./L and the E_bC_{50} was 0.052 mg a.s./L (nominal).

The following algae toxicity study on the blue-green alga Anabaena cylindrica performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.6.2/6* *Kubitza J., 2002m* *Effect of Metiram (applied as formulated product BAS 222 28 F) on the growth of Anabaena cylindrica* *2002/1005283*
Guidelines:	*OECD 201*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a 72-hours static toxicity laboratory study, the growth of blue-green alga *Anabaena cylindrica* was investigated. The following nominal concentrations of metiram (tested as BAS 222 28 F) were applied: 0 (control), 0.137, 0.226, 0.37, 0.617, 1.028 and 1.644 mg a.s./L. Assessment of growth was conducted daily. The percentage growth inhibition, relative to the control, was calculated for each test concentration based upon mean growth rates and biomass based on number of cells.

The biological results are based on nominal concentrations. No morphological effects on the algae could be observed at the lowest test concentration. At 0.226 mg a.s./L and 0.370 mg a.s./L a few and at 0.617 mg a.s./L and higher more or less all cells appeared frayed and translucent.

In a 72-hour algae test with *Anabaena cylindrica,* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.360 mg a.s./L and the E_bC_{50} was 0.264 mg a.s./L (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2000-2; content of a.s. metiram (BAS 222 F, Reg. No. 250 284): 68.5% analyzed (nominal: 70%).

B. STUDY DESIGN

Test species: Filamentous blue-green alga *Anabaena cylindrica* Lemmermann, SAG 1403-2, Cyanophyta, Cyanophyceae, Nostoceae; stock obtained from "Sammlung von Algenkulturen" Göttingen, Germany.

Test design: Static system; test duration 72 hours; 6 test concentrations, each with 5 replicates plus a control with 10 replicates; daily assessment of growth.

Endpoints: Effects on growth rate and biomass.

Test concentrations: Control, 0.137, 0.226, 0.37, 0.617, 1.028 and 1.644 mg metiram/L (nominal); corresponding to 0, 0.2, 0.33, 0.54, 0.9, 1.5 and 2.4 mg BAS 222 28 F/L.

Test conditions: Glass Erlenmeyer flasks; nutrient solution according to OECD 201, pH 8.0 at test initiation; initial cell densities 3 x 10^4 cells/mL; temperature 24 °C ± 2 °C; continuous light at about 2300 lux; continuous shaking.

Analytics: Analytical verification of test item concentrations was conducted using GC/MS method CP 402.

Statistics: Descriptive statistics, probit-analysis for determination of the EC-values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentration was conducted in the stock solution at test initiation. The measured value for metiram was 108.8%, confirming nominal data. Therefore, biological results are based on nominal concentrations of the test item.

Biological results: No morphological effects on the algae could be observed at the lowest test concentration. At 0.226 mg a.s./L and 0.370 mg a.s./L a few and at 0.617 mg a.s./L and higher more or less all cells appeared frayed and translucent. The effects on algal growth are summarized in Table 8.2.6.2-6.

Table 8.2.6.2-6: Effect of metiram (tested as BAS 222 28 F) on the growth of the filamentous blue-green alga *Anabaena cylindrica*

Concentration BAS 222 28 F (nominal) [mg/L]	**Control**	**0.2**	**0.33**	**0.54**	**0.9**	**1.5**	**2.4**
Concentration metiram (nominal) [mg a.s./L]	**Control**	**0.137**	**0.226**	**0.37**	**0.617**	**1.028**	**1.644**
Inhibition in 72 h (growth rate) [%]	--	0.1	15.6	71.2	79.3	94.1	100
Inhibition in 72 h (biomass) [%]	--	-1.9	40.1	88.3	93.4	100	100
	Endpoints [mg a.s./L] (nominal)						
E_rC_{50} (0-72 h)	0.360 (95% confidence limits: 0.351 – 0.369)						
E_rC_{10} (0-72 h)	0.180 (95% confidence limits: 0.173 - 0.188)						
E_bC_{50} (0-72 h)	0.264 (95% confidence limits: 0.259 – 0.270)						
E_bC_{10} (0-72 h)	0.159 (95% confidence limits: 0.154 - 0.164)						

III. CONCLUSION

In a 72-hour algae test with *Anabaena cylindrica,* the E_rC_{50} of metiram (applied as BAS 222 28 F) was determined to be 0.360 mg a.s./L and the E_bC_{50} was 0.264 mg a.s./L (nominal).

New supplemental data (not evaluated previously on EU level):

The following alga study on the freshwater diatom Navicula pelliculosa performed with metiram (tested as BAS 222 28 F) is not required for registration in the EU and it has not been evaluated previously on EU level. The study was conducted due to U.S. data requirements and it is provided for completeness.
In accordance to the EFSA Aquatic Guidance Document (EFSA, 2013) and OECD guideline 201 (2011) the 72 h endpoints obtained in the 96 h study are considered as relevant endpoint for the aquatic risk assessment, therefore both the 72 h and 96 h endpoints are reported in the study summary below.

Report:	CA 8.2.6.2/7 Minderhout T. et al., 2008a Polyram (BAS 222 28 F): A 96-hour toxicity test with the freshwater diatom (Navicula pelliculosa) 2008/7015288
Guidelines:	OECD 201, EPA 850.5400, EEC 92/69 C 3
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

In a 96-hour static acute toxicity laboratory study, the effect of metiram (tested as BAS 222 28 F) on the growth of the freshwater diatom *Navicula pelliculosa* was investigated. The following nominal concentrations were applied: 0.0093, 0.016, 0.026, 0.043, 0.072 and 0.120 mg metiram/L (corresponding to geometric mean measured concentrations of 0.0036, 0.0064, 0.0069, 0.021, 0.046 and 0.071 mg a.s./L). Additionally, a dilution water control was set up. Assessment of growth was conducted 24 h, 48 h, 72 h and 96 h after test initiation.

The biological results are based on geometric mean measured concentrations. After 96 hours of exposure, no morphological effects on algae were observed at concentrations up to and including the highest test item concentration of 0.071 mg a.s./L. After 72 hours of exposure, cell density, biomass, growth rate and yield were statistically significantly reduced at all test item concentrations. After 96 hours of exposure, growth rate was statistically significantly affected at the four highest test item concentrations, while cell density, biomass and yield were statistically significantly reduced at all test item concentrations.

In a 96-hour algae test with *Navicula pelliculosa*, the 96 h E_rC_{50} and E_yC_{50} values for metiram (tested as BAS 222 28 F) were determined to be 0.015 mg a.s./L and 0.0069 mg a.s./L, respectively, based on geometric mean measured concentrations. The 72 h E_rC_{50} and E_yC_{50} values were 0.011 mg a.s./L. and 0.0032 mg a.s./L, respectively (geometric mean measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 1653; content of a.s.: metiram (BAS 222 F; Reg. No 250284): 72.5% (nominal: 70.0%); density: 1.740 g/cm^3.

B. STUDY DESIGN

Test species: Freshwater diatom, *Navicula pelliculosa*; stock obtained from the University of Texas at Austin, USA.

Test design: Static system; test duration 96 hours; 6 test item concentrations, each with 4 replicates per treatment and control; daily assessment of growth.

Endpoints: EC_{10} and EC_{50} with respect to cell density, biomass (area under the growth curve), growth rate and yield after exposure over 72 and 96 hours.

Test concentrations: Control, 0.0093, 0.016, 0.026, 0.043, 0.072 and 0.120 mg a.s./L (nominal), corresponding to geometric mean measured concentrations of < limit of quantification (LOQ), 0.0036, 0.0064, 0.0069, 0.021, 0.046 and 0.071 mg a.s./L.

Test conditions: 250 mL Erlenmeyer flasks; test volume 100 mL; freshwater algal medium with silica constituents; pH 7.4 - 7.5 at test initiation and pH 7.5 - 8.0 at test termination; temperature: 23.8 °C - 24.3 °C; initial cell densities 1 x 10^4 cells/mL; continuous light at 3880 - 4450 lux; continuous shaking at 100 rpm.

Analytics: Analytical verification of test item concentrations was conducted using a GC-method with MS detection.

Statistics: Descriptive statistics; non-linear regression or linear interpolation for determination of EC_x values for cell density, biomass, growth rate and yield; Dunnett's test ($\alpha = 0.05$) for determination of NOEC values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at the beginning and at the end of the test. The mean measured values of metiram ranged from 96.8% to 125.1% of nominal at test initiation and from 6.6% to 32.9% of nominal at test termination. The following biological results are based on geometric mean concentrations.

Biological results: After 96 hours of exposure, no morphological effects on algae were observed at concentrations up to and including the highest test item concentration of 0.071 mg a.s./L. After 72 hours of exposure, cell density, biomass, growth rate and yield were statistically significantly reduced at all test item concentrations (Dunnett's test, $p < 0.05$). After 96 hours of exposure, growth rate was statistically significantly affected at the four highest test item concentrations, while cell density, biomass and yield were statistically significantly reduced at all test item concentrations (Dunnett's test, $p < 0.05$). The effects on algal growth are summarized in Table 8.2.6.2-7.

Table 8.2.6.2-7: Effect of metiram (tested as BAS 222 28 F) on the growth of the freshwater diatom *Navicula pelliculosa*

Concentration [mg a.s./L] (nominal)	**0.0093**	**0.016**	**0.026**	**0.043**	**0.072**	**0.120**
Concentration [mg a.s./L] (geometric mean measured)	**0.0036**	**0.0064**	**0.0069**	**0.021**	**0.046**	**0.071**
Inhibition in 72 h (cell density) [%]	52 *	56 *	86 *	96 *	97 *	97 *
Inhibition in 72 h (biomass) [%]	54 *	63 *	87 *	97 *	97 *	99 *
Inhibition in 72 h (growth rate) [%]	17 *	19 *	45 *	75 *	78 *	81 *
Inhibition in 72 h (yield) [%]	53 *	56 *	87 *	98 *	98 *	98 *
Inhibition in 96 h (cell density) [%]	17 *	22 *	51 *	96 *	98 *	98 *
Inhibition in 96 h (biomass) [%]	43 *	49 *	77 *	97 *	98 *	99 *
Inhibition in 96 h (growth rate) [%]	4.1	5.3	16 *	72 *	85 *	89 *
Inhibition in 96 h (yield) [%]	17 *	22 *	52 *	97 *	99 *	99 *
	Endpoints [mg a.s./L] (geometric mean measured) $					
EC_{50} (72 h)	0.003 (95% confidence limits: 0.0018 - 0.0049)					
E_bC_{50} (72 h)	0.0027 (95% confidence limits: 0.0018 - 0.0041)					
E_rC_{50} (72 h)	0.011 (95% confidence limits: 0.0077 - 0.017)					
E_yC_{50} (72 h)	0.0032 (95% confidence limits: 0.0021 - 0.0049)					
EC_{50} (96 h) #	0.0069 (95% confidence limits: 0.0067 - 0.010)					
E_bC_{50} (96 h) #	0.0064 (95% confidence limits: 0.0018 - 0.0065)					
E_rC_{50} (96 h)	0.015 (95% confidence limits: 0.011 - 0.020)					
E_yC_{50} (96 h) #	0.0069 (95% confidence limits: 0.0067 - 0.010)					

* Statistically significant differences compared to the control (Dunnett's test, $p < 0.05$).
$ EC_{50} values and 95% confidence limits were calculated using non-linear regression, unless indicated differently.
\# The EC_{50} and corresponding 95% confidence limits were calculated using linear interpolation.

III. CONCLUSION

In a 96-hour algae test with *Navicula pelliculosa*, the 96 h E_rC_{50} and E_yC_{50} values for metiram (tested as BAS 222 28 F) were determined to be 0.015 mg a.s./L and 0.0069 mg a.s./L, respectively, based on geometric mean measured concentrations. The 72 h E_rC_{50} and E_yC_{50} values were 0.011 mg a.s./L. and 0.0032 mg a.s./L, respectively (geometric mean measured).

The following alga study on the freshwater alga Anabaena flos-aquae performed with metiram (tested as BAS 222 28 F) is not required for registration in the EU and it has not been evaluated previously on EU level. The study was conducted due to U.S. data requirements and it is provided for completeness.
In accordance to the EFSA Aquatic Guidance Document (EFSA, 2013) and OECD guideline 201 (2011) the 72 h endpoints obtained in the 96 h study are considered as relevant endpoint for the aquatic risk assessment, therefore both the 72 h and 96 h endpoints are reported in the study summary below.

Report:	CA 8.2.6.2/8 Minderhout T. et al., 2008b Polyram (BAS 222 28 F): A 96-hour toxicity test with the freshwater alga (Anabaena flos-aquae) 2008/7015287
Guidelines:	OECD 201, EPA 850.5400, EEC 92/69 C 3
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

In a 96-hour static acute toxicity laboratory study, the effect of metiram (tested as BAS 222 28 F) on the growth of the freshwater blue-green alga *Anabaena flos-aquae* was investigated. The following nominal concentrations were applied: 0.039, 0.065, 0.108, 0.180 and 0.300 mg metiram/L (corresponding to geometric mean measured concentrations of 0.013, 0.024, 0.039, 0.092 and 0.153 mg a.s./L). Additionally, a dilution water control was set up. Assessment of growth was conducted 24 h, 48 h, 72 h and 96 h after test initiation.

The biological results are based on geometric mean measured concentrations. After 96 hours of exposure, no morphological effects on algae were observed at concentrations up to and including the highest test item concentration of 0.153 mg a.s./L. After 72 hours of exposure, cell density, biomass and yield were statistically significantly reduced at all test item concentrations compared to the control. Statistically significant inhibition of growth rate compared to the control was observed at the four highest test item after 72 hours. After 96 hours of exposure, cell density and yield were statistically significantly affected at the two highest test item concentrations, while biomass was statistically significantly reduced at the four highest test item concentrations and growth rate was significantly affected at the three highest tested concentrations.

In a 96-hour algae test with *Anabaena flos-aquae*, the 96 h E_rC_{50} and E_yC_{50} values for metiram (tested as BAS 222 28 F) were determined to be 0.097 mg a.s./L and 0.031 mg a.s./L, respectively, based on geometric mean measured concentrations. The 72 h E_rC_{50} and E_yC_{50} values were 0.061 mg a.s./L and 0.012 mg a.s./L, respectively (geometric mean measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 1653; content of a.s.: metiram (BAS 222 F; Reg. No 250284): 72.5% (nominal: 70.0%); density: 1.740 g/cm^3.

B. STUDY DESIGN

Test species: Freshwater blue-green alga, *Anabaena flos-aquae*; specification: UTCC 67; in-house cultures; stock obtained from "University of Toronto", Canada.

Test design: Static system; test duration 96 hours; 5 test item concentrations plus a control, each with 3 replicates per treatment and control group; daily assessment of growth.

Endpoints: EC_{50} with respect to cell density, biomass (area under the growth curve), growth rate and yield after exposure over 72 and 96 hours.

Test concentrations: Control, 0.039, 0.065, 0.108, 0.180 and 0.300 mg a.s./L (nominal), corresponding to geometric mean measured concentrations of < limit of quantification (LOQ), 0.013, 0.024, 0.039, 0.092 and 0.153 mg a.s./L.

Test conditions: 250 mL Erlenmeyer flasks; test volume 100 mL; freshwater algal medium; pH 7.6 - 7.8 at test initiation and pH 7.7 - 7.8 at test termination; temperature: 23.5 °C - 24.1 °C; initial cell densities 1 x 10^4 cells/mL; continuous light; light intensity 2010 - 2300 lux; continuous shaking at 100 rpm.

Analytics: Analytical verification of test item concentrations was conducted using a GC-method with MS detection.

Statistics: Descriptive statistics; non-linear regression or linear interpolation for determination of EC_x values for cell density, biomass, growth rate and yield; Dunnett's test ($\alpha = 0.05$) for determination of NOEC values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at the beginning and at the end of the test. The mean measured values of metiram ranged from 74.6% to 107.2% of nominal at test initiation and from 14.4% to 24.6% of nominal at test termination. The following biological results are based on geometric mean measured concentrations.

Biological results: After 96 hours of exposure, no morphological effects on algae were observed at concentrations up to and including the highest test item concentration of 0.153 mg a.s./L. After 72 hours of exposure, cell density, biomass and yield were statistically significantly reduced at all test item concentrations compared to the control (Dunnett's test, $p < 0.05$). Statistically significant inhibition of growth rate compared to the control was observed at the four highest test item concentrations after 72 hours (Dunnett's test, $p < 0.05$). After 96 hours of exposure, cell density and yield were statistically significantly affected at the two highest test item concentrations, while biomass was statistically significantly reduced at the four highest test item concentrations and growth rate was significantly affected at the three highest tested concentrations (Dunnett's test, $p < 0.05$). The effects on algal growth are summarized in Table 8.2.6.2-8.

Table 8.2.6.2-8: Effect of metiram (tested as BAS 222 28 F) on the growth of the freshwater blue-green alga *Anabaena flos-aquae*

Concentration [mg a.s./L] (nominal)	0.039	0.065	0.108	0.180	0.300
Concentration [mg a.s./L] (geometric mean measured)	**0.013**	**0.024**	**0.039**	**0.092**	**0.153**
Inhibition in 72 h (cell density) [%]	54 *	74 *	80 *	93 *	100 *
Inhibition in 72 h (biomass) [%]	51 *	65 *	82 *	86 *	100 *
Inhibition in 72 h (growth rate) [%]	16	31 *	38 *	67 *	100 *
Inhibition in 72 h (yield) [%]	54 *	75 *	81 *	94 *	100 *
Inhibition in 96 h (cell density) [%] #	-33	31	65	88 *	100 *
Inhibition in 96 h (biomass) [%]	18	54 *	75 *	89 *	100 *
Inhibition in 96 h (growth rate) [%] #	-4.4	7.1	21 *	44 *	100 *
Inhibition in 96 h (yield) [%]	-33	31	66	88 *	100 *
Endpoints [mg a.s./L] (geometric mean measured)					
EC_{50} (72 h) $	0.012 (95% confidence limits: 0.006 - 0.024)				
E_bC_{50} (72 h) $	0.013 (95% confidence limits: 0.0069 - 0.026)				
E_rC_{50} (72 h) +	0.061 (95% confidence limits: 0.0073 - 0.135)				
E_yC_{50} (72 h) $	0.012 (95% confidence limits: 0.0059 - 0.023)				
EC_{50} (96 h) $	0.031 (95% confidence limits: 0.019 - 0.052)				
E_bC_{50} (96 h) $	0.023 (95% confidence limits: 0.016 - 0.033)				
E_rC_{50} (96 h)	0.097 (95% confidence limits: 0.058 - 0.120)				
E_yC_{50} (96 h)	0.031 (95% confidence limits: 0.019 - 0.051)				

Negative values indicate stimulated growth compared to the control.
* Statistically significant differences compared to the control (Dunnett's test, $p < 0.05$).
+ The EC_{50} and 95% confidence limits were calculated using linear interpolation.
$ EC_{50} values and 95% confidence limits were calculated using non-linear regression.

III. CONCLUSION

In a 96-hour algae test with *Anabaena flos-aquae*, the 96 h E_rC_{50} and E_yC_{50} values for metiram (tested as BAS 222 28 F) were determined to be 0.097 mg a.s./L and 0.031 mg a.s./L, respectively, based on geometric mean measured concentrations. The 72 h E_rC_{50} and E_yC_{50} values were 0.061 mg a.s./L and 0.012 mg a.s./L, respectively (geometric mean measured).

The following alga study on the marine diatom Skeletonema costatum performed with metiram (tested as BAS 222 28 F) is not required for registration in the EU and it has not been evaluated previously on EU level. The study was conducted due to U.S. data requirements and it is provided for completeness.
In accordance to the EFSA Aquatic Guidance Document (EFSA, 2013) and OECD guideline 201 (2011) the 72 h endpoints obtained in the 96 h study are considered as relevant endpoint for the aquatic risk assessment, therefore both the 72 h and 96 h endpoints are reported in the study summary below.

Report: CA 8.2.6.2/9
Minderhout T. et al., 2008c
Polyram (BAS 222 28 F): A 96-hour toxicity test with the marine diatom (Skeletonema costatum)
2008/7018037
Guidelines: OECD 201, EPA 850.5400, EEC 92/69 C 3
GLP: yes
(certified by United States Environmental Protection Agency)

Executive Summary

In a 96-hour static acute toxicity laboratory study, the effect of metiram (tested as BAS 222 28 F) on the growth of the marine diatom *Skeletonema costatum* was investigated. The following nominal concentrations were applied: 0.0093, 0.016, 0.026, 0.043, 0.072, 0.120 mg metiram/L (corresponding to geometric mean measured concentrations of 0.0053, 0.0031, 0.013, 0.036, 0.055 and 0.090 mg a.s./L. Additionally, a dilution water control was set up. Assessment of growth was conducted 24 h, 48 h, 72 h and 96 h after test initiation.

The biological results are based on geometric mean measured concentrations. After 96 hours of exposure, no morphological effects on algae were observed at concentrations up to and including the highest test item concentration of 0.090 mg a.s./L. After 72 hours of exposure, cell density, biomass and yield were statistically significantly reduced at all test item concentrations compared to the control. Statistically significant inhibition of growth rate compared to the control was observed at the four highest test item concentrations after 72 hours. After 96 hours of exposure, cell density, growth rate and yield were statistically significantly affected at the four highest test item concentrations, while biomass was statistically significantly reduced at all test item concentrations.

In a 96-hour algae test with *Skeletonema costatum*, the 96 h E_rC_{50} and E_yC_{50} values for metiram (tested as BAS 222 28 F) were determined to be 0.0085 mg a.s./L and 0.0071 mg a.s./L, respectively, based on geometric mean measured concentrations. The 72 h E_rC_{50} and E_yC_{50} values were 0.007 mg a.s./L and 0.0062 mg a.s./L, respectively (geometric mean measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 1653; content of a.s.: metiram (BAS 222 F; Reg. No 250284): 72.5% (nominal: 70.0%); density: 1.740 g/cm^3.

B. STUDY DESIGN

Test species: Marine diatom, *Skeletonema costatum*; stock obtained from "Provasoli - Guillard National Center for Culture of Marine Phytoplankton (CCMP)".

Test design: Static system; test duration 96 hours; 6 test item concentrations, each with 3 replicates per treatment and control; daily assessment of growth.

Endpoints: EC_{10} and EC_{50} with respect to cell density, biomass (area under the growth curve), growth rate and yield after exposure over 72 and 96 hours.

Test concentrations: Control, 0.0093, 0.016, 0.026, 0.043, 0.072, 0.120 mg a.s./L (nominal), corresponding to geometric mean measured concentrations of < limit of quantification (LOQ), 0.0053, 0.0031, 0.013, 0.036, 0.055 and 0.090 mg a.s./L.

Test conditions: 250 mL Erlenmeyer flasks; test volume 100 mL; saltwater algal medium; pH 8.0 - 8.1 at test initiation and pH 7.9 - 8.2 at test termination; temperature: 19.1 °C - 19.8 °C; initial cell densities 1 x 10^4 cells/mL; photoperiod: 16 hours light; light intensity 3940 - 4620 lux; continuous shaking at 100 rpm.

Analytics: Analytical verification of test item concentrations was conducted using a GC-method with MS detection.

Statistics: Descriptive statistics; non-linear regression or linear interpolation for determination of EC_x values for cell density, biomass, growth rate and yield; Dunnett's test ($\alpha = 0.05$) for determination of NOEC values.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in each concentration at the beginning and at the end of the test. The mean measured values of metiram ranged from 78.1% to 128.1% of nominal at test initiation and from 4.8% to 53.5% of nominal at test termination. Percent recoveries in the second lowest test item treatment group, resulted in a geometric mean measured concentration that was lower than the measured concentration for the lowest treatment group (geometric mean of 0.0053 mg a.s./L in the lowest treatment group compared to 0.0031 mg a.s./L in the second lowest treatment group). The following biological results are based on geometric mean measured concentrations.

Biological results: Because recoveries of the test item in the second lowest test item treatment group were lower than in the lowest treatment group, the second lowest treatment group of 0.0031 mg a.s./L was excluded from statistical analysis of the biological results.
After 96 hours of exposure, no morphological effects on algae were observed at concentrations up to and including the highest test item concentration of 0.090 mg a.s./L. After 72 hours of exposure, cell density, biomass and yield were statistically significantly reduced at all test item concentrations compared to the control (Dunnett's test, $p < 0.05$). Statistically significant inhibition of growth rate compared to the control was observed at the four highest test item concentrations after 72 hours (Dunnett's test, $p < 0.05$). After 96 hours of exposure, cell density, growth rate and yield were statistically significantly affected at the four highest test item concentrations, while biomass was statistically significantly reduced at all test item concentrations (Dunnett's test, $p < 0.05$). The effects on algal growth are summarized in Table 8.2.6.2-9.

Table 8.2.6.2-9: Effect of metiram (tested as BAS 222 28 F) on the growth of the marine diatom *Skeletonema costatum*

Concentration [mg a.s./L] (nominal)	**0.0093**	**0.016**	**0.026**	**0.043**	**0.072**	**0.120**
Concentration [mg a.s./L] (geometric mean measured)	**0.0053**	**0.0031 #**	**0.013**	**0.036**	**0.055**	**0.090**
Inhibition in 72 h (cell density) [%]	19 *	70	91 *	93 *	91 *	94 *
Inhibition in 72 h (biomass) [%]	25 *	77	98 *	92 *	96 *	98 *
Inhibition in 72 h (growth rate) [%]	8.7	53	90 *	99 *	94 *	100 *
Inhibition in 72 h (yield) [%]	21 *	77	97 *	100 *	98 *	100 *
Inhibition in 96 h (cell density) [%]	9.8	81	97 *	98 *	98 *	98 *
Inhibition in 96 h (biomass) [%]	17 *	80	99 *	98 *	99 *	100 *
Inhibition in 96 h (growth rate) [%]	2.7	48	96 *	100 *	100 *	100 *
Inhibition in 96 h (yield) [%]	10	84	100 *	100 *	100 *	100 *
Endpoints [mg a.s./L] (geometric mean measured) #						
EC_{50} (72 h)	0.0059 (95% confidence limits: 0.0026 - 0.014)					
E_bC_{50} (72 h)	0.0053 (95% confidence limits: 0.0023 - 0.013)					
E_rC_{50} (72 h)	0.007 (95% confidence limits: 0.0028 - 0.018)					
E_yC_{50} (72 h)	0.0062 (95% confidence limits: 0.0018 - 0.022)					
EC_{50} (96 h)	0.0063 (95% confidence limits: 0.003 - 0.013)					
E_bC_{50} (96 h)	0.0062 (95% confidence limits: 0.0029 - 0.013)					
E_rC_{50} (96 h)	0.0085 (95% confidence limits: 0.0056 - 0.013)					
E_yC_{50} (96 h)	0.0071 (95% confidence limits: 0.0064 - 0.008)					

\# Results of the second lowest treatment group were excluded from statistical analysis.
* Statistically significant differences compared to the control (Dunnett's test, $p < 0.05$).

III. CONCLUSION

In a 96-hour algae test with *Skeletonema costatum*, the 96 h E_rC_{50} and E_yC_{50} values for metiram (tested as BAS 222 28 F) were determined to be 0.0085 mg a.s./L and 0.0071 mg a.s./L, respectively, based on geometric mean measured concentrations. The 72 h E_rC_{50} and E_yC_{50} values were 0.007 mg a.s./L and 0.0062 mg a.s./L, respectively (geometric mean measured).

The following alga study on the marine diatom Skeletonema costatum performed with metiram (tested as BAS 222 28 F) is not required for registration in the EU and it has not been evaluated previously on EU level. The study was conducted due to U.S. data requirements and it is provided for completeness.
In accordance to the EFSA Aquatic Guidance Document (EFSA, 2013) and OECD guideline 201 (2011) the 72 h endpoints obtained in the 96 h study are considered as relevant endpoint for the aquatic risk assessment, therefore both the 72 h and 96 h endpoints are reported in the study summary below.

Report:	CA 8.2.6.2/10 Dobbins L. et al., 2013a Polyram (BAS 222 28F): A 96-hour toxicity test with the marine diatom (Skeletonema costatum) 2013/7000406
Guidelines:	OECD 201
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

In a 96-h static toxicity laboratory study, the effect of metiram (tested as BAS 222 28 F) on growth of the marine diatom *Skeletonema costatum* was investigated. The following nominal concentrations were applied: 0 (control) 0.0015, 0.0044, 0.013, 0.040 and 0.120 mg metiram/L. Assessment of growth was conducted 24 h, 48 h, 72 h and 96 h after test initiation.

The biological results are based on nominal concentrations. No morphological effects on algae were observed in the control and at any test item treatment. After 72 and 96 hours of exposure, statistically significant effects on all measured parameters compared to the control were observed at the three highest tested concentrations.

In a 96-h algae test with *Skeletonema costatum*, the 96 h E_rC_{50} and the E_yC_{50} values of metiram (tested as BAS 222 28 F) were determined to be 0.0259 mg a.s./L and 0.0237 mg a.s./L, respectively, based on nominal concentrations. The 72 h E_rC_{50} and E_yC_{50} values were 0.0252 mg a.s./L and 0.0201 mg a.s./L, respectively (nominal).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 80449975L0; content of a.s.: metiram (BAS 222 F, Reg. no. 250284): 72.3% (nominal: 70.0%); density: 1.740 g/cm^3.

B. STUDY DESIGN

Test species: Marine diatom, *Skeletonema costatum*, in-house culture; stock originally obtained from the "Provasoli - Guillard National Center for Culture of Marine Phytoplankton".

Test design: Static system (96 hours); 5 test concentrations plus control with 4 replicates for each test item concentration and 6 replicates for the control; daily assessment of growth.

Endpoints: EC_{50} with respect to growth rate and yield after exposure over 96 hours.

Test concentrations: Control, 0.0015, 0.0044, 0.013, 0.040 and 0.120 mg metiram/L (nominal).

Test conditions: 250 mL flasks; test volume: 100 mL; saltwater algal medium; pH 8.0 at test initiation and pH 7.9 - 9.0 at test termination; temperature: 18.5 °C - 20.6 °C; initial cell densities: 1 x 10^4 cells/mL; photoperiod: 14 hours light : 10 hours dark, light intensity: 4100 lux - 4670 lux, continuous shaking at 100 rpm.

Analytics: Analytical verification of test item concentrations was conducted using a GC-method with MS detection.

Statistics: Descriptive statistics, linear interpolation for determination of EC_{50} values, Dunnett's test ($\alpha = 0.05$) for determination of the NOEC value.

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted in the four highest test concentrations at the beginning and at the end of the test. The lowest test item concentration was not measured due to this concentration being below the level of quantitation (LOQ = 3.34 µg/L). At test initiation, measured concentrations of metiram ranged from 80% to 151% of nominal concentrations. At test termination, recoveries of metiram BAS 222 28 F were between < LOD (limit of detection = 1.94 µg/L) and 30% of nominal. The concentration decrease in the treatment groups over the 96-hour exposure period was attributed to breakdown of the test substance by hydrolysis. Because, analytical recoveries in the samples of the lowest test item concentration could not be determined, the following biological results are based on nominal concentrations.

Biological results: No morphological effects on algae were observed in the control and in any test item treatment. After 72 and 96 hours of exposure, statistically significant effects on all measured parameters compared to the control were observed at the three highest tested concentrations (Dunnett's test, $\alpha = 0.05$). The effects on algal growth and yield are summarized in Table 8.2.6.2-10.

Table 8.2.6.2-10: Effect of metiram (tested as BAS 222 28 F) on the growth of the marine diatom *Skeletonema costatum*

Concentration [mg a.s./L] (nominal)	0.0015	0.0044	0.013	0.040	0.120
Inhibition in 72 h (cell density) [%]	4	1	32 *	100 *	100 *
Inhibition in 72 h (area under the growth curve) [%] [#]	9	-2	36 *	100 *	100 *
Inhibition in 72 h (growth rate) [%]	1	0	9 *	100 *	100 *
Inhibition in 72 h (yield) [%]	4	1	32 *	100 *	100 *
Inhibition in 96 h (cell density) [%]	9	2	17 *	100 *	100 *
Inhibition in 96 h (area under the growth curve) [%]	8	0	28 *	100 *	100 *
Inhibition in 96 h (growth rate) [%]	2	1	4 *	100 *	100 *
Inhibition in 96 h (yield) [%]	9	2	17 *	100 *	100 *
Endpoints [mg metiram/L] (nominal)					
EC_{50} (72 h)	0.0203 (95% confidence limits: 0.0169 - 0.0229)				
E_bC_{50} (72 h)	0.0191 (95% confidence limits: 0.0157 - 0.0220)				
E_rC_{50} (72 h)	0.0252 (95% confidence limits: 0.0246 - 0.0257)				
E_yC_{50} (72 h)	0.0201 (95% confidence limits: 0.0167 - 0.0228)				
EC_{50} (96 h)	0.0238 (95% confidence limits: 0.0202 - 0.0259)				
E_bC_{50} (96 h)	0.0213 (95% confidence limits: 0.0193 - 0.0232)				
E_rC_{50} (96 h)	0.0259 (95% confidence limits: 0.0254 - 0.0264)				
E_yC_{50} (96 h)	0.0237 (95% confidence limits: 0.0208 - 0.0260)				

[#] Negative values indicate stimulated growth compared to the control.
* Statistically significant differences compared to the control (Dunnett's test, $\alpha = 0.05$).

III. CONCLUSION

In a 96-h algae test with *Skeletonema costatum*, the 96 h E_rC_{50} and the E_yC_{50} values of metiram (tested as BAS 222 28 F) were determined to be 0.0259 mg a.s./L and 0.0237 mg a.s./L, respectively, based on nominal concentrations. The 72 h E_rC_{50} and E_yC_{50} values were 0.0252 mg a.s./L and 0.0201 mg a.s./L, respectively (nominal).

CA 8.2.7 Effects on aquatic macrophytes

New supplemental data (not evaluated previously on EU level):

The following alga study on the duckweed Lemna gibba performed with metiram (tested as BAS 222 29 F) is not required for registration in the EU and it has not been evaluated previously on EU level. The study was conducted due to U.S. data requirements and it is provided for completeness.

Report: CA 8.2.7/1
Porch J.R. et al., 2010a
Metiram: A 7-day static-renewal toxicity test with duckweed (Lemna gibba G3)
2010/7013131
Guidelines: EPA 850.4400, OECD 221, ASTM E 1415-91
GLP: yes
(certified by United States Environmental Protection Agency)

Executive Summary

In a 7-day semi-static toxicity laboratory study, the effect of metiram (tested as BAS 222 29 F) on the growth of the duckweed *Lemna gibba* was investigated. The following nominal concentrations were applied: 0.050, 0.100, 0.200, 0.400 and 0.800 mg metiram/L, corresponding to mean measured concentrations of 0.0194, 0.0497, 0.0795, 0.194 and 0.517 mg a.s./L. Additionally, a dilution water control and a solvent control were set up. Assessment of growth and other effects were conducted on days 0, 3, 5 and 7 days after test initiation. The percentage growth inhibition, relative to the pooled control, was calculated for each test concentration based upon mean growth rates and final yield for the parameters frond number and dry weight (biomass).

The biological results are based on mean measured concentrations. The duckweed population in the control vessels showed exponential growth. After 7 days of exposure, dead fronds were observed at the highest test item concentration, chlorotic fronds were detected in the control, the solvent control and at the test item concentrations of 0.0795 and 0.194 mg a.s./L, while necrotic fronds were observed in both control groups. No statistically significant effects on plant growth were detected up to and including the highest test item concentration of 0.517 mg a.s./L.

In a 7-day aquatic plant test with *Lemna gibba,* the E_rC_{50} and the E_yC_{50} values for metiram were both determined to be > 0.517 mg a.s./L based on frond number and dry weight (mean measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 29 F, batch no. 300013; content of a.s.: metiram (BAS 222 F; Reg. No 250284): 91.6% (nominal: 85%); density: 1.860 g/cm³.

B. STUDY DESIGN

Test species: Duckweed (*Lemna gibba* G3); cultures maintained in-house; stock obtained from "United States Department of Agriculture".

Test design: Semi-static system (7 days); 7 treatment groups (5 test item concentrations plus control and solvent control) with 3 replicates for the test item treatments and the control groups; 4 plants per replicate; total number of fronds at test initiation: 12 per replicate; assessment of growth and other effects on days 0, 3, 5 and 7; determination of dry weight at test termination.

Endpoints: EC_{50} with respect to growth rate and yield after exposure over 7 days.

Test concentrations: Control (dilution water), solvent control (0.1 mL N,N-dimethylformamide/L), 0.050, 0.100, 0.200, 0.400 and 0.800 mg metiram/L (nominal), corresponding to mean measured concentrations of < limit of quantification (LOQ), < LOQ, 0.0194, 0.0497, 0.0795, 0.194 and 0.517 mg a.s./L.

Test conditions: 250 mL glass beakers, test volume 100 mL, M-Hoagland's medium, pH 4.8 - 5.5; temperature: 24.2 - 25.2 °C, continuous light, light intensity: 5130 - 5640 lux.

Analytics: Analytical verification of the test item was conducted using a GC-method with MS detection.

Statistics: Descriptive statistics, t-test for comparison of the control data; Dunnett's test for determination of the NOEC values ($p < 0.05$).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of the test item concentrations was conducted in samples of fresh solutions at test initiation, in samples of fresh and spent solutions on days 3 and 5 and in spent solutions at test termination for all test item concentrations. The analyzed contents of metiram in fresh samples taken at test initiation and on day 3 and 5, ranged from 55.4% to 180.4% of nominal concentrations. The measured concentrations in the old solutions taken on day 3, 5 and 7 were between 1.0% and 10.2% of nominal. The following biological results are based on mean measured concentrations.

Biological results: No statistically significant differences were determined between the control and the solvent control data (t-test). Thus, the data from both control groups were pooled and the pooled data set was used for statistical evaluation of treatment related effects. The duckweed population in the control vessels showed exponential growth, increasing from 12 fronds per vessel to an average of 118 fronds per vessel (pooled control), corresponding to a 9.8 x multiplication. The control dry weight increased to an average of 24.1 mg per vessel (pooled control) at test termination. After 7 days of exposure, dead fronds (0.3%) were observed at the highest test item concentration, chlorotic fronds were detected in the control, the solvent control and at the test item concentrations of 0.0795 and 0.194 mg a.s./L (1.1%, 0.9%, 0.3% and 0.3%, respectively), while necrotic fronds were observed in both control groups (0.3%). No statistically significant effects on plant growth were detected up to and including the highest test item concentration of 0.517 mg a.s./L (Dunnett's test, $p < 0.05$). Effects on growth rate and yield are summarized in Table 8.2.7-1.

Table 8.2.7-1: Effect of metiram (tested as BAS 222 29 F) on the growth of duckweed *Lemna gibba*

Concentration [mg a.s./L] (nominal)	**0.050**	**0.100**	**0.200**	**0.400**	**0.800**
Concentration [mg a.s./L] (mean measured)	**0.0194**	**0.0497**	**0.0795**	**0.194**	**0.517**
Inhibition after 7 d [%] [#, a)] (growth rate based on frond no.)	0.1	1.0	-4.9	-1.3	3.5
Inhibition after 7 d [%] [#, a)] (growth rate based on dry weight)	6.2	1.6	5.5	1.4	5.2
Inhibition after 7 d [%] [#, a)] (yield based on frond no.)	0.6	2.5	-13	-3.2	8.5
Inhibition after 7 d [%] [#, a)] (yield based on dry weight)	-19	-4.6	-16	-3.7	14
	Endpoints [mg metiram/L] (mean measured)				
E_rC_{50} (7 d) & E_yC_{50} (7 d) based on frond no and dry weight	> 0.517				

[#] Negative values indicate stimulated growth compared to the control.
[a)] Percent inhibition was calculated relative to the pooled control.

III. CONCLUSION

In a 7-day aquatic plant test with *Lemna gibba,* the E_rC_{50} and the E_yC_{50} values for metiram were both determined to be > 0.517 mg a.s./L based on frond number and dry weight (mean measured).

The following alga study on the duckweed Lemna gibba performed with the metiram metabolite ethylenethiourea is not required for registration in the EU and it has not been evaluated previously on EU level. The study was conducted due to U.S. data requirements and it is provided for completeness.

Report:	CA 8.2.7/2 Softcheck K.A., 2008a Ethylenethiourea - 7-day toxicity test with duckweed (Lemna gibba) following OPPTS draft guideline 850.4400 2008/7015436
Guidelines:	EPA 850.4400
GLP:	yes (certified by United States Environmental Protection Agency)

Executive Summary

In a 7-day semi-static toxicity laboratory study, the effect of ethylenethiourea (metabolite of metiram) on the growth of the duckweed *Lemna gibba* was investigated. The following nominal concentrations were applied: 0 (control), 63, 130, 250, 500 and 1000 mg ethylenethiourea/L (corresponding to mean measured concentrations of 0, 59, 120, 230, 480 and 960 mg/L). Assessment of growth and other effects was conducted 3, 5 and 7 days after test initiation.

The biological results are based on mean measured concentrations. No morphological changes were observed in the control and at mean measured test item concentration of up to and including 480 mg ethylenethiourea/L. At the highest tested concentration of 960 mg/L, fronds were observed to be curled throughout the exposure. No statistically significant effects on plant growth were detected for all measured parameters at any test item concentration tested.

In a 7-day aquatic plant test with *Lemna gibba*, the E_rC_{50} for ethylenethiourea (metabolite of metiram) based on frond number and the E_bC_{50} values based on frond number and dry weight were determined to be all > 960 mg/L (mean measured).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: Ethylenethiourea (BF 222-ETU, Reg. no. 146 099; metabolite of metiram), ID no. TSN030606-0001; Lot. No. 04816CH; purity: 100%.

B. STUDY DESIGN

Test species: Duckweed (*Lemna gibba*), inocula 6 days old cultures; cultures maintained in-house; strain 310 obtained from University of Toronto, Toronto, Canada.

Test design: Semi-static system (7 days); 6 treatment groups (5 test item concentrations, control) with 3 replicates for the test item treatments and the control; plants with 3 to 4 fronds, giving a total number of 15 fronds per replicate at test initiation; assessment of growth and other effects on days 3, 5 and 7.

Endpoints: EC_{05} and EC_{50} with respect to frond density, frond growth rate and frond biomass (dry weight) after exposure over 7 days.

Test concentrations: Control, 63, 130, 250, 500 and 1000 mg ethylenethiourea/L (nominal); corresponding to 0, 59, 120, 230, 480 and 960 mg/L (mean measured).

Test conditions: 270 mL crystallizing dishes, test volume 100 mL, 20x-AAP nutrient medium, pH 7.5 - 9.2; water temperature: 24 °C - 25 °C, continuous light at 5100 lux- 6600 lux.

Analytics: Analytical verification of the test item concentrations was conducted using an HPLC-method with UV detection.

Statistics: Descriptive statistics; Kruskal-Wallis' Test or Williams' Test for determination of the NOEC values ($p < 0.05$).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of the test item concentrations was conducted at test initiation (fresh solution), on day 1 (aged solutions) and on day 3 (aged solutions). Mean measured concentrations of ethylenethiourea ranged from 92% to 96% of nominal concentrations. The following biological results are based on mean measured concentrations.

Biological results: No morphological changes were observed in the control and at mean measured test item concentration of up to and including 480 mg ethylenethiourea/L. At the highest tested concentration of 960 mg/L, fronds were observed to be curled throughout the exposure. No statistically significant effects on plant growth were detected for all measured parameters at any test item concentration tested (Kruskal-Wallis' Test or Williams' Test, $p < 0.05$). Effects on *Lemna* growth are summarized in Table 8.2.7-2.

Table 8.2.7-2: Effect of ethylenethiourea (metabolite of metiram) on the growth of duckweed *Lemna gibba*

Concentration [mg/L] (nominal)	**63**	**130**	**250**	**500**	**1000**
Concentration [mg/L] (mean measured)	**59**	**120**	**230**	**480**	**960**
Inhibition after 7 d [%] * (frond density)	-14	-13	-11	-11	28
Inhibition after 7 d [%] * (growth rate based on frond no.)	-4	-2	-2	4	11
Inhibition after 7 d [%] * (biomass based on dry weight)	-34	-24	-22	-7	5
	Endpoints [mg ethylenethiourea/L] (mean measured)				
E_rC_{50} (7 d) based on frond no.	> 960				
E_bC_{50} (7 d) based on frond no. & dry weight	> 960				

* Negative values indicate stimulated growth compared to the control

III. CONCLUSION

In a 7-day aquatic plant test with *Lemna gibba*, the E_rC_{50} for ethylenethiourea (metabolite of metiram) based on frond number and the E_bC_{50} values based on frond number and dry weight were determined to be all > 960 mg/L (mean measured).

CA 8.2.8 Further testing on aquatic organisms

Data already submitted during previous Annex I inclusion process (already evaluated on EU level):

The following toxicity study on African clawed frog (Xenopus laevis) tadpoles performed with ethylenethiourea (ETU; metabolite of metiram) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.2.8/1*
	[redacted] 2002e
	Reg.No. 146 099 - Xenopus laevis metamorphosis assay
	2002/1003402
Guidelines:	*German UBA Project FKZ 200 67 409*
GLP:	*yes*
	(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)

Executive Summary

In a semi-static exposure system *Xenopus laevis* tadpoles (development stage 48-50) were exposed to ethylenethiourea (ETU; metabolite of metiram) over a 28 d period. The nominal exposure concentrations were 5, 10, 22, 50 and 100 mg ETU/L plus a control, a positive and a negative control. Tadpoles were observed daily for survival and toxic signs, and on days 0, 5, 12, 19, 26 and 28 days after start of exposure for development, body length and tail length.

The results are based on nominal concentrations. ETU caused no mortality or symptoms over the whole exposure period in any of the concentrations or control groups. Development was statistically significantly delayed compared to control in the 3 highest test concentrations (≥ 22 mg/L). Body and tail length were not statistically significantly different to the untreated control for any of the test concentrations.

In a semi-static exposure system *Xenopus laevis* tadpoles were exposed to ethylenethiourea (ETU; metabolite of metiram) over a 28-day period. The overall NOEC was determined to be 10 mg/L based on nominal concentrations.

. MATERIAL AND METHODS

A. MATERIALS

Test item:	Ethylenethiourea (ETU, Reg. No 146099; metabolite of metiram), batch-no. 01743-136, substance-no: 00/533-2; purity: 99.9%.

B. STUDY DESIGN

Test species:	African clawed frog (*Xenopus laevis*), development stage: 49.0 (48-50); body length: 23.8 (17 – 31) mm, animal supplier: test organisms were bred in the testing facility.
Test design:	Semi-static system, test duration: 28 d; renewal of test solution 3 times weekly; 30 animals per replicate, 1 replicate/concentration, positive control with L-Thyroxine (0.001 mg/L), negative control with Propylthiouracil (75 mg/L), control with test water only, 3 replicates for controls. Daily assessment of mortality and visual symptoms; determination of developmental stage, body length and tail length on days 0, 5, 12, 19, 26 and 28.
Endpoints:	NOEC based on mortality, toxic symptoms, development stage, body length and tail length.
Test concentrations:	control, negative control, positive control, 5, 10, 22, 50 and 100 mg ETU/L (nominal).
Test conditions:	Glass aquaria (30 m x 20 m x 20 m), M4 medium (test volume: 10 L); temperature 23 ± 0.5 °C; pH 7.1 – 8.0; oxygen content: 6.2 – 8.8 mg/L; conductivity: 550 - 650 µS/cm; total hardness: 2.20 – 3.20 mmol/L; feeding with commercial fish diet (Sera micron, sera GmbH, Heinsheim, Germany); slight aeration of aquaria.
Analytics:	The test item concentrations were analyzed with HPLC-UV method CF-A 446.
Statistics:	For the developmental stage, body length and tail length statistical evaluation was done using Dunnett's test; for comparison of negative and positive control group using Student's t-test.

II. RESULTS AND DISCUSSION

Analytical measurements: At the start of the test the analytical results for ETU are within a range of 101.7% to 107.4% of nominal concentrations. At test termination the concentrations vary between 96.7% and 103.2% of the nominal contents. Measured values of the interim analyses were between 96.7% and 114.5%. As the amounts of test compound were well documented and confirmed the correct application of test substance, the biological results are based on nominal concentrations.

Biological results: ETU caused no mortality or symptoms over the whole exposure period in any of the concentrations or control groups. Development was statistically significantly delayed in the 3 highest concentrations groups (≥ 22 mg/L). In the positive control group the development was statistically significantly increased, in the negative control group the development was statistically significantly decreased in comparison to the untreated control group. Body and tail length were not statistically significantly different to the untreated control for any of the test concentrations. In the positive control group body and tail length were statistically significantly decreased in comparison to the untreated control from day 19 (body length) and 12 (tail length) of exposure until termination of exposure. In the negative control a statistically significant reduction of body and tail length could be observed from day 5 to day 26 of exposure, towards the end of exposure the differences decreased. The results are summarized in Table 8.2.8-1.

Table 8.2.8-1: Chronic toxicity of ethylenethiourea (ETU; metabolite of metiram) on African clawed frogs (*Xenopus laevis*)

Concentration [mg ETU/L] nominal	**Control**	**Control pos.**	**Control neg.**	**5**	**10**	**22**	**50**	**100**
Mortality (28 d) [%]	0	0	0	0	0	0	0	0
Toxic signs (during the study)	none	none	none	none	none	none	none	none
Mean developmental stage (28 d)	59.0	61.6	52.0	59.1	58.3	55.5*	52.5*	52.0*
Mean body length (28 d) [mm]	53.0	43.1**	53.3	53.7	55.3	56.6	56.9	56.6
Mean tail length (28 d) [mm]	37.0	28.0**	37.9	37.9	39.6	40.4	40.3	40.7
	Endpoints [mg ETU/L] (nominal)							
NOEC (28 d)	**10**							

* Statistically significant compared to control (Dunnett's test, p ≤ 0.05)
** Statistically significant compared to control (Student's t-test, p ≤ 0.05)

III. CONCLUSION

In a semi-static exposure system *Xenopus laevis* tadpoles were exposed to ethylenethiourea (ETU; metabolite of metiram) over a 28-day period. The overall NOEC was determined to be 10 mg/L based on nominal concentrations.

New supplemental data (not evaluated previously on EU level):

From the performed literature search the following peer-reviewed scientific study investigating lethal and sublethal effects of ethylenethiourea (ETU; metabolite of metiram) on Xenopus laevis tadpoles was considered relevant and reliable (with restrictions, RI 2) for the aquatic risk assessment of metiram (for details please see the literature search and evaluation files also provided within the submission for Annex I Renewal). The data had not been used or evaluated during the previous Annex I inclusion process and thus, relevant information and the results for ethylenethiourea of this study are described in the following

Report:	*CA 8.2.8/2* *Opitz R. et al., 2004a* *Description and initial evaluation of a Xenopus metamorphosis assay for detection of thyroid system-disrupting activities of environmental compounds* *2005/1043780*
Guidelines:	*none*
GLP:	*no*

Executive Summary

The influence of ethylenethiourea (ETU) on the thyroid system of African clawed frogs (*Xenopus laevis*) was investigated in a ring test with six scientific research institutions participating.

In a total of ten 28-day semi-static toxicity laboratory studies, tadpoles of African clawed frogs (*Xenopus laevis*) were exposed to nominal concentrations of 5, 10, 25, 50 and 100 mg ethylenethiourea (ETU)/L in groups of 30 animals per replicate in glass aquaria containing 10 L water. Additionally a solvent control (DMSO), a positive control (thyroxine) and a negative control (propylthiouracil) were set up. Tadpoles were observed daily for survival and symptoms of toxicity.

The biological results are based on nominal concentrations. No relevant mortalities were observed at up to and including the highest concentration tested of 100 mg ETU/L when compared to the solvent control. In all ten test trials performed with ETU, results from developmental stage determinations revealed a concentration dependent inhibition of metamorphic development. The two highest ETU concentrations of 50 and 100 mg/L caused a complete inhibition of metamorphic development in all test trials. In seven test trials, significant retardation of tadpole development was observed at 25 mg ETU/L, whereas one trial showed a significantly lower development already at the concentration of 10 mg ETU/L in comparison to solvent control tadpoles. In contrast to the two highest ETU concentrations, the lower ETU concentration of 25 mg/L did not completely inhibit metamorphic development but metamorphosis proceeded much slower compared to the solvent control groups.

At 10 mg ETU/L only weak inhibitory effects were observed. Assessment of tadpole growth by means of body length measurements was limited to the initial 14 days of exposure (initial growth).In eight of ten trials, no statistically significant effects of ETU on initial growth rates were observed. Mean tail length was statistically significantly increased at the three highest test concentrations of 25, 50 and 100 mg ETU/L. As inhibitory effects of ETU on tail resorption occurred in only five of ten test trials, tail-length measurements proved to be a far less sensitive parameter to detect inhibitory activities of ETU on metamorphic development during Xenopus metamorphosis assay (XEMA).

In ten 28-day semi-static toxicity studies with ethylenethiourea (ETU; metabolite of metiram) performed by six independent scientific institutions, the resulting overall LOEC for African clawed frog (*Xenopus laevis*) based on developmental effects was determined to be 25 mg ETU/L (nominal). The derived overall NOEC is 10 mg ETU/L.

I. MATERIAL AND METHODS

A. MATERIALS

Test item:	Ethylenethiourea (ETU; BAS 222 F; Reg. no. 146 099), purchased from Sigma; different lots were used at the test facilities; purity: not reported.
Reference item:	Negative control with propylthiouracil (75 and 100 mg PTU/L); positive control with thyroxine (0.001 mg T4/L); purchased from Sigma; different lots were used at the test facilities; purity: not reported.

B. STUDY DESIGN

Test species:	African clawed frog (*Xenopus laevis*); tadpoles (Nieuwkoop and Faber (NF) stage 48 - 50); whole body length > 16 mm; derived from laboratory-bred *X. laevis.*
Test design:	Semi-static system (28 days); renewal of test solution 3 times weekly; 30 tadpoles per replicate; 2 replicates per treatment and 3 replicates for the solvent control; daily assessment of mortality and symptoms of toxicity; determination of developmental stages, whole body length and tail length at test initiation and on days 7, 14, 21 and 28 using a binocular dissection microscope; for body length measurements the chamber was placed on a length scale.

Research institutions:	1 Department of Inland Fisheries, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin 12587, Germany 2 BASF Aktiengesellschaft, Product Safety—Regulations, Toxicology, and Ecology, 67056 Ludwigshafen, Germany 3 Department of Zoology, University of Heidelberg, Heidelberg 69120, Germany 4 Department of Ecology and Evolution, University of Frankfurt, Frankfurt am Main 60054, Germany 5 Towa Kagaku, Hiroshima 730-0841, Japan 6 AstraZeneca Global Safety, Health, and Environment, Brixham Environmental Laboratory, Freshwater Quarry, Brixham, Devon TQ5 8BA, United Kingdom
Endpoints:	LOEC based on developmental stage; Mortality and sub-lethal effects (developmental stage, whole body length, tail length, abnormal behavior, malformations).
Test concentrations:	Solvent control (0.01% dimethylsulfoxide (DMSO)), negative control, positive control; 5, 10, 25, 50 and 100 mg ETU/L (nominal).
Test conditions:	11 L glass aquaria, test volume: approx. 10 L, dilution water: 2.5 g of commercial salt mixture (Tropic Marine Meersalz; Tagis, Dreieich, Germany) added to 10 L of deionized water; renewal of test solutions 3 times per week; temperature: 22 ± 1 °C; pH 7.0 ± 0.5; photoperiod: 12 h light : 12 h dark; fed daily with commercial tadpole food (Sera, Heinsberg, Germany): 200 mg/day from exposure days 0 to 5 and 300 mg/day from exposure days 6 to 28.
Analytics:	Not reported.
Statistics:	Descriptive statistics; Tail length and whole body length: Dunnett's test ($p < 0.05$) to compare data from the solvent control group to all treatment groups; when data were not distributed normally, nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test ($p < 0.05$) was used. Developmental stages: Kruskal-Wallis ($p < 0.05$) test to determine significant differences between treatment groups and Dunn's multiple comparison test ($p < 0.05$) for comparisons with the solvent control group.

II. RESULTS AND DISCUSSION

Analytical measurements: Chemical analyses of actual water concentrations of T4, PTU and ETU were performed only in one of six laboratories in order to confirm the stability of all compounds during the semi-static exposure regime. The analytical results were not reported. The following biological results are based on nominal concentrations.

Biological results:
Mortality: After 28 days of exposure, mortalities of tadpoles in the control groups were very low or not detectable. No relevant mortalities were observed at up to and including the highest concentration tested of 100 mg ETU/L when compared to the solvent control. No substance-related mortalities were observed in the groups exposed to thyroxine and propylthiouracil.

Developmental stages: Development of the positive control groups (thyroxin) was markedly accelerated while the development in the negative control groups (propylthiouracil) was inhibited in comparison to the solvent control groups. During the exposure period of 28 days, tadpoles in the solvent control group displayed development to prometamorphic and early climax stages. Furthermore considerable interindividual variability in tadpole development occurred. In all ten test trials performed with ETU, results from developmental stage determinations revealed a concentration dependent inhibition of metamorphic development. The two highest ETU concentrations of 50 and 100 mg/L caused a complete inhibition of metamorphic development in all test trials (Dunn's test, $p < 0.05$). In seven test trials, significant retardation of tadpole development was observed at 25 mg ETU/L, whereas one trial showed a significantly lower development already at the concentration of 10 mg ETU/L in comparison to solvent control tadpoles (Dunn´s test, $p < 0.05$). In contrast to the two highest ETU concentrations, the lower ETU concentration of 25 mg/L did not completely inhibit metamorphic development but metamorphosis proceeded much slower compared to the solvent control groups. At 10 mg ETU/L only weak inhibitory effects were observed.

Body length / tail length: Assessment of tadpole growth by means of body length measurements was limited to the initial 14 days of exposure (initial growth). In eight of ten trials, no statistically significant effects of ETU on initial growth rates were observed. Mean tail length was statistically significantly increased at the three highest test concentrations of 25, 50 and 100 mg ETU/L. As inhibitory effects of ETU on tail resorption occurred in only five of ten test trials, tail-length measurements proved to be a far less sensitive parameter to detect inhibitory activities of ETU on metamorphic development during Xenopus metamorphosis assay (XEMA). While initial growth of the positive control groups (thyroxin) was not statistically significantly different from the solvent control groups, significant reduction in initial growth rates of tadpoles was observed for both PTU concentrations (Dunnett's test, $p = 0.05$). Regarding mean tail length, thyroxin treated tadpoles showed a significantly reduced mean tail length in comparison to the solvent control ($p = 0.05$).). While significant reduction in mean tail length was observed in nine and five PTU groups on day 14 and 21, respectively, mean tail length in four of ten PTU test groups was significantly increased at test termination ($p = 0.05$). The results are summarized in Table 8.2.8-2 to Table 8.2.8-5.

Table 8.2.8-2: Mortality [%] of *X. laevis* tadpoles exposed to ethylenethiourea (ETU) in different test trials after 28 days

Laboratory	Replicate test	Concentration [mg ETU/L] (nominal)							
		Solvent control	Positive control	Negative control	5.0	10	25	50	100
1	R1	0	2.2	2.2	0	0	0	0	0
	R2	0	1.1	0	0	0	0	0	3.3
2	R1	0	0	1.1	0	0	0	1.7	0
	R2	0	0	1.1	0	1.7	0	0	0
3	R1	0	0	1.1	0	0	1.7	0	1.7
	R2	0	0	0	1.7	0	1.7	0	0
4	R1	2.2	5.6	3.3	3.3	5.0	5.0	3.3	3.3
	R2	2.2	3.3	6.6	1.7	3.3	3.3	1.7	0
5	R1	0	5.0	1.7	0	1.7	3.3	1.7	0
6	R1	2.2	2.2	2.2	1.7	1.7	0	1.7	3.3

Table 8.2.8-3: Developmental stage of *X. laevis* tadpoles exposed to ethylenethiourea (ETU) in different test trials after 28 days

Lab	Repl. test	Concentration [mg ETU/L] (nominal)								LOEC [mg ETU/L] (nominal)
		Solvent control	Pos. control	Neg. control	5.0	10	25	50	100	
1	R1	59 (55–65)	62 (57–66) #	52 (48–54) #	59 (54–65)	58 (54–63)	54.5 (51–59) #	52 (50–54) #	52 (50–54) #	25
	R2	60 (53–65)	63 (59-66) #	53 (51–54) #	59 (56–64)	58 (53–65)	57 (49–62) #	53 (52–54) #	53 (52–54) #	25
2	R1	55 51–64)	59 (58-62 #	51 (50–54) #	55 (54–62)	55 (52–60)	55 (50–59)	51 (50–55) #	54 (50–54) #	50
	R2	57 (55–64)	59 (59-65) #	53 (51–54) #	56 (54–64)	57 (54–62)	56 (52–59)	53 (51–54) #	53 (51–54) #	50
3	R1	62 (57–66)	64 (59-66) #	53 (52–54) #	62 (57–65)	62 (54–65)	54 (52–63) #	53 (52–53) #	53 (50–53) #	25
	R2	58 (54–66)	61 (57-66) #	53 (52–54) #	57 (56–64)	57 (54–66)	53 (52–58) #	53 (52–54) #	53 (52–54) #	25
4	R1	58 (55–61)	60 (58-61) #	52 (50–53) #	57 (53–59)	55 (51–57) #	54 (50–58) #	52 (51–54) #	52 (50–53) #	10
	R2	56 (51–61)	57 (57-62) #	53 (51–54) #	56 (52–59)	56 (52–	55 (51–58)	52 (51–54) #	52 (51–54) #	50

Lab	Repl. test	Concentration [mg ETU/L] (nominal)								LOEC [mg ETU/L] (nominal)
		Solvent control	Pos. control	Neg. control	5.0	10	25	50	100	
						59)				
5	R1	56.5 (53–60)	57 (56-63) #	52 (49–53) #	57 (54–62)	57 (55–61)	53 (51–56) #	51 (50–54) #	52 (50–53) #	25
6	R1	60 (53–65)	63 (58-66) #	53 (51–55) #	61 (54–66)	62 (51–65)	54 (51–58) #	53 (51–54) #	53 (51–55) #	25

Values shown are the median stage with the total range of stages in parentheses.
Statistically significant compared to solvent control (Dunn´s test, $p < 0.05$).

Table 8.2.8-4: Whole body length [mm] of *X. laevis* tadpoles exposed to ethylenethiourea (ETU) in different test trials after 14 days

Lab	Replicate test	Concentration [mg ETU/L] (nominal)							
		Solvent control	Pos. control	Neg. control	5.0	10	25	50	100
1	R1	49.0 ± 4.9	46.8 ± 5.6	35.9 ± 6.3 *	48.3 ± 5.0	48.2 ± 4.9	49.0 ± 4.2	48.1 ± 3.6	48.2 ± 5.6
	R2	48.6 ± 4.9	46.7 ± 4.6	45.8 ± 5.1 *	48.1 ± 3.5	47.3 ± 3.9	48.2 ± 4.3	47.2 ± 4.4	47.1 ± 3.4
2	R1	39.5 ± 5.5	35.2 ± 4.8 *	33.6 ± 5.2 *	39.6 ± 3.9	39.0 ± 4.9	42.4 ± 5.9 *	39.9 ± 6.6	35.1 ± 4.9 *
	R2	46.7 ± 4.1	45.0 ± 3.8	41.2 ± 5.7 *	44.9 ± 4.7	45.9 ± 4.6	46.0 ± 3.8	45.1 ± 3.8	45.2 ± 4.5
3	R1	49.5 ± 3.6	48.0 ± 4.4	45.1 ± 4.8 *	48.8 ± 4.5	49.2 ± 4.5	49.5 ± 4.5	48.2 ± 3.8	48.0 ± 4.5
	R2	49.3 ± 4.2	47.5 ± 4.0	46.2 ± 4.3 *	48.4 ± 4.0	49.0 ± 3.6	49.2 ± 4.2	48.2 ± 4.4	48.3 ± 3.3
4	R1	33.1 ± 6.1	33.1 ± 7.5	29.6 ± 6.1 *	33.1 ± 7.6	35.1 ± 7.6	36.4 ± 8.6	36.4 ± 6.0	33.3 ± 6.2
	R2	48.2 ± 5.6	48.8 ± 5.3	47.4 ± 4.5	47.5 ± 4.2	48.8 ± 4.6	46.7 ± 5.2	46.3 ± 4.7	47.7 ± 4.4
5	R1	39.5 ± 5.4	40.7 ± 5.1	36.3 ± 4.9 *	40.7 ± 4.2	40.2 ± 5.1	40.9 ± 4.6	40.7 ± 5.4	40.0 ± 4.7
6	R1	51.7 ± 6.2	49.2 ± 6.7	41.6 ± 8.1	52.5 ± 6.4	53.9 ± 6.3	55.2 ± 6.9	55.2 ± 7.3	52.4 ± 8.9

Values shown are means and standard deviations.
* Significantly different from the solvent control (Dunnett´s test, $p < 0.05$)

Table 8.2.8-5: Tail length [mm] of *X. laevis* tadpoles exposed to ethylenethiourea (ETU) in different test trials after 28 days

Lab	Replicate test	Concentration [mg ETU/L] (nominal)							
		Solvent control	Pos. control	Neg. control	5.0	10	25	50	100
1	R1	31.0 ± 10.9b	21.0 ± 14.5 *	34.6 ± 6.7	32.4 ± 10.4	33.5 ± 7.0	38.3 ± 4.3*	38.1 ± 3.8*	37.9 ± 5.1*
	R2	28.6 ± 12.8	17.4 ± 15.7 *	35.3 ± 4.3 *	32.9 ± 8.2	32.8 ± 8.6	38.0 ± 5.2*	37.9 ± 4.5*	37.8 ± 3.7*
2	R1	35.7 ± 5.3	32.8 ± 4.8 *	34.6 ± 5.2	36.7 ± 3.7	36.8 ± 4.5	37.9 ± 4.3	36.9 ± 5.3	35.6 ± 4.9
	R2	39.3 ± 4.4	33.4 ± 9.5 *	38.6 ± 4.6	39.4 ± 4.8	40.3 ± 3.3	41.7 ± 2.8*	40.0 ± 4.0	40.8 ± 4.1
3	R1	21.2 ± 14.7	14.3 ± 14.5 *	36.6 ± 3.3 *	21.7 ± 14.3	27.1 ± 12.6	39.4 ± 3.5*	38.2 ± 3.5*	38.1 ± 3.4*
	R2	34.4 ± 11.6	27.4 ± 12.9 *	37.1 ± 3.1	37.1 ± 6.0	36.8 ± 7.4	39.8 ± 3.3*	38.9 ± 4.1	39.2 ± 3.6*
4	R1	31.2 ± 4.8	28.7 ± 5.8 *	26.7 ± 5.9 *	31.9 ± 5.5	31.1 ± 6.0	31.1 ± 6.6	31.8 ± 7.5	30.5 ± 6.2
	R2	41.5 ± 4.8	39.8 ± 3.8	41.0 ± 3.7	41.4 ± 3.8	41.6 ± 3.9	41.0 ± 4.3	40.6 ± 4.5	40.4 ± 3.9
5	R1	32.5 ± 4.1	31.8 ± 4.3	32.8 ± 5.9	33.0 ± 3.7	32.4 ± 4.3	34.0 ± 3.6	32.9 ± 5.4	32.7 ± 5.2
6	R1	32.7 ± 13.1	17.3 ± 12.8 *	38.7 ± 7.7	29.6 ± 14.6	32.4 ± 13.1	44.0 ± 7.0*	44.7 ± 6.9*	43.7 ± 7.6*

Values shown are means and standard deviations.
* Significantly different from the solvent control (Dunnett's test, $p < 0.05$).

III. CONCLUSION

In ten 28-day semi-static toxicity studies with ethylenethiourea (ETU; metabolite of metiram) performed by six independent scientific institutions, the resulting overall LOEC for African clawed frog (*Xenopus laevis*) based on developmental effects was determined to be 25 mg ETU/L (nominal). The derived overall NOEC is 10 mg ETU/L.

***From the performed literature search the following peer-reviewed scientific review was considered relevant for the aquatic risk assessment of metiram (for details please see the literature search and evaluation files also provided within the submission for Annex I Renewal). The review systematically compares the relative sensitivity of amphibians and fish to chemicals and investigates the relationship between fish and amphibian acute and chronic sensitivity. The review had not been considered or evaluated during the previous Annex I inclusion process and thus, the relevant information is described in the following summary*.**

Report:	*CA 8.2.8/3* *Weltje L. et al., 2012a* *Comparative acute and chronic sensitivity of fish and amphibians: A critical review of data* *2013/1416700*
Guidelines:	*none*
GLP:	*no*

Executive Summary

Acute toxicity data for 55 chemicals (including 32 plant protection product active substances) and chronic toxicity data for 52 chemicals (including 20 plant protection product active substances) were obtained from the U.S. Environmental Protection Agency (U.S. EPA) ECOTOX database and were supplemented with data from the scientific and regulatory literature.

Pairwise comparisons of 96 h LC_{50} values and key NOECs from amphibian and fish studies were undertaken for each chemical, and the ratio of amphibian to fish sensitivity was calculated.

The analysis of acute and chronic toxicity data demonstrates that in most cases fish are more sensitive than amphibians to chemicals and thus are appropriate representative species to cover the sensitivity of aquatic vertebrates in current risk assessment procedures.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: 55 chemicals (including 32 plant protection product active substances) for analysis of acute toxicity data; 52 chemicals (including 20 plant protection product active substances) for analysis of chronic toxicity data.

B. STUDY DESIGN

Test species: Acute: *Oncorhynchus mykiss* or *Pimephales promelas* and amphibian species providing the most sensitive endpoint.
Chronic:
Fish species: *Oryzias latipe, Pimephales promelas, Zoarces viviparous, Oncorhynchus mykiss, Ictalurus punctatus, Cyprinodon variegatus, Salmo trutta, Salmo salar, Tilapia guineensis, Etheostoma fonticola, Danio rerio, Lepomis macrochirus, Poecilia reticulata, Salvelinus namaycush, Gambusia holbrooki, Oryzias latipes, Jordanella floridae.*
Amphibian species: *Xenopus laevis, Rana sylvatica, Rana catesbeiana, Bufo arenarum, Rana arvalis, Pseudacris regilla, Ambystoma barbouri, Ambystoma maculatum, Rana temporaria, Osteopilus septentrionalis, Rana sphenocephala, Rana pipiens, Bufo americanus, Rana hexadactyla, Rana aurora, Xenopus tropicalis, Spea multiplicata, Bombina orientalis, Hyla chrysoscelis, Bufo fergusonii.*

Test design: Acute toxicity data: pairs of 96 h LC_{50} values comprising any amphibian species and either rainbow trout (*Oncorhynchus mykiss)* or fathead minnow (*Pimephales promelas*). *Oncorhynchus mykiss* data were selected preferentially over *P. pr*omelas data. When data on several amphibian species were available, only data for the most sensitive amphibian species were selected for analysis and in the case that LC_{50} values were available for different amphibian life stages (embryos, tadpoles or adults) the lowest LC_{50} value was selected.

Chronic toxicity data: Static renewal and flow-through systems; studies that reported apical endpoints of potential population relevance (i.e. survival, growth, development (including metamorphosis), or reproduction); exposure duration for fish species: 21 - 396 days; amphibian species: 10 - 210 days; the lowest long-term population-relevant NOEC values were selected as key NOECs.

Endpoints: 96 h LC_{50} values, NOEC and LOEC values based on development, growth, survival and reproduction.

Analytics: Acute toxicity data: if differentiated in the study only tests that reported measured concentrations of test chemicals were included, some amphibian studies included in the analysis did not differentiate between LC_{50} values based on measured concentrations or those based on nominal concentrations. Chronic toxicity data: studies based on nominal rather than measured concentrations were not excluded from the analysis.

Statistics: Statistical correlation between amphibian and fish LC_{50} and NOEC values was investigated using Spearman's correlation.

II. RESULTS AND DISCUSSION

For acute data, pairwise comparisons of the LC_{50} values from amphibian and fish studies were undertaken for the chemical for which a pair of 96 h LC_{50} values was available. Ratios greater than 100 indicate that the standard EU acute toxicity assessment factor of 100 routinely applied to the results of acute fish tests during risk assessment would not be sufficient to cover the sensitivity of aquatic life stages of amphibians.

For chronic data, pairwise comparisons of key NOECs (lowest long-term population-relevant NOEC) from amphibian and fish studies were undertaken for each chemical, and the ratio of amphibian to fish sensitivity was calculated. Ratios of greater than one (fish NOEC/amphibian NOEC) indicate that amphibians are relatively more sensitive than fish. Factors greater than 10 indicate that the standard EU assessment factor of 10 routinely applied to the results of chronic fish tests during risk assessment would not be sufficient to cover the sensitivity of aquatic life stages of amphibians. Where spacing factors between amphibian and fish studies were of unequal size, an additional ratio was calculated of the respective maximum acceptable toxicant concentration (MATC), which is calculated as the geometric mean of the NOEC and LOEC value, as this measure is less sensitive to differences in spacing factors.

The analysis showed that fish and amphibian toxicity data are highly correlated and that fish are generally more sensitive than amphibians (acute and chronic). Relatively few instances were seen of amphibians showing marked higher sensitivity than fish after either acute or chronic exposure. Regarding acute toxicity, amphibians were between 10-fold and 100-fold more sensitive than fish for only 4 out of 55 substances. However, application of the standard EU assessment factor of 100 to the regulatory fish LC_{50} value would have covered the amphibian LC_{50} value. In only two instances amphibian sensitivity was more than 100-fold greater compared to fish sensitivity, however analysis of those cases showed that in general, amphibians are of comparable acute sensitivity to fish in laboratory toxicity studies and, on average, slightly less sensitive than fish. Therefore, the overall picture from this analysis is that fish are generally more acutely sensitive than amphibians, and the standard EU risk assessment factor of 100 accounts for potential species sensitivity differences between fish and amphibians.

Regarding chronic toxicity, some studies showed a higher sensitivity in amphibians than in fish, however they were mostly considered to be unreliable (unbounded NOECs, effects not based on apical endpoints, large spacing factors between NOEC and LOEC, problematic test mediums, and unsuitable test designs). Therefore, the overall picture from this analysis is that amphibians are not generally more chronically sensitive than fish and the standard EU risk assessment factor of 10 accounts for potential species sensitivity differences between fish and amphibians.

III. CONCLUSION

The analysis of acute and chronic toxicity data demonstrates that in most cases fish are more sensitive than amphibians to chemicals and thus are appropriate representative species to cover the sensitivity of aquatic vertebrates in current risk assessment procedures.

From the performed literature search the following peer-reviewed scientific study on Xenopus laevis was considered relevant and reliable (with restrictions; RI 2) for the aquatic risk assessment of metiram and its metabolite ethylenethiourea (for details please see the literature search and evaluation files also provided within the submission for Annex I Renewal). In this study, X. laevis tadpoles were exposed to the metiram metabolite ethylenethiourea and perchlorate and subsequently studied for histological and molecular changes. The data had not been used or evaluated during the previous Annex I inclusion process and thus, relevant information and results of this study are described in the following summary. In the following summary only the experimental data and results for ethylenethiourea are presented.

Report:	*CA 8.2.8/4* *Opitz R. et al., 2008a* *Perchlorate and Ethylenethiourea induce different histological and molecular alterations in a non-mammalian vertebrate model of thyroid goitrogenesis* *2008/1102096*
Guidelines:	*none*
GLP:	*no*

Executive Summary

In a 12 days flow-through toxicity laboratory study, stage 51 tadpoles (*Xenopus laevis)* were exposed to 50 mg ethylenethiourea (ETU)/L (nominal) in groups of 22 tadpoles in 7 L exposure tanks. Additionally, a dilution water control was set up. 4 replicates were employed for both the test item treatment and the control. Histological changes in the thyroid gland and molecular changes in the thyroid gland, the pituitary gland and in brain tissue of *Xenopus laevis* tadpoles were examined during the 12 day exposure period.
Assessment of life stage and measurements of whole body length (WBL) and hind limb length (HLL) were conducted on days 1, 3, 5, 8 and 12. Samples for temporal profiles of tshb-A mRNA expression in pituitaries of tadpoles and for thyroid gland histology were taken on days 1, 3, 5, 8 and 12. A candidate gene approach was used to characterize profiles of thyroid gene expression in tadpoles from ETU and control treatments. Candidate genes were selected according to their putative role or functional relevance for specific cellular processes related to thyroid hormone (TH) synthesis, secretion and metabolism, protein biosynthesis and vesicular transport, cell growth and proliferation, angiogenesis and stress responses. As a first step, the relative expression of 60 candidate genes in thyroid tissues was sampled on study day 12. In a second step, a subset of candidate genes was further characterized for temporal expression profiles between study day 1 and 12. Samples for gene expression analysis in tadpole brain from stage 55 tadpoles were taken on study day 8.

The biological results are based on the nominal concentrations. No significant differences between the control and the ETU-treatment were detected for WBL on any sampling day. No significant differences in developmental endpoints (developmental stage, HLL) were observed between the control and the ETU-treatment on study days 1, 3, 5 and 8. On study day12, all control tadpoles showed development to stage 56 whereas ETU treatment caused developmental arrest of tadpoles at stages 54/55.
Increased tshb-A expression in pituitaries relative to the control group was detected in ETU-treated tadpoles on study days 8 and 12. Thus, a lag period of 5 - 8 days preceded the detection of significant increases in tshb-A expression in ETU-treated tadpoles.
A moderate thyroid enlargement was observed on study day 5 in the test item treatment group and became more prominent after 8 days of treatment. By study day 12, ETU-treated tadpoles presented thyroids that can be characterized as small goiters because of a several-fold increase in size relative to time-matched controls. Early histological changes in ETU treated tadpoles comprised a mild to moderately severe depletion of stainable colloid within 3 - 5 days of treatment, respectively, followed by follicular cell hypertrophy within 5 days and follicular cell hyperplasia within 8 days of treatment. Furthermore, ETU treatment resulted in a progressive enlargement of follicles with distended luminal cavities.
In ETU-treated tadpoles, thyroidal expression of 49 candidate genes was significantly different from the control group, including 43 genes with increased expression and 6 genes with decreased expression in the ETU treatment group. The three genes related to TH synthesis (slc5a5, tpo, tshr) showed a lag period of five days until a significant up-regulation in tadpoles treated with ETU. Genes related to protein metabolism and transport were significantly up-regulated within 24 h of treatment and their expression remained elevated throughout the experimental period of 12 days (eif4a1 and hspa5), while sar1a up-regulation was only observed after a lag period of 5 days in ETU-treated tadpoles and rnp24 represented a late response gene showing significant up-regulation only after 8 and 12 days of treatment. Both transcripts related to cell proliferation (pcna and mcm2) were rapidly up-regulated in thyroids by ETU treatment, mcm2 mRNA within 24 h and pcna mRNA within 3 days. Relative expression levels of tie-2 (related to angiogenesis) were reduced after a lag period of 5 days in the ETU treatment group. A statistically significant up-regulation of gstp1 (stress response related) was observed for the ETU treatment compared to the control and was detectable from day 5 onwards. In brain tissue of ETU-treated tadpoles, significantly lower mRNA expression at study day 8 was detected for several genes known to be up-regulated by TH including thrb (thyroid hormone receptor β), bteb1-A (basic transcription element-binding protein 1), pcna, mcm2, and kif2C. For dapl1, a gene down-regulated by TH in tadpole brain, mRNA expression was increased in brain of ETU-treated tadpoles.

In a 12 day flow-through toxicity study tadpoles of *Xenopus laevis* exposed to 50 mg ETU/L were studied for histological and molecular changes. Inhibition of thyroid hormone (TH) synthesis by ETU was evident from developmental retardation, reduced expression of TH-regulated genes and up-regulation of tshb-A mRNA in the pituitary of tadpoles. Thyroid histopathology revealed alterations including colloid resorption, follicular cell hypertrophy, thyroid hyperplasia, and goiter formation within 3, 5, 8 and 12 days of treatment, respectively. Analysis of expression of 60 candidate genes in the thyroid gland showed that ETU-treatment modulated the expression of 49 transcripts relative to the control (43 genes with increased expression and 6 genes with decreased expression). Several genes related to TH synthesis and protein metabolism were significantly affected by ETU-treatment.

I. MATERIAL AND METHODS

A. MATERIALS

Test item:	Ethylenethiourea (ETU) (Reg. no. 146099, metabolite of metiram) obtained from Sigma; some relevant information (batch no., purity) is missing in the study report.

B. STUDY DESIGN

Test species:	Tadpoles (*Xenopus laevis*); in-house breeding of adult animals; stage 51 tadpoles at day 14 post-fertilization.
Test design:	Flow-through system (12 days); 22 tadpoles per tank; 4 replicates for the test item treatment and the control. Assessment of growth and development (life stage determination, measurements of WBL and HLL) on days 1, 3, 5, 8 and 12. Samples for tshb-A mRNA expression in pituitaries of tadpoles and for thyroid gland histology taken on days 1, 3, 5, 8 and 12. Expression of candidate genes in thyroid tissue: 1.) Assessment of the relative expression of 60 candidate genes in thyroid tissues sampled on study day 12; 2.) further characterization of a subset of 14 candidate genes for temporal expression profiles based on thyroid tissue samples taken on study days 1, 3, 5, 8 and 12. Samples for gene expression in tadpole brain tissue from stage 55 tadpoles sampled on study day 8. Relative quantifications of gene expression were performed by real-time PCR using total RNA
Endpoints:	Growth and development of tadpoles, histological alterations in the thyroid gland, gene expression in pituitaries, thyroid tissue and brain tissue.
Test concentrations:	Dilution water control and 50 mg ETU/L (nominal).
Test conditions:	7 L exposure tanks; flow-rate: 25 mL/min); dilution water: non-chlorinated tap water (iodide concentration 10 µg/L); temperature: 22 ± 1 °C; pH 7.8 ± 0.2; photoperiod 12 h light : 12 h dark; daily feeding with commercial fish food (Sera Micron) with a daily ration increasing from 30 to 50 mg/animal during the course of the experiment.
Analytics:	Analytical verification of test item concentrations was conducted using an HPLC system with UV detection.
Statistics:	Descriptive statistics; Dunn's multiple comparison test ($p < 0.05$); Tukey-Kramer multiple comparison test ($p < 0.05$).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations in test solutions was conducted on various dates. On all time points analyzed, measured concentrations of ETU did not differ by more than 10% from the nominal concentration of 50 mg ETU/L. As analytical measurements confirmed correct application of the test item, the following biological results are based on nominal concentrations.

Biological results:
Growth and development: No significant differences between the control and the ETU-treatment were detected for WBL on any sampling day. No significant differences in developmental endpoints (developmental stage, HLL) were observed between the control and the ETU-
treatment on study days 1, 3, 5, and 8. On study day12, all control tadpoles had reached stage 56 whereas ETU treatment caused developmental arrest of tadpoles at stages 54/55.

Expression of tshb-A mRNA in pituitaries: On study day 1, tshb-A levels were similar for the control and the ETU-treatment group. Between study day 1 and 12, tshb-A expression increased 2.1- and 4.1-fold in the control and the ETU group, respectively. When expression levels were compared between tadpoles matched for sampling time, increased tshb-A expression relative to the control group was detected in ETU-treated tadpoles on study days 8 and 12. Thus, a lag period of 5 - 8 days preceded the detection of significant increases in tshb-A expression in ETU-treated tadpoles.

Thyroid gland histology: In the control group, the progressive increase in glandular size during the experimental period was accompanied by increases in follicle number and in size of individual follicles. Thyroid size of tadpoles treated with ETU was similar to controls on study days 1 and 3. A moderate thyroid enlargement was observed on study day 5 in the test item treatment group and became more prominent after 8 days of treatment. By study day 12, ETU-treated tadpoles presented thyroids that can be characterized as small goiters because of a several-fold increase in size relative to time-matched controls.
During the time course of the experiment, a distinct sequence of histological changes was observed in thyroids of ETU-treated tadpoles. Early histological changes comprised a mild to moderately severe depletion of stainable colloid within 3 - 5 days of treatment, respectively, followed by follicular cell hypertrophy within 5 days and follicular cell hyperplasia within 8 days of treatment. Furthermore, ETU treatment resulted in a progressive enlargement of follicles with distended luminal cavities.

Expression of candidate genes in thyroid tissue: In ETU-treated tadpoles, thyroidal expression of 49 genes was significantly different from the control group, including 43 genes with increased expression and 6 genes with decreased expression in the ETU group.

The three genes related to TH synthesis (slc5a5, tpo, tshr) showed a very similar temporal expression profile which was characterized by a lag period of five days until a significant up-regulation was evident in tadpoles treated with ETU. Different temporal expression profiles were detected when analyzing four candidate genes (eif4a1, hspa5, sar1a, rnp24) related to protein metabolism and transport. Expression of eif4a1 and hspa5 mRNAs was significantly up-regulated within 24 h of treatment and their expression remained elevated throughout the experimental period of 12 days.

The expression profile of sar1a closely resembled the pattern found for slc5a5, tpo, and tshr as up-regulation of sar1a mRNA was only observed after a lag period of 5 days in ETU-treated tadpoles. In comparison, rnp24 represented a late response gene showing significant up-regulation only after 8 and 12 days of treatment. Both transcripts pcna and mcm2 were rapidly up-regulated in thyroids by ETU treatment, mcm2 mRNA within 24 h and pcna mRNA within 3 days. Mean values for vegf-A mRNA expression showed some increases early during treatment, but the differences were not statistically significant. In contrast, relative expression levels of tie-2mRNAwere reduced after a lag period of 5 days in the ETU treatment group. A statistically significant up-regulation of gstp1 mRNA was observed for the ETU treatment compared to the control and was detectable from day 5 onwards.

Gene expression in tadpole brain: In brain tissue of ETU-treated tadpoles, significantly lower mRNA expression was detected for several genes known to be up-regulated by TH including thrb, bteb1-A, pcna, mcm2 and kif2C. For dapl1, a gene down-regulated by TH in tadpole brain, mRNA expression was increased in brain of ETU-treated tadpoles.

The results are summarized in Table 8.2.8-6.

Table 8.2.8-6: Effects on growth, development and gene expression in thyroid tissue, brain tissue and pituitary tissue of *Xenopus laevis* tadpoles after exposure to 50 mg ETU/L

Study day			1 d	3 d	5 d	8 d	12 d
Growth and development (n = 24 for day 1, n = 16 for days 3, 5,8 and 12)	Median developmental stages		-	-	-	-	↓ *** a)
	Mean WBL [mm]		-	-	-	-	-
	Mean HLL [mm]		-	-	-	-	↓ *** b)
Pituitary tissue	relative expression levels of tshb-A mRNA (n = 8)		-	-	-	↑ *** b)	↑ *** b)
relative expression levels of genes from thyroid tissue (n = 8)	TH synthesis	solute carrier transporter 5a5 (slc5a5)	-	-	↑ *** a)	↑ *** a)	↑ *** a)
		thyroid peroxidase (tpo)	-	-	↑ ** b)	↑ *** b)	↑ *** b)
		thyroid-stimulating hormone receptor (tshr)	-	-	↑ *** b)	↑ *** b)	↑ *** b)
	Protein metabolism and vesicular transport	eukaryotic translation initiation factor 4A1 (eif4a1)	↑ *** a)	↑ * a)	↑ ** a)	↑ *** a)	↑ *** a)
		heavy-chain binding protein (hspa5)	↑ *** a)	↑ *** a)	↑ *** a)	↑ *** a)	↑ *** a)
		sar1a protein (sar1a)	-	-	↑ ** b)	↑ *** b)	↑ *** b)
		coated vesicle membrane protein rnp24 (rnp24)	-	-	-	↑ *** b)	↑ *** b)
	Cell proliferation	minichromosome maintenance protein 2 (mcm2)	↑ ** b)	↑ * b)	↑ * b)	↑ *** b)	↑ ** b)
		proliferating cell nuclear antigen (pcna)	-	↑ *** b)	↑ ** b)	↑ ** b)	↑ ** b)
	Angiogenesis	vascular endothelial growth factor A (vegf-A)	-	-	-	-	-

		endothelium-specific receptor tyrosine kinase 2 (tie-2)	-	-	↓ * [b)]	↓ *** [b)]	↓ *** [b)]
	Compound-specific	transcription factor gadd153 (gadd153)	-	-	-	-	-
		asparagine synthetase (asns)	-	-	-	↑ * [a)]	-
		glutathione S-transferase (gstp1)	-	-	↑ ** [a)]	↑ *** [a)]	↑ *** [a)]
Relative expression levels of mRNAs in brain tissue at day 8 (n = 8)	thyroid hormone receptor β (thrb)		↓ * [b)]				
	basic transcription element-binding protein 1 (btebl-A)		↓ *** [b)]				
	minichromosome maintenance protein 2 (mcm2)		↓ ** [b)]				
	proliferating cell nuclear antigen (pcna)		↓ *** [b)]				
	kinesin family member 2C (kif2c)		↓ ** [b)]				
	death-associated protein-like 1 (dapl1)		↑ *** [b)]				

↑ - increase in ETU treatment group compared to the control
↓ - decrease in ETU treatment group compared to the control
- no statistically significant differences compared to the control ($p < 0.05$).
* Statistically significant differences compared to the control ($p < 0.05$).
** Statistically significant differences compared to the control ($p < 0.01$).
*** Statistically significant differences compared to the control ($p < 0.001$).
[a)] Dunn's test
[b)] Tukey-Kramer multiple comparison test

III. CONCLUSION

In a 12 day flow-through toxicity study tadpoles of *Xenopus laevis* exposed to 50 mg ETU/L were studied for histological and molecular changes. Inhibition of thyroid hormone (TH) synthesis by ETU was evident from developmental retardation, reduced expression of TH-regulated genes and up-regulation of tshb-A mRNA in the pituitary of tadpoles. Thyroid histopathology revealed alterations including colloid resorption, follicular cell hypertrophy, thyroid hyperplasia, and goiter formation within 3, 5, 8 and 12 days of treatment, respectively. Analysis of expression of 60 candidate genes in the thyroid gland showed that ETU-treatment modulated the expression of 49 transcripts relative to the control (43 genes with increased expression and 6 genes with decreased expression). Several genes related to TH synthesis and protein metabolism were significantly affected by ETU-treatment.

From the performed literature search the following peer-reviewed scientific study investigating the toxicity of the metiram metabolite ethylenethiourea to Xenopus laevis tadpoles was considered relevant for the aquatic risk assessment of metiram and its metabolite ethylenethiourea. Due to missing analytical measurements in this study, it was classified as "not reliable" (RI 3). For details please see the literature search and evaluation files also provided within the submission for Annex I Renewal. Nevertheless, the study is considered to provide some additional information on chronic effects of ethylenethiourea on amphibians. The data have not been used or evaluated during the previous Annex I inclusion process and thus, relevant information and results of the study are described in the following summary.

Report:	*CA 8.2.8/5* *Opitz R. et al., 2006a* *Evaluation of histological and molecular endpoints for enhanced detection of thyroid system disruption in Xenopus laevis tadpoles* *2006/1051113*
Guidelines:	*none*
GLP:	*no*

Executive Summary

In a 90 days semi-static toxicity laboratory study, pre-metamorphic tadpoles (*Xenopus laevis)* at stage 51 were exposed to 0 (solvent control), 1.0, 2.5, 10, 25 and 50 mg ethylenethiourea (ETU)/L (nominal) in groups of 20 tadpoles in glass aquaria containing 10 L water. Tadpoles were assessed daily for mortality, abnormal behavior and animals showing fore limb emergence (FLE). On the day of the first observation of FLE (exposure day 20), developmental stage and whole body length (WBL) were determined for all tadpoles. From exposure day 20 onward, individual tadpoles displaying FLE were removed from the tanks and WBL was measured. Histological analyses of the thyroid glands and gene expression analysis (TSHα and TSHβ mRNA in pituitary tissue, TRβA mRNA in brain tissue) with samples from tadpoles exhibiting FLE at stage 58. Tadpoles exposed to 50 mg ETU/L did not develop to stage 58, therefore stage 53/54-animals were assessed on days 28 and 90.

The biological results are based on the nominal concentrations. Analysis of developmental stages on exposure day 20 and monitoring of time to FLE revealed retardation and complete arrest of tadpole development at 25 and 50 mg ETU/L, respectively. Development was not affected by 1.0, 2.5 and 10 mg ETU/L. Histological alterations in the thyroid gland were observed in FLE-displaying tadpoles after exposure to 2.5, 10 and 25 mg ETU/L, as well as in developmentally arrested tadpoles exposed to 50 mg ETU/L. Prevalence and severity of histological changes increased in a concentration-dependent manner. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) showed increased mRNA expression of the α- and β-subunits of thyroid-stimulating hormone (TSHα, TSHβ) in pituitary tissue of tadpoles exposed to 25 and 50 mg ETU/L. Analysis of TRβA mRNA expression levels in brain tissue was the least sensitive endpoint with significantly lower expression levels only detected in brain tissue of tadpoles of the highest test item treatment group.

In a 90 day semi-static toxicity study tadpoles of *Xenopus laevis* exposed to ETU showed retardation and complete arrest of tadpole development at the two highest test item concentrations of 25 and 50 mg ETU/L. Histological alterations in the thyroid gland were observed at 2.5, 10, 25 and 50 mg ETU/L and prevalence and severity of histological changes increased in a concentration-dependent manner. Expression of the α- and β-subunits of the thyroid-stimulating hormone in pituitary tissue was significantly increased at the two highest test item concentrations of 25 and 50 mg ETU/L. Significantly lower expression levels of TRβA mRNA in brain tissue were only detected in tadpoles of the highest test item concentration.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: Ethylenethiourea (ETU) (Reg. no. 146 099; metabolite of metiram) obtained from Sigma; some relevant information (batch no., purity) is missing in the study report.

B. STUDY DESIGN

Test species: Tadpoles (*Xenopus laevis*); stage 51 tadpoles at day 14 post-fertilization; supplied by Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany.

Test design: Semi-static system (90 days); 20 tadpoles per aquarium (density 2 tadpoles/L); 3 replicates per test item concentration and solvent control; renewal of test solutions three times per week. Daily assessment of mortality and abnormal behavior; daily monitoring for tadpoles showing FLE; on the day of the first observation of FLE (exposure day 20), developmental stage and WBL were determined for all tadpoles (developmental stage was assessed by visual inspection, WBL measurements from the tip of snout to tail end). From exposure day 20 onward, individual tadpoles displaying FLE were removed from the tanks and WBL was measured. Histological analyses of the thyroid glands and gene expression analysis (TSHα and TSHβ mRNA in pituitary tissue, TRβA mRNA in brain tissue) by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) when individual tadpoles exhibited fore limb emergence at stage 58. Tadpoles exposed to 50 mg ETU/L did not develop to stage 58, therefore stage 53/54-animals were assessed on days 28 and 90.

Endpoints: Growth and development of tadpoles, histological alterations in the thyroid gland, gene expression (mRNA expression of TSHα and TSHβ in pituitary tissue, mRNA expression of TRβA in brain tissue).

Test concentrations: Solvent control (0.005% dimethylsulfoxide (DMSO)), 1.0, 2.5, 10, 25 and 50 mg ETU/L (nominal).

Test conditions: Glass aquaria (11 L), test volume 10 L, test aquaria were placed in a thermos-regulated water bath (22 ± 1 °C); dilution water: synthetic medium, formulated by adding 2.5 g of the commercial salt mixture "Tropic Marin Meersalz" to 10 liters of deionized water; pH: 7.0 ± 0.5; photoperiod 12 h light : 12 h dark; daily feeding with dry commercial fish food (Sera Micron), initial food ration 200 mg/tank, but increase of daily food ration during exposure to account for tadpole growth.

Analytics: Analytical verification of test item concentrations was not conducted.

Statistics: Descriptive statistics; Dunn's multiple comparison test ($p < 0.05$); Dunnett's test ($p < 0.05$); Tukey-Kramer multiple comparison test ($p < 0.05$).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was not conducted.

Biological results:
Effects on survival, growth and development: No signs of systemic toxicity were observed during the 90-day exposure of *Xenopus laevis* tadpoles to ETU. No gross morphological or behavioral abnormalities were detected at any treatment group, and only one tadpole died in one replicate tank of the 50 mg ETU/L treatment group on day 71. Growth of tadpoles, when assessed by means of WBL measurements on exposure day 20, was also not affected by ETU treatment. Exposure of tadpoles to ETU resulted in a concentration-dependent inhibition of metamorphic development. Determination of developmental stage of all test organisms on exposure day 20 revealed that 50 mg ETU/L caused a complete inhibition of metamorphic development at late pre-metamorphic stages 53/54. Significant developmental retardation was also detected for the 25 mg ETU/L treatment; however, metamorphosis was not completely inhibited, but if compared to the solvent control group proceeded much slower as evident from the observation of earlier developmental stages on exposure day 20. Treatment of tadpoles with 1.0, 2.5 and 10 mg ETU/L did not cause distinct differences in day 20 developmental stages.

Histological effects: Visual examination of thyroid gland gross morphology of ETU-exposed specimens revealed increases in glandular size at 10, 25 and 50 mg ETU/L. Histological examination of ETU-treated tadpoles revealed a variety of histological alterations, which are summarized in Table 8.2.8-7. Histological changes observed in thyroid glands from the 1.0 and 2.5 mg ETU/L treatment group included mild increases in peripheral vacuolation of the colloid (1.0 and 2.5 mg ETU/L) and mild follicle distension (2.5 mg ETU/L). The prevalence of peripheral colloid vacuolation was increased at 2.5 mg ETU/L. At 10 mg ETU/L, many thyroid glands showed a marked increase in follicle size, accompanied by partial colloid depletion. In some tadpoles, treatment with 10 mg ETU/L resulted in a foamy appearance and pale staining of the colloid. The follicular epithelium still consisted of a single layer of follicular cells. No signs of follicular cell hypertrophy were evident in thyroid glands from tadpoles exposed to concentrations up to and including 10 mg ETU/L. In the 10 mg ETU/L treatment group, epithelial cells appeared flattened compared to the control group. Thyroid glands of tadpoles exposed to 25 and 50 mg ETU/L showed a high prevalence of follicular cell hypertrophy and hyperplasia. Colloid depletion was generally enhanced in thyroid glands from these treatment groups as was evident from a foamy and faintly-staining colloid. Follicle size was markedly increased at 25 and 50 mg ETU/L, leading to pronounced diffuse enlargement of the glands. Results from epithelial cell height measurements revealed a biphasic response pattern to ETU exposure. Compared to the solvent control group, statistically significant increases in mean epithelial cell heights were observed in thyroid glands from tadpoles exposed to 25 and 50 mg ETU/L (Dunnett's test, $p < 0.05$).

Gene Expression Analysis: When determined in FLE-displaying tadpoles, no significant differences compared to the solvent control were detected in mRNA expression levels of TSHα and TSHβ in pituitary tissue at test item concentration of 1.0, 2.5 and 10 mg ETU/L, while mRNA expression of both TSH subunits was significantly increased in FLE-displaying tadpoles exposed to 25 mg ETU/L and in developmentally arrested tadpoles of the 50 mg ETU/L treatment group (Tukey-Kramer multiple comparison test, $p < 0.05$). For stage 53/54-arrested tadpoles from the 50 mg ETU/L treatment group, pituitary gene expression was analyzed separately for tissue samples collected on exposure days 28 and 90, respectively. At both time points, mRNA expression levels of TSHα and TSHβ were statistically significantly lower than in FLE-displaying tadpoles from the 25 mg ETU/L treatment group (Tukey-Kramer multiple comparison test, $p < 0.05$). No significant differences of TRβA mRNA expression levels in brain tissue were detectable at up to and including the second highest test item concentration of 25 mg ETU/L compared to the solvent control. Significantly lower expression levels of TRβA mRNA were only detected in brain tissue of stage 53/54-arrested tadpoles of the highest test item treatment group (Tukey-Kramer multiple comparison test, $p < 0.05$).

The results for all endpoints are summarized in Table 8.2.8-7

Table 8.2.8-7: Effects on growth and development of *Xenopus laevis* tadpoles and histological alterations in the thyroid gland after 90 day of exposure to ETU

Concentration [mg/L] (nominal)		Solvent control	1.0	2.5	10	25	50
Gross morphological parameters	Developmental stage on exposure day 20 [a)]	57 (55 - 58)	57 (56 - 58)	57 (55 - 58)	57 (55 - 58)	55 (53 - 57) *	54 (53 - 54) *
	WBL on day 20 [b)] [mm]	54.1 ± 4.4	55.5 ± 3.3	55.0 ± 3.5	54.0 ± 3.1	53.6 ± 3.8	54.4 ± 4.4
	Median time to FLE [days]	24	24	25	26	38 *	d.a.
	WBL at FLE [b)] [mm]	59.1 ± 3.7	59.6 ± 3.1	59.7 ± 3.8	59.2 ± 3.1	68.0 ± 9.7 **	69.6 ± 12.1 **, [c)]
Histological alterations in the thyroid gland	Histological alterations from developmental stage 51 until forelimb emergence (stage 58) #	PV	PV	PV, PD, FD, TE,	PV, PD, FD, TE, SC	PV, PD, FD, TE, SC, CH, HP	PV, PD, FD, TE, SC, CH, HP
	Epithelial cell height	--	-	-	↓ [d)]	↑ [d)]	↑ [d)]
Gene Expression Analysis	TSHα and TSHβ mRNA in pituitary tissue	--	-	-	-	↑ [e)]	↑ [e)]
	TRβA mRNA expression levels in brain tissue	--	-	-	-	-	↓ [e)]

d.a. - developmental arrest of tadpoles at stages 53/54.
\# Histological alterations at stage 58: PV - peripheral vacuolation of colloid, PD - partial depletion of colloid, FD - distension of follicles, TE -enlargement of thyroid gland, SC - foamy staining of colloid, CH - epithelial cell hypertrophy, HP - hyperplasia.
* Statistically significantly different compared to the solvent control (Dunn's test, $p < 0.05$).
** Statistically significantly different compared to the solvent control (Dunnett's test, $p < 0.05$).
\- No statistically significant effects compared to solvent control observed.
↓ Statistically significant decrease in the test item treatment compared to the solvent control.
↑ Statistically significant increase in the test item treatment compared to the solvent control.
a) Values given as median stage with the total range of stages in parentheses.
b) Values given as means and standard deviations.
c) Values given as means and standard deviations from day 90 measurements in developmentally arrested tadpoles.
d) Dunnett's test ($p < 0.05$).
e) Tukey-Kramer multiple comparison test ($p < 0.05$).

III. CONCLUSION

In a 90 day semi-static toxicity study tadpoles of *Xenopus laevis* exposed to ETU showed retardation and complete arrest of tadpole development at the two highest test item concentrations of 25 and 50 mg ETU/L. Histological alterations in the thyroid gland were observed at 2.5, 10, 25 and 50 mg ETU/L and prevalence and severity of histological changes increased in a concentration-dependent manner. Expression of the α- and β-subunits of the thyroid-stimulating hormone in pituitary tissue was significantly increased at the two highest test item concentrations of 25 and 50 mg ETU/L. Significantly lower expression levels of TRβA mRNA in brain tissue were only detected in tadpoles of the highest test item concentration.

From the performed literature search the following peer-reviewed scientific study investigating the ecological impact of metiram application (tested as formulated product BAS 222 28 F) to outdoor freshwater microcosms was considered relevant and reliable (with restrictions; RI 2) for the aquatic risk assessment of metiram (for details please see the literature search and evaluation files also provided within the submission for Annex I Renewal). The data have not been used or evaluated during the previous Annex I inclusion process and thus, relevant information and results of the study are described in the following summary.

Report: CA 8.2.8/6
Lin R. et al., 2012a
Effects of the fungicide Metiram in outdoor freshwater microcosms: Responses of invertebrates, primary producers and microbes
2012/1366942
Guidelines: none
GLP: no

Executive Summary

The ecological impact of metiram (tested as formulated product BAS 222 28 F) was studied in a 59 days outdoor freshwater microcosm study with 14 enclosures placed in an experimental ditch. The microcosms were treated three times (interval 7 days) with metiram. Intended nominal metiram concentrations in the overlying water were 0 (control), 4, 12, 36, 108 and 324 µg a.s./L. 2 replicates per test item treatment and 4 replicates for the control were employed. The control enclosures received water only. Responses of zooplankton, macroinvertebrates, phytoplankton, macrophytes, microbes and community metabolism endpoints were investigated.

The biological results are based on nominal concentrations. Significant treatment-related effects on the **zooplankton community** were detected at the highest test concentration on day 3 and in the 108 µg a.s./L and 324 µg a.s./L enclosures on days 10, 17 and 24. The most sensitive populations in the microcosms comprised representatives of Rotifera with a NOEC of 12 µg a.s./L on isolated sampling days and a NOEC of 36 µg a.s./L on consecutive samplings. Treatment-related effects of metiram on the **macroinvertebrate community** could not be demonstrated by means of multivariate PRC analysis. The only macroinvertebrate taxon for which statistical significant differences were observed on two consecutive samplings in the post-treatment period was Dytiscidae larvae (NOEC = 108 µg a.s./L), but this taxon occurred in low densities (always < 5 individuals per sample) and the effect concerned a treatment-related increase. Metiram application did not result in adverse effects on **phytoplankton community.** Although statistically significant responses could be observed for 42 taxa, the majority of these taxa showed a statistical significant response on an isolated sampling day only. The blue-green alga *Anabaena* sp. was one of the few phytoplankton taxa that showed a treatment-related decline in abundance on two consecutive sampling days (day 17 NOEC = 108 µg a.s./L and day 24 NOEC = 36 µg a.s./L), however this decline was followed by recovery.

No significant treatment- related effects on **above-sediment biomass** could be observed in treated enclosures when compared to controls. For the dominant **aquatic hyphomycetes on alder leaf material** statistically significant treatment-related effects could not be demonstrated. A statistically significant effect of metiram on **total fungal biomass** (increase) associated with alder leaf litter could be observed on sampling day 3 only (NOEC = 4 µg a.s./L). This effect, however, did not show a clear concentration-response relationship. PRC analysis indicated that **sediment bacterial community structure** differed significantly between control and metiram-treated enclosures (NOEC = 108 µg a.s./L). However, because these differences between control and treated enclosures were present pre-application (i.e. day -4) they cannot be attributed to the metiram treatments. PRC analyses detected no significant effect of metiram application on the **sediment fungal community structure**.

In a 59 days outdoor freshwater microcosm study, consistent treatment-related effects of metiram on phytoplankton and macroinvertebrate communities and on the sediment microbial community could not be demonstrated or were minor. There was no evidence that metiram affected the biomass, abundance or functioning of aquatic hyphomycetes on decomposing alder leaves. The most sensitive populations in the microcosms comprised representatives of Rotifera (zooplankton) with a NOEC of 12 µg a.s./L on isolated sampling days and a NOEC of 36 µg a.s./L on consecutive samplings and hence the overall community and population level NOEC ($NOEC_{microcosm}$) was 12 - 36 µg a.s./L. At higher treatment levels, including the test systems that received the highest dose, ecological recovery of affected measurement endpoints was fast (effect period < 8 weeks).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, content of a.s.: metiram (BAS 222 F, Reg. no. 250284): 70.39% (w/w; nominal: 70.0%; density: 1.112 g/cm^3. Some relevant information (batch no.) is missing in the study report.

B. STUDY DESIGN

Test system: Outdoor freshwater microcosms consisting of 14 enclosures (polycarbonate, translucent cylinders, diameter: 1.05 m; surface area 0.865 m^2; height: 0.9 m) placed in an experimental ditch located at the Sinderhoeve Experimental Station, Renkum, the Netherlands. The enclosures were pushed approximately 0.15 m into the sandy loam sediment of the ditch (water depth: approx. 0.5 m) and were installed into the experimental ditch 25 days before start of treatment. The enclosures simulated a shallow, mesotrophic, macrophyte-dominated freshwater community, fish were not present. 30 individuals of *Gammarus pulex* and 28 individuals of *Asellus aquaticus* were introduced in each enclosure.

Test design: Static system (59 days), 5 test item concentrations plus control, 2 replicates per test item treatment and 4 replicates for the control; 3 applications of test item (on days 0, 7 and 14); the control enclosures received water only.
Endpoints investigated in microcosm study:
Physico-chemical parameters: pH, dissolved oxygen, temperature, and electrical conductivity on days -1, 3, 10, 17, 24, 31, 48, 59; alkalinity on days -1, 17, 59; nutrients on days -1, 59.
Macroinvertebrates (species composition, abundance) on days -12, 15, 29, 43, 57; **Zooplankton** (species composition, abundance) on days -1, 3, 10, 17, 24, 31, 48, 59; **Phytoplankton** (species composition, abundance, chlorophyll a) on days -1, 3, 10, 17, 24, 31, 48, 59; **Macrophytes (**above ground biomass) on days -14, 62; **Microbes and decomposition (**fungal biomass on leaves, fungal species abundance on leaves, leaf decomposition on days -4, 3, 10, 17, 31, 52 and sediment bacterial and fungal community structure on days -4, 3, 10, 17, 24, 31, 48, 59).
Sampling methods:
Artificial substrates, in the form of litter bags and pebble baskets, were used to monitor the **macroinvertebrate community**. Two litter bags (initially containing 2 g dry weight of Populus leaves) and two pebble baskets were incubated in the enclosures for approximately 2 weeks prior to sampling.
Zooplankton and phytoplankton sampling with depth-integrated water samples (approximately 10 L volume).
Concentrations of **total phytoplankton chlorophyll *a*** were measured using a BBE AlgaeTorch.

Pre-application **above-sediment macrophyte biomass** was assessed by sampling three representative plots (0.25 m^2) inside the study ditch but outside of the enclosures. The above-sediment macrophyte biomass was sampled from each enclosure at the end of the study.
Fungal biomass, aquatic hyphomycete abundance and leaf decomposition were assessed using 15 fine (600 µm) and 15 coarse mesh (0.5 cm x 0.5 cm) bags per enclosure containing 5 or 8 g of air-dried alder leaf material (pre-conditioned in an experimental ditch for 4 weeks), which were allocated to the enclosures 4 days before the first application.
Effects on **microbes in the sediment** were studied by taking 3 cores of the upper 3 cm of sediment from each control enclosure and from the two highest treatment enclosures on the respective sampling dates.

Endpoints: NOEC values at taxon level, NOEC values at the community level of zooplankton, macroinvertebrate, phytoplankton, sediment bacterial and sediment fungal communities.

Test concentrations: Control, 4, 12, 36, 108 and 324 µg a.s./L (nominal).

Test conditions: Water temperature: 18 - 19 °C at the start of the experiment and during the metiram application period, temperatures gradually declined from sampling day 17 onwards, the lowest water temperature measured was approx. 12 °C; air temperature: 12.1 - 22.4 °C; pH of control enclosures: 7.2 - 9.0; dissolved oxygen: > 4 mg/L; alkalinity in control enclosures: 1.08 - 1.24 mmol/L; total soluble nitrogen: 0.4 - 1.1 mg/L; nitrate/nitrite, ammonium, ortho-phosphate and total phosphate concentrations were below detection limits.

Analytics: Analytical verification of metiram concentrations was conducted using a LC method with tandem mass spectrometric detection (samples of the dosing solutions and samples taken 2 h after each application) or using a LC method with MS/MS detection (water samples collected on day 17 and on day 59. Analytical measurements of metiram metabolites were conducted using a LC method with MS/MS detection.

Statistics: William's test for NOEC calculations at taxon or parameter level ($p < 0.05$); principle response curves (PRC) method for analysis of effects on the zooplankton, macroinvertebrate, phytoplankton, sediment bacterial and sediment fungal communities, in addition to the overall significance of the effects of the treatment regime (Monte Carlo permutation tests; $p < 0.05$), each treatment was also compared to the controls to identify the NOEC at the community level using William's test ($p < 0.05$).

II. RESULTS AND DISCUSSION

Analytical measurements: Analytical verification of test item concentrations was conducted by analysing the metiram concentration of the dosing solutions before application and in water samples taken from each enclosure 2 h after each application. In addition, water samples were collected on day 17 (3 days after the last metiram application) and on day 59 (end of experiment) from enclosures that received the three highest treatment levels and were analysed for concentrations of metiram and its degradates ethylene-thiourea (ETU), ethylene-urea (EU), hydantoin (HY), carbamid, ethylene bisisothiocarbamate (EBIS) and $C_8H_8N_4S_2$ (TDIT).

Metiram concentrations in the dosing solutions were on average 92.7% of the intended concentration (range of 79.0% to 113.4%), but concentrations in water samples collected approximately 2 h after the first application were only 36.6% (range of 16.0% to 65.1%) of the initial concentration, highlighting the rapid disappearance of metiram from the water compartment. The water samples collected 2 h after the second and third application could not be analyzed due to technical problems during metiram analysis.
On day 17, the average concentration in water samples collected from the 324 µg a.s./L enclosures was 0.14 mg a.s./L (0.04% of the initial concentration) and no metiram was detected in samples from the 108 µg a.s./L and 36 µg a.s./L enclosures (< 0.05 µg a.s./L).

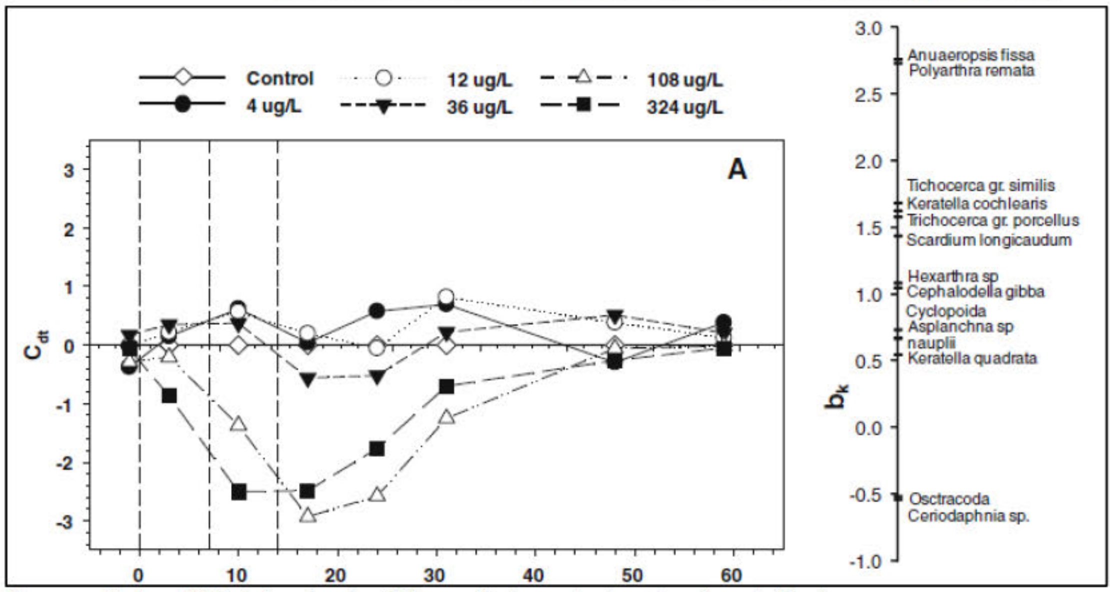
Average concentrations of the metabolites EU and ETU in the day 17 water samples from the 324, 108 and 36 mg a.s./L enclosures, were 38.8 mg EU/L and 12.2 µg ETU/L, 15.6 µg EU/L and 0.6 µg ETU/L and 4.3 µg EU/L and 0.13 µg ETU/L, respectively. All other degradates analyzed were below detection limits (i.e. < 20 µg/L for HY; < 0.2 µg/L for EBIS, carbimid and TDIT). At the end of the experiment the concentrations of metiram and all metabolites analysed were below detection limits (metiram< 0.05 µg/L; EU < 1.0 µg/L; HY < 20 mg/L; ETU, EBIS, carbimid and TDIT < 0.2 µg/L). These data illustrate that metiram dissipates very fast (estimated water dissipation DT_{50} of approximately 1 - 6 h) and that its metabolites are not persistent in the water compartment. The following biological results are based on nominal concentrations.

Biological results:
Physico-chemical measurements
A significant, but small, treatment-related increase in **electronic conductivity** was observed at the highest treatment-level on the days 10 and 17. The **pH** values of the control enclosures were significantly lower than that of treatment enclosures in the pre-treatment period (day -1) and immediately after first metiram application (day 3). However, on sampling days 10, 17 and 48, pH values showed a small, but statistically significant, treatment-related decrease and on day 59 there was a significant increase in pH although all deviations were less than 1 pH unit. A small but significant decline in **dissolved oxygen** was observed on day 17 in the 324 µg a.s./L treatment, and on day 48 dissolved oxygen levels were significantly higher in all treated enclosures relative to controls. All treatment-related differences in dissolved oxygen concentration were less than 1 - 2 mg/L. On day 17, a small but significant treatment-related increase in **alkalinity** was measured, while at the end of the experiment (day 59) a small but significant treatment-related decrease was observed. Treatment-related effects on **nutrient concentrations** in the water column could not be demonstrated.

Zooplankton responses

The most abundant zooplankton taxa in decreasing order were: *Anuraeopsis fissa* (Rotifera), copepod nauplii (Copepoda), *Polyarthra remata* (Rotifera), *Trichocerca gr. similis* (Rotifera), *Keratella cochlearis* (Rotifera), *Lecane gr. luna* (Rotifera), Cyclopoida (Copepoda), *Ceriodaphnia* sp. (Cladocera), *Trichocerca gr. porcellus* (Rotifera) and *Squatinella rostrum* (Rotifera). The number of zooplankton taxa was significantly reduced relative to controls at the highest dose 10 days after the first application. A statistically non-significant decline in zooplankton richness was observed in the post-exposure period (days 17 - 24) in the two highest doses. Significant treatment-related effects on the zooplankton community were detected at the highest test concentration on day 3 and in the 108 µg a.s./L and 324 µg a.s./L enclosures on days 10, 17 and 24 (see Figure 8.2.8-1). The most sensitive populations in the microcosms comprised representatives of Rotifera with a NOEC of 12 µg a.s./L on isolated sampling days and a NOEC of 36 µg a.s./L on consecutive samplings.

Figure 8.2.8-1: Principal response curve diagram for the zooplankton dataset (the vertical dotted lines indicate days of metiram application)



C_{dt} - canonical coefficient showing the difference between treatments and control in time
B_k - species weight that indicates the affinity of the taxon with the PRC

Macroinvertebrate responses

The most abundant macroinvertebrate taxa in decreasing order were: *Dero* sp.(Oligochatea), *Chaoborus* sp. (Insecta), Chironomini (Insecta), *Mesostoma* sp. (Turbellaria), *Lumbriculus* sp. (Oligochaeta), *Orthocladinae* (Insecta), *Ceratopogonidae* (Insecta), *Caenis* sp. (Insecta), *Zygoptera* (Insecta) and *Dugesia* lugubris (Turbellaria). A small decrease in the number of macroinvertebrate taxa relative to controls could be observed on day 15 (one day after the third metiram application) in the enclosures that received the highest test item concentration.

Treatment-related effects of metiram on the macroinvertebrate community could not be demonstrated by means of multivariate PRC analysis (Monte Carlo permutation test, $p = 0.83$). Although statistically significant differences between treatments and controls could be observed for 15 of the 63 macroinvertebrate taxa ($NOEC_{macroinvertebrates}$ = 36 µg a.s./L), these deviations predominantly occurred on isolated sampling days. The only macroinvertebrate taxon for which statistical significant differences were observed on two consecutive samplings in the post-treatment period (days 43 and 57) was Dytiscidae larvae (NOEC = 108 µg a.s./L), but this taxon occurred in low densities (always < 5 individuals per sample) and the effect concerned a treatment-related increase.

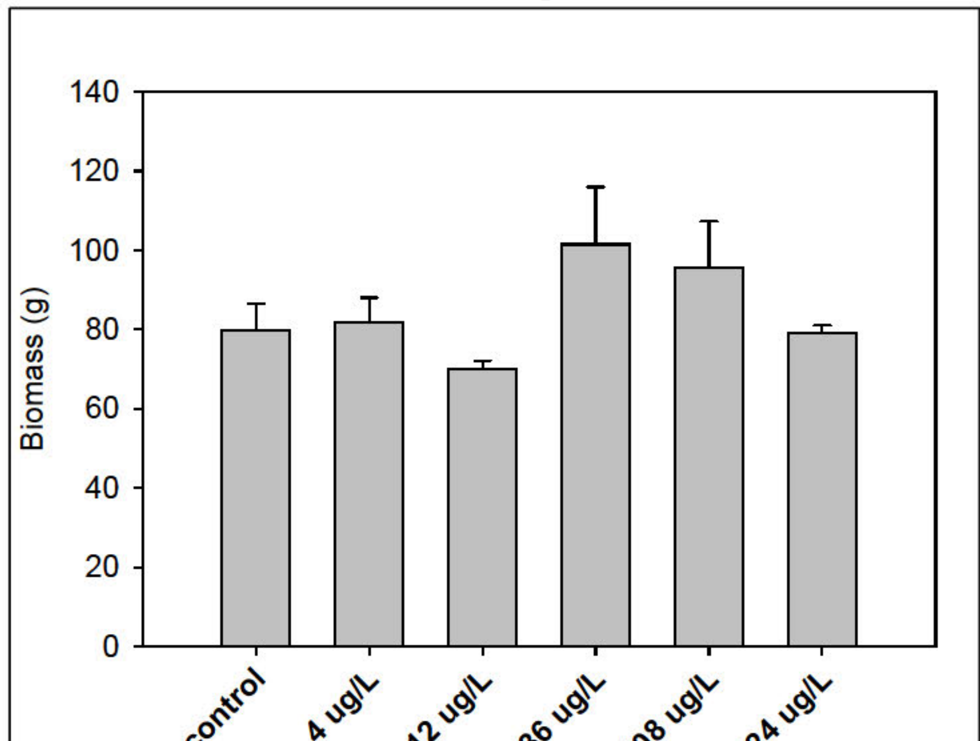
Phytoplankton responses

The most abundant phytoplankton taxa in decreasing order were: *Volvox* (Chlorophyta), *Scenedesmus arcuatus* (Chlorophyta), *Tetraedron minimum* (Chlorophyta), Pennales (Diatomeae), Pseudanabaenaceae (Cyanophyta), *Phacotus lendneri* (Chlorophyta), *Aphanocapsa* (Cyanophyta), *Anabaena* (Cyanophyta), *Oocystis* (Chlorophyta) and *Aphanothece* (Cyanophyta). A small decrease in the number of phytoplankton taxa relative to controls was observed on day 17 at the highest test item concentration. There was little evidence of a treatment-related response in total chlorophyll *a* biomass with significant reductions only observed on day 31 at the highest concentration. PRC analysis demonstrated that metiram treatment did not explain a significant component of the variation in phytoplankton community composition (Monte Carlo permutation test, $p = 0.544$). Nevertheless, statistically significant treatment-related effects could be calculated for 42 of the 109 phytoplankton taxa (not including the abundance of main taxonomic groups), although the vast majority of these taxa (37 out of 42) showed a statistical significant response on an isolated sampling day only and mostly concerned low density taxa (< 10 individuals/ml). In addition, statistically significant responses were related to both decreases (15 cases) and increases (27 cases) in abundance and were mostly observed in the highest treatment only.
The blue-green alga *Anabaena* sp. was one of the few phytoplankton taxa that showed a treatment-related decline in abundance on two consecutive sampling days (day 17 NOEC = 108 µg a.s./L and day 24 NOEC = 36 µg a.s./L), however this decline was followed by recovery.

Biomass of macrophytes

Prior to the metiram application the above-sediment macrophyte biomass was estimated to be 58.6 ± 13.4 g dry weight per enclosure (geomean ± SD; n = 3). At the end of the study the above-sediment macrophyte biomass in control enclosures had increased to 79.8 ± 6.8 g dry weight (geomean ± SD; n = 4), but no significant treatment- related effects on above-sediment biomass could be observed in the treated enclosures when compared to controls (see Figure 8.2.8-2).

Figure 8.2.8-2: Above sediment dry weight biomass (mean + SD) of macrophytes in the enclosures at the end of the metiram experiment



Microbial endpoints and alder leaf decomposition

The dominant **aquatic hyphomycetes on pre-conditioned alder leaf material** were *Angillospora longissima* and *Tetracladium setigerum*. For both species statistically significant treatment-related effects could not be demonstrated (William's test, $p > 0.05$) despite the trend in lower abundance for *T. setigerum* in most enclosures that received metiram.

A statistically significant effect of metiram on **total fungal biomass** (increase) associated with alder leaf litter could be observed on sampling day 3 only (NOEC = 4 µg a.s./L;). This effect, however, did not show a clear concentration-response relationship.

PRC analysis indicated that **sediment bacterial community structure** differed significantly between control and metiram-treated enclosures (NOEC = 108 µg a.s./L) (Monte Carlo permutation test, $p < 0.05$). However, because these differences between control and treated enclosures were present pre-application (i.e. day -4) they cannot be attributed to the metiram treatments. PRC analyses detected no significant effect of metiram application on the **sediment fungal community structure** (Monte Carlo permutation test, $p > 0.05$).

Ecological recovery

Fast recovery was found for all affected endpoints (effect period < 8 weeks). Short-term exposure to metiram (overall dissipation DT_{50} 1 - 6 h) and the short generation time of the sensitive populations affected explains the fast ecological recovery despite repeated application.

III. CONCLUSION

In a 59 days outdoor freshwater microcosm study, consistent treatment-related effects of metiram on phytoplankton and macroinvertebrate communities and on the sediment microbial community could not be demonstrated or were minor. There was no evidence that metiram affected the biomass, abundance or functioning of aquatic hyphomycetes on decomposing alder leaves. The most sensitive populations in the microcosms comprised representatives of Rotifera (zooplankton) with a NOEC of 12 µg a.s./L on isolated sampling days and a NOEC of 36 µg a.s./L on consecutive samplings and hence the overall community and population level NOEC ($NOEC_{microcosm}$) was 12 - 36 µg a.s./L. At higher treatment levels, including the test systems that received the highest dose, ecological recovery of affected measurement endpoints was fast (effect period < 8 weeks).

References

EFSA (2013) EFSA Scientific Opinion. Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. EFSA Journal 2013; 11(7): 3290.

European Commission (2013) Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with the Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. OJ L 93, 3.4.2013, p. 1–84.

OECD (2011) OECD Guidelines for the Testing of Chemicals, Guideline 201, Freshwater Algae and Cyanobacteria, Growth Inhibition Test. OECD Publishing. Adopted: 23 March 2006, Annex 5 corrected: 28 July 2011. pp. 25.

CA 8.3 Effects on arthropods

CA 8.3.1 Effects on bees

Since Annex I inclusion of metiram, new toxicity studies on the active substance (tested with the solo-formulation BAS 222 28 F) have been performed and as a result there are new endpoints which are now used in the risk assessment. Summaries of these new studies are provided below.

Details on the EU agreed studies which have been already evaluated within the Annex I inclusion of metiram are provided in the EU Review documents of metiram (Monograph, Vol. 3, Annex B.9, July 2000; Addendum to the Monograph, Annex B.9, June 2002 and April 2004; EC Review Report (SANCO/4059/2001-rev 3.3), June 2005).

Furthermore, summaries on the EU agreed studies which are still relevant for the risk assessment are also provided below. For better transparency and traceability of the active substance history, the results of all studies are summarized in Table 8.3.1-1.

Table 8.3.1-1: Toxicity of metiram to arthropods

Substance	Test species	Endpoint	Value	Reference (BASF DocID)	EU agreed
metiram [1]	honeybee	72 h acute oral LD_{50}	> 80.0 µg a.s./bee	Anonymous 1987/10164	yes
		72 h acute contact LD_{50}	> 80.0 µg a.s./bee		
metiram [2]	honeybee	48 h acute oral LD_{50}	> 110.1 µg a.s./bee	Schmitzer 2005/1012879	no, new study
		48 h acute contact LD_{50}	> 100.0 µg a.s./bee		
	bumblebee	96 h acute oral LD_{50}	> 374 µg a.s./bumblebee	Amsel 2014/1083451	no, new study
		96 h acute contact LD_{50}	> 400 µg a.s./bumblebee		
	honeybee	10 d chronic LD_{50}	> 49.248 µg a.s./bee/day	Kleebaum 2012/1189907	no, new study
		10 d chronic NOED	≥ 49.248 µg a.s./bee/day		
		10 d chronic LC_{50}	> 2.563 g a.s./kg food		
		10 d chronic NOEC	≥ 2.563 g a.s./kg food		
		72 h acute oral larvae LD_{50}	75.3 µg a.s./larva	Kleebaum 2012/1189906	no, new study
		72 h acute oral larvae NOED	24.8 µg a.s./larva		
		72 h acute oral larvae LC_{50}	2.222 g a.s./kg food		
		72 h acute oral larvae NOEC	0.731 g a.s./kg food		
		semi-field tunnel test	no unacceptable lethal or sub-lethal effects on honeybee colonies exposed to 1.4 kg a.s./ha	Classen 2012/1111495	no, new study

[1] Endpoint taken from EC Review Report (2005). Study was performed with BAS 222 28 F (80% metiram complex).

[2] Studies was conducted with the solo-formulation BAS 222 28 F (containing 70% metiram, nominally).

CA 8.3.1.1 Acute toxicity to bees

CA 8.3.1.1.1 Acute oral toxicity

The following acute study on honeybees (Apis mellifera) performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.3.1.1.1/1* *Anonymous, 1987a* *Results of the laboratory investigation of BAS 222 28 F on toxicity to bees* *1987/10164*
Guidelines:	*Drescher W. et al. (Dec. 1975) Provisional guidelines for testing crop protection agents for their danger to bees 23-1*
GLP:	*no*

Executive Summary

In a limit test, worker bees (*Apis mellifera*) were exposed to BAS 222 28 F. The toxicity of the test product was determined in an oral test at a nominal concentration of 100 µg/bee (equivalent to 80 µg a.s./bee). Additionally, honeybees were treated with Nexit liquid as reference item or with a solution of water and sugar as control. The test was conducted at three different test sites with 3 replicates; each of the test cages contained 10 bees. Assessment of mortality was done after 24 hours.

Mortality of 0%, 3% and 10% was observed after 24 hours at the three different test sites. No behavioral abnormalities of the bees could be observed.

Toxicity of BAS 222 28 F on honeybees was tested in an acute oral toxicity test. The LD_{50} value (72 h) was >100 µg BAS 222 28 F/bee, corresponding to > 80 µg a.s./bee.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, content of a.s.: Metiram 80% complex (BAS 222 F, Reg. No. 250 284): 80.0%.

B. STUDY DESIGN

Test species: Honeybee (*Apis mellifera*), field bees, caught while sweeping off the outer honeycombs.

Test design: Limit test for oral toxicity; duration 72 h, 3 replicates, each replicate consisting of 10 bees per cage; assessment of mortality after 72 hours. The test was conducted at three different test sites: 1. Bayrische Landesanstalt für Bienenzucht, Erlangen; 2. Zoologisches Institut, Universität Saarbrücken; 3. Niedersächsisches Landesinstitut für Bienenforschung, Celle.

Endpoints: LD_{50} value, behavioral abnormalities.

Reference item: Nexit liquid (0.005% at test sites 1 and 2, 0.025% at test site 3).

Test concentrations: Control, 100 µg/bee, concentration of spray mixture 1.6%.

Test conditions: Temperature: 20 °C - 25 °C, feeding with 50% sugar solution.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

Mortality of 0%, 3% and 10% was observed after 72 hours at the three different test sites. No behavioral abnormalities of the bees could be observed. The results are summarized below in Table 8.3.1.1.1-1.

Table 8.3.1.1.1-1: Toxicity of BAS 222 28 F to honeybees (*Apis mellifera*) in an oral toxicity test

Treatment	Uptake of test item	Mean mortality [%]
[µg/bee]	[µg/bee]	72 h
test site 1: Control	--	0
test site 1: 100	100	0
test site 2: Control	--	0
test site 2: 100	100	10
test site 3: Control	--	0
test site 3: 100	100	3
		Endpoint
LD_{50}		> 100 µg/bee (corresponding to > 80 µg a.s./bee)

III. CONCLUSION

Toxicity of BAS 222 28 F on honeybees was tested in an acute oral toxicity test. The LD_{50} value (72 h) was >100 µg BAS 222 28 F/bee, corresponding to > 80 µg a.s./bee.

The following acute study on honeybees (Apis mellifera) performed with metiram (tested as BAS 222 28 F) is provided in support of the risk assessment and has not been evaluated previously on EU level.

Report: CA 8.3.1.1.1/2
Schmitzer S., 2006a
Effects of BAS 222 28 F (acute contact and oral) on honey bees (Apis mellifera L.) in the laboratory
2005/1012879
Guidelines: OECD 213, OECD 214
GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Laendlichen Raum und Verbraucherschutz)

Executive Summary

In a limit test, worker bees (*Apis mellifera*) were exposed to BAS 222 28 F. The toxicity of the test product was determined in an oral test at a nominal concentration of 100 µg a.s./bee, resulting in an actual uptake of 110.1 µg a.s./bee. Additionally, honeybees were treated with Dimethoate as reference standard at 0.04, 0.08, 0.17 and 0.34 µg Dimethoate/bee (nominal) or with a solution of water and sugar as a control. The test was conducted with 5 replicates; each of the test cages contained 10 bees. Assessment of mortality was done after 4, 24 and 48 hours.

No mortality was observed after 48 hours in the test item. In the control treatment a mortality rate of 2% after 48 hours was observed. No behavioral abnormalities of the bees could be observed.

The oral LD_{50} value (48 h) of BAS 222 28 F was > 157 µg product/bee, equivalent to >110.1 µg a.s./bee.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2002-1; content of a.s.: metiram (BAS 222 F, Reg. No. 250 284): 70.0% (70.5% analyzed).

B. STUDY DESIGN

Test species: Honeybee (*Apis mellifera*), worker bees (4 - 6 weeks old); deriving from a healthy and queen-right colony, bred in-house.

Test design: Limit test for oral toxicity; duration 48 h, 5 replicates, each replicate consisting of 10 bees per cage; assessment of mortality after 4, 24 and 48 hours.

Endpoints: LD_{50} value, behavioral abnormalities.

Reference item: Perfekthion (dimethoate, nominal 400 g/L).

Test concentrations: Control, 100 µg a.s./bee (nominally equivalent to 143 µg product/bee); resulting in an actual uptake of 110.1 µg a.s./bee (nominally equivalent to 157 µg product/bee).

Test conditions: Temperature: 25 °C; relative humidity: 58% - 61%, photoperiod: 24 h darkness.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

No mortality was observed after 48 hours in the test item. In the control treatment a mortality rate of 2% after 48 hours was observed. No behavioral abnormalities of the bees could be observed. The results are summarized below Table 8.3.1.1.1-2.

Table 8.3.1.1.1-2: Toxicity of BAS 222 28 F to honeybees (*Apis mellifera*) in an oral toxicity test

Treatment	Uptake of test item	Mean mortality [%]	
[µg a.s./bee]	[µg a.s./bee]	24 h	48 h
Control	--	0	2
100	110.1	0	0
Endpoint [µg a.s./bee]			
LD_{50}		> 110.1	

III. CONCLUSION

The oral LD_{50} value (48 h) of BAS 222 28 F was >157 µg product/bee, equivalent to >110.1 µg a.s./bee.

The following acute study on bumblebees (Bombus terrestris) performed with metiram (tested as BAS 222 28 F) is provided in support of the risk assessment and has not been evaluated previously on EU level.

Report:	CA 8.3.1.1.1/3 Amsel K., 2014b Acute toxicity of BAS 222 28 F to the bumblebee Bombus terrestris L. under laboratory conditions 2014/1083451
Guidelines:	OECD 213 (1998), OECD 214 (1998), EFSA Guidance Document on bees (2013)
GLP:	yes (certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

Executive Summary

In an oral toxicity test, bumblebees (young adult worker bumblebees of *Bombus terrestris*) were exposed to BAS 222 28 F. The toxicity of the test item was determined at nominal doses of 36, 71, 143, 286 and 572 µg BAS 222 28 F/bumblebee (equivalent to 25, 50, 100, 200 and 400 µg a.s./bumblebee), resulting in an actual uptake of 35, 70, 141, 283 and 534 µg BAS 222 28 F/bumblebee. Additionally, bumblebees were treated with dimethoate as a reference item at dose rates ranging from 0.31 to 2.5 µg dimethoate/bumblebee (nominal) or with a 50% (w/v) sucrose solution as a control.

After 96 hours of oral exposure, 0.0% mortality was observed in the control. In the test item treatment, no mortality occurred after oral consumption of 35, 70, 141, 283 and 534 µg BAS 222 28 F/bumblebee. Furthermore, no behavioral abnormalities of surviving bumblebees occurred throughout the oral toxicity test.

In an oral toxicity study with BAS 222 28 F on bumblebees, the LD_{50} value (96 h) was estimated to be > 534 µg BAS 222 28 F/bumblebee, corresponding to > 374 µg consumed a.s./bumblebee.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 222 28 F; batch no. FRE-000977; content of a.s.: metiram (BAS 222 F, Reg. No. 250 284): 70 g/L (nominal), 69.7 g/L (analyzed); density: 1.74 g/cm^3.

B. STUDY DESIGN

Test species: *Bombus terrestris* (bumblebee), young adult worker bumblebees derived from a healthy and queen-right micro-hive; source: Biobest Belgium N.V., Ilse Velden 18, 2260 Westerlo Belgium; collected in the morning prior to use; starving period: 2 hours.

Test design: In a 96 hour test, adults of worker bumblebees of *Bombus terrestris* were exposed orally to BAS 222 28 F via food (50 % (w/v) aqueous sucrose solution). In total, 3treatment groups were set up (5 dose rates of the test item, 1 untreated control and 4 dose rates of the reference item) with 30 replicates per dose and 1 bumblebee per replicate. Assessment of bumblebee mortality and behavioral effects was done after 4, 24, 48, 72 and 96 hours.

Endpoint: Mortality, behavioral impairments.

Reference item: BAS 152 11 I (dimethoate, nominal 400.0 g/L).

Test concentrations: Water control (deionized water), Tween control (1% (v/v) Tween solution), Reference item at dose rates of 2.5, 5.0, 10.0 and 20.0 µg dimethoate/bumblebee,
Test item at dose rates:

Nominal product dose rate (µg/bumblebee)	Nominal a.s. dose rate (µg/bumblebee)	Consumed product (µg/bumblebee)	Consumed a.s. (µg/bumblebee)
36	25	35	25
71	50	70	49
143	100	141	98
286	200	283	198
572	400	534	374

Test conditions: Temperature: 25.2 °C – 25.5 °C, relative humidity: 56% – 58%, photoperiod: 24 h darkness; food: 50% (w/v) sucrose solution.

Statistics: Descriptive statistics; Fisher's Exact Binomial Test with Bonferroni Correction for mortality data (one-sided greater, $\alpha = 0.05$).

II. RESULTS AND DISCUSSION

After 96 hours of oral exposure, 0.0% mortality was observed in the control. In the test item treatment, no mortality occurred after oral consumption of 35, 70, 141, 283 and 534 µg BAS 222 28 F/bumblebee. Furthermore, no behavioral abnormalities of surviving bumblebees occurred throughout the oral toxicity test. The results are summarized in Table 8.3.1.1.1-3.

Table 8.3.1.1.1-3: Toxicity of BAS 222 28 F to *Bombus terrestris* (bumblebee) in an oral toxicity test

Treatment	Uptake of test item	Mortality [%]			
[µg BAS 222 28 F/bee]		24 h	48 h	72 h	96 h
Control	--	0.0	0.0	0.0	0.0
36	35	0.0	0.0	0.0	0.0
71	70	0.0	0.0	0.0	0.0
143	141	0.0	0.0	0.0	0.0
286	283	0.0	0.0	0.0	0.0
572	534	0.0	0.0	0.0	0.0
	Endpoint (based on actual uptake)				
	[µg a.s./bee]		**[µg BAS 222 28 F/bee]**		
LD_{50} (96 h)	> 374		> 534		

III. CONCLUSION

In an oral toxicity study with BAS 222 28 F on bumblebees, the LD_{50} value (96 h) was estimated to be > 534 µg BAS 222 28 F/bumblebee, corresponding to > 374 µg consumed a.s./bumblebee.

CA 8.3.1.1.2 Acute contact toxicity

The following acute study on honeybees (Apis mellifera) performed with metiram (tested as BAS 222 28 F) has already been evaluated on EU level during previous Annex I inclusion process for metiram. Nevertheless a study summary is provided below.

Report:	*CA 8.3.1.1.2/1* *Anonymous, 1987a* *Results of the laboratory investigation of BAS 222 28 F on toxicity to bees* *1987/10164*
Guidelines:	*Drescher W. et al. (Dec. 1975) Provisional guidelines for testing crop protection agents for their danger to bees 23-1*
GLP:	*no*

Executive Summary

In a limit test, worker bees (*Apis mellifera*) were exposed to BAS 222 28 F. The toxicity of the test product was determined by exposing the bees via direct overspray and via treated piece of paper folded to fit the test cage. Spray solution per cage at a concentration of 1.6%. Additionally, honeybees were treated with Nexit liquid as reference item or with water as a control. The test was conducted at three different test sites with 3 replicates; each containing 10 bees. Assessment of mortality was done after 24, 48 and 72 hours.

Mortality ranging from 0% to 13% was observed in the test item group and the control after 72 hours. After 48 hours mortality of 3% occurred in one of the control treatments. No behavioral abnormalities of the bees could be observed.

Toxicity of BAS 222 28 F on honeybees was tested in an acute contact toxicity test. The LD_{50} value (72 h) was > 100 BAS 222 28 F/bee, corresponding to > 80 µg a.s./bee.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, content of a.s.: Metiram 80% complex (BAS 222 F, Reg. No. 250 284): 80.0%.

B. STUDY DESIGN

Test species: Honeybee (*Apis mellifera*), field bees, caught while sweeping off the outer honeycombs.

Test design: Limit test for contact toxicity; the bees were exposed via direct overspray and via treated piece of paper folded to fit the test cage. The duration of both exposure scenarios was 72 h, each contained 3 replicates, each replicate consisting of 10 bees per cage; assessment of mortality after 24, 48 and 72 hours. The test was conducted at three different test sites: 1. Bayrische Landesanstalt für Bienenzucht, Erlangen; 2. Zoologisches Institut, Universität Saarbrücken; 3. Niedersächsisches Landesinstitut für Bienenforschung, Celle.

Endpoints: LD_{50} value, behavioral abnormalities.

Reference item: Nexit liquid (0.005% at test sites 1 and 2, 0.025% at test site 3).

Test concentrations: Control, 100 µg/bee, concentration of spray mixture 1.6%.

Test conditions: Temperature: 20 °C - 25 °C, feeding with 50% sugar solution.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

Mortality ranging from 0% to 13% was observed in the test item group and the control after 72 hours. After 48 hours mortality of 3% occurred in one of the control treatments. No behavioral abnormalities of the bees could be observed. The results are summarized below in Table 8.3.1.1.2-1.

Table 8.3.1.1.2-1: Toxicity of BAS 222 28 F to honeybees (*Apis mellifera*) in a contact toxicity test

Treatment	Mean mortality [%]		
[µg/bee]	24 h	48 h	72 h
Exposure via treated paper			
test site 1: Control	0	0	0
test site 1: 100	0	0	0
test site 2: Control	0	0	7
test site 2: 100	0	0	13
test site 3: Control	0	0	13
test site 3: 100	0	0	13
Exposure via direct overspray			
test site 1: Control	0	3	3
test site 1: 100	0	0	0
test site 2: Control	0	0	13
test site 2: 100	0	0	13
test site 3: Control	0	0	3
test site 3: 100	0	0	10
	Endpoint [µg a.s./bee]		
LD_{50}	> 100 µg/bee (corresponding to > 80 µg a.s./bee)		

III. CONCLUSION

Toxicity of BAS 222 28 F on honeybees was tested in an acute contact toxicity test. The LD_{50} value (72 h) was > 100 µg BAS 222 28 F/bee, corresponding to > 80 µg a.s./bee.

The following acute study on honeybees (Apis mellifera) performed with metiram (tested as BAS 222 28 F) is provided in support of the risk assessment and has not been evaluated previously on EU level.

Report: CA 8.3.1.1.2/2
Schmitzer S., 2006a
Effects of BAS 222 28 F (acute contact and oral) on honey bees (Apis mellifera L.) in the laboratory
2005/1012879
Guidelines: OECD 213, OECD 214
GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Laendlichen Raum und Verbraucherschutz)

Executive Summary

In a limit test, worker bees (*Apis mellifera*) were exposed to BAS 222 28 F. The toxicity of the test product was determined in a contact test at a nominal concentration of 100 µg a.s./bee (nominally equivalent to 143 µg product/bee). Additionally, honeybees were treated with Dimethoate as reference standard at 0.1, 0.15, 0.20 and 0.30 µg Dimethoate/bee or with water as a control. The test was conducted with 5 replicates; each of the test cages contained 10 bees. Assessment of mortality was done after 4, 24 and 48 hours.

No mortality was observed in the test item group and the control. No behavioral abnormalities of the bees could be observed.

The contact LD_{50} value (48 h) of BAS 222 28 F was >143 µg product/bee, equivalent to >100 µg a.s./bee.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2002-1; content of a.s.: metiram (BAS 222 F, Reg. No. 250 284): 70.0% (70.5% nominal).

B. STUDY DESIGN

Test species: Honeybee (*Apis mellifera*), worker bees (4 - 6 weeks old); deriving from a healthy and queen-right colony, bred in-house.

Test design: Limit test for contact toxicity; duration 48 h, 5 replicates, each replicate consisting of 10 bees per cage; assessment of mortality after 4, 24 and 48 hours.

Endpoints: LD_{50} value, behavioral abnormalities.

Reference item: Perfekthion (dimethoate, nominal 400 g/L).

Test concentrations: Control, 100 µg a.s./bee (nominally equivalent to 143 µg product/bee).

Test conditions: Temperature: 25 °C; relative humidity: 60% - 66%, photoperiod: 24 h darkness.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

No mortality was observed in the test item group and the control. No behavioral abnormalities of the bees could be observed. The results are summarized below Table 8.3.1.1.2-2.

Table 8.3.1.1.2-2: Toxicity of BAS 222 28 F to honeybees (*Apis mellifera*) in a contact toxicity test

Treatment	Mean mortality [%]	
[µg a.s./bee]	24 h	48 h
Control	0	0
100	0	0
Endpoint [µg a.s./bee]		
LD_{50}	> 100	

III. CONCLUSION

The contact LD_{50} value (48 h) of BAS 222 28 F was >143 µg product/bee, equivalent to >100 µg a.s./bee.

The following acute study on bumblebees (Bombus terrestris) performed with metiram (tested as BAS 222 28 F) is provided in support of the risk assessment and has not been evaluated previously on EU level.

Report:	CA 8.3.1.1.2/3 Amsel K., 2014a Acute toxicity of BAS 222 28 F to the bumblebee Bombus terrestris L. under laboratory conditions 2014/1083451
Guidelines:	OECD 213 (1998), OECD 214 (1998), EFSA Guidance Document on bees (2013)
GLP:	yes (certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

Executive Summary

In a contact toxicity test, young adult worker bumblebees of *(Bombus terrestris)* were exposed to BAS 222 28 F. The toxicity of the test item was determined at nominal doses of 36, 71, 143, 286 and 572 µg BAS 222 28 F/bumblebee (equivalent to 25, 50, 100, 200 and 400 µg a.s./bumblebee). Additionally, bumblebees were treated with dimethoate as a reference item at dose rates ranging from 2.5 to 20.0 µg dimethoate/bumblebee (nominal) and furthermore with deionized water and Tween solution controls.

After 96 hours of contact exposure, no mortality occurred in the control groups, neither the 1% Tween (v/v) solution nor deionized water. In the test item treatments, no mortality occurred after thoracic application in any of the treatment groups after 96 hours. No behavioral abnormalities of surviving bumblebees occurred throughout the contact toxicity test.

In a contact toxicity study with BAS 222 28 F on bumblebees, the LD_{50} value (96 h) was determined to be > 572 µg BAS 222 28 F/bumblebee, corresponding to > 400 µg a.s./bumblebee.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 222 28 F; batch no. FRE-000977; content of a.s.: metiram (BAS 222 F, Reg. No. 250 284): 70 g/L (nominal), 69.7 g/L (analyzed); density: 1.74 g/cm^3.

B. STUDY DESIGN

Test species: *Bombus terrestris* (bumblebee), young adult worker bumblebees derived from a healthy and queen-right micro-hive; source: Biobest Belgium N.V., Ilse Velden 18, 2260 Westerlo Belgium; collected in the morning prior to use.

Test design: In a 96 hour test, young adult worker bumblebees of *Bombus terrestris* were exposed to 5 doses of BAS 222 28 F placed on the dorsal bumblebee thorax. The test solution contained 1% (*v/v*) wetting agent Tween. In total, 3 treatment groups were set up (5 dose rates of the test item, 2 untreated controls (deionized water and Tween) and 4 dose rates of the reference item) with 3 replicates per dose and 10 bumblebees per replicate. Assessment of bumblebee mortality and behavioral effects was done after 4, 24, 48, 72 and 96 hours.

Endpoint: Mortality, behavioral impairments.

Reference item: BAS 152 11 I (dimethoate, nominal 400.0 g/L).

Test concentrations: Water control (deionized water), Tween control (1% (v/v) Tween solution), Reference item at dose rates of 2.5, 5.0, 10.0 and 20.0 µg dimethoate/bumblebee,
Test item at dose rates:

Nominal product dose rate (µg/bumblebee)	Nominal a.i. dose rate (µg/bumblebee)
36	25
71	50
143	100
286	200
572	400

Test conditions: Temperature: 25.2°C – 25.5°C, relative humidity: 56% – 58%, photoperiod: 24 h darkness, food: 50% (w/v) sucrose solution.

Statistics: Descriptive statistics; Fisher's Exact Binomial Test with Bonferroni Correction for mortality data (one-sided greater, α = 0.05).

II. RESULTS AND DISCUSSION

After 96 hours of contact exposure, no mortality occurred in the control groups, neither the 1% Tween (v/v) solution nor deionized water. In the test item treatments, no mortality occurred after thoracic application in any of the treatment groups after 96 hours. No behavioral abnormalities of surviving bumblebees occurred throughout the contact toxicity test. The results are summarized in Table 8.3.1.1.2-3.

Table 8.3.1.1.2-3: Toxicity of BAS 310 55 I to *Bombus terrestris* (bumblebee) in a contact toxicity test

Treatment [µg BAS 222 28 F/bee]	Mortality [%]			
	24 h	48 h	72 h	96 h
Control	0.0	0.0	0.0	0.0
36	0.0	0.0	0.0	0.0
71	0.0	0.0	0.0	0.0
143	0.0	0.0	0.0	0.0
286	0.0	0.0	0.0	0.0
572	0.0	0.0	0.0	0.0
	Endpoint			
	[µg BAS 222 28 F/bee]		[µg a.s./bee]	
LD_{50} (96 h)	> 572		> 400	

III. CONCLUSION

In a contact toxicity study with BAS 222 28 F on bumblebees, the LD_{50} value (96 h) was determined to be > 572 µg BAS 222 28 F/bumblebee, corresponding to > 400 µg a.s./bumblebee.

CA 8.3.1.2 Chronic toxicity to bees

The following chronic study on honeybees (Apis mellifera) performed with metiram (tested as BAS 222 28 F) is provided in support of the risk assessment and has not been evaluated previously on EU level.

Report:	CA 8.3.1.2/1 Kleebaum K., 2014a Chronic toxicity of BAS 222 28 F to the honeybee Apis mellifera L. under laboratory conditions 2012/1189907
Guidelines:	Decourty et al. (2005), Suchail et al. (2001), CEB No. 230 (2010)
GLP:	yes (certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

Executive Summary

In a 10-day chronic oral toxicity test, 2 - 3 day old worker honeybees (*Apis mellifera*) were exposed to a daily application of BAS 222 28 F diluted in the bee food (50% w/v aqueous sucrose solution). The chronic toxicity of the test item was determined at nominal doses of 2.6, 6.4, 16.0, 39.9 and 99.8 µg a.s./bee/day (effective doses were 1.4, 4.0, 9.2, 21.0 and 49.2 µg a.s./bee/day), corresponding to concentrations of 0.066, 0.164, 0.410, 1.025 and 2.563 g a.s./kg food, respectively. Additionally, honeybees were treated with Dimethoate EC 400 as reference item at nominal doses ranging from 4.3 to 20.1 ng a.s./bee/day. Untreated diet served as a control.

The control group showed a mean mortality of 5.0% after 10 days of testing. In the test item group bees consumed doses between 1.4 and 49.2 µg a.s./bee/day and revealed mortalities that ranged between 1.7% and 8.3%. These mortalities are not statistically significantly increased compared to the control group; therefore no LD_{50}/LC_{50} could be calculated.
The NOED was determined to be > 49.2 µg consumed a.s./bee/day, and the NOEC > 2.563 g a.s./kg food, respectively. During the testing period no behavioral abnormalities could be observed in the test item group.

In a 10-day chronic toxicity feeding study with tested dosages between 1.4 and 49.248 µg consumed a.s./bee/day of BAS 222 28 F, no LD_{50}/LC_{50} could be determined.
The NOED was determined to be > 49.248 µg consumed a.s./bee/day, and the NOEC > 2.563 g a.s./kg food, respectively.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. FRE-000825; content of a.s.: metiram (BAS 222 F, Reg. No. 250 284): 71.25% w/w (nominal: 70.0% w/w).

B. STUDY DESIGN

Test species: *Apis mellifera carnica* P. (honeybee); 2 – 3 day old bees; derived from a healthy and queen-right colony; source: Bienenfarm Kern GmbH, Leipzig, Germany.

Test design: In a 10-day test, young adults of *Apis mellifera* L. were exposed daily to 5 doses of BAS 222 28 F in treated food (50% w/v aqueous sucrose solution). In total, 3 treatment groups were set up: 5 doses of the test item, one untreated control and 4 doses of the reference item with 3 replicates per dose and 20 bees per replicate. Assessments of bee mortality and behavioral effects were done daily during the study.

Endpoints: Mortality, behavioral impairments.

Reference item: Dimethoate 400 EC (analyzed content of a.s.: 411.7 g/L).

Test doses: Control: untreated diet (50% w/v aqueous sucrose solution).

Test item treatments	
Doses [µg a.s./bee/day]	**Concentrations [g a.s./kg food]**
2.6	0.066
6.4	0.164
16.0	0.410
39.9	1.025
99.8	2.563
Reference item	
Doses [ng dimethoate/bee/day]	**Concentrations [mg dimethoate/kg food]**
4.3	0.1
7.2	0.2
12.0	0.3
20.1	0.5

Test conditions: Temperature: 34°C – 35°C; relative humidity: 67% - 72%, photoperiod: 24 h darkness; food: 50% w/v aqueous sucrose solution.

Statistics: Descriptive statistics; for mortality data Fisher's Exact Binomial Test with Bonferroni Correction (one-sided greater, $\alpha = 0.05$). The median lethal dose/concentration of test and reference item were calculated with Probit analysis using linear maximum likelihood regression.

II. RESULTS AND DISCUSSION

In a chronic oral toxicity test, the test item BAS 222 28 F was daily administered to the honeybees via food at the following concentrations: 0.066, 0.164, 0.410, 1.025 and 2.563 g a.s./kg food. The actual daily mean doses were 1.4, 4.0, 9.2, 21.0 and 49.2 µg a.s./bee/day after 10 days, corresponding to nominal doses of 2.6, 6.4, 16.0, 39.9 and 99.8 µg a.s./bee/day.

The control group showed a mean mortality of 5.0% after 10 days of testing. In the test item group bees consumed doses between 1.4 and 49.2 µg a.s./bee/day and revealed mortalities that ranged between 1.7% and 8.3%. These mortalities are not statistically significantly increased compared to the control group, therefore no LD_{50}/LC_{50} could be calculated.
The NOED was determined to be > 49.2 µg consumed a.s./bee/day, and the NOEC > 2.563 g a.s./kg food, respectively (Fisher's Exact Binominal Test with Bonferroni Correction (one-sided greater, $\alpha = 0.05$). During the testing period no behavioral abnormalities could be observed in the test item group. The results are summarized in Table 8.3.1.2-1.

Table 8.3.1.2-1: Cumulative mortality and toxicity endpoints of honeybees (*Apis mellifera* L.) exposed to BAS 222 28 F in a chronic oral toxicity test

Treatment [BAS 222 28 F]			Mortality after 10 days	
Actual daily mean doses [µg a.s./bee/day]	**Overall doses [µg a.s./bee/day]**	**Concentration [g a.s./kg food]**	**Cumulative mortality [%]**	**Corrected cumulative mortality [%]**
Control	Control	Control	5.0	--
1.4	2.6	0.066	3.3	0.0
4.0	6.4	0.164	8.3	3.5
9.2	16.0	0.410	1.7	0.0
21.0	39.9	1.025	3.3	0.0
49.2	99.8	2.563	8.3	3.5
Endpoints			**10 days**	
Test item doses [µg a.s./bee/day]		**LD_{50}**	n.d.	
		NOED [1)]	≥ 49.248	
Test item concentrations [g a.s./kg food]		**LC_{50}**	n.d.	
		NOEC [1)]	≥ 2.563	

1) Fisher's Exact Binominal Test with Bonferroni Correction (one-sided greater, $\alpha = 0.05$).
n.d.: not determined.

In the reference item treatment a LD_{50} was determined to be 10.501 ng dimethoate/bee/day, which corresponds to an LC_{50} of 0.415 mg dimethoate/kg food.

III. CONCLUSION

In a 10-day chronic toxicity feeding study with tested dosages between 1.4 and 49.248 µg consumed a.s./bee/day of BAS 222 28 F no LD_{50}/LC_{50} could be determined.
The NOED was determined to be > 49.248 µg consumed a.s./bee/day, and the NOEC > 2.563 g a.s./kg food, respectively.

CA 8.3.1.3 Effects on honeybee development and other honeybee life stages

The following acute study on honeybee larvae (Apis mellifera) performed with metiram (tested as BAS 222 28 F) is provided in support of the risk assessment and has not been evaluated previously on EU level.

Report:	CA 8.3.1.3/1 Kleebaum K., 2014a Acute toxicity of BAS 222 28 F to honeybee larvae (Apis mellifera L.) under laboratory conditions (in vitro) 2012/1189906
Guidelines:	OECD 237 (2013) Honey bee (Apis mellifera) larval toxicity test single exposure
GLP:	yes (certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

Executive Summary

In an acute oral larval toxicity test, four day old honeybee larvae (*Apis mellifera*) were exposed to one application of BAS 222 28 F diluted in the larvae food. The toxicity of the test item was determined with doses of 6.2, 12.4, 24.8, 49.5 and 99.1 µg a.s./larva (corresponding to 8.8, 12.6, 35.4, 70.8 and 141.5 µg product/larva). The concentrations of test item in the diet were 0.2, 0.4, 0.7, 1.5 and 2.9 g a.s./kg food. Additionally, honeybee larvae were treated with dimethoate tech. as the reference item. Untreated diet served as a control.

After 72 hours of exposure, a mortality of 8.3% was observed in the control. In the test item groups, larvae fed with 49.5 and 99.1 µg a.s./larva revealed a mortality of 41.7% and 66.7%, respectively, which was statistically significant in comparison to the control group. After 72 hours of exposure, deviations from the normal food consuming behavior occurred in 6.1% of the remaining individuals of the control treatment and in 6.4%, 6.1%, 13.7%, 73.6% and 93.3% of the remaining individuals treated with 6.2, 12.4, 24.8, 49.5 and 99.1 µg a.s./larva. Affected larvae had food left in their grafting cells and a smaller body size when compared to the average control larvae. The same symptoms were observed in the remaining larvae after being treated with reference item.

In an acute oral larval toxicity study with BAS 222 28 F on honeybee larvae, the LD_{50} value (72 h) was determined to be 75.3 µg a.s./larva (equivalent to LC_{50} (72 h) = 2.222 g a.s./kg food). The NOED was determined to be 24.8 µg a.s./larva (equivalent to NOEC = 0.731 g a.s./kg food).

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. FRE-000825; content of a.s.: metiram (BAS 222 F, Reg. No. 250 284): 71.25% w/w (nominal: 70.0% w/w).

B. STUDY DESIGN

Test species: *Apis mellifera* L. subspecies *carnica* P. (honeybee); synchronized first instar larvae (one day old); derived from three healthy and queen-right colonies; source: Bienenfarm Kern GmbH, Leipzig, Germany.

Test design: One day old honeybee larvae of *Apis mellifera* were transferred from brood combs to polystyrene grafting cells in 48-well cell culture plates 3 days before start of the treatment. After this, in a 72 hour acute test, the 4 day old larvae were exposed to a single application of BAS 222 28 F diluted in the larvae food (aqueous sugar solution mixed with royal jelly). In total, 3 treatment groups were set up: 5 doses of the test item, one untreated control group and 4 doses of the reference item with 3 replicates per dose and 12 larvae per replicate. After the day of application, additional feeding of the larvae took place 24 and 48 hours later. Assessments of larval mortality were done after 24, 48 and 72 hours. Additionally, other observations such as small body size or large quantities of remaining food after 72 hours were noted. In an analytical phase of the study, the concentration of the active substance in the test item stock solution was determined.

Endpoints: Mortality (LD_{50}), quantitative observations: body size, remaining food.

Reference item: Dimethoate technical (analyzed purity: 99.8%).

Test doses: Control (50% aqueous sugar solution with 50% royal jelly)
Test item treatments:

Nominal dose/concentration	
Doses [µg/larva]	**Concentrations [g a.s./kg food]**
6.2	0.2
12.4	0.4
24.8	0.7
49.5	1.5
99.1	2.9

Reference item treatments: 1.1, 2.2, 4.4 and 8.8 µg dimethoate/larva.

Test conditions: Temperature: 34.0° C – 34.5° C; relative humidity: 85% - 96%, photoperiod: 24 h darkness; food: 50% aqueous sugar solution with 50% royal jelly.

Statistics: Descriptive statistics; for mortality data Fisher's Exact Binomial Test with Bonferroni Correction (one-sided greater, α = 0.05). The median lethal dose/concentration of test and reference item were calculated with Probit or Weibull analysis, respectively.

II. RESULTS AND DISCUSSION

After 72 hours of exposure, a mortality of 8.3% was observed in the control. In the test item groups, larvae fed with 49.5 and 99.1 µg a.s./larva revealed a mortality of 41.7% and 66.7%, respectively, which was statistically significant in comparison to the control group.
After 72 hours of exposure, deviations from the normal food consuming behavior occurred in 6.1% of the remaining individuals of the control treatment and in 6.4%, 6.1%, 13.7%, 73.6% and 93.3% of the remaining individuals treated with 6.2, 12.4, 24.8, 49.5 and 99.1 µg a.s./larva.
Affected larvae had food left in their grafting cells and a smaller body size when compared to the average control larvae. The same symptoms were observed in the remaining larvae after being treated with reference item. The results are summarized in Table 8.3.1.3-1.

Table 8.3.1.3-1: Toxicity of BAS 222 28 F to *Apis mellifera* (honeybee) in an acute oral larval toxicity test after 72 hours

Dosage [µg a.s./larva]	Concentration [g a.s./kg food]	Mortality [%]		Other observations 1) [%]
		absolute	corrected 2)	
Control	Control	8.3	--	6.1
6.2	0.183	13.9	6.1	6.4
12.4	0.365	5.6	0.0	6.1
24.8	0.731	16.7	9.1	13.7
49.5	1.461	41.7 *	36.4	73.6
99.1	2.923	66.7 *	63.6	93.3
		Endpoints [µg/bee]		
LD_{50} [µg a.s./larvae] (95% confidence limits)		75.3 (44.2 – 128.3)		
NOED [µg a.s./larvae]		24.8		
LC_{50} [g a.s./kg food] (95% confidence limits)		2.222 (1.770 – 2.788)		
NOEC [µg a.s./kg food]		0.731		

* Statistically significantly different compared to the control (Fisher's Exact Binomial Test, one-sided greater; α = 0.05).
1) Other observations were large quantities of remaining food, smaller body size of larvae.
2) According to Schneider-Orelli (1947).

The LD_{50} value (72 h) for the reference item in the acute oral larval toxicity test was determined to be 4.421 µg dimethoate/larva.

III. CONCLUSION

In an acute oral larval toxicity study with BAS 222 28 F on honeybee larvae, the LD_{50} value (72 h) was determined to be 75.3 µg a.s./larva (equivalent to LC_{50} = 2.222 g a.s./kg food). The NOED was determined to be 24.8 µg a.s./larva (equivalent to NOEC = 0.731 g a.s./kg food).

The following semi-field brood study (tunnel test) on honeybees (Apis mellifera) performed with metiram (tested as BAS 222 28 F) is provided in support of the risk assessment and has not been evaluated previously on EU level.

Report:	CA 8.3.1.3/2 Classen C., 2012a Semi-field brood study to evaluate potential effects of BAS 222 28 F on the brood development of honeybees (Apis mellifera L.) 2012/1111495
Guidelines:	OECD 75 (2007)
GLP:	yes (certified by Landesanstalt fuer Umwelt, Messungen und Naturschutz Baden-Wuerttemberg, Karlsruhe, Germany)

Executive Summary

A tunnel test was carried out to determine the effects of BAS 222 28 F on honeybee colonies under semi-field conditions. For this purpose, BAS 222 28 F was applied at a rate of 2.0 kg (equivalent to 1.4 kg BAS 222 F/ha). BAS 222 28 F/ha to flowering *Phacelia tanacetifolia* with foraging honeybees being present in the crop. Additionally, a water treated control and a toxic reference item were included in the study. Each of the three treatment groups had four replicates (= tunnels) with one bee colony per replicate. Mortality of the bees was assessed daily from 3 days before to 26 days after treatment (DAT), respectively. Foraging activity was monitored daily from 3 days before to 7 DAT. Behavioral abnormalities were recorded from 3 days before to 21 DAT. Condition of the colonies (strength, brood and food development) was assessed 2 days prior to application and on DAT 3, 8, 13, 20 and 26. Brood status assessments (brood indices and brood compensation indices) were carried out on BFD (brood area fixing day) 0, 5, 10, 16 and 22.

There was statistically significant difference of the mortality between the control and the test item group observed on DAT 4 and 5. However, as the recorded number of dead honeybees in the test item group on the respective days was comparable to the remaining days during the exposure period it can be assumed that these findings are in a natural range.

The overall foraging activity of all treatment groups before application was on a comparable level, indicating that the honeybees had well adapted to the new environmental conditions. After the application the mean foraging activity of the test item group was slightly reduced for 45 minutes. Afterwards the foraging activities were comparable to the control. On all following days mean foraging activities of the test item and the reference groups were comparable during the exposure period when compared to control. No statistically significant differences between the control, the test item and reference group were observed.

No test item related effect on the honeybees behavior occurred. After the application the development (colony strength, brood and food) of the control and the test item colonies were in a natural range during the post-application period and there was no indication of a test item related effect. The mean brood termination rate, the brood-index and the brood compensation-index of the test item group were comparable to those of the control group.

Under semi-field conditions (tunnel test), BAS 222 28 F was applied at a rate of 2.0 kg/ha (equivalent to 1.4 kg BAS 222 F/ha) on *Phacelia tanacetifolia* during honeybee flight. No unacceptable effects on mortality, foraging activity, colony development, colony strength or bee brood were observed. Overall, based on the results of this study, BAS 222 28 F does not adversely affect honeybee colonies.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 80449975L0, content of a.s: metiram (BAS 222 F, Reg. No. 250 284): 72.3% (nominal 70.0%).

B. STUDY DESIGN

Test species: *Apis mellifera* L. (honeybee), healthy colonies (free of clear visual symptoms of diseases), each consisting of approximately 5720 - 9490 bees, 10 combs including 3 - 5 brood combs with sufficient food supply.

Test plots: The test site was located near Heddesheim, Southern Germany. The field site was covered by flowering *Phacelia tanacetifolia.*

Test design: The semi-field study was carried out based on the OECD Guidance document No. 75 (2007). 12 tunnel tents were set up on a field grown with *Phacelia tanacetifolia* (each measuring 6 m x 18 m; approx. 84 m^2 crop area). The study comprised 3 treatment groups with 4 replicates each (control (C), test item (T), reference item (R)). In each tunnel tent (“tunnel”) a honeybee colony was introduced 5 days before application. The application took place at good weather conditions during bee flight. After seven days of exposure the colonies were moved to a monitoring site for additional 19 days.

Endpoint: Mortality on days after treatment (DAT) -3 to 7: sum of dead honeybees on linen and in dead bee traps, DAT 8 to 26: dead honeybees in dead bee traps
Foraging activity of the bees: inside the tunnels
Behavior inside and outside the tunnels
Condition of the colonies (strength, brood and food development)
Detailed brood assessments (brood termination-rate, brood-index, brood compensation-index)

Reference item: Insegar, 0.6 kg/ha (a.s.: 250 g fenoxycarb/kg) in 400 L water/ha.

Application rates: BAS 228 22 F: 2.0 kg/ha. For the control group, tap water (400 L/ha) was used; all substances were applied in 400 L/ha water.

Test conditions: Semi-field conditions. On the day of application no rainfall occurred. During the exposure phase, rainfall occurred on DAT 1, 2, 4, 5 and 6 with volumes of < 0.5 to 3 L/m². The rainfall which was recorded on DAT 1 occurred in the night after the day of application. The minimum temperatures in the exposure period and post-exposure ranged from 9.3°C to 18.1°C, the range of the maximum temperatures was 17.2°C to 32.6°C.
At the monitoring site no rain was recorded on 4 days (DAT 10, DAT 11, DAT 12, DAT 21), on the remaining days the rainfall ranged from < 0.5 L/m² to 22.0 L/m². Heavy rainfall occurred on DAT 13 (22.0 L/m²), DAT 15 (13.0 L/m²) and DAT 22 (18.0 L/m²). The minimum temperatures at the monitoring site ranged from 10.2°C to 17.7°C, the maximum temperatures ranged from 15.6°C to 26.4°C.

Statistics: Descriptive statistics; Shapiro Wilks-test followed by Mann-Whitney-U-test.

II. RESULTS AND DISCUSSION

The results are summarized in Table 8.3.1.3-2.

Table 8.3.1.3-2: Effects of BAS 222 28 F on honeybee mortality, foraging activity and honeybee brood under semi-field conditions (tunnel test)

Parameter	Control	2.0 kg BAS 222 28 F/ha	Reference item
Mean mortality of worker bees of colony / day [n]			
pre-application phase [1]	24.4	43.4	23.4
exposure phase in the tunnels [1]	27.5	37.3	26.2
post-exposure phase outside the tunnels [2]	21.9	14.0	20.8
overall after application	23.3	20.9	22.4
Mean mortality of pupae of colony / day [n]			
pre-application phase [1]	0.2	0.4	0.2
exposure phase in the tunnels [1]	0.2	0.1	0.3
post-exposure phase outside the tunnels [2]	0.3	0.1	22.8 *
overall after application	0.3	0.1	16.1
Mean foraging activity/ m² of colony/ day [n]			
pre-application phase	13.3	13.4	13.6
exposure phase in the tunnels	18.2	16.9	15.5
Detailed brood development of eggs at BFD 22 [%]			
mean brood termination rate	22.25	30.00	57.45
mean brood-index	3.89	3.50	2.13
mean brood compensation-index	4.14	3.80	3.37

[1] Sum of dead individuals found in dead bee traps and on linen sheets in the tunnels.
[2] Dead individuals found in dead bee traps, only.
* Statistically significantly different (comparing treatment against control, Mann-Whitney-U-test, α=0.05).
BFD = brood area fixing day.

Mortality
The mortality of worker bees and pupae during the pre-application phase was generally low in all treatment groups, indicating comparable and well adapted colonies. After the application the mortality of worker bees in the test item group was comparable to that of the control. Nevertheless, statistically significant differences between the control and the test item group were observed on DAT 4 and 5 (Mann-Whitney-U-test, $\alpha = 0.05$). However, as the recorded number of dead honeybees in the test item group on the respective days was comparable to the remaining days during the exposure period (which did not differ statistically significant compared to the control) it can be assumed that these findings are in a natural range. The mortality within each treatment group before and after the application was not statistically significant different. The worker bee mortality of the test item replicates at the monitoring site after exposure was not statistically significant different compared to the control (Mann-Whitney-U-test, $\alpha = 0.05$). The pupae mortality of the test item replicates was not increased at any time. In contrast, the pupae mortality of the reference item replicates was dramatically increased, indicating the suitability of the test system to detect potential effects of the test item on the honeybee brood.

Foraging activity
The overall foraging activity of all treatment groups before application was on a comparable level, indicating that the honeybees had well adapted to the new environmental conditions. After the application the mean foraging activity of the test item group was slightly reduced for 45 minutes. Afterwards the foraging activities were comparable to the control. On all following days mean foraging activities of the test item and the reference groups were comparable during the exposure period when compared to control. No statistically significant differences between the control, the test item and reference group were observed (Mann-Whitney-U-test, $\alpha = 0.05$).

Bee behavior
No test item related effect on the honeybees behavior occurred.

Condition of the colonies
The pre-application brood check indicated that honeybee colonies were healthy, all brood stages were present, colony strengths were comparable and a sufficient amount of nectar and pollen was available in all colonies. After the application the development (colony strength, brood and food) of the control and the test item colonies were in a natural range during the post-application period and there was no indication of a test item related effect. In contrast, compared to the control a lower proportion of brood was observed for the reference item group. The development of the colony strength was comparable between the control and the reference item group.

Detailed brood development
The mean brood termination rate, the brood-index and the brood compensation-index of the test item group were comparable to those of the control group. Consequently, no statistically significant differences were observed between both control and test item treatment group (Mann-Whitney-U-test, $\alpha = 0.05$). In contrast to the control and test item, the reference item caused a moderate increase of the brood termination and low brood indices. Compensation indices indicate the recovery of the reference item colonies.

Development of colony strength

The mean strength (± SD) of the colonies at pre-application assessment was 7540 ± 1565 bees/colony in the control, 7881 ± 1279 bees/colony in the test item group and 7264 ± 1287 bees/colony in the reference item group and thus on a similar level in all treatment groups. The strength of the control and the test item colonies developed in a similar way. At the first assessment the colonies of both treatment groups showed their minimum strength. Afterwards, the number of bees of the control and the test item group colonies increased until the assessment on DAT 20, where the colonies reached their maximum strength (control: 13618 ± 2906 bees, test item: 13163 ± 3631 bees). At the last colony assessment (DAT 26) the number of bees was slightly reduced compared to that at the colony assessment on DAT 20.

Development of the mean colony strength:

Treatment group	DAT -2	DAT +3	DAT +8	DAT +13	DAT +20	DAT +26
Control	7540	7963	10173	13065	13618	13098
BAS 222 28 F	7881	8710	10546	12968	13163	12220
Reference item	7264	8434	10189	10124	6776	7215

Brood and food storage area development

During the entire course of the field phase the number of brood cells of the control and the test item colonies was very similar and can be considered in a range of expected variability.

Development of the mean amount of all brood cells:

Treatment group	DAT -2	DAT +3	DAT +8	DAT +13	DAT +20	DAT +26
Control	19850	21400	17350	18750	22100	27000
BAS 222 28 F	22700	22300	16850	16250	20350	23900
Reference item	19350	15350	12550	11600	12950	11933[1)]

[1)] Due to the loss of its queen after DAT 22, colony R1 was excluded for any calculations after DAT 20/BFD 22.

The supply of food was ensured throughout the study period. At no time did the number of food storage cells decreased under the values estimated at the initial colony assessment. After moving the colonies to the monitoring location the number of cells containing food increased strongly. This was caused by the good supply of pollen and nectar by flowering chestnuts.

Development of the mean amount of food storage cells:

Treatment group	DAT -2	DAT +3	DAT +8	DAT +13	DAT +20	DAT +26
Control	5650	7050	7050	23850	27550	24500
BAS 222 28 F	6050	8100	7350	28450	28550	23100
Reference item	7700	9300	10350	20000	17550	12350

Brood termination-rate
The mean termination-rates at the last brood assessment on BFD 22 were 22.25 ± 15.30% for the control group, 30.00 ± 10.67% for the test item group (replicate T4 was not considered), and 57.45 ± 28.30% for the reference group. The high standard deviation suggests that the termination-rates of the replicates within each treatment group were fluctuating. For example, the termination-rates of the control group ranged from 8.75% to 43.00%, that of the test item group from 22.69% to 42.25% and the termination-rates of the reference item group ranged from 16.38% to 81.02%.

Mean brood termination-rates [%]:

Treatment group	BFD 5	BFD 10	BFD 15	BFD 22
Control	17.10	21.25	22.19	22.25
BAS 222 28 F	21.77	28.92	30.00	30.00
Reference item	52.76	56.88	57.45	57.45

BFD = brood area fixing day.

Brood-index
The brood-indices correlate with the termination-rates; the higher the termination-rates, the lower the brood-indices and vice versa. Consequently, the mean brood-index of the test item group was higher on BFD 5 but lower on BFD 10 to 22 when compared to the control and not statistically significant different (Mann-Whitney-U-test, $\alpha = 0.05$). Thus, regarding the brood indices, the test item did not cause adverse effects on the development of the bee brood.

Mean brood-indices:

Treatment group	BFD 0	BFD 5	BFD 10	BFD 16	BFD 22
Control	1.0	2.04	3.19	3.30	3.89
BAS 222 28 F	1.0	2.08	2.86	2.92	3.50
Reference item	1.0	1.15	1.78	2.10	2.13

Compensation-Index
The compensation-index is an indicator for the recovery of the colony.
Generally the compensation-indices of all treatment groups were slightly higher than the corresponding brood-indices at almost all days, indicating that cells with terminated brood were refilled with new eggs, which developed successfully. The mean compensation-indices of the test item group were lower compared to that of the control group, but statistically significant differences were not observed (Mann-Whitney-U-test, $\alpha = 0.05$).

Mean compensation-indices:

Treatment group	BFD 0	BFD 5	BFD 10	BFD 16	BFD 22
Control	1.0	2.07	3.25	3.31	4.14
BAS 222 28 F	1.0	2.09	2.92	2.97	3.80
Reference item	1.0	1.24	2.10	2.47	3.37

III. CONCLUSION

Under semi-field conditions (tunnel test), BAS 222 28 F was applied at a rate of 2.0 kg/ha (equivalent to 1.4 kg BAS 222 F/ha) on *Phacelia tanacetifolia* during honeybee flight. No unacceptable effects on mortality, foraging activity, colony development, colony strength or bee brood were observed. Overall, based on the results of this study, BAS 222 28 F does not adversely affect honeybee colonies.

CA 8.3.1.4 Sub-lethal effects

No new studies are available.

CA 8.3.2 Effects on non-target arthropods other than bees

No new studies are available.

CA 8.3.2.1 Effects on Aphidius rhopalosiphi

No new studies are available.

CA 8.3.2.2 Effects on Typhlodromus pyri

No new studies are available.

CA 8.4 Effects on non-target soil meso- and macrofauna

Since Annex I inclusion of the active substance metiram (BAS 222 F), new studies on soil macro-organisms have been performed with the representative formulation and relevant metabolites in soil. As a result there are new endpoints, which are considered in the respective risk assessment. The new acute toxicity study on earthworms performed with metiram (tested as BAS 222 28 F) is not a data requirement for the risk assessment and is therefore summarized in chapter CA 8.7 as additional information.

Details on the EU agreed studies which have been already evaluated within the Annex I inclusion of metiram are provided in the EU Review documents of metiram (Monograph, Vol. 3, Annex B.9, July 2000; Addendum to the Monograph, Annex B.9, June 2002 and April 2004; EC Review Report (SANCO/4059/2001-rev 3.3), June 2005). These acute toxicity studies on earthworms are not a data requirement for the risk assessment and are therefore summarized in chapter CA 8.7 as additional information.

For better transparency and traceability of the active substance history, the results of all studies are summarized in Table 8.4-1.

Table 8.4-1 Toxicity to non-target soil meso- and macrofauna of metiram and relevant metabolites

Substance	Species	Endpoint	Value [mg/kg dry soil]	Reference (BASF Doc ID)	EU agreed
metiram [2)]	*Eisenia fetida*	LC_{50}	> 1000	Adolphi, 1985/10074	yes
metiram as contained in BAS 222 28 F [2)#]	*Eisenia fetida*	LC_{50}	> 700	Sattler, 2008/1005501	no, new study
ETU = M222F002 = Reg. no. 146099 [2)#]	*Eisenia fetida*	LC_{50}	> 1000	Staab, 2001/1000882	yes
EU = M222F003 = Reg. no. 27270 [2)#]	*Eisenia fetida*	LC_{50}	> 886	Staab, 2001/1000881	yes
metiram as contained in BAS 222 28 F	*Eisenia fetida*	NOEC	≥ 23.3	Luehrs, 2002/1008555	no, new study
ETU = M222F002 = Reg. no. 146099	*Eisenia fetida*	NOEC	≥ 24 [3)]	Ganssmann, 2012/1202289	no, new study
TDIT = M222F007 = Reg. no. 4670450	*Eisenia fetida*	NOEC $NOEC_{CORR}$	80 40 [1)]	Friedrich, 2013/1003167	no, new study
EBIS = M222F004 = Reg. no. 243959	*Eisenia fetida*	NOEC $NOEC_{CORR}$	40 20 [1)]	Friedrich, 2013/1003166	no, new study
metiram as contained in BAS 222 28 F	*Folsomia candida*	NOEC	22.4	Luehrs, 2010/1075821	no, new study
metiram as contained in BAS 222 28 F	*Hypoaspis aculeifer*	NOEC EC_{10}	87.5 54.19	Royer, 2010/1075822	no, new study

1) The study was carried out with 10% peat and no log P_{ow} values are available for the metabolites TDIT and EBIS. Therefore, the endpoint was divided by a safety factor of 2, assuming a worst case scenario.

2) Acute studies are listed for reference, but not used in the following risk assessment as acute studies are no longer required according to EU Commission Regulation No.283/2013.

3) The study was carried out with 10% peat and no log P_{ow} values are available for the metabolites ETU and EU. However, considering the K_{oc} values (ETU: 4, EU: 9.75), the metabolites are highly hydro-soluble and thus do not bind to organic material like peat. Therefore, no soil factor was used to correct the endpoints.

\# Study is presented as additional information (see CA 8.7).

CA 8.4.1 Earthworms – sub-lethal effects

The following chronic toxicity study on earthworms performed with metiram (tested as BAS 222 28 F) is provided in support of the risk assessment and has not been evaluated previously on EU level.

Report:	CA 8.4.1/1 Luehrs U., 2002a Effects of BAS 222 28 F on reproduction and growth of earthworms Eisenia fetida in artificial soil 2002/1008555
Guidelines:	BBA VI 2-2, ISO 11268-2 (1998)
GLP:	yes (certified by Hessisches Ministerium fuer Umwelt, Landwirtschaft und Forsten, Wiesbaden)

Executive Summary

The effects of BAS 222 28 F on mortality, biomass development and reproduction of the earthworm *Eisenia fetida* were investigated in a 56-days extended laboratory study. Five application rates (1.25, 2.5, 5.0, 12.5 and 25.0 kg BAS 222 28 F/ha) were incorporated into the soil with four replicates per treatment (each containing 10 worms). An untreated control with 4 replicates was included. The reference item was tested in a parallel study. Assessment of adult worm mortality and biomass development was carried out after 28 days; assessment of reproduction rate (number of juveniles) was carried out after 56 days.

No mortality of parent earthworms was observed in the control and all test item treatment groups. No statistically significant effects on body weight and reproduction were observed from 2.5 up to 25.0 kg BAS 222 28 F/ha.

In a 56-day reproduction study with BAS 222 28 F, no statistically significant effects on mortality, growth and reproduction of earthworms (*Eisenia fetida*) were observed up to a rate of 25.0 kg BAS 222 28 F/ha, the highest rate tested. Therefore the NOEC was determined to be 25.0 kg BAS 222 28 F/ha (equivalent to 33.3 mg/kg dry soil, which is equal to 23.3 mg a.s./kg dry soil).

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 2001-1; content of a.s.: metiram (BAS 222 F, Reg. No. 250284): 70% nominal, 72.1% analyzed.

B. STUDY DESIGN

Test species: Earthworm (*Eisenia fetida*), adult worms (with clitellum and weight of 300 - 500 mg), 8 to 9 months old; source: in-house culture.

Test design: Different concentrations of the test item are mixed homogeneously into the soil. 6 treatment groups (5 test item rates, control); 4 replicates/group with 10 worms each; 4 replicates for the control. Assessment of worm mortality, behavioral effects and weight change after 28 days of exposure, after additional 28 days (56 days after application) determination of number of offspring.

Endpoints: Mortality, weight change, behavioral effects, reproduction rate.

Reference item: Derosal SC 360 (31.1% w/w, carbendazim), with an application rate of 998 g carbendazim/ha. The effects of the reference item were investigated in parallel to the test item.

Test rates: Control, 1.25, 2.5, 5.0, 12.5 and 25.0 kg BAS 222 28 F/ha equivalent to 01.67, 3.33, 6.67, 16.67 and 33.33 mg BAS 222 28 F/kg dry soil.

Test conditions: Artificial soil according to OECD 207; pH 5.6 at test initiation, 5.7 - 6.0 at test termination; water content at test initiation 31.2% - 32.1% soil dry weight (corresponding to 52.5% - 54.0% of its water holding capacity), 28.5 - 34.1 at test termination (corresponding to 48.0% - 57.4% of its water holding capacity); temperature: 19°C - 21°C; photoperiod: 16 hours light : 8 hours dark, light intensity: 455-683 lux. Feeding with cattle manure.

Statistics: Descriptive statistics; two-sided Dunnett's test for test item groups (α = 0.05), Student t-test for reference substance group.

II. RESULTS AND DISCUSSION

No mortality of parent earthworms was observed in the control and all test item treatment groups. No statistically significant effects on body weight and reproduction were observed from 2.5 kg/ha up to 25.0 kg/ha, the highest rate tested (Dunnett's test, α = 0.05). At 1.25 kg product/ha the body weight was statistically significantly higher compare to control. This was considered to be not treatment related as at higher test rates the weight change of earthworms was not statistically significant influenced. The results are summarized in Table 8.4.1-1.

Table 8.4.1-1: Effects of BAS 222 28 F on earthworm (*Eisenia fetida*) in a 56-day reproduction study

BAS 222 28 F [kg/ha]	Control	1.25	2.5	5.0	12.5	25.0
Mortality (28 d) [%]	0	0	0	0	0	0
Weight change (28 d) [%]	38.7	50.5 *	40.4	39.3	44.0	45.7
Number of juveniles (56 d)	278	188 *	219	252	222	214
Reproduction in percent of control (56 d) [%]	100	67.6	78.8	90.6	79.9	77.0
	Endpoints [kg BAS 222 28 F/ha]					
NOEC (56 d)	≥ 25.0 (corresponding to ≥ 33.3 mg/kg dry soil)					
LC_{50}	not determined					

[1)] Statistically significant compared to the control (Dunnett's test for weight change and for reproduction data, $\alpha = 0.05$).

III. CONCLUSION

In a 56-day reproduction study with BAS 222 28 F, no statistically significant effects on mortality, growth and reproduction of earthworms (*Eisenia fetida*) were observed up to a rate of 25.0 kg BAS 222 28 F/ha, the highest rate tested. Therefore the NOEC was determined to be 25.0 kg BAS 222 28 F/ha (equivalent to 33.3 mg/kg dry soil, which is equal to 23.3 mg a.s./kg dry soil).

The following chronic toxicity study on earthworms performed with the metabolite ETU is provided in support of the risk assessment and has not been evaluated previously on EU level.

Report:	CA 8.4.1/2 Ganssmann M., 2013a Effects of Reg.No. 146099 (metabolite of BAS 222 F, Metiram, BF 222-ETU) on reproduction and growth of earthworms Eisenia fetida in artificial soil 2012/1202289
Guidelines:	OECD 222 - Earthworm reproduction Test (2004), ISO 11268-2 (1998)
GLP:	yes (certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Executive Summary

The effects of Reg. No. 146 099 (metabolite of BAS 222 F, metiram, BF 222-ETU) on mortality, biomass development and reproduction of the earthworm *Eisenia fetida* (Annelida: Oligochaeta) were investigated in a 56-day extended laboratory study. Five concentrations (1.5, 3, 6, 12 and 24 mg Reg. No. 146 099/kg dry soil) were incorporated into the soil (10 % peat) with four 4 replicates per test item treatment and 8 replicates for the control treatment (each containing 10 worms). The reference item was tested in a separate study. Assessment of adult worm mortality, body weight and feeding activity was carried out after 28 days; assessment of reproduction was carried out after 56 days.

After 28 days of exposure, no mortality was observed in the control group. The mortality in the test item concentrations ranged between 0% and 2.5%, which was not statistically significantly different compared to the control. The weight change of adult worms was about 12.4% – 24.4 % in the treated variants and 15.8% in the control group. Body weight was significantly different compared to the control at the highest test item concentration of 24 mg/kg, however this was not considered as an adverse effect. The reproduction rates were not significantly different compared to the control up to and including the highest test concentration of 24 mg /kg soil No behavioral abnormalities were observed in any of the treatment groups. The feeding activity in all the treated groups was comparable to the control.

In a 56-day reproduction study with Reg. No. 146 099 on earthworms, the NOAEC for weight change and the NOEC for reproduction was determined to be ≥ 24 mg Reg. No. 146 099/kg dry soil. The EC_{50} for reproduction was > 24 mg Reg. No. 146 099/kg dry soil.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: Reg. No. 146 099 (metabolite of BAS 222 F (metiram), BF 222-ETU), batch No. WF13952, analyzed purity: 99.7%.

B. STUDY DESIGN

Test species: Earthworm (*Eisenia fetida)*, adult worms (with clitellum and weight of 300 mg – 600 mg), age: approximately 9 months old; source: in-house culture.

Test design: In a 56-day test different concentrations of Reg. No. 146 099 were mixed homogeneously into the soil (10 % peat); 6 treatment groups (5 test item rates, control); 4 replicates per test item treatment group and 8 replicates for the control, with 10 worms each; assessment of worm mortality, behavioral effects and weight change after 28 days of exposure, after additional 28 days (56 days after application) determination of reproduction rate.

Endpoints: Mortality, weight change, feeding activity, reproduction rate, determination of NOEC.

Reference item: Luxan Carbendazim 50 FLOW (carbendazim, 500 g/L nominal). The effects of the reference item were investigated in a separate study.

Test rates: Control, 1.5, 3, 6, 12 and 24 mg Reg. No. 146 099/kg dry soil.

Test conditions: Artificial soil according to OECD 222 (10 % peat); pH 5.9 - pH 6.0 at test initiation, pH 6.0 - pH 6.1 at test termination; water content of soil: 52.1% - 55.4% of max. water holding capacity (WHC) at test initiation, 58.7% - 64.7% of max. WHC at test termination; temperature: 18°C - 22°C; photoperiod: 16 h light : 8 h dark, light intensity: 400 - 800 lux; food: cattle manure.

Statistics: Descriptive statistics; Fisher's Exact test for mortality data, Williams t-test for weight change and reproduction data ($\alpha = 0.05$).

II. RESULTS AND DISCUSSION

After 28 days of exposure, no mortality was observed in the control group. The mortality in the test item concentrations ranged between 0% and 2.5%, which was not statistically significantly different compared to the control (Fisher's Exact Binominal test, $\alpha = 0.05$). The weight change of adult worms was about 12.4% – 24.4 % in the treated variants and 15.8% in the control group. Body weight was significantly different (increased) compared to the control at the highest test item concentration of 24 mg/kg, however this was not considered as an adverse effect (Williams t-test, $\alpha = 0.05$). The reproduction rates were not significantly different compared to the control up to and including the highest test concentration of 24 mg /kg soil No behavioral abnormalities were observed in any of the treatment groups. The feeding activity in all the treated groups was comparable to the control. The results are summarized in Table 8.4.1-2.

Table 8.4.1-2: Effect of Reg. No. 146 099 on earthworms (*Eisenia fetida*) in a 56-day reproduction study

Reg. No. 146 099 [mg/kg dry soil]	Control	1.5	3	6	12	24
Mortality (28 d) [%]	0.0	0.0	0.0	2.5	0.0	0.0
Weight change (28 d) [%]	15.8	14.5	12.4	20.3	22.5	24.4 *
Number of juveniles (56 d)	229	263	256	254	268	228
Reproduction [% of control] (56 d)	--	114.7	111.5	110.8	116.8	99.6
	Endpoints [mg Reg. No. 146 099/kg dry soil]					
$NOAEC_{weight}$ (28 d)	≥ 24					
$NOEC_{reproduction}$ (56 d)	≥ 24					
EC_{50}	> 24					

* Statistically significantly different from control (Williams t-test, $\alpha = 0.05$, one-sided smaller).

In the separate study with the reference item Luxan Carbendazim 50 FLOW (carbendazim, 500 g/L nominal), there were statistically significant effects on reproduction at a concentration of 1.30 mg carbendazim/kg soil and higher; the EC_{50} for reproduction was calculated as 1.7 mg carbendazim/kg soil.

III. CONCLUSION

In a 56-day reproduction study with Reg. No. 146 099 on earthworms, the NOAEC for weight change and the NOEC for reproduction was determined to be ≥ 24 mg Reg. No. 146 099/kg dry soil. The EC_{50} for reproduction was > 24 mg Reg. No. 146 099/kg dry soil.

The following chronic toxicity study on earthworms performed with the metabolite TDIT is provided in support of the risk assessment and has not been evaluated previously on EU level.

Report:	CA 8.4.1/3 Friedrich S., 2013a Sublethal toxicity of Reg.No. 4670450 (metabolite of BAS 222 F, Metiram, BF 222-TDIT) to the earthworm Eisenia fetida in artificial soil 2013/1003167
Guidelines:	OECD 222 (2004)
GLP:	yes (certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

Executive Summary

The effects of Reg. No. 4 670 450 (metabolite of BAS 222 F, metiram, BF 222-TDIT) on mortality, biomass development and reproduction of the earthworm *Eisenia fetida* (Annelida: Oligochaeta) were investigated in a 56-day extended laboratory study. Five concentrations (10, 20, 40, 80 and 160 mg Reg. No. 4 670 450/kg dry soil) were incorporated into the soil (10 % peat) with four 4 replicates per test item treatment and 8 replicates for the untreated control (each containing 10 worms). The reference item was tested in a separate study. Assessment of adult worm mortality, body weight and feeding activity was carried out after 28 days; assessment of reproduction was carried out after 56 days.

After 28 days of exposure, no mortality was observed in the control group. The mortality in the test item concentrations ranged between 0% and 2.5%, which was not statistically significantly different compared to the control. The weight change of adult worms was about 30.4% – 35.2% in the treated variants and 31.3% in the control group. Only the reproduction rate was significantly different compared to the control at the highest test item concentration of 160 mg/kg. No behavioral abnormalities were observed in any of the treatment groups. The feeding activity in all the treated groups was comparable to the control.

In a 56-day reproduction study with Reg. No. 4 670 450 on earthworms, the NOEC for weight change and was determined to be 160 mg Reg. No. 4 670 450/kg dry soil. The NOEC for reproduction was determined to be 80 mg Reg. No. 4 670 450/kg dry soil. The EC_{50} for reproduction was 131 mg Reg. No. 4 670 450/kg dry soil.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: Reg. No. 4 670 450 (metabolite of BAS 222 F (metiram), BF 222-TDIT), batch No. L83-138, analyzed purity: 91.4%.

B. STUDY DESIGN

Test species: Earthworm (*Eisenia fetida)*, adult worms (with clitellum and weight of 300 mg – 473 mg), age: approximately 3 months old; source: "W. Neudorff GmbH KG" followed by in-house culture.

Test design: In a 56-day test different concentrations of Reg. No. 4 670 450 were mixed homogeneously into treated artificial soil (10 % peat); 6 treatment groups (5 test item rates, control); 4 replicates per test item treatment group and 8 replicates for the control, with 10 worms each; assessment of worm mortality, behavioral effects and weight change after 28 days of exposure, after additional 28 days (56 days after application) determination of reproduction rate.

Endpoints: Mortality, weight change, feeding activity, reproduction rate, determination of NOEC.

Reference item: Nutdazim 50 FLOW (carbendazim, SC 500). The effects of the reference item were investigated in a separate study.

Test rates: Control, 10, 20, 40, 80 and 160 mg Reg. No. 4 670 450/kg dry soil.

Test conditions: Artificial soil according to OECD 222 (10 % peat); pH 6.20 - 6.27 at test initiation, pH 5.80 – pH 5.91 at test termination; water content of soil: 54.8% - 55.1% of max. water holding capacity (WHC) at test initiation, 54.0% - 54.3% of max. WHC at test termination; temperature: 18.2°C - 21.3°C; photoperiod: 16 h light : 8 h dark, light intensity: 540 lux; food: horse manure, straw and peat.

Statistics: Descriptive statistics; Fisher's Exact Binomial test for mortality data (α = 0.05, one-sided greater), Williams t-test for weight change and reproduction data (α = 0.05, one-sided smaller), Probit analysis for determination of EC_{50}.

II. RESULTS AND DISCUSSION

After 28 days of exposure, no mortality was observed in the control group. The mortality in the test item concentrations ranged between 0% and 2.5%, which was not statistically significantly different compared to the control (Fisher's Exact Binomial test, α = 0.05). The weight change of adult worms was about 30.4% – 35.2% in the treated variants and 31.3% in the control group. Only the reproduction rate was significantly different compared to the control at the highest test item concentration of 160 mg/kg (Williams t-test, α = 0.05). No behavioral abnormalities were observed in any of the treatment groups. The feeding activity in all the treated groups was comparable to the control. The results are summarized in Table 8.4.1-3

Table 8.4.1-3: Effect of Reg. No. 4 670 450 on earthworms (*Eisenia fetida*) in a 56-day reproduction study

Reg. No. 4 670 450 [mg/kg dry soil]	Control	10	20	40	80	160
Mortality (28 d) [%]	0	2.5	0	0	0	0
Weight change (28 d) [%]	31.3	30.4	35.2	33.1	32.8	32.4
Number of juveniles (56 d)	136.9	146.0	122.3	133.3	112.5	48.5 *
Reproduction [% of control] (56 d)	100	106.7	89.3	97.4	82.2	35.4
	Endpoints [mg Reg. No. 4 670 450/kg dry soil]					
$NOEC_{weight}$ (28 d)	≥ 160					
$NOEC_{reproduction}$ (56 d)	80					
EC_{50}	131					

* Statistically significantly different from control (Williams t-test, α = 0.05, one-sided smaller).

In the separate study with the reference item Nutdazim 50 FLOW (carbendazim, SC 500), the number of juveniles after 56 days was reduced by 72.7% and 98.8% at 5 and 10 mg/kg dry soil, respectively.

III. CONCLUSION

In a 56-day reproduction study with Reg. No. 4 670 450 on earthworms, the NOEC for weight change and mortality was determined to be 160 mg Reg. No. 4 670 450/kg dry soil. The NOEC for reproduction was determined to be 80 mg Reg. No. 4 670 450/kg dry soil. The EC_{50} for reproduction was 131 mg Reg. No. 4 670 450/kg dry soil.

The following chronic toxicity study on earthworms performed with the metabolite EBIS is provided in support of the risk assessment and has not been evaluated previously on EU level.

Report:	CA 8.4.1/4 Friedrich S., 2013b Sublethal toxicity of Reg.No. 243959 (metabolite of BAS 222 F, Metiram, BF 222-EBIS) to the earthworm Eisenia fetida in artificial soil 2013/1003166
Guidelines:	OECD 222 (2004)
GLP:	yes (certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

Executive Summary

The effects of Reg. No. 243 959 (metabolite of BAS 222 F, metiram, BF 222-EBIS) on mortality, biomass development and reproduction of the earthworm *Eisenia fetida* (Annelida: Oligochaeta) were investigated in a 56-day extended laboratory study. Five concentrations (10, 20, 40, 80 and 160 mg Reg. No. 243 959/kg dry soil) were incorporated into the soil (10 % peat) with four 4 replicates per test item treatment and 8 replicates for the control treatment (each containing 10 worms). The reference item was tested in a separate study. Assessment of adult worm mortality, body weight and feeding activity was carried out after 28 days; assessment of reproduction was carried out after 56 days.

After 28 days of exposure, a mortality of 1.3% was observed in the control group. The mortality in the test item concentrations ranged between 0% and 5.0%, which was not statistically significantly different compared to the control. The weight change of adult worms was about 21.1% – 24.5% in the treated variants and 23.4% in the control group, which was not statistically significantly different compared to the control. The reproduction rate was significantly different compared to the control at the two highest test item concentrations of 80 and 160 mg/kg. No behavioral abnormalities were observed in any of the treatment groups. The feeding activity in all the treated groups was comparable to the control.

In a 56-day reproduction study with Reg. No. 243 959 on earthworms, the NOEC for weight change was determined to be 160 mg Reg. No. 243 959/kg dry soil. The NOEC for reproduction was determined to be 40 mg Reg. No. 243 959/kg dry soil. The EC_{50} for reproduction was 113 mg Reg. No. 243959/kg dry soil.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: Reg. No. 243 959 (metabolite of BAS 222 F (metiram), BF 222-EBIS), batch No. L83-136, analyzed purity: 96.7%.

B. STUDY DESIGN

Test species: Earthworm (*Eisenia fetida)*, adult worms (with clitellum and weight of 300 mg – 450 mg), age: approximately 3 months old; source: "W. Neudorff GmbH KG" followed by in-house culture.

Test design: In a 56-day test different concentrations of Reg. No. 243 959 were mixed homogeneously into the soil (10 % peat); 6 treatment groups (5 test item rates, control); 4 replicates per test item treatment group and 8 replicates for the control, with 10 worms each; assessment of worm mortality, behavioral effects and weight change after 28 days of exposure, after additional 28 days (56 days after application) determination of reproduction rate.

Endpoints: Mortality, weight change, feeding activity, reproduction rate, determination of NOEC.

Reference item: Nutdazim 50 FLOW (carbendazim, SC 500). The effects of the reference item were investigated in a separate study.

Test rates: Control, 10, 20, 40, 80 and 160 mg Reg. No. 243 959/kg dry soil.

Test conditions: Artificial soil according to OECD 222 (10 % peat); pH 6.11 - 6.20 at test initiation, pH 5.87 – pH 5.93 at test termination; water content of soil: 54.8% - 54.9% of max. water holding capacity (WHC) at test initiation, 54.3% - 54.8% of max. WHC at test termination; temperature: 18.1°C - 20.8°C; photoperiod: 16 h light : 8 h dark, light intensity: 580 lux; food: horse manure.

Statistics: Descriptive statistics; Fisher's Exact Binomial test for mortality data (α = 0.05, one-sided greater), Williams' t-test for weight change and reproduction data (α = 0.05, one-sided smaller), Probit analysis for determination of EC_{50}.

II. RESULTS AND DISCUSSION

After 28 days of exposure, a mortality of 1.3% was observed in the control group. The mortality in the test item concentrations ranged between 0% and 5.0%, which was not statistically significantly different compared to the control. The weight change of adult worms was about 21.1% – 24.5% in the treated variants and 23.4% in the control group, which was not statistically significantly different compared to the control. The reproduction rate was significantly different compared to the control at the two highest test item concentrations of 80 and 160 mg/kg. No behavioral abnormalities were observed in any of the treatment groups. The feeding activity in all the treated groups was comparable to the control. The results are summarized in Table 8.4.1-4.

Table 8.4.1-4: Effect of Reg. No. 243 959 on earthworms (*Eisenia fetida*) in a 56-day reproduction study

Reg. No. 243 959 [mg/kg dry soil]	Control	10	20	40	80	160
Mortality (28 d) [%]	1.3	0.0	5.0	0.0	5.0	2.5
Weight change (28 d) [%]	23.4	24.5	23.9	21.1	24.1	22.5
Number of juveniles (56 d)	122.0	125.8	112.8	118.3	92.3 *	29.5 *
Reproduction [% of control] (56 d)	100	103.1	92.4	96.9	75.6	24.2
	Endpoints [mg Reg. No. 243 959/kg dry soil]					
$NOEC_{weight}$ (28 d)	≥ 160					
$NOEC_{reproduction}$ (56 d)	40					
EC_{50}	113					

* Statistically significantly different from control (Williams t-test, $\alpha = 0.05$, one-sided smaller).

In the separate study with the reference item Nutdazim 50 FLOW (carbendazim, SC 500), the number of juveniles after 56 days was reduced by 72.7% and 98.8% in 5 and 10 mg/kg dry soil, respectively.

III. CONCLUSION

In a 56-day reproduction study with Reg. No. 243 959 on earthworms, the NOEC for weight change was determined to be 160 mg Reg. No. 243 959/kg dry soil. The NOEC for reproduction was determined to be 40 mg Reg. No. 243 959/kg dry soil. The EC_{50} for reproduction was 113 mg Reg. No. 243959/kg dry soil.

CA 8.4.2 Effects on non-target soil meso- and macrofauna (other than earthworms)

The following chronic toxicity study on springtails performed with metiram (tested as BAS 222 28 F) is provided in support of the risk assessment and has not been evaluated previously on EU level.

Report: CA 8.4.2/1
Luehrs U., 2010a
Effects of BAS 222 28 F on reproduction of the Collembola Folsomia candida in artificial soil with 5% peat
2010/1075821

Guidelines: OECD 232 (2009), ISO 11267 (1999), EEC 91/414 amended by EEC 96/12

GLP: yes
(certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Executive Summary

The effects of BAS 222 28 F on mortality and reproduction of the Collembola *Folsomia candida* were investigated in a laboratory study over 28 days. The test item was incorporated into the soil at concentrations of 4, 8, 16, 32 and 64 mg BAS 222 28 F /kg dry soil. For the control treatment, the soil was left untreated. The artificial test soil had an organic content of 5% (peat). Assessment of mortality of the adults and reproduction (number of juveniles) was carried out after 28 days.

Mortality rates of up to 28% were observed at the test item concentrations up to and including 64 mg BAS 222 28 F/kg, which were not statistically significant different compared to 13% in the control. In the control, a mean of 861 juveniles was counted. In the treatment groups, a mean number of juveniles between 456 to 781 was counted, which corresponds to reproduction rates between 53% and 91% of the control group. Statistically significant differences on reproduction compared to the control were recorded at concentrations of 8, 16 and 64 mg BAS 222 28 F/kg dry soil. The effects at 8 and 16 mg BAS 222 28 F/kg dry soil were considered not to be treatment related due to the lack of a dose- response relationship (no statistically significant effect at 32 mg BAS 222 28 F/kg dry soil. No abnormal behavior or conditions of the surviving Collembola were observed at any tested concentration.

In a 28-day reproduction study with BAS 222 28 F on springtails, the overall NOEC was 32 mg BAS 222 28 F/kg dry soil. The NOEC based on mortality was ≥ 64 mg BAS 222 28 F/kg dry soil.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 25095588Q0; content of a.s.: metiram (BAS 222 F, Reg. no. 250 284): 70.39%; (nominal: 70%).

B. STUDY DESIGN

Test species: *Folsomia candida* (springtails), juveniles, 10 - 12 days old; source: in-house culture.

Test design: In a 28-day test, adults of *Folsomia candida* were exposed to 5 soil concentrations of BAS 222 28 F. Different concentrations of the test item were mixed homogeneously into the soil which was filled in glass vessels before the springtails were introduced on top of the soil surface. The test substrate was artificial soil according to OECD 232 (5% peat). In total, 7 treatment groups were set up (5 concentrations of the test item, an untreated control and reference item) with 4 replicates for the test item treatment groups and 8 replicates for the control with 10 collembolans per replicate. Assessment of adult collembolans mortality, behavioral effects and reproduction (number of juveniles) was done after 28 days.

Endpoints: NOEC (LC_{50}, EC_{50}).

Reference item: Boric acid (99.6% analyzed). The effects of the reference item were investigated in a separate study.

Test rates: Control, 4, 8, 16, 32 and 64 mg BAS 222 28 F/kg dry soil.

Test conditions: Artificial soil according to OECD 232 (5% peat); pH 5.6 - 5.7 at test initiation and pH 5.7 - 6.1 at test termination; water content at study initiation: 50.7% - 59.3% of maximum water holding capacity (WHC) and 44.2% - 53.5% of maximum WHC at test termination; temperature: 18 °C - 22 °C; photoperiod: 16 h light : 8 h dark, light intensity: 400 lux - 800 lux; food: approx. 2 mg dry yeast at the beginning of the test and on day 14.

Statistics: Descriptive statistics. Fisher’s Exact test for mortality data and Dunnett’s t-test for reproduction data (one-sided smaller, $\alpha = 0.05$).

II. RESULTS AND DISCUSSION

Mortality rates of up to 28% were observed at the test item concentrations up to and including 64 mg BAS 222 28 F/kg, which were not statistically significant different compared to 13% in the control (Fisher's Exact test, one-sided greater; α = 0.05). In the control, a mean of 861 juveniles was counted. In the treatment groups, a mean number of juveniles between 456 to 781 was counted, which corresponds to reproduction rates between 53% and 91% of the control group. Statistically significant differences on reproduction compared to the control were recorded at concentrations of 8, 16 and 64 mg BAS 222 28 F/kg dry soil (Dunnett's t-test, one-sided smaller; α = 0.05). The effects at 8 and 16 mg BAS 222 28 F/kg dry soil were considered not to be treatment related due to the lack of a dose-response relationship (no statistically significant effect at 32 mg BAS 222 28 F/kg dry soil (Dunnett's t-test, one-sided smaller; α = 0.05)). No abnormal behavior or conditions of the surviving Collembola were observed at any tested concentration. The results are summarized in Table 8.4.2-1.

Table 8.4.2-1: Effects of BAS 222 28 F on *Folsomia candida* in a 28-day reproduction study

BAS 222 28 F [kg/ha]	Control	4	8	16	32	64
Mortality (28 d) [%]	13	23	18	13	18	28
Number of juveniles (28 d)	861	764	677 *	697 *	781	456 *
Reproduction in percent of control (28 d) [%]	--	89	79	81	91	53
	Endpoints [kg BAS 222 28 F/ha]					
$NOEC_{reproduction}$	32					
$NOEC_{mortality}$	≥ 64					
$EC50_{reproduction}$ [1)]	> 64					
LC_{50}[1)]	> 64					

* Statistically significant differences compared to the control (Fisher`s Exact test for mortality data, Dunnett's t-test for reproduction data, α = 0.05).
[1)] Estimated value.

III. CONCLUSION

In a 28-day reproduction study with BAS 222 28 F on springtails, the overall NOEC was 32 mg BAS 222 28 F/kg dry soil. The NOEC based on mortality was ≥ 64 mg BAS 222 28 F/kg dry soil.

The following chronic toxicity study on soil mites performed with metiram (tested as BAS 222 28 F) is provided in support of the risk assessment and has not been evaluated previously on EU level.

Report:	CA 8.4.2/2 Luehrs U., 2010b Effects of BAS 222 28 F on reproduction of the predatory mite Hypoaspis aculeifer in artificial soil with 5% peat 2010/1075822
Guidelines:	EEC 91/414 amended by EEC 96/12, OECD 226 (2008)
GLP:	yes (certified by Hessisches Ministerium fuer Umwelt, Energie, Landwirtschaft und Verbraucherschutz, Wiesbaden)

Executive Summary

The effects of BAS 222 28 F on mortality and reproduction of the predatory mite *Hypoaspis aculeifer* were investigated in a laboratory study over 14 days. Five application rates (62.5, 125, 250, 500 and 1000 mg BAS 222 28 F/kg dry soil) were incorporated into the soil (5% peat) with 4 replicates per treatment (each containing 10 mites). An untreated control with 8 replicates was included. Assessment of adult mortality and reproduction rate was carried out after 16 days, after mites were extracted over 2 days by a heat gradient.

Mortality of up to 10% in the groups treated with BAS 222 28 F was observed, which was not statistically significantly different compared to the control where 8% mortality occurred. The reproduction rates were not statistically significantly different compared to control up to a concentration of 125 mg BAS 222 28 F/kg dry soil. At 250 mg BAS 222 28 F/kg dry soil and above, a statistically significant reduction of reproduction was observed.

In a 14-day predatory mite (*Hypoaspis aculeifer*) study with BAS 222 28 F, no unacceptable effects on survival and reproduction could be determined at concentrations up to 125 mg BAS 222 28 F/kg dry soil. The overall NOEC was 125 mg BAS 222 28 F/kg dry soil, which is equal to 87.5 mg a.s./kg dry soil. The EC_{10} was estimated to be 77.41 mg BAS 222 28 F/kg dry soil, corresponding to 54.19 mg a.s./kg dry soil.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 25095588Q0; content of a.s.: metiram (BAS 222 F, Reg. no. 250 284): 70.39%; (nominal: 70%).

B. STUDY DESIGN

Test species: Predatory mite (*Hypoaspis aculeifer*), adult females, approx. 8 days after reaching the adult stage (29 days after placing adult females in clean rearing vessel); source: Öre-Bioprotect GmbH, Germany.

Test design: 14-day test in treated artificial soil according to OECD 226 (5% peat); artificial soil filled in glass vessels was treated with different concentrations of the test item before predatory mites were introduced on top of the soil; 6 treatment groups (control, 5 test item concentrations); 8 replicates for control and 4 replicates for test item, each containing 10 female predatory mites; assessment of adult mortality and reproduction rate (number of juveniles) after 14 days.

Endpoints: Mortality and reproduction rate, determination of LC_{50}, EC_{50}.

Reference item: Perfekthion (dimethoate, 400 g/L nominal). The effects of the reference item were investigated at least once a year in a separate study.

Test rates: Control, 62.5, 125, 250, 500 and 1000 mg BAS 222 28 F/kg dry soil (equivalent to 43.75, 87.5, 175.0, 350.0 and 700.0 mg metiram/kg dry soil).

Test conditions: Temperature: 18 - 22 °C; pH 5.7 - 5.8 at test initiation, pH 5.7 - 5.9 at test termination; water content 20.6% - 21.7% (51.5 - 54.2% of the max. water holding capacity (WHC)) at experimental start, 19.5% - 20.6% (48.8% - 51.5% of max. WHC) at experimental end; photoperiod: 16 h light : 8 h dark, light intensity: 400 - 800 lux; food: cheese mite (*Tyrophagus putrescentiae*) *ad libitum* at test start and two to three times a week.

Statistics: Descriptive statistics. Mortality: Fisher's Exact test (one-sided greater, $\alpha = 0.05$). Reproduction: Dunnett's t-test (one-sided smaller, $\alpha = 0.05$). Probit analysis for determination of EC_{50}.

II. RESULTS AND DISCUSSION

Mortality of up to 10% in the groups treated with BAS 222 28 F was observed, which was not statistically significantly different compared to the control where 8% mortality occurred (Fisher's Exact test, $\alpha = 0.05$). The reproduction rates were not statistically significantly different compared to control up to a concentration of 125 mg BAS 222 28F/kg dry soil. At 250 mg BAS 222 28 F/kg dry soil and above, a statistically significant reduction of reproduction was observed (Dunnett's t-test, $\alpha = 0.05$). The results are summarized in Table 8.4.2-2.

Table 8.4.2-2: Effect of BAS 222 28 F on predatory mite (*Hypoaspis aculeifer*) in a 14-day reproduction study

BAS 222 28 F [kg/ha]	Control	62.5	125	250	500	1000
Mortality (14 d) [%]	8	3	3	3	10	8
Number of juveniles (14 d)	249	230	180	85 *	63 *	0 *
Reproduction in percent of control (14 d) [%]	--	92	72	34 *	25 *	0 *
	Endpoints [kg BAS 222 28 F/ha]					
NOEC $_{mortality}$ (14 d)	1000					
LC_{50}	> 1000					
NOEC $_{reproduction}$ (14 d)	125					
EC_{50}	204.2 (95% confidence limit: 134.4 – 307.3)					

* Statistically significant differences compared to the control (Dunnett's t-test, α= 0.05).

Based on a request by the RMS, EC_x values have been recalculated, applying a 3-parameter Weibull (type 2) model. The results are summarized in table 8.4.2-3. For full details, please refer to document 2017/1045558.

Table 8.4.2-3: EC_x estimation

	Endpoints [kg BAS 222 28 F/ha]
EC_{10}	77.41 (95% confidence limit: 27.64 – 127.18)
EC_{20}	101.34 (95% confidence limit: 48.05 – 154.63)

The reference item dimethoate inhibited the reproduction statistically significantly compared to control at a concentration of 4.0 mg dimethoate/kg dry soil. The EC_{50} for reproduction was 3.24 mg dimethoate/kg dry soil.

III. CONCLUSION

In a 14-day predatory mite (*Hypoaspis aculeifer*) study with BAS 222 28 F, no unacceptable effects on survival and reproduction could be determined at concentrations up to 125 mg BAS 222 28 F /kg dry soil. The overall NOEC was 125 mg BAS 222 28 F/kg dry soil, which is equal to 87.5 mg a.s./kg dry soil. The EC_{10} was estimated to be 77.41 mg BAS 222 28 F/kg dry soil, corresponding to 54.19 mg a.s./kg dry soil.

CA 8.4.2.1 Species level testing

Not triggered; no new studies are available.

CA 8.5 Effects on nitrogen transformation

Since Annex I inclusion of the active substance metiram (BAS 222 F) a new study on nitrogen transformation has been performed with the representative formulation BAS 222 28 F. As a result, there is a new endpoint, which is considered in the risk assessment.

Details on the EU agreed studies which have been already evaluated within the Annex I inclusion of metiram are provided in the EU Review documents of metiram (Monograph, Vol. 3, Annex B.9, July 2000; Addendum to the Monograph, Annex B.9, June 2002 and April 2004; EC Review Report (SANCO/4059/2001-rev 3.3), June 2005).

An overview on studies and endpoints is given in Table 8.5-1.

Table 8.5-1 Toxicity to nitrogen transformation of metiram and its metabolites

Substance	Endpoint	NOEC	Reference (BASF DocID)	EU agreed
metiram as contained in BAS 222 28 F [1)]	Effects on nitrogen transformation	51.2 mg a.s./kg dry soil	Dohmen, 1990/0223	yes
metiram as contained in BAS 222 28 F [2)]	Effects on nitrogen transformation	24.3 mg a.s./kg dry soil	Schulz 2010/1144224	no, new study
EU (= M222F003 = BF 222-EU = Reg. no. 27 270)	Effects on nitrogen transformation	5.6 mg/kg dry soil	Krieg, 2001/1000108	yes
ETU (= M222F002 = BF 222-ETU = Reg. no. 146 099)	Effects on nitrogen transformation	5.6 mg/kg dry soil	Krieg, 2001/1000109	yes

1) Formulation contains 80% metiram.
2) Formulation contains 70% metiram.

The following study on nitrogen transformation performed with metiram (tested as BAS 222 28 F) has already been evaluated on the EU level during a previous Annex I inclusion process for metiram. Nevertheless, a study summary is provided below.

Report:	*CA 8.5/1* *Dohmen G.P., 1990a* *Effect of BAS 222 28 F on nitrification* *1990/0223*
Guidelines:	*BBA VI 1-1*
GLP:	*yes* *(certified by Ministerium fuer Arbeit, Soziales, Familie und Gesundheit, Postfach 3180, 6500 Mainz)*

Executive Summary

The effect of BAS 222 28 F on nitrogen transformation was tested in two sandy loam soils. BAS 222 28 F was applied to samples of the soils, in a laboratory, at nominal application rates of 5.33 mg/kg and 64.00 mg/kg dry soil, equivalent to 4.0 kg/ha and 48 kg/ha. The treated soils and untreated controls were incubated at approx. 20 °C in the dark for 28 days. Triplicate samples of each treatment were removed for analysis of NH_4-nitrogen and NO_3-nitrogen, using an 'Expandable Ion Analyzer EA', 7, 14, 21, 28, 42, 56 and 70 days after application sandy loam A and 7, 14, 21, 28 and 35 after application sandy loam B.

No adverse effects of BAS 222 28 F on nitrogen transformation in two sandy loam soils were observed in both test item concentrations in sandy loam soil A after 56 days and in sandy loam soil B after 35 days.

Based on the results of this study, in accordance with OECD guideline 216, BAS 222 28 F caused no long-term effects on the soil nitrogen transformation (measured as NO_3-N production) in two field soils tested up to a concentration of 64.00 mg/kg dry soil (51.2 mg a.s./kg dry soil), equivalent to a field application rate of 48 kg BAS 222 28 F per ha.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 90-1; content of a.s.: Metiram (BAS 222 F, Reg. No. 250284): 80% (nominal).

B. STUDY DESIGN

Test species: Biologically active agricultural soil: sandy loam A: No. 90/145/01, pH 6.5, 1.1% C_{org}, WHC 30%; sandy loam B: No. 90/060/01, pH 7.3, 1.7% C_{org}, WHC 40%.

Test design: Determination of the N-transformation (NO_3-nitrogen production) after addition of $(NH_4)SO_4$ in soil. Comparison of test item treated soil with a non-treated soil and a reference item treated soil. 3 replicates per treatment and concentration. NH_4-nitrogen formed from the $(NH_4)SO_4$, from organically bound nitrogen and NO_3-nitrogen from the nitrification process was determined by using an 'Expandable Ion Analyzer EA' (Orion research, Cambridge). Sampling scheme: 0, 7, 14 21, 28, 42/35 and 56 days after treatment, subsamples were withdrawn from the bulk batches and subjected to the measurement.

Endpoints: Effects on the NO_3-nitrogen production after 56 days (sandy loam soil A) and 35 day (sandy loam soil B) of exposure.

Test rates: Control, 5.33 mg BAS 222 28 F per kg dry soil (corresponding to an application rate of 4.0 kg BAS 222 28 F/ha) and 64.00 mg BAS 222 28 F per kg dry soil (corresponding to 48.0 kg BAS 222 28 F per kg soil). Test concentrations related to a soil depth of 5 cm and a soil density of 1.5 g/cm^3.

Reference item: N-Serve 24E (Nitrapyrine, 21.9 %). The reference item was applied at a rate of 6.33 µl/kg dry soil.

Test conditions: Sand loam soil A: Soil moisture: 44% of its maximum water holding capacity. Sand loam soil B: Soil moisture: 47% of its maximum water holding capacity. Soil samples were incubated at 20.0 °C +/- 2.0 °C while stored in 1.5 L glass jars in the dark.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

No adverse effects of BAS 222 28 F on nitrogen transformation in two sandy loam soils were observed in both test item concentrations in sandy loam soil A after 56 days and in sandy loam soil B after 35 days. Results are summarized in Table 8.5-2.

Table 8.5-2: Effects of BAS 222 28 F on soil micro-organisms (nitrogen transformation) on days 7, 14, 21, 28, 35/42 and 56

Soil (days)	Control	5.33 mg BAS 222 28 F per kg dry soil equivalent to 4.0 kg/ha		64.00 mg BAS 222 28 F per kg dry soil equivalent to 48.0 kg/ha	
	NO_3-N [mg/100 g dry soil]	NO_3-N [mg/100 g dry soil]	% Deviation from the control [1)]	NO_3-N [mg/100 g dry soil]	% Deviation from the control [1)]
Sandy loam soil A (7 d)	7.18	0.5	- 93.0	0.00	- 100.0
Sandy loam soil A (14 d)	13.86	8.07	- 41.8	0.00	- 100.0
Sandy loam soil A (21 d)	15.36	12.76	- 25.5	0.00	- 100.0
Sandy loam soil A (28 d)	16.17	17.12	+ 5.9	0.00	- 100.0
Sandy loam soil A (42 d)	14.65	18.21	+ 24.3	3.45	- 76.5
Sandy loam soil A (56 d)	14.79	16.84	+13.9	15.33	+ 3.7
Sandy loam soil B (7 d)	16.99	3.56	- 79.0	± 0.00	- 100.0
Sandy loam soil B (14 d)	18.63	18.63	± 0.0	± 0.00	- 100.0
Sandy loam soil B (21 d)	18.60	18.43	- 0.9	0.66	- 96.5
Sandy loam soil B (28 d)	18.14	18.14	± 0.0	7.03	- 61.8
Sandy loam soil B (35 d)	18.23	19.01	+ 4.3	18.90	+ 3.7

1) Based on NO_3-nitrogen production; - = inhibition, + = stimulation.

In the study the reference item N-Serve 24E (Nitrapyrine, 21.9 %) produced a clear effect at 6.33 µl/kg dry soil.

III. CONCLUSION

Based on the results of this study, in accordance with OECD guideline 216, BAS 222 28 F caused no long-term effects on the soil nitrogen transformation (measured as NO_3-N production) in two field soils tested up to a concentration of 64.00 mg/kg dry soil (51.2 mg a.s./kg dry soil), equivalent to a field application rate of 48 kg BAS 222 28 F per ha.

Study Comments: IIIA 8.5/1	
Agreed endpoint: IIIA 8.5/1	

The following study on nitrogen transformation performed with metiram (tested as BAS 222 28 F) is provided in support of the risk assessment and has not been evaluated previously on EU level.

Report:	CA 8.5/2 Schulz L., 2011a Effects of BAS 222 28 F on the activity of soil microflora (nitrogen transformation test) 2010/1144224
Guidelines:	OECD 216 (2000)
GLP:	yes (certified by Saechsisches Staatsministerium fuer Umwelt und Landwirtschaft, Dresden, Germany)

Executive Summary

In a soil microbial activity study, the effects of BAS 222 28 F on the nitrogen transformation were investigated in a silty loamy sand soil. BAS 222 28 F was applied to samples of the soil at nominal test concentrations of 3.47 and 34.67 mg/kg dry soil. BAS 222 28 F treated soils and untreated controls were incubated at approx. 20 °C in the dark for 28 days.

No adverse effects of BAS 222 28 F on nitrogen transformation could be observed at both test concentrations after 28 days. Only negligible deviations from the control of +4.9% (test concentration of 3.47 mg BAS 222 28 F/kg dry soil) and +18.5% (test concentration of 34.67 mg BAS 222 28 F/kg dry soil) were measured at the end of the 28-day incubation period (time interval 0-28).

Based on the results of this study, BAS 222 28 F caused no adverse effects (deviation from control < 25 %, OECD 216) on the nitrogen transformation in a silty loamy sand soil tested up to a concentration of 34.67 mg BAS 222 28 F/kg dry soil (equivalent to 24.3 mg a.s./kg dry soil) at the end of the 28-day incubation period.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 222 28 F; batch no. 25095588Q0; content of a.s.: metiram (BAS 222 F, Reg. No. 250 284): 70.39% (70.0% nominal).

B. STUDY DESIGN

Test soil: Biologically active agricultural soil: silty loamy sand (DIN 4220) / sandy loam (USDA): pH 6.4, 1.43% C_{org}, 36.41% water holding capacity (WHC).

Test design: Determination of the N-transformation (NO_3-nitrogen production) in soil enriched with lucerne meal (concentration in soil 0.5%). Comparison of test item treated soil with a non-treated soil. NH_4-nitrogen formed from organically bound nitrogen in the soil and NO_3-nitrogen from the nitrification process was determined by using an Autoanalyzer II (Bran and Luebbe). Sampling scheme: 0, 7, 14 and 28 days after treatment, sub-samples (3 replicates) were withdrawn from the bulk batches and subjected to measurement.

Endpoints: Effects on NO_3-nitrogen production 0, 7, 14, and 28 days after application.

Test concentrations: Control, 3.47 and 34.67 mg BAS 222 28 F/kg dry soil.

Reference item: Dinoterb (purity: 99.9%). The reference item was tested in a separate study at rates of 6.80, 16.00 and 27.00 mg/kg dry soil.

Test conditions: Temperature: 18.5 °C - 21.3 °C; pH 6.4; soil moisture: approx. 45% of its maximum water holding capacity, measured water content: 15.24 - 16.01 g/100 g dry soil. Soil samples were incubated while stored in glass flasks in the dark.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

No adverse effects of BAS 222 28 F on nitrogen transformation could be observed at both test concentrations after 28 days. Only negligible deviations from the control of +4.9% (test concentration of 3.47 mg BAS 222 28 F/kg dry soil) and +18.5% (test concentration of 34.67 mg BAS 222 28 F/kg dry soil) were measured at the end of the 28-day incubation period (time interval 0-28). The results are summarized in Table 8.5-3.

Table 8.5-3: Effects BAS 222 28 F on soil micro-organisms (nitrogen transformation) on days 0, 7, 14 and 28 of incubation

Soil (days)	Control	3.47 mg BAS 222 28 F/kg dry soil		34.67 mg BAS 222 28 F/kg dry soil	
	NO_3-N [mg/kg dry soil]	NO_3-N [mg/kg dry soil]	% Deviation from the control [1)]	NO_3-N [mg/kg dry soil]	% Deviation from the control [1)]
Loamy sand soil (0 -7 d)	10.23	11.73	+14.7	2.83	-72.3
Loamy sand soil (0 - 14 d)	15.53	18.00	+15.9	14.50	-6.7
Loamy sand soil (0 - 28 d)	26.60	27.90	+4.9	31.53	+18.5

[1)] Based on NO_3-nitrogen production; - = inhibition; + = stimulation

In a separate study the reference item Dinoterb produced a stimulation of nitrogen transformation of +37.6%, +51.4% and +27.1% at 6.80 mg, 16.00 mg and 27.00 mg/kg dry soil, respectively, 28 days after application.

III. CONCLUSION

Based on the results of this study, BAS 222 28 F caused no adverse effects (deviation from control < 25 %, OECD 216) on the nitrogen transformation in a silty loamy sand soil tested up to a concentration of 34.67 mg BAS 222 28 F/kg dry soil (equivalent to 24.3 mg a.s./kg dry soil) at the end of the 28-day incubation period.

Study Comments: IIIA 8.5/2	
Agreed endpoint: IIIA 8.5/2	

The following study on nitrogen transformation performed with BF 222-EU has already been evaluated on the EU level during a previous Annex I inclusion process for metiram. Nevertheless, a study summary is provided below.

Report:	*CA 8.5/3* *Krieg W., 2001a* *Effect of BF 222-EU on nitrogen transformation of the soil microflora* *2001/1000108*
Guidelines:	*OECD 216*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a soil microbial activity study, the effects of BF 222-EU (metabolite of BAS 222 F) on soil micro-organism activity (nitrogen transformation) were investigated in a lucerne-enriched loamy sand soil. BF 222-EU was applied to samples of the soil at nominal application rates of 0.56 mg/kg and 5.6 mg/kg dry soil. BF 222-EU treated soil and the untreated control was incubated at approx. 20 °C in the dark for 28 days. Triplicate samples of each treatment were removed for analysis of NH_4-nitrogen and NO_3-nitrogen 7, 14 and 28 days after application.

Based on the results of this study, in accordance with OECD guideline 216, BF 222-EU caused no short-term and no long-term effects on the soil nitrogen transformation (measured as NO_3-N production) in a field soil tested up to a concentration of 5.6 mg/kg dry soil, equivalent to a field application rate of 4.20 kg BF 222-EU per ha.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BF 222-EU (metabolite of BAS 222 F, Reg. No. 27 270), Lot No.: 01743-161, purity: 88.6%.

B. STUDY DESIGN

Test soil: Biologically active agricultural soil: loamy sand soil, pH 6.2, C_{org}: 18.2 mg C/100 g dry weight (OxiTop® method), WHC: 32%.

Test design: Determination of the N-transformation (NO_3-nitrogen production) in soil enriched with lucerne meal (concentration in soil 0.5%). Comparison of test item treated soil was done with the non-treated soil; 3 replicates per treatment and concentration. NH_4-nitrogen formed from organically bound nitrogen and NO_3-nitrogen from the nitrification process was determined by using calibrated ion-sensitive electrodes and the 'Expandable Ion Analyzer EA 940' (Orion Research, Cambridge). Sampling scheme: 0, 7, 14 and 28 days after treatment, sub-samples were withdrawn from the bulk batches and subjected to measurement.

Endpoints: Effects on NO_3-nitrogen production 7, 14 and 28 days after application.

Test concentrations: Control, 0.56 mg BF 222-EU per kg dry soil (corresponding to a single application rate of 0.42 kg/ha) and 5.6 mg BF 222-EU per kg dry soil (corresponding to 4.20 kg/ha). Test concentrations related to a soil depth of 5 cm and a soil density of 1.5 g/cm^3. The 0.56 mg a.s./ kg dry soil treatment group reflected the maximum transformation rate (15%) of the active substance BAS 222 F into the metabolite BF 222-EU.

Reference item: N-Serve. Effects of the reference item were reported in a separate study.

Test conditions: Soil moisture: 45% of its maximum water holding capacity, pH 6.94 – 7.47. Soil samples were incubated at 18 - 22 °C while stored in glass bottles in the dark.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

No adverse influence of BF 222-EU on the nitrate transformation could be observed during the whole study at both application rates. Compared to the control, only negligible deviations of -0.44% (0.56 mg BF 222-EU per kg dry soil) and +2.21% (5.6 mg BF 222-EU per kg dry soil) were measured after 28 days. The results are summarized in Table 8.5-4.

Table 8.5-4: Effects of BF 222-EU on soil micro-organisms (nitrogen transformation) on days 7, 14 and 28 of incubation

Soil	Control	0.56 mg BF 222-EU per kg dry soil equivalent to 0.42 kg/ha		5.6 mg BF 222-EU per kg dry soil equivalent to 4.2 kg/ha	
	NO_3-N [mg/kg dry soil]	NO_3-N [mg/kg dry soil]	% Deviation from the control [1)]	NO_3-N [mg/kg dry soil]	% Deviation from the control [1)]
Loamy sand (7 d)	5.74	5.69	-0.87	6.17	+7.49
Loamy sand (14 d)	3.40	3.40	0.00	3.59	+5.59
Loamy sand (28 d)	2.26	2.25	-0.44	2.31	+2.21

[1)] Based on NO_3-nitrogen production; - = inhibition; + = stimulation.

In a separate study the reference item N-Serve produced an effect on the N-transformation of -69.4% in loamy sand soil and -53.9% in sandy loam soil at a rate of 20 mg/kg soil.

III. CONCLUSION

Based on the results of this study, in accordance with OECD guideline 216, BF 222-EU caused no short-term and no long-term effects on the soil nitrogen transformation (measured as NO_3-N production) in a field soil tested up to a concentration of 5.6 mg/kg dry soil, equivalent to a field application rate of 4.20 kg BF 222-EU per ha.

Study Comments: IIIA 8.5/3	
Agreed endpoint: IIIA 8.5/3	

The following study on nitrogen transformation performed with BF 222-ETU has already been evaluated on the EU level during a previous Annex I inclusion process for metiram. Nevertheless, a study summary is provided below.

Report:	*CA 8.5/4* *Krieg W., 2001b* *Effect of BF 222-ETU on nitrogen transformation of the soil microflora* *2001/1000109*
Guidelines:	*OECD 216*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a soil microbial activity study, the effects of BF 222-ETU (metabolite of BAS 222 F) on soil micro-organism activity (nitrogen transformation) were investigated in a lucerne-enriched loamy sand soil. BF 222-ETU was applied to samples of the soil at nominal application rates of 0.56 mg/kg and 5.6 mg/kg dry soil. BF 222-ETU treated soil and the untreated control was incubated at approx. 20 °C in the dark for 28 days. Triplicate samples of each treatment were removed for analysis of NH_4-nitrogen and NO_3-nitrogen 7, 14 and 28 days after application.

Based on the results of this study, in accordance with OECD guideline 216, BF 222-ETU caused no long-term effects on the soil nitrogen transformation (measured as NO_3-N production) in a field soil tested up to a concentration of 5.6 mg/kg dry soil, equivalent to a field application rate of 4.20 kg BF 222-ETU per ha.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BF 222-ETU (metabolite of BAS 222 F, Reg. No. 146 099), Lot-No. 01743-165, purity: 99.9%.

B. STUDY DESIGN

Test soil: Biologically active agricultural soil: loamy sand soil, pH 6.2, C_{org}: 18.2 mg C/100 g dry weight (OxiTop® method), WHC: 32%.

Test design: Determination of the N-transformation (NO_3-nitrogen production) in soil enriched with lucerne meal (concentration in soil 0.5%). Comparison of test item treated soil was done with the non-treated soil; 3 replicates per treatment and concentration. NH_4-nitrogen formed from organically bound nitrogen and NO_3-nitrogen from the nitrification process was determined by using calibrated ion-sensitive electrodes and the 'Expandable Ion Analyzer EA' (Orion research, Cambridge).
Sampling scheme: 0, 7, 14 and 28 days after treatment, sub-samples were withdrawn from the bulk batches and subjected to measurement.

Endpoints: Effects on NO_3-nitrogen production 7, 14 and 28 days after application.

Test concentrations: Control, 0.56 mg BF 222-ETU per kg dry soil (corresponding to a single application rate of 0.42 kg/ha) and 5.6 mg BF 222-ETU per kg dry soil (corresponding to 4.20 kg/ha). Test concentrations related to a soil depth of 5 cm and a soil density of 1.5 g/cm^3. The 0.56 mg a.s./ kg dry soil treatment group reflected the maximum transformation rate (15%) of the active substance BAS 222 F into the metabolite BF 222-EU.

Reference item: N-Serve. Effects of the reference item were reported in a separate study.

Test conditions: Soil moisture: 45% of its maximum water holding capacity, pH 6.97 – 7.49. Soil samples were incubated at 18 - 22 °C while stored in glass flasks in the dark.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

No adverse influence of BF 222-ETU on the nitrate transformation could be observed after 28 days at both application rates. Compared to the control, slightly reduced nitrogen transformation rates of +5.05% and +5.96% in the 0.56 mg/kg and 5.60 mg/kg dry soil, respectively, were measured after 28 days. The results are summarized below Table 8.5-5.

Table 8.5-5: Effects of BF 222-ETU on soil micro-organisms (nitrogen transformation) on days 7, 14 and 28 of incubation

Soil	Control	0.56 mg BF 222-ETU per kg dry soil equivalent to 0.42 kg/ha		5.6 mg BF 222-ETU per kg dry soil equivalent to 4.20 kg/ha	
	NO_3-N [mg/kg dry soil]	NO_3-N [mg/kg dry soil]	% Deviation from the control [1)]	NO_3-N [mg/kg dry soil]	% Deviation from the control [1)]
Loamy sand (7 d)	5.43	5.77	+6.26	3.86	-28.91
Loamy sand (14 d)	3.17	3.37	+6.31	3.37	+6.31
Loamy sand (28 d)	2.18	2.29	+5.05	2.31	+5.96

1) Based on NO_3-nitrogen production; - = inhibition; + = stimulation

In a separate study the reference item N-Serve produced an effect on the N-transformation of -69.4% in loamy sand soil and -53.9% in sandy loam soil at a rate of 20 mg/kg soil.

III. CONCLUSION

Based on the results of this study, in accordance with OECD guideline 216, BF 222-ETU caused no long-term effects on the soil nitrogen transformation (measured as NO_3-N production) in a field soil tested up to a concentration of 5.6 mg/kg dry soil, equivalent to a field application rate of 4.20 kg BF 222-ETU per ha.

Study Comments: IIIA 8.5/4	
Agreed endpoint: IIIA 8.5/4	

CA 8.6 Effects on terrestrial non-target higher plants

No new studies are available.

CA 8.6.1 Summary of screening data

No new studies are available.

CA 8.6.2 Testing on non-target plants

No new studies are available.

CA 8.7 Effects on other terrestrial organisms (flora and fauna)

The following studies are no data requirement for the risk assessment and are presented as additional information to the dossier.

The following acute toxicity study on earthworms, performed with metiram (tested as BAS 222 28 F), is provided in support of the risk assessment and has not been evaluated previously on the EU level.

Report:	CA 8.7/1 Sattler F., 2008a Effect of BAS 222 28 F on the mortality of the earthworm Eisenia fetida in artificial soil with 5% peat 2008/1005501
Guidelines:	ISO 11268-1
GLP:	yes (certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, Mainz, Germany)

Executive Summary

Adult earthworms, of the species *Eisenia fetida*, were exposed to BAS 222 28 F. The test item was mixed into artificial soil at rates of 198, 296, 444, 667 and 1000 mg BAS 222 28 F/kg dry soil. For the control treatment, the soil was left untreated. The test soil had an organic content of 5% instead of 10% (as sphagnum peat) to increase the bioavailability of the test item.

The worms were placed on the surface of the soil. Four replicates were prepared for each treatment group and the control, each containing 10 worms. Assessments of mortality and behavioral effects were made 7 and 14 days after treatment. Assessment of worm weight was made after 14 days.

After 14 days of exposure no mortality was observed at test item concentrations up to 444 mg BAS 222 28 F/kg dry soil. Low mortality of 2.5% was observed in the two highest test item concentrations of 667 and 1000 mg BAS 222 28 F/kg dry soil. No statistically significant mortality was observed in the two highest test item concentrations. Biomass development was not statistically significant different compared to the control up to and including 296 mg BAS 222 28 F/kg dry soil.

In a 14-d toxicity study with BAS 222 28 F on earthworms (*Eisenia fetida)* the LC_{50} was > 1000 mg BAS 222 28 F/kg dry soil (equivalent to >700 mg a.s./kg dry soil). The NOEC related to biomass was 296 mg BAS 222 28 F/kg dry soil.

I. MATERIALS AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 1653; content of a.s.: metiram (BAS 222 F, Reg. No. 250284): 70% nominal, 72.5% analyzed.

B. STUDY DESIGN

Test species: Earthworm (*Eisenia fetida)*, source: in-house culture, adult worms (with clitellum and weight 300 mg - 600 mg), between two months and one year old.

Test design: 14-d exposure in treated artificial soil (5% peat); different concentrations of the test item are mixed homogeneously into the soil which is filled in glass jars before the earthworms are introduced on top of the soil; 6 treatment groups (5 test item rates, control); 4 replicates/group with 10 worms each. Earthworm mortality and behavioral effects were assessed after 7 and 14 d, measurement of weight change as sublethal parameter after 14 d.

Endpoints: LC_{50} (50% mortality of earthworms after exposure over 14 days), NOEC, behavioral effects, weight change.

Reference item: 2-chloroacetamide. The effects of the reference item were investigated in a separate study.

Test concentrations: Control, 198, 296, 444, 667 and 1000 mg BAS 222 28 F/kg dry soil.

Test conditions: Artificial soil according to ISO 11268-1 (with a reduced content of peat of 5%); pH 6.0 at test initiation; water content at test initiation: 19.0% (of dry soil), corresponding to 50% of the max. WHC; 18.8% (of dry soil) at test termination; temperature: 20.0 °C - ±2 °C; constant illumination of 400 lux – 800 lux at a light/dark cycle of 16h : 8 h.

Statistics: Descriptive statistics, Dunnett's t-test for weight change and Fishers-exact-test for mortality ($\alpha = 0.05$).

II. RESULTS AND DISCUSSION

The LC_{50} was determined to be > 1000 mg BAS 222 28 F/kg dry soil.

After 14 days of exposure no mortality was observed at test item concentrations up to 444 mg BAS 222 28 F/kg dry soil. Low mortality of 2.5% was observed in the two highest test item concentrations of 667 and 1000 mg BAS 222 28 F/kg dry soil. No statistical significant mortality was observed in the two highest test item concentrations. The biomass development was not statistically significant different compared to the control up to and including 296 mg BAS 222 28 F/ kg dry soil (see Table 8.7-1).

Table 8.7-1: Effect of BAS 222 28 F on earthworm (*Eisenia fetida)* mortality and biomass (14 d)

BAS 222 28 F [mg/kg dry soil]	Control	198	296	444	667	1000
Mortality [%]	0.0	0.0	0.0 n s	0.0 n s	2.5 n s	2.5 n s
Weight change [%]	-10.76	-12.37 n s	-14.70 n s	-18.04 *	-24.86 *	-27.58 *
Endpoints [mg/kg dry soil]						
$NOEC_{weight\ change}$	296					
LC_{50}	> 1000					

* = Statistically significant differences compared to the control (Fishers-exact-test for mortality, Dunnett's t-test for weight data; $\alpha = 0.05$).
n.s. = No statistically significant differences compared to the control (Fisher-exact-test for mortality, Dunnett's t-test for weight data; $\alpha = 0.05$).

III. CONCLUSION

In a 14-d toxicity study with BAS 222 28 F on earthworms (*Eisenia fetida)* the LC_{50} was > 1000 mg BAS 222 28 F/kg dry soil (equivalent to >700 mg a.s./kg dry soil). The NOEC related to biomass was 296 mg BAS 222 28 F/kg dry soil.

The following acute toxicity study on earthworms performed with BF 222-EU has already been evaluated on the EU level during previous Annex I inclusion process for metiram. Nevertheless, a study summary is provided below.

Report:	*CA 8.7/2* *Staab F., 2001a* *Effect of BF 222-EU on the mortality of the earthworm Eisenia foetida* *2001/1000881*
Guidelines:	*OECD 207*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In an acute toxicity laboratory study, adult earthworms of the species *Eisenia fetida* (Annelida: Oligochaeta), were exposed to BF 222-EU (metabolite of BAS 222 F). The test item was mixed into artificial soil at rates of 175.0, 262.5, 393.7, 590.7 and 886.0 mg/kg dry soil (actual concentration). For the control treatment, the soil was left untreated. The worms were introduced on top of the soil. Four replicates were prepared for the treatment groups, each containing 10 worms. Assessment of mortality was carried out 7 and 14 days after treatment. Assessments of weight change and behavioral effects as sub-lethal parameter were made after 14 days.

After 14 days of exposure, no mortality was observed at any of the test item groups. The biomass rose in all treatments especially in the treatment groups with higher test concentrations, but no statistically significant effects on body weight were observed in the whole concentration range. No other particular behavioral or morphological changes were observed.

In a 14-d toxicity study with earthworms (*Eisenia fetida*) the LC_{50} of BF 222-EU was >886.0 mg/kg dry soil. The NOEC was determined to be 886.0 mg/kg dry soil (with respect to worm mortality and biomass).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BF 222-EU (Metabolite of BAS 222 F, Reg. No. 27/270), batch no. 01743-161, purity: 88.6%.

B. STUDY DESIGN

Test species: Earthworm (*Eisenia fetida*), adult worms (with clitellum and weight between 250 mg and 600 mg), age: less than 1 year old, source: from in-house culture.

Test design: 14-d exposure in treated artificial soil with 10% peat according to OECD 207. Different concentrations of the test item were mixed homogeneously into the soil, which was then used to fill glass vessels after which the earthworms were introduced on top of the soil. 6 treatment groups (5 test item concentrations, control); 4 replicates/ treatment group with 10 worms each. Assessment of worm mortality was carried out after 7 and 14 d, assessment of weight change and behavioral effects as sub-lethal parameter was made after 14 d.

Endpoints: NOEC, LC_{50} (50% mortality of earthworms after exposure over 14 days), behavioral effects, weight change.

Test concentrations: Control, 197.5, 296.3, 444.4, 666.7and 1000.0 mg BF 222-EU/kg dry soil. With respect to the low purity of the batch the actual concentrations of BF 222-EU were calculated to be 175.0, 262.5, 393.7, 590.7 and 886.0 mg BF 222-EU/kg dry soil.

Reference item: 2-Chloroacetamide. The effects of the reference item were evaluated in a separate study.

Test conditions: Artificial soil according to OECD 207; pH 5.8; water content: 34.8% soil dry weight at test initiation (corresponding to 60% of the max. water holding capacity), 32.5% at test termination (mean out of 3 replicates), temperature: 20 °C - 21 °C; continuous illumination.

Statistics: Descriptive statistics. ANOVA followed by Dunnett's t-test for biomass development.

II. RESULTS AND DISCUSSION

After 14 days of exposure, no mortality was observed at any of the test item treatment groups.
The biomass rose in all treatments especially in the treatment groups with higher test concentrations, but no statistically significant effects on body weight were observed in the whole concentration range. No other particular behavioral or morphological changes were observed. The results are summarized in Table 8.7-2

Table 8.7-2: Effect of BF 222-EU on earthworm (*Eisenia fetida)* mortality and biomass (14 d)

Concentration [mg/kg dry soil][1)]	**Control**	**175.0**	**262.5**	**393.7**	**590.7**	**886.0**
Mortality [%]	0.0	0.0	0.0	0.0	0.0	0.0
Weight change [%]	0.71	4.01 n s	5.85 n s	10.86 n s	10.01 n s	10.58 n s
	Endpoints [mg BF 222-EU/kg dry soil]					
LC_{50}	>886.0					
NOEC	886.0					

1) actual concentrations based on calculation of 100% purity of BF 222-EU
n.s. = no statistically significant differences compared to the control (Dunnett's t-test for weight change; $\alpha = 0.05$)

III. CONCLUSION

In a 14-d toxicity study with earthworms (*Eisenia fetida*) the LC_{50} of BF 222-EU was >886.0 mg/kg dry soil. The NOEC was determined to be 886.0 mg/kg dry soil (with respect to worm mortality and biomass).

The following acute toxicity study on earthworms performed with BF 222-ETU has already been evaluated on the EU level during previous Annex I inclusion process for metiram. Nevertheless, a study summary is provided below.

Report:	*CA 8.7/3* *Staab F., 2001b* *Effect of metabolite ETU on the mortality of the earthworm Eisenia foetida* *2001/1000882*
Guidelines:	*OECD 207*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In an acute toxicity laboratory study, adult earthworms of the species *Eisenia fetida* (Annelida: Oligochaeta), were exposed to BF 222-ETU (metabolite of BAS 222 F). The test item was mixed into artificial soil at rates of 197.5, 296.3, 444.4, 666.7 and 1000 mg/kg dry soil. For the control treatment, the soil was left untreated. The worms were introduced on top of the soil. Four replicates were prepared for the treatment groups, each containing 10 worms. Assessments of mortality and behavioral effects were carried out 7 and 14 days after treatment. Assessment of weight change as sub-lethal parameter was made after 14 days.

After 14 days of exposure a mean mortality of 10% was observed in the 296.3 mg/kg dry soil treatment. In other variants including the control and the groups treated with higher concentrations of BF 222-ETU mortality of 0.0% or 2.5% occurred. No significantly relevant reduction in biomass could be determined.

In a 14-d toxicity study with earthworms (*Eisenia fetida*) the LC_{50} of BF 222-ETU was >1000.0 mg/kg dry soil. The NOEC was determined to be 1000.0 mg/kg dry soil (with respect to worm mortality and biomass).

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BF 222-ETU (Metabolite of BAS 222 F, Reg. No. 146/099), batch no. 01743-165, purity: 99.9%.

B. STUDY DESIGN

Test species: Earthworm (*Eisenia fetida*), adult worms (with clitellum and weight between 250 mg and 600 mg), age: less than 1 year old, source: from in-house culture.

Test design: 14-d exposure in treated artificial soil with 10% peat according to OECD 207. Different concentrations of the test item were mixed homogeneously into the soil, which was then used to fill glass vessels after which the earthworms were introduced on top of the soil. 6 treatment groups (5 test item concentrations, control); 4 replicates/ treatment group with 10 worms each. Assessments of worm mortality were made after 7 and 14 d, assessments of weight change and behavioral effects as sub-lethal parameter were made after 14 d.

Endpoints: NOEC, LC_{50} (50% mortality of earthworms after exposure over 14 days), behavioral effects, weight change.

Test concentrations: Control, 197.5, 296.3, 444.4, 666.7 and 1000.0 mg BF 222-ETU/kg dry soil.

Reference item: 2-Chloroacetamide. The effects of the reference item were evaluated in a separate study.

Test conditions: Artificial soil according to OECD 207; pH 5.8; water content: 34.8% soil dry weight at test initiation (corresponding to 60% of the max. water holding capacity), 34.8% at test termination (mean out of 3 replicates), temperature: 20 °C - 21 °C; continuous illumination.

Statistics: Descriptive statistics. ANOVA followed by Dunnett's t-test for biomass development.

II. RESULTS AND DISCUSSION

After 14 days of exposure a mean mortality of 10% was observed in the 296.3 mg/kg dry soil treatment. In other treatments including the control and the groups treated with higher concentrations of BF 222-ETU mortality of 0% or 2.5% occurred. No significantly relevant reduction in biomass could be determined. No other particular behavioral or morphological changes were observed. The results are summarized in Table 8.7-3.

Table 8.7-3: Effect of BF 222-ETU on earthworm (*Eisenia fetida)* mortality and biomass (14 d)

Concentration [mg/kg dry soil]	Control	197.5	296.3	444.4	666.7	1000.0
Mortality [%]	2.5	0.0	10.0	2.5	0.0	2.5
Weight change [%]	-8.96	-3.98 n s	1.77 n s	1.64 n s	7.44 n s	4.15 n s
	Endpoints [mg BF 222-ETU/kg dry soil]					
LC_{50}	>1000.0					
NOEC	1000.0					

n.s. = no statistically significant differences compared to the control (Dunnett-test t for weight change; $\alpha = 0.05$)

III. CONCLUSION

In a 14-d toxicity study with earthworms (*Eisenia fetida*) the LC_{50} of BF 222-ETU was >1000.0 mg/kg dry soil. The NOEC was determined to be 1000.0 mg/kg dry soil (with respect to worm mortality and biomass).

The following study on carbon transformation performed with metiram (tested as BAS 222 28 F) has already been evaluated on the EU level during previous Annex I inclusion process for metiram. Nevertheless, a study summary is provided below.

Report:	*CA 8.7/4* *Gerhardt R., 1990a* *Effect of BAS 222 28 F on soil respiration* *1990/0190*
Guidelines:	*BBA VI 1-1*
GLP:	*yes* *(certified by Ministerium fuer Arbeit, Soziales, Familie und Gesundheit, Postfach 3180, 6500 Mainz)*

Executive Summary

The effect of BAS 222 28 F on carbon transformation was investigated in two sandy loam soils. BAS 222 28 F was applied to samples of the soils A and B in a laboratory at nominal application rates of 5.33 mg/kg and 64.00 mg/kg dry soil, equivalent to 4.0 kg/ha and 48 kg/ha. BAS 222 28 F treated soils and untreated controls were incubated at apporox. 20°C in the dark. Four replicate samples of each treatment were removed for analysis of carbon transformation (oxygen consumption), using a "Sapromat B12" system, 7, 14, 21, 28 and 42 days after application.

No adverse effects of BAS 222 28 F on carbon transformation in soil were observed in both test item concentrations in sandy loam soil A after 42 days and in sandy loam soil B after 28 days.

Based on the results of this study, in accordance with OECD guideline 217, BAS 222 28 F caused long-term effects on carbon transformation in two field soils tested up to a concentration of 64.0 mg/kg dry soil, equivalent to a field application rate of 48 kg BAS 222 28 F per ha.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BAS 222 28 F, batch no. 90-1; content of a.s.: Metiram (BAS 222 F, Reg. No. 250284): 80% (nominal).

B. STUDY DESIGN

Test species: Biologically active agricultural soil: sandy loam A (No. 90/145/01), pH 6.5, 1.1% C_{org}, WHC 30%; sandy loam B (No. 90/060/01), pH 7.3, 1.7% C_{org}, WHC 40%.

Test design: Determination of carbon-transformation in soil after addition of glucose (concentration in soil: 0.1%). Comparison of test item treated soil with a non-treated treated soil. Four replicates per concentration. A "Sapromat B12" (Fa. J.M. Voith, Heidenheim/Brenz) system was used to measure the oxygen consumption over a period of 12 hours at different sampling intervals. Sampling scheme: 0, 7, 14, 21, 28, 42 and 63 days after treatment, subsamples were withdrawn from the bulk batches and subjected to the measurement.

Test rates: Control, 5.33 mg BAS 222 28 F per kg dry soil (corresponding to an application rate of 4.0 kg BAS 222 28 F/ha) and 64.00 mg BAS 222 28 F per kg dry soil (corresponding to 48.0 kg BAS 222 28 F per kg soil). Test concentrations related to a soil depth of 5 cm and a soil density of 1.5 g/cm^3.

Endpoints: Effects on O_2 consumption after 42 days (sandy loam soil A) and 28 day (sandy loam soil B) of exposure.

Reference item: none.

Test conditions: Soil A: Soil moisture: 40% of its maximum water holding capacity, soil B: Soil moisture: 47% of its maximum water holding capacity. Soil samples were incubated at 20 °C +/- 2 °C in plastic bottles.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

No adverse effects of BAS 222 28 F on carbon transformation in soil were observed in both test item concentrations in sandy loam soil A after 42 days and in sandy loam soil B after 28 days. The results are summarized below [see Table 8.7-4].

Table 8.7-4: Effects of BAS 222 28 F on soil micro-organisms (carbon transformation) on days 7, 14, 21, 28 and 42

Soil (days)	Control	5.33 mg BAS 222 28 F per kg dry soil equivalent to 4.0 kg/ha		64.00 mg BAS 222 28 F per kg dry soil equivalent to 48.0 kg/ha	
	O_2 consumption [mg/12h/100 g dry soil]	O_2 consumption [mg/12h/100 g dry soil]	% Deviation from the control [1)]	O_2 consumption [mg/12h/100 g dry soil]	% Deviation from the control [1)]
Sandy loam soil A (7 d)	13.5	14.1	+ 4.4	23.4	+ 73.3
Sandy loam soil A (14 d)	15.4	14.9	- 3.2	40.3	+ 161.7
Sandy loam soil A (21 d)	10.7	9.9	- 7.5	27.5 [2)]	+ 157.0
Sandy loam soil A (28 d)	10.8	9.1	- 15.7	20.8	+ 92.6
Sandy loam soil A (42 d)	10.9	10.3	- 5.5	12.5	+ 14.7
Sandy loam soil B (7 d)	23.3	23.8 [2)]	+ 2.1	30.0	+ 28.8
Sandy loam soil B (14 d)	25.3 [2)]	23.5	- 7.1	35.4	+ 39.9
Sandy loam soil B (21 d)	23.1	20.1	- 13.0	29.4	+ 27.3
Sandy loam soil B (28 d)	24.4	20.1	- 17.6	24.7 [2)]	+ 1.2

1) Based on O_2 consumption; - = inhibition, + = stimulation
2) mean value of only three replicates

III. CONCLUSION

Based on the results of this study, in accordance with OECD guideline 217, BAS 222 28 F caused long-term effects on carbon transformation in two field soils tested up to a concentration of 64.0 mg/kg dry soil, equivalent to a field application rate of 48 kg BAS 222 28 F per ha.

The following study on carbon transformation performed with BF 222-ETU has already been evaluated on the EU level during previous Annex I inclusion process for metiram. Nevertheless, a study summary is provided below.

Report:	*CA 8.7/5* *Krieg W., 2001c* *Effect of BF 222-ETU on carbon transformation of the soil microflora* *2001/1000909*
Guidelines:	*OECD 217*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a soil microbial activity study, the effects of BF 222-ETU (metabolite of BAS 650 F) on soil micro-organism activity (carbon transformation) were investigated in a loamy sand soil. BF 222-ETU was applied to samples of the soil at nominal application rates of 0.56 mg/kg and 5.6 mg/kg dry soil. BF 222-ETU treated soil and the untreated control were incubated at approx. 20 °C in the dark for 29 days. Four replicates per treatment were removed for analysis of carbon transformation 7, 14 and 29 days after application.

Based on the results of this study, in accordance with OECD guideline 217, BF 222-ETU caused no short-term and long-term effects on carbon transformation in a field soil tested up to a concentration of 5.6 mg/kg dry soil, equivalent to a field application rate of 4.20 kg BF 222-ETU per ha.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BF 222-ETU (Metabolite of BAS 222 F, Reg. No. 146 099), Lot-No. 01743-165, purity: 99.9%.

B. STUDY DESIGN

Test soil: Biologically active agricultural soil: loamy sand soil, pH 6.2, C_{org}: 18.2 mg C/100 g dry weight (OxiTop® method), WHC: 32%.

Test design: Determination of the C-transformation in soil after addition of glucose (concentration in soil 0.4%). Comparison of test item treated soil was done with the untreated control; 4 replicates per treatment and concentration. A "BSB-digi" respirometer system was used to measure the O_2-consumption over a period of minimally 20 hours at different sampling intervals. Sampling scheme: 0, 7, 14 and 29 days after treatment, sub-samples were withdrawn from the bulk batches and subjected to measurement.

Endpoints: Effects on O_2-consumption 7, 14 and 29 days after application.

Test concentrations: Control, 0.56 mg BF 222-ETU per kg dry soil (corresponding to a single application rate of 0.42 kg/ha) and 5.6 mg BF 222-ETU per kg dry soil (corresponding to 4.20 kg/ha). Test concentrations related to a soil depth of 5 cm and a soil density of 1.5 g/cm^3. The 0.56 mg a.s./ kg dry soil treatment group reflected the maximum formation rate (15%) of the active substance BAS 222 F into the metabolite BF 222-ETU.

Reference item: Dinoterb. Effects of the reference item were reported in a separate study.

Test conditions: Soil moisture: 45% of its maximum water holding capacity, pH 6.53 – 6.76. Soil samples were incubated at 18 °C - 22 °C while stored in glas bottles in the dark.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

No adverse influence of BF 222-ETU on the carbon transformation could be observed during the whole study at both application rates. Only slightly reduced respiration rates of -7.04% in the 0.56 mg BF 222-EU/kg dry soil treatment and -5.63% in the 5.6 mg BF 222-EU/kg dry soil treatment were measured after 29 days. The results are summarized below [see Table 8.7-5].

Table 8.7-5: Effects of BF 222-ETU on soil micro-organisms (carbon transformation)on days 7, 14 and 29 of incubation

Soil	Control	0.56 mg BF 222-ETU per kg dry soil equivalent to 0.42 kg/ha		5.6 mg BF 222-ETU per kg dry soil equivalent to 4.20 kg/ha	
	O_2 consumption [mg/kg dry soil/h]	O_2 consumption [mg/kg dry soil/h]	% Deviation from the control [1)]	O_2 consumption [mg/kg dry soil/h]	% Deviation from the control [1)]
Loamy sand soil (7 d)	8.8	7.9	-10.23	8.2	-6.82
Loamy sand soil (14 d)	7.3	6.8	-6.85	6.6	-9.59
Loamy sand soil (29 d)	7.1	6.6	-7.04	6.7	-5.63

1) Based on O_2 consumption; - = inhibition; + = stimulation

In a separate study the reference item Dinoterb (20.0 mg/kg dry soil) produced an effect of the C-transformation of -40.0% and -56.3%.

III. CONCLUSION

Based on the results of this study, in accordance with OECD guideline 217, BF 222-ETU caused no short-term and long-term effects on carbon transformation in a field soil tested up to a concentration of 5.6 mg/kg dry soil, equivalent to a field application rate of 4.20 kg BF 222-ETU per ha.

The following study on carbon transformation performed with BF 222-EU has already been evaluated on the EU level during previous Annex I inclusion process for metiram. Nevertheless, a study summary is provided below.

Report:	*CA 8.7/6* *Krieg W., 2001d* *Effect of BF 222-EU on carbon transformation of the soil microflora* *2001/1000910*
Guidelines:	*OECD 217*
GLP:	*yes* *(certified by Landesanstalt fuer Pflanzenbau und Pflanzenschutz Rheinland-Pfalz, Mainz)*

Executive Summary

In a soil microbial activity study, the effects of BF 222-EU (metabolite of BAS 222 F) on soil micro-organism activity (carbon transformation) were investigated in a loamy sand soil. BF 222-EU was applied to samples of the soil at nominal application rates of 0.56 mg/kg and 5.6 mg/kg dry soil. BF 222-EU treated soil and the untreated control were incubated at approx. 20 °C in the dark for 29 days. Four replicates per treatment were removed for analysis of carbon transformation 7, 14 and 29 days after application.

Based on the results of this study, in accordance with OECD guideline 217, BF 222-EU caused no short-term and long-term effects on carbon transformation in a field soil tested up to a concentration of 5.6 mg/kg dry soil, equivalent to a field application rate of 4.20 kg BF 222-EU per ha.

I. MATERIAL AND METHODS

A. MATERIALS

Test item: BF 222-EU (Metabolite of BAS 222 F, Reg. No. 27 270), Lot. No. 01743-161), purity: 88.6%.

B. STUDY DESIGN

Test soil: Biologically active agricultural soil: loamy sand soil, pH 6.2, C_{org}: 18.2 mg C/100 g dry weight (OxiTop® method), WHC: 32%.

Test design: Determination of the C-transformation in soil after addition of glucose (concentration in soil 0.4%). Comparison of test item treated soil was done with the untreated control; 4 replicates per treatment and concentration. A "BSB-digi" respirometer system was used to measure the O_2-consumption over a period of minimally 20 hours at different sampling intervals.
Sampling scheme: 0, 7, 14 and 29 days after treatment, sub-samples were withdrawn from the bulk batches and subjected to measurement.

Endpoints: Effects on O_2-consumption 7, 14 and 29 days after application.

Test concentrations: Control, 0.56 mg BF 222-EU per kg dry soil (corresponding to a single application rate of 0.42 kg/ha) and 5.6 mg BF 222-EU per kg dry soil (corresponding to 4.20 kg/ha). Test concentrations related to a soil depth of 5 cm and a soil density of 1.5 g/cm^3. The 0.56 mg a.s./ kg dry soil treatment group reflected the maximum formation rate (15%) of the active substance BAS 222 F into the metabolite BF 222-EU.

Reference item: Dinoterb. Effects of the reference item were reported in a separate study.

Test conditions: Soil moisture: 45% of its maximum water holding capacity, pH 6.60 – 6.91. Soil samples were incubated at 18 °C - 22 °C while stored in glas bottlesin the dark.

Statistics: Descriptive statistics.

II. RESULTS AND DISCUSSION

No adverse influence of BF 222-EU on the carbon transformation could be observed during the whole study at both application rates. Only slightly reduced respiration rates of -3.95% in the 0.56 mg BF 222-EU/kg dry soil and the 5.6 mg BF 222-EU/kg dry soil treatment were measured to the control after 29 days. The results are summarized below [see Table 8.7-6].

Table 8.7-6: Effects of BF 222-EU on soil micro-organisms (carbon transformation) on days 7, 14 and 29 of incubation

Soil	Control	0.56 mg BF 222-EU per kg dry soil equivalent to 0.42 kg/ha		5.6 mg BF 222-EU per kg dry soil equivalent to 4.20 kg/ha	
	O_2 consumption [mg/kg dry soil /h]	O_2 consumption [mg/kg dry soil/h]	% Deviation from the control [1)]	O_2 consumption [mg/kg dry soil/h]	% Deviation from the control [1)]
Loamy sand soil (7 d)	9.3	8.8	-5.38	8.8	-5.38
Loamy sand soil (14 d)	7.4	6.8	-8.11	7.1	-4.05
Loamy sand soil (29 d)	7.6	7.3	-3.95	7.3	-3.95

1) Based on O_2 consumption; - = inhibition; + = stimulation

In a separate study the reference item Dinoterb (20 mg/kg dry soil) produced an effect of the C-transformation of -40.0% and -56.3%.

III. CONCLUSION

Based on the results of this study, in accordance with OECD guideline 217, BF 222-EU caused no short-term and long-term effects on carbon transformation in a field soil tested up to a concentration of 5.6 mg/kg dry soil, equivalent to a field application rate of 4.20 kg BF 222-EU per ha.

CA 8.8 Effects on biological methods for sewage treatment

The results of the already peer-reviewed and accepted study are still valid and they are summarized in Table 8.8-1. No new study has been performed.

Table 8.8-1: Effects on biological methods for sewage treatment

Test item	Study type	Endpoint [mg a.s./L]	Reference (BASF DocID)	EU agreed
BAS 222 F (metiram)	Respiration inhibition test (inhibition of oxygen consumption activated sludge from wastewater plant)	EC_{50} (18 h) = 2.4 NOEC (18 h) = 0.7	1988/10296	yes

CA 8.9 Monitoring data

According to the knowledge of the applicant, there are currently no published ecotoxicological monitoring data available for metiram or its metabolites, which would provide additional knowledge on the ecotoxicological assessment not covered by this dossier.

Metiram

Document M-CA, Section 9

LITERATURE DATA

Compiled by:

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 9 LITERATURE DATA

A literature search on metiram and the common product trade names was performed by the BASF Group Information Center.

In addition a literature search on all metabolites has been performed and for common metabolites ETU, EU and EBIS, the results have been exchanged with the European Mancozeb Consortium, as a common set of data is used to generate a common list of endpoints.

The Literature Search Report on metiram describes the general search and evaluation process as well as details on search profiles, search histories and summary tables.

The complete search report is provided in K-CA 9 (BASF DocID 2015/1125776).

The first step of the search result processing based on summary records was done by the Information Center and involved the separation into "hits" and "ballast" (obviously irrelevant records). The “ballast” was not further processed.

The “hits” were further evaluated by the scientific experts and categorized into “not relevant”, “not reliable”, and “used for dossier”. This is documented in EXCEL files which are attached to the search report in K-CA 9 with the file names as listed below (alphabetical order):

Analytics:	Metiram Literature Analytics
Ecotoxicology:	Metiram Literature Ecotox aquatic
	Metiram Literature Ecotox general
	Metiram Literature Ecotox terrestric
	Metiram Literature Ecotox wildlife
E-fate:	Metiram Literature E-fate
Consumer Safety:	Metiram Literature Metabolism and Residues in Animals
	Metiram Literature Metabolism and Residues in Plants
Toxicology:	Metiram Literature Toxicology

All hits, considered reliable and contributing to the risk assessment were therefore further discussed in the dossier.

Metiram

Document M-CA, Section 10

CLASSIFICATION AND LABELLING OF THE ACTIVE SUBSTANCE

Compiled by:

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 10 CLASSIFICATION AND LABELLING OF THE ACTIVE SUBSTANCE

There is no harmonized classification for metiram.

BASF proposes the following self classification, based on already peer reviewed data and on new studies. **Studies not yet peer reviewed are reported in bold.**

Physico - chemical properties

Table 10-1: Physico-chemical properties

Study Type	Results (triggered classification and labelling)	Reference
Explosivity	Not explosive (-)	**DocID 2012/1254517**
Oxidizing properties	Not oxidizing (-)	**DocID 2012/1254517**
Flammability	Not highly flammable (-)	**DocID 2012/1254517**
Content of hydrocarbon	The content of hydrocarbon is below 10% (w/w)	See Doc JCA
Viscosity (kinematic)	Not applicable	

Table 10-2: Toxicology

Study Type	Results (triggered classification and labelling)	Reference
Acute oral	LD50 > 6810 mg/kg bw (-)	DocID 1992/10669
Acute dermal	LD_{50} > 2000 mg/kg (-)	DocID 1979/032
Acute inhalation	LC_{50} > 5.7 mg/L air (4h) (-)	DocID1983/064
Skin irritation	Not irritating to the skin (-)	DocID 2002/1005292
Eye irritation	Not irritating to eyes (-)	DocID 2002/1005291, DocID 2002/1006988
Skin sensitization (Maximization Test)	Skin sensitiser **(Skin sensitization Cat. 1; Warning; H317)**	DocID 1982/068
In vitro NRU Phototoxicity Test, Balb/c 3T3 cells	**Phototoxic in vitro**	**DocID 2013/1358979**

Table 10-2: Toxicology

Study Type	**Results (triggered classification and labelling)**	**Reference**
Reproductive toxicity	Classification and labelling resulting from the classification and labelling of the active substance metiram **(-)**	DocID 1981/132
Repeated dose toxicity	Classification and labelling resulting from the classification and labelling of the active substance metiram **(STOT RE 2; H373)***	DocID 1977/043 DocID 1992/11224
Cancerogenicity	Classification and labelling resulting from the classification and labelling of the active substance metiram **(-)**	DocID 1981/280 DocID 1989/0001 DocID 2002/1007052 DocID 2002/1006228 DocID 2002/1011511 DocID 1979/033
Mutagenicity	Classification and labelling resulting from the classification and labelling of the active substance metiram **(-)**	DocID 1977/027 DocID 1985/020 DocID 1985/210 DocID 1985/238 DocID 1990/0285 DocID 1986/082 DocID 1984/209 **DocID 2014/1313080** **DocID 2014/1315333** **DocID 2014/1315334** DocID 1979/069

* There is evidence of hindlimb ataxia in rats dosed with a dietary dose of 960 ppm (corresponding to 61 – 89 mg/kg bw) in a 90-day with atrophy of skeletal muscles (no evidence of degenerative neurotoxicity). In further 28- and 90-day studies reversibility of both effects was observed. Target organ: Skeletal muscle. As the trigger for classification with STOT RE 2 is effects seen at doses between 10 - 100 mg/kg bw in 90-day rat studies, STOT RE 2 is warranted.

Ecotoxicology/Environment

Table 10-3: Ecotoxicology/Environment

Study Type (duration)	**Results (triggered risk phrase)**	**Reference**
Oncorhynchus mykiss (96 h)	96 h LC_{50} = 0.336 [1)] mg metiram/L (Acute aquatic hazard Cat. 1; Warning; H400)	DocID 1994/10920 Amendment: DocID 1995/10143
Pimephales promelas (ELS, 33 d) [$]	33 d NOEC = 0.013 [2)] mg metiram/L (Chronic aquatic hazard Cat. 1; Warning; H410)	**DocID 2010/7012795**
Crassostrea virginica (96 h) [$]	48 h EC_{50} = 0.140 [2)] mg metiram/L (Acute aquatic hazard Cat. 1; Warning; H400)	**DocID 2010/7010901**
***Americamysis bahia* (40 d)** [$]	**40 d NOEC = 0.0031 [2)] mg metiram/L (Chronic aquatic hazard Cat. 1; Warning; H410, M(chronic) = 1 [+])**	**DocID 2010/7015187**
***Skeletonema costatum* (96 h)** [$]	**72 h E_rC_{50} = 0.007 [1)] mg metiram/L (Acute aquatic hazard Cat. 1; Warning; H400, M(acute) = 100)**	**Doc ID 2008/7018037**
	72 h NOAErC = 0.0053 [1)] mg metiram/L (Chronic aquatic hazard Cat. 1; Warning; H410)	
Lemna gibba (7 d) [$]	7 d E_rC_{50} > 0.517 [2)] mg metiram/L	**DocID 2010/7013131**
	7 d NOEC = 0. 517 [2)] mg metiram/L	
Biodegradation	Metiram * is not readily biodegradable	**BASF DocID 1992/10646**

1) Study was conducted with the solo-formulation BAS 222 28 F (containing 70% metiram, nominally); metiram endpoints were re-calculated based on the analyzed purity of the tested formulation batch.

2) Study was conducted with the solo-formulation BAS 222 29 F (containing 85% metiram, nominally); endpoints for BAS 222 29 F were calculated based on the analyzed purity in the tested formulation batch (*i.e.* 91.6%).

$ Study was not submitted during Annex I inclusion process of the active substance (for details see chapter CA 8.2).

* Metiram is not readily biodegradable; however, it degrades rapidly with $DT_{50} < 1$ d (see Review Report (SANCO/4059/2001-rev 3.3), June 2005)

+ assuming rapid degradation of metiram

The following is proposed in accordance with Regulation (EC) No 1272/2008:

Table 10-4: Proposed Hazard and Precautionary Statements

Pictogram(s)		
Signal word	Warning. Dangerous for the environment	
Hazard statements	H317	May cause an allergic skin reaction.
	H373	May cause damage to organs through prolonged or repeated exposure§
	H400	Very toxic to aquatic life.
	H410	Very toxic to aquatic life with long lasting effects
	EUH401	To avoid risks to human health and the environment, comply with the instructions for use.
Precautionary Statements		
General	P102	Keep out of reach of children
Prevention	P260	Do not breath dust
	P272	Contaminated work clothing should not be allowed out of the workplace
	P280	Wear protective gloves
Response	P303+P352	If on skin (on hair): wash with plenty of soap and water
	P333+P311	If skin irritation or rash occurs: Call a Poison Center or doctor/physician
	P362+P364	Take off contaminated clothing and wash before reuse
	P391	Collect spillage
Storage	P411	Store at temperatures not exceeding 30°C
Disposal	P501	Dispose of contents/container to hazardous or special waste collection point