Draft Assessment Report

Bacillus amyloliquefaciens strain MBI600

Volume 1

Rapporteur Member State : France

Version History of Volume 1

Date	**Reason for revision**
December 2014	Initial DAR

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Level 1

Bacillus amyloliquefaciens strain MBI600

1. Statement of subject matter and purpose for which the monograph was prepared

1.1. Purpose for which the monograph was prepared (Dossier Document A)

This Draft Assessment Report has been prepared to support the approval of the new active substance *Bacillus amyloliquefaciens* strain MBI600 according to Regulation (EC) 1107/2009

1.2. Summary and assessment of information relating to the collective assessment of dossiers (Dossier Document B)

BASF Agricultural Specialities Ltd is the sole notifier of *Bacillus amyloliquefaciens* strain MBI600.

1.3. Identity of the micro-organism (annex IIM 1)

1.3.1. Name and address of the applicant

Applicant: BASF Agricultural Specialities Ltd (Formerly Becker Underwood Ltd.)

Harwood Industrial Estate
Harwood Road
Littlehampton
BN17 7AU
United Kingdom

Contact Point: [redacted]

Representative: [redacted]

Contact Point: [redacted]

1.3.2. Producer(s)

Confidential information-See Annex C.

1.3.3. Name and species description, strain characterization

Taxonomic name and strain

Species:	*Bacillus amyloliquefaciens*
First description:	The first description of the species is from J. Fukumoto (1943) (IIM, 1.3.1/01-Fukumoto, J. 1943). In 1987, a group of scientists, including Fergus G. Priest of Heriot Watt University, established it as a separate species to *Bacillus subtilis* (IIM, 1.3.1/02, Priest, F.G and al., 1987).
Strain:	MBI600
Genus:	*Bacillus*
Family:	*Bacillaceae*
Division:	Bacteria

1.3.4. Composition of material used for manufacturing of the formulated product

Purity of the Microbial Active Substance *Bacillus amyloliquefaciens* MBI600

The nominal purity: 5.1×10^{11} CFU/g (80 % w/w *B. amyloliquefaciens* MBI600).
The minimum purity: 5.0×10^{11} CFU/g (78.2 % w/w *B. amyloliquefaciens* MBI600).
The maximum purity: 5.5×10^{11} CFU/g (86.2 % w/w *B. amyloliquefaciens* MBI600).
See Volume 4 for more details.

1.3.5. Accession number in culture collection

The strain is deposited in the National Collection of Industrial, Marine and Food Bacteria Ltd (NCIMB), Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA, Scotland.

Accession number: NCIMB 12376

The strain is further deposited in the USA in the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, USA.

Safe deposit agreement: 2009-SD-00011
Safe deposit number: SD-1414

1.4. Identity of the plant protection product (Annex IIM 3.1; IIIM 1)

1.4.1. Current, former and proposed trade names and development code numbers

The representative formulation is Subtilex®.
Trade name: BUEXP1780S

1.4.2. Manufacturer(s) of the preparation and the micro-organism

Company 1: BASF Agricultural Specialities Ltd. (Formerly Becker Underwood Inc.)
Harwood Industrial Estate Harwood Road
Littlehampton
BN17 7AU
United Kingdom
Contact: [redacted]
Phone: [redacted]
Fax:
E-Mail: [redacted]

Company 2: BASF Corporation (Formerly Becker Underwood Inc.)
801 Dayton Avenue
Ames
Iowa 50010

USA
Contact: [redacted]
Phone: [redacted]
Fax:
E-Mail: gordon.sargent@basf.com

1.4.3. Type of preparation and code

Wettable Powder (WP)

1.4.4. Function (Annex IIIM 1.6)

Fungicide

1.4.5. Composition of the formulated product (Annex IIM 1.7.2)

Content of the technical Microbial Active Substance *Bacillus amyloliquefaciens* MBI600 in the representative formulation

The nominal purity: [redacted]
The minimum purity: 5.5×10^{10} CFU/g (5.0 % w/w).
The maximum purity: -

See Volume 4 for more details.

1.5. Uses of the plant protection product (Annex IIM 3; IIIA 3)

1.5.1. Field of use

B. amyloliquefaciens strain MBI600 is intended to be used in vineyards.

1.5.2. Effects on harmful organisms

Bacillus amyloliquefaciens disrupts the growth of the hyphae and prevents spore germination, following contact with the fungal pathogen at the leaf surface.
In addition, *Bacillus amyloliquefaciens* exhibits fungicidal properties via production of iturin A and surfactin, which are antagonistic to the fungal pathogen.
Bacillus amyloliquefaciens strain MBI600 colonises developing shoot systems of plants, suppressing by competition (space and nutrients) disease organisms and induces systemic resistance response of the plant, indicated by enhanced peroxidase production.

1.5.3. Summary of intended uses

Crop and /or situation (a)	Member state or Country	Product name	F G or I (b)	Pest or Group of pests controlled (c)	Formulation		Application				Application rate per treatment			PHI (days) (l)	Remarks (m)
					Type (d-f)	Minimum conc. of MPCA (i)	method kind (f-h)	growth stage & season (j)	number min max	interval between applications (min)	CFU MPCA/hl min max	water l/ha min max	CFU MPCA/ha min max		
Grapes	SEU	BUEXP1780S = SUBTILEX	F	*Botrytis* spp., Grey mould	WP	5.5 x 10^{10} cfu/g 50 g/kg	Spray tractor-mounted air assisted or knapsack sprayers	All	1 - 10	7 days	0.014 - 0.0055	400 - 1000	0.055 kg MPCA/ ha	-	Equivalent to 0.5 kg MPCP /ha

Explanation of a -m
a: The EU classification for crops (90/642/EEC).
b: Outdoor or field use (F), glasshouse application (G) or indoor application (I)
c: e.g. biting and sucking insects, soil born insects, foliar fungi, weeds
d: e.g. wettable powder (WP), emulsifiable concentrate (EC), granule (GR), water soluble powder (SP)
e: GCPF Codes - GIFAP Technical Monograph No 2, 1989
f: all abbreviations used must be explained
g: Method, e.g. high volume spraying, low volume spraying, spreading, dusting, drench,
h: Kind, e.g. overall, broadcast, aerial spraying, row, individual plant, between the plants - type of equipment used must be indicated.
i: g/kg, g/l or appropriate term for micro-organisms
j: Growth stage at last treatment (BBCH Monograph, Growth stage of plants, 1997, Blackwell, ISBN 3-8263-3152-4)
k: The minimum and maximum number of application possible under practical conditions of use must be provided
l: PHI - minimum pre-harvest interval
m: Remarks may include: Extent of use/economic importance/restrictions

1.5.4. Information on authorization in EU Member States

Authorised uses (crops, harmful organisms, rates of application, number of applications, timings of applications - growth stages and where appropriate, season)	**Actual uses, if current practice is known to deviate from the authorised uses (crops, harmful organisms, rates of application, number of applications, timings of applications - growth stages and where appropriate, season)**
NA	NA

Level 2

Bacillus amyloliquefaciens strain MBI600

2. Reasoned statement of the overall conclusions drawn by the rapporteur member state

2.1. Identity, Biological properties, Details of Uses, Further Information

2.1.1. Identity of the micro-organism

Articles and report studies provided demonstrate that *Bacillus amyloliquefaciens MBI 600* was previously identified as *Bacillus subtilis MBI 600.*
Identification on the basis of 16S rRNA gene sequencing is not sufficient for the discrimination at the strain level. The applicant is currently undertaking a genome sequencing project on MBI600, and this information would be available by December 2014.
A new molecular method of identification for *Bacillus amyloliquefaciens MBI 600* at the strain level is required.

Furthermore, a new maximum certified value of the microbial active substance *Bacillus amyloliquefaciens* MBI 600 in accordance with batches of the analytical profile of MPCA should be provided which can be set at the maximum value founded in the batches (e.g.:1.4 x 10^{12} CFU/g).

In addition, some data on the characterization, the quantification, specification values for impurities in the five batches of technical MPCA are still required in the confidential part (see volume 4). **The quantification of the impurities should use validated methods.**

Finally, the analysis certificates of the results indicated for the determination of contaminants in the formulation SUBTILEX are still required.

2.1.2. Biological, physical, chemical and technical properties

Biological properties:

- *General information*

Life cycle consists in a cycle of several discernible phases: germination, outgrowth, multiplication and sporulation. *Bacillus amyloliquefaciens* strain MBI600 is not dependent upon a host, but rather the availability of decomposable organic matter.
The endospore is the prevalent stage in all environmental compartments and plays a dominant role in the biology and the life-cycle of *Bacillus amyloliquefaciens*. In fact, the endospore is the most heat tolerant bacterial life form, enduring temperatures in excess of 80°C or even 100°C (Schlegel 1985).
Generally *Bacillus amyloliquefaciens* reproduces under aerobic conditions, although in the presence of glucose and nitrate anaerobic growth occurs.
The pH-range for growth was found to be pH 4.5 to 9.5-10.0. The temperature range for growth of *B. amyloliquefaciens* is 10 to 50°C. *B. amyloliquefaciens* was found to reproduce under aerobic and saline conditions.

- *Mode of action*

Bacillus amyloliquefaciens disrupts the growth of the hyphae and prevents spore germination, following contact with the fungal pathogen at the leaf surface.
In addition, *Bacillus amyloliquefaciens* exhibits fungicidal properties via production of iturin A and surfactin, which are antagonistic to the fungal pathogen.
Bacillus amyloliquefaciens strain MBI600 colonises developing shoot systems of plants, suppressing by competition (space and nutrients) disease organisms and induces systemic resistance response of the plant, indicated by enhanced peroxidase production.
The *Bacillus* genus is composed of two major (*B. subtilis* and *B. cereus* groups, respectively) and several minor groups with *B. amyloliquefaciens* belonging to the *B. subtilis* group. Two species contain human pathogens: *B. anthracis* and *B. cereus.* These belong to the *B. cereus* group, a group with a highly similar morphology.
The phylogenetic tree shows that *B. amyloliquefaciens* is not related to the *B. cereus* group.
The mode of action of *B. amyloliquefaciens* MBI600 is partly based on the production and secretion of lipopeptides surfactin and iturin. These substances are strong surfactants showing membrane damaging properties *in vitro*.

Bacillus amyloliquefaciens strain MBI600 is susceptible to a wide spectrum of antibiotics commonly used in human and veterinary medicine.

Physical and chemical properties:
The product Subtilex® is a WP (Wettable Powder) formulation containing 110 g/kg (5.5×10^{10} CFU/g) of technical MPCA *Bacillus amyloliquefaciens* MBI600. The formulation is Light brown free-flowing homogeneous powder with a faint yeasty odour. It is not explosive, has no oxidising properties and is not flammable. It has slightly alkaline pH value around 8 for a 1% dilution. As the wettability is outside the acceptable limit before swirling, the formulation should be used under a continuous agitation. The content of MPCA after 12 weeks at 35°C is below the minimum certified value. The Notifier explains that the study will be- re-run in September 2014 due to a problem with the sampling method. These new data should be provided as soon as possible using a validated method.

Data on microbial contaminants according to OECD issue paper, Oct. 2011 before and after storage (12 weeks at 35 °C) were not provided and are required as soon as possible using validated methods.

Study of storage during 2 years at ambient temperature in the commercial packaging is required as soon as possible using validated methods. The study should include microbial contaminants according OECD guidelines 65 (Oct. 2011).

2.1.3. Details of uses and further information

Crop and /or situation (a)	Member state or Country	Product name	F G or I (b)	Pest or Group of pests controlled (c)	Formulation		Application				Application rate per treatment			PHI (days) (l)	Remarks (m)
					Type (d-f)	Minimum conc. of MPCA (i)	method kind (f-h)	growth stage & season (j)	number min max	interval between applications (min)	CFU MPCA/hl min max	water l/ha min max	CFU MPCA/ha min max		
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Explanation of a -m
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j: Growth stage at last treatment (BBCH Monograph, Growth stage of plants, 1997, Blackwell, ISBN 3-8263-3152-4)
k: The minimum and maximum number of application possible under practical conditions of use must be provided
l: PHI - minimum pre-harvest interval
m: Remarks may include: Extent of use/economic importance/restrictions

To support the approval of *Bacillus amyloliquefaciens* MBI600 as active substance under regulation (EC) 1107/2009, efficacy data for the representative use against grey mould (*Botrytis cinerea*) on grapevines were presented for the product SUBTILEX (BUEXP1780S).
28 efficacy trials illustrate the efficacy of SUBTILEX and the interest of *Bacillus amyloliquefaciens* MBI600.

2.1.4. Proposals for classification and labelling

Classification and labelling of chemical substances based on the criteria according to Regulation (EC) No 1272/2008 and Directive 67/548/EEC and are not applicable to micro-organisms.
However micro-organisms should be regarded as potential sensitisers and the following hazard statement has to be applied:
'Micro-organisms may have the potential to provoke sensitising reactions'.

Preparation Subtilex:

Classification

In accordance with Directives 67/548/EEC and 1999/45/EC the following classification is proposed for the preparation:

Hazard symbol:	None
Indication of danger:	None
Risk phrases:	None

According to Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16th December 2008, the following classification is proposed for the preparation:

Hazard class:	None
Signal word:	None
Hazard statements:	None

Labelling
'Contains *Bacillus amyloliquefaciens,* micro-organisms may have the potential to provoke sensitising reactions'.

This labelling phrase implies that PPE have to be worn when handling the product or applying the product :

- Gloves (nitrile, EN 374-3)
- Working coveralls
- Disposable filtering facepiece respirator to at least EN149 FFP3 or equivalent

2.2. Analytical methods

Enumeration method Whittaker, M. (2013, 2014) for the determination of *Bacillus amyloliquefaciens* strain MBI600 in the MPCA and in the MCPP are available and validation data are considered sufficient.
Methods for the determination of microbial contaminants in the MPCA and MCPP are available and validation data are considered sufficient. Content of microbial contaminants in MPCA and MCPP are in accordance with OECD guidelines 65.
If the screening of the technical active substance demonstrate impurities higher than 1g/kg or relevant impurities, validation methods for their determination are required.

2.3. Impact on human and animal health

2.3.1. Effects having relevance to human and animal health arising from exposure to the micro-organism or to impurities, additives, contaminating micro-organisms contained in the material used for manufacturing of formulated products

Bacillus amyloliquefaciens MBI600 (formerly identified as *Bacillus subtilis* MBI600) was isolated from broad bean leaves in United Kingdom. *Bacillus amyloliquefaciens* MBI600 is neither a mutant nor a genetically modified strain.

Bacillus amyloliquefaciens MBI600 has been used in plant protection products since 1984 in USA and since 2007 in Canada.

Different strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* are approved for in Articles 5 and 6 of Directive 91/414/EEC1 concerning the placing of plant protection products on the market. Further uses include feed and food additives and biotechnological application i.e. enzyme production.

The Panel on Biological Hazards of EFSA[1] has considered that *Bacillus amyloliquefaciens* is suitable for Qualified Presumption of Safety (QPS) assessment for biological agents intentionally added to food and feed with the qualification of absence of toxigenic activity. Case reports on infection or other incidents in humans related to *Bacillus amyloliquefaciens* or *Bacillus subtilis* were scarce and occurred mainly in immune suppressed people.
Moreover, for all QPS bacterial taxonomic units, the strains should not harbour any acquired antimicrobial resistance genes to clinically relevant antibiotic. Two studies of antibiotic resistance were performed on *Bacillus amyloliquefaciens* MBI600. Results were detailed in the Volume 3 B2.1.9. *Bacillus amyloliquefaciens* strain MBI600 is susceptible to a wide spectrum of antibiotics commonly used in human and veterinary medicine.

Bacillus amyloliquefaciens is not listed in the directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work.
No evidence of adverse health effects have been observed in any of the employees involved in the production, handling and application of *Bacillus amyloliquefaciens* strain MBI600.

Toxicological studies performed with the active substance *Bacillus amyloliquefaciens* strain MBI600 revealed a low toxicity via the oral, dermal and intravenous routes and does not show any evidence of pathogenicity or infectivity. After intratracheal instillation of *Bacillus amyloliquefaciens* strain MBI600, deaths were seen shortly after administration, which is not consistent with an infection. Moreover, no trends of increase of body temperature at 24 hours were observed and no observable abnormalities were lesions were revealed at necropsy. This study did not show evidence of pathogenicity or infectivity, a pattern of clearance was established. Mortality in inhalation toxicity studies on microbial pesticides is not unusual, and these deaths seem to be the result of physical action rather than inherent toxicity of the substance. Furthermore, no mortality was observed in an intravenous study and in the inhalation toxicity study performed with the technical microorganism as currently marketed with a more realistic exposure pathway.
Bacillus amyloliquefaciens strain MBI600 is not irritating to skin or eyes. As many micro-organisms, this strain may have the potential to provoke sensitising reactions after skin and/or inhalation contact.
The fermentation broth of *Bacillus subtilis* strain MBI600 was not mutagenic in this Ames test performed on several strains of *Salmonella typhimurium* and *Escherichia coli* either in the presence or absence of metabolic activation in the strains tested.

Bacillus amyloliquefaciens species are known to be a producer of a large range of secondary metabolites, especially non ribosomal cyclic lipopeptides. According to a screening test performed on *Bacillus amyloliquefaciens* MBI600, surfactin and iturin were the principal secondary metabolites produced in broth fermentation conditions. These substances belong to the lipopeptides family surface-active compounds targeting biological membranes but not the genome.

Two sets of studies were provided by the applicant:

- Studies performed in 1989 by [redacted]: these studies were run with fermentation broth. The broth was designed to provide optimal conditions for *Bacillus amyloliquefaciens* MBI600 to

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EFSA Journal (2007) 587, 1-16 Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA Opinion of the Scientific Committee.

produce metabolites. The studies therefore tested the toxicity of spores, other life-stages and a representative metabolite profile which the organism would produce under optimal conditions.

- Studies performed in 2011 and 2012 on *Bacillus amyloliquefaciens* strain MBI600 Technical. Tested batches were representative of current commercial lots.

Table 2.3.1-1: Summary of the toxicity studies performed with *Bacillus amyloliquefaciens* strain MBI600

Study	Item/Vehicle	Dose levels	Results
Skin sensitisation GLP/OECD 406 Guinea pig	fermentation broth of *Bacillus amyloliquefaciens* StrainMBI600/ Water or mixture of FCA with water	Epidermal induction: 0.1ml/site at 5% test item solution in vehicle challenge: 0.2ml site at 50% test item solution in vehicle	100% sensitisation response. Responses generally persisted up to the end of the observation period, with necrosis and severe oedema observed in some test animals
Acute oral Toxicity/Pathogenicity Study in Rats GLP/ EPA FIFRA 152A-10	fermentation broth of *Bacillus amyloliquefaciens* StrainMBI600/ Distilled water	10^9 viable spores/animal	LD 50 >10^9 viable spores Large numbers of spores survived passage through the intestinal tract, but were rapidly eliminated within 7 days. Clearance of live microorganism from all tissues analysed was complete 21 days after the dosing. No evidence of pathogenicity or infectivity.
Acute oral Toxicity/Pathogenicity Study in Rats GLP/OCSPP 870.1100 OECD 425	*Bacillus amyloliquefaciens* strain MBI600 technical (Batch BS7211)/ Water	5000 mg/kg bw 4.04 x 10^{11} spores/kg bw	LD_{50} > 4.04 10^{11} spores/kg bw
Acute pulmonary Toxicity/Pathogenicity Study in Rats GLP/ EPA FIFRA 152A-12	fermentation broth of *Bacillus amyloliquefaciens* StrainMBI600/ -	3.5 x 10^8 viable spores/animal	Mortality (shortly after administration) No evidence of pathogenicity or infectivity Clear pattern of clearance
Acute inhalation Toxicity GLP/OCSPP 870.1300 OECD 403	*Bacillus amyloliquefaciens* strain MBI600 technical (Batch BS7211)/-	5.31 mg/L 4.29 x 10^9 spores/mL	No mortality LC_{50}> 5.31 mg/L
Acute intravenous toxicity and infectivity/pathogenicity in Rats GLP/EPA FIFRA 152A-13	fermentation broth of *Bacillus amyloliquefaciens* StrainMBI600/ Physiological saline	4.5 x 10^7 viable spores/animal	No evidence of pathogenicity or infectivity Clear pattern of clearance
Bacterial reverse mutation test of the supernatant of the fermentation broth of *Bacillus amiloliquefaciens* StrainMBI600	Supernatant of the fermentation broth of *Bacillus subtilis* strain MBI600 / -	4.88, 19.5, 78.1, 313, 1250 and 5000 µg/plate	Not mutagenic in absence and presence of S9 mix
Acute dermal toxicity study in rabbit GLP/EPA FIFRA 152A-11	fermentation broth of *Bacillus amyloliquefaciens* StrainMBI600/ -	2,0ml/ kg bw	No mortality Not irritating
Acute dermal toxicity study in rats GLP/OCSPP 870.1200, OCDE 402	*Bacillus amyloliquefaciens* strain MBI600 technical (Batch BS7211)/ moistened with 1.0	5050 mg/kg bw equivalent to 4.08 x 10^{11} CFU/kg bw	No mortality Not irritating LD_{50} >5050 mg/kg bw equivalent to 4.08 x 10^{11} CFU/kg bw

	ml/g de-ionised water		
Acute dermal irritation study in rabbit GLP/OCSPP 870.2500, OCDE 404	*Bacillus amyloliquefaciens* strain MBI600 technical (Batch BS7211)/ moistened with 1.5 ml/g de-ionised water	500 mg/ animal	Slightly irritating
Primary eye irritation and infectivity in rabbit GLP/EPA FIFRA 152A-14	fermentation broth of *Bacillus amyloliquefaciens* StrainMBI600/-	0.1 ml/animal equivalent to 10^9 viable spores/animal	Slightly irritating
Acute eye irritation study in rabbit GLP/ OCSPP 870.2400, OECD 405	*Bacillus amyloliquefaciens* strain MBI600 technical (Batch BS7211)/-	100 mg/animal	Slightly irritating

2.3.2. Impact on human health arising from exposure to the micro-organims or to impurities, additives, contaminating micro-organisms contained in the material used for manufacturing of formulated products

Subtilex® is formulated as a wettable powder (WP) and contains as active substance spores of *Bacillus amyloliquefaciens* MBI600 (11% w/w). Subtilex® is to be used on grape vines to control Botrytis. Subtilex® may be applied via a tractor mounted air assisted sprayer or a knapsack sprayer at a rate of 0.5 kg/ha of formulated product, which is equivalent to a minimum of 2.75×10^{13} cfu/ha. It is applied in 400 to 1000 L/ha of water, thus the concentration is between 2.75×10^{10} and 6.88×10^{10} cfu/L.
All toxicological studies performed with the MPCP Subtilex® (*Bacillus amyloliquefaciens* strain MBI600 11% w/w) revealed no effects to human or animal health. It is of low toxicity via the oral, dermal and inhalation routes. It is not irritating to skin or eyes and is not a skin sensitizer (however sensitising tests are not considered suitable for micro-organisms based products). The MPCA, *Bacillus amyloliquefaciens* strain MBI600 does not show any evidence of pathogenicity or infectivity.

Proposed labelling phrase for the formulated product Subtilex® is:

"Contains *Bacillus amyloliquefaciens* strain MBI600. Micro-organisms may have the potential to provoke sensitising reactions."

Therefore appropriate personal protective equipment should be worn when handling Subtilex®.

Subtilex® is intended on grape vines to control Botrytis. The derivation of an AOEL is not necessary based on the lack of adverse effect related to the microorganism in the available studies. Therefore, operator, worker, resident and bystander exposure estimates to the microorganism are not needed.

Operator exposure is considered acceptable when PPE and RPE are used. Worker exposure is considered acceptable when PPE is used. Bystander and resident risk is considered acceptable under the proposed conditions of use.

Table 2.3.2-1 Summary of toxicity studies performed on the formulated product Subtilex®.

Study	Test item	Dose levels	Finding

Acute oral toxicity GLP/ OCSPP 870.1100 OECD 425 rat	Subtilex® Batch n°53357	5000 mg/kg bw	LD_{50} > 5000 mg/kg bw
Acute inhalation GLP/ OCSPP 870.1300 OECD 403 rat	Subtilex® Batch n°53357	5.23 mg/L	LC_{50}> 5.23 mg/L
Acute percutaneous toxicity GLP/ OCSPP 870.1200 OECD 402 rat	Subtilex® Batch n°53357	5050 mg/kg bw	LD_{50} > 5050 mg/kg bw
Skin irritation GLP/ OCSPP 870.2500 OECD 404 rabbit	Subtilex® Batch n°53357	500 mg/animal	Not irritating
Eye irritation GLP/ OCSPP 870.2400 OECD 405 rabbit	Subtilex® Batch n°53357	100 mg/animal	Slightly irritating
Skin sensitisation GLP/OPPTS 870.2600 Buehler test 3 applications Guinea pig	Subtilex® Batch n°53357	400 mg/animal	Not sensitising

2.4. Residues

B. amyloliquefaciens MBI 600 is a naturally occurring, indigenous wild type and was isolated from the surface of broad bean leaves (*Vicia faba*) in UK.
B. amyloliquefaciens is ubiquitous in the environment. Colonisation of different foodstuffs is commonly reported but of no concern because the species are generally accepted to be non-pathogenic.
Bacillus amyloliquefaciens strain MBI600 is intended to be used as foliar treatment on grapevine. It works by preventing further growth of fungi already present, but in addition forms a protective layer on the surface of the plant to prevent further fungal growth. It has been demonstrated that environmental conditions on leaf surfaces are usually unfavourable for bacterial growth and that it is expected that the number of cfu will persist at the initial level of application but will not increase.
Antifungal compounds or other substances are involved in antagonistic activity of *B. amyloliquefaciens* strain MBI600 and will probably only be produced at the treatment site during direct interaction with the target pathogen and only for a period of a few days. The substances will not accumulate and their persistence is considerably shorter than that of the microorganism itself.
EFSA has considered *Bacillus amyloliquefaciens* along with its assessment of *Bacillus* species and has concluded an absence of emetic food poisoning toxins with surfactant activity and an absence of enterotoxic activity. Based on this assessment *Bacillus amyloliquefaciens* is included in the list of microorganisms which warrant a Qualified Presumption of Safety. According to the provide data *B. amyloliquefaciens* strain MBI 600 is eligible to QPS status and therefore a risk for consumer is not expected following its use as plant protection product.

2.5. Fate and behaviour in the environment

Due to the close relationship and physiological similarity of *B. subtilis* and *B. amyloliquefaciens* information on the fate and behaviour of *B. amyloliquefaciens* in different environmental compartments can be deduced from data on *B. subtilis*.
A review summary of scientific literature has been presented in the dossier and for some articles the original papers are missing from the dossier. Furthermore, some of the cited articles were previously assessed by

Germany as RMS for the active substance *B. amyloliquefaciens* subsp. *plantarum* strain D747. Therefore, the notifier proposes the same summaries as presented in the volume 3 B.8 of *B. amyloliquefaciens* subsp. *plantarum* strain D747 for *B. amyloliquefaciens* strain MBI 600. To be consistent with the previous assessment on these articles, RMS (FR) followed the conclusions of the Rapporteur Member State Germany. Concerning the articles not already assessed by DE and for which a full copy is not available or provided too late in the process, a brief summary are presented by RMS as informative data, but cannot be used to finalise the risk assessment. Therefore, a data gap for scientific literature quoted by the notifier in its assessment has been identified.

Soil

Bacillus species, including *B. amyloliquefaciens,* are commonly found in soils, including agricultural settings, and are naturally present on fresh products. *B. amyloliquefaciens* strain MBI600 spores introduced into soils are likely to germinate, grow and exhibit plant protective activity in the rhizosphere, as sufficient concentration of nutrients for induction of germination are available. Due to their ability to produce antimicrobial metabolites including iturin A and surfactin vegetative cells can compete with autochthon microorganisms to some extent, although studies have been shown a significant difference between fate of introduced cells in sterile and non-sterile soil. MBI600 added as cells to sterilised soil soon converted to spores and survived well in this form, either remaining at the level of introduction or increasing in number. In non-sterile soil this was not the case, and survival of the introduced bacteria was poor, with low spore formation. Survival is facilitated by sporulation, which is low in natural soils.

Survival of vegetative cells also depends on abiotic factors like pH, temperature and UV-light. Spores introduced into soils with poor nutrient content and without plants (without rhizosphere) will not germinate. Sporulation of vegetative cells occurs if environmental conditions become unfavourable. As spores are very resistant to abiotic environmental factors and do not compete with autochthon microorganisms for nutrients, they can survive in soil for a long period of time.

Grazing by protozoa and by bacterial predators is one of the main reduction factors of vegetative cells and spores in soil. Infection by bacteriophages is further factors responsible for the decline of vegetative cells. High clay contents of soil are said to be protective concerning grazing and infection.

However, influence of environmental factors cause a steady decline of vegetative cells and spores until an equilibrium is reached. The concentration of equilibrium is not fixed, but seems to be dependent on local environmental conditions.

In order to perform a risk assessment for the soil non-target organisms, the actual concentration of *B. amyloliquefaciens* strain MBI600 is calculated for soil, based on a cumulative application rate of 5 kg product/ha (for 10 applications), assuming (equivalent to 550 g *B. amyloliquefaciens* strain MBI600/ha and 2.75×10^{14} cfu/ha), and no degradation occurs after application. The resultant load of *B. amyloliquefaciens* strain MBI600 will be related to the top 5 cm of soil to achieve the highest theoretical soil concentration. The initial PEC value (PEC soil) for *B. amyloliquefaciens* strain MBI600 is 6.67 mg Subtilex®/kg dry weight soil corresponding to 0.73 mg of MPCA/kg soil and 3.67×10^{8} cfu/kg soil. Based on available data, the natural abundance of *B. subtilis* varied from 3×10^{4} CFU/g dry soil in desert soils to 7.6×10^{4} CFU/g dry soil in a pine forest soil.

Some information has been provided on the potential transfer of genetic material from *Bacillus amyloliquefaciens* to other organisms. It is concluded that even if genetic exchange occurs, it will only affect single cells in a population of soil bacteria. Proliferation will only happen if the new recombinant strain has a selective advantage compared to indigenous strains. This scenario is very unlikely as it would necessitate a single gene that confers a competitive advantage to all other microorganisms competing for the same ecological niche.

Water

Contamination of surface waters by applications of plant protection products containing spores of *B. amyloliquefaciens* can mainly occur via spray drift. Multiplication and persistence in water is not expected. Spores are known to be metabolically inactive. Thus, they do not consume nutrients and do not grow/multiply. Members of this group of bacilli are not regarded as autochthonous inhabitants of aquatic environments and do not find optimal conditions for growth, as waters are often poor in organic carbon.

All studies cited above reveal that vegetative cells of *B. subtilis* introduced into different non-sterile aqueous environments including nutrient-rich sewage declined rapidly. Therefore, proliferation is not likely to occur. Bacterial cells and especially spores may survive, but will be subject to natural competition in the diverse micro-flora of natural waters.

In order to perform a risk assessment for the aquatic non-target organisms, the actual predicted initial environmental concentration of Subtilex® calculated for surface water, based on a single application of 0.5 kg Subtilex®/ha (0.055 kg *B. amyloliquefaciens* strain MBI600 /ha), and resulting from spray drift at 3 m of 8.02 % are 13.37 µg product/L, 1.47 µg *B. amyloliquefaciens* strain MBI600 /L and 7.35×10^5 CFU/L. For 10 cumulative applications, the PECsw are 133.7 µg product/L, 14.7 µg *B. amyloliquefaciens* strain MBI600 /L and 7.35×10^6 CFU/L.

Some information has been provided in relation to potential interferences of *Bacillus amyloliquefaciens* with the analytical systems for the control of the quality of drinking water provided for in Directive 98/83/EC. Based on the notifier's statement, it is concluded, that *B. amyloliquefaciens* strain MBI600 will not interfere with the microbiological methods used for drinking water monitoring.

Air

Spores are suitable for aerial distribution as they are easily blown by wind and under conditions of use drift spacious transport may occur. However, germination and multiplication of MBI600 in the air, aerosols or clouds can be excluded due to lack of organic matter substrate and lack of mineral matrix to adhere to. In addition, during aerolization vegetative cells of MBI600 would be exposed to severe environmental stress factors (desiccation, UV-radiation, temperature) making survival of vegetative cells unlikely.

Mobility

No data on the soil mobility of *B. amyloliquefaciens* cells was provided by the notifier. The notifier indicated that the multiplication and persistence in water of *B. amyloliquefaciens* cells is unlikely to happen. Germany as RMS of the active *B. amyloliquefaciens* subsp. *plantarum* strain D747 indicated that leakage of *B. amyloliquefaciens* spores through soil can occur (Jiang *et al*., 2005[2]). Therefore, contamination of groundwater cannot be excluded. However, DE states as *B. amyloliquefaciens* is an ubiquitous soil microorganism groundwater contamination probably happens naturally. Spores reaching groundwater environments do not germinate and grow due to insufficient nutrient availability. Dilution as a result of continuous water flux and predation by groundwater (micro-) organisms will cause a continuous decline of the spore populations. Thus, a groundwater accumulation is unlikely. The RMS (FR) view is similar. Nevertheless, the notifier should better address this issue through a more exhaustive literature search.

2.6. Effects on non-target species

2.6.1. Effects on terrestrial vertebrates

Bacillus amyloliquefaciens MBI600 is a ubiquitous micro-organism in the environment, found in soil, plants and animal foodstuffs, with which birds and mammals will naturally be in contact. Any potential risk to birds and mammals from Subtilex® will occur due to ingestion of food (plants and insects) containing residues of the microbial pest control agent (MPCA) *Bacillus amyloliquefaciens* MBI600. A standard risk assessment has been conducted to determine a worst-case dietary exposure level in accordance with the EFSA Guidance for the Risk Assessment for Birds and Mammals (2009)[3].

Table 6.1-1: Acute DDD for birds and mammals – screening step

[2] *Jiang, G., Noonan, M.J., Buchan, G.D., Smith, N. (2005): Transport and deposition of* Bacillus subtilis *through an intact soil column. Australian Journal of Soil Research, 43, 695-703*

[3] *Guidance of EFSA Risk assessment for Birds and Mammals, EFSA Journal 2009; 7(12):1438*

Crop	Indicator species	Rate (kg as/ha)	Max. No. Applns.	MAF_{90} [2)]	Shortcut value (SV) for acute assessment [1)]	DDD (mg/kg/d)
Vines	Small omnivorous bird	0.055	1	not relevant	95.3	5.24
Vines	Small omnivorous bird	0.055	10	not relevant	95.3	52.4
Vines	Small herbivorous mammal	0.055	1	not relevant	136.4	7.5
Vines	Small herbivorous mammal	0.055	10	not relevant	136.4	75

1) Short cut value based on the 90th percentile of residues provided in EFSA Guidance document 2009.
2) Multiple application factor used for chemical substance is not relevant for microorganisms.

The screening step gives TER values above the trigger of 10 for acute risk for 10 cumulative applications and the active substance was demonstrated in two studies with the bobwhite quail, and a rat study to have no pathogenicity to the test species. Thus the risk to birds and mammals is considered as acceptable from the proposed use of Subtilex®.

Table 6.1-2: Acute Toxicity/exposure ratios for birds and mammals– screening step

Test substance	Crop, use pattern	Crop scenario, indicator species	Toxicity endpoint (mg a.s./kg bw)	DDD (mg a.s./kg bw)	TER	TER risk assessment trigger
Bacillus amyloliquefaciens MBI600	Vines	Small omnivorous bird	>4000	52.4	>76	10
Bacillus amyloliquefaciens MBI600	Vines	Small herbivorous mammal	>5000	75	>67	10

2.6.2. Effects on aquatic species

Bacillus amyloliquefaciens MBI600 is not toxic to a fish *Oncorhynchus mykiss* at 100 mg/L for 96-h (9.9 x 10^{10} cfu/L), an invertebrate *Daphnia magna* at 100 mg/L for 48-h (1.9 x 10^{10} cfu/L) and a green algae *Pseudokirchneriella subcapitata* at 100 mg for 72 h (5.8 x 10^{11} cfu/L). Based on a 30-d study with *Cyprinus carpio*, it can be conclude that *Bacillus amyloliquefaciens* MBI600 is not pathogenic to fish and is unlikely to cause adverse long-term effects. However, *Bacillus amyloliquefaciens* MBI600 may affect reproduction of *Daphnia magna* in a 21-d study with a no effect level at 2.7 x 10^{7} cfu/L.
The TER exceeds the trigger values specified in COMMISSION REGULATION (EU) No 546/2011 for a single application and 10 cumulative applications, with the exception of the long-term TER for invertebrate calculated for 10 cumulative applications (TER = 3.7). However, the unrealistic exposure estimate for 10 cumulative applications is below the no effect level on the reproduction to *Daphnia magna*. It is therefore concluded that Subtilex® does not present an unacceptable risk to aquatic organisms when used according to the proposed GAP.

Table 6.2-1: TER_A for aquatic organisms

Species	Endpoint	Toxicity (mg as/L)	PEC_{SW}	TER	TER Trigger value
Oncorhyncus mykiss	96-hour LC_{50}	>100 mg MPCA/L > 9.9 x 10^{10} cfu/L	14.7 µg MPCA/L 7.35 x 10^{6} cfu/L (10 applications)	> 6803 > 13469	100
Daphnia magna	48-hour EC_{50}	>100 mg MPCA/L > 1.9 x 10^{10} cfu/L	14.7 µg MPCA/L 7.35 x 10^{6} cfu/L (10 applications)	> 6803 > 2585	100

Daphnia magna	21-days EC_{50}	2.7 x 10^7 cfu/L	7.35 x 10^6 cfu/L (10 applications)	**3.7**	10
Daphnia magna	21-days EC_{50}	2.7 x 10^7 cfu/L	7.35 x 10^5 cfu/L (1 application)	37 *	10
Pseudokirchneriella subcapitata	72-hour EC_{50}	>100 mg MPCA/L (>1.1 x 10^{11} cfu/L) > 5.8 x 10^{11} cfu/L	14.7 µg MPCA/L 7.35 x 10^6 cfu/L (10 applications)	> 6803 > 79000	10

* RMS notes when considering the maximum test item specifications, the calculated TER for a single application could exceed the TER trigger value. However, the maximum test item specification is not available (see data gap reported in Volume 4).

2.6.3. Effects on bees and other arthropod species

The microbial pest control agent *Bacillus amyloliquefaciens* MBI600 is a naturally occurring, predominantly soil borne bacterium. It is a ubiquitous soil microorganism and occurs without geographical restriction. It is spread to associated environments including plants and plant materials (straw and composts) by dust or other means. In the target crop, grapevines, terrestrial arthropods other than bees will be constantly exposed to *Bacillus amyloliquefaciens*, present at ambient background levels.

The standard risk assessment scheme for chemicals is not considered applicable to micro-organisms. The ratio of a maximum application rate of the MPCA to an LD_{50} value would not represent the potential risk of a micro-organism. In the following risk assessments for Subtilex®, the proposed field application rate for MBI600 (2.75 x 10^{13} cfu/ha in a volume of 400 L water) has been compared with the test item treatment solutions for the non-target arthropod studies in terms of cfu/L and assuming an application volume of 400 L/ha to represent an equivalent field application rate according to the proposed GAP.
Two non-GLP studies were conducted with *Bacillus amyloliquefaciens* MBI600 to determine effects on four non-target arthropods (IIM 8.8/01 by Mori (1997b) and 8.8/02 by Wada (1996)). The first of these studies examined effects on three non-target arthropods from three different arthropod orders (the predatory coccinellid beetle, *Harmonia axyridis*; the predatory neuropteran, *Chrysoperla carnea* and the predatory mite, *Phytoseiulus persimilis*) and the second examined effects on silkworm larvae. The studies demonstrated little or no effect of *Bacillus amyloliquefaciens* MBI600 on the four non-target arthropod species tested at test item treatment application rates, equivalent to 1 x 10^{12} cfu/L (IIM 8.8/01) or 1.66 x 10^{12} cfu/L (IIM 8.8/02).

The test item treatment concentration is equivalent to a field rate of 4 x 10^{14} cfu/ha in the first study and 6.64 x 10^{14} cfu/ha in the second study, assuming application in 400 L volume as the proposed GAP. Both are higher than the proposed single field application rate for *Bacillus amyloliquefaciens* MBI600 of 2.75 x 10^{13} cfu/ha. It is therefore considered that exposure to *Bacillus amyloliquefaciens* MBI600 following application of Subtilex® according to the proposed GAP will not represent a risk to non-target arthropods.

No additional acute/short-term or higher tier studies have been performed or considered necessary.

2.6.4. Effects on earthworms and other soil non-target macro-organisms

The maximum peak PEC_S value of 3.67 x 10^8 cfu/kg soil was estimated following 10 applications of Subtilex™ to vines at 2.75 x 10^{13} cfu MBI 600/ha. This value has been used in the TER calculations:

Table 6.4-1: TER_{LT} for earthworms

Species	**Endpoint**	**Toxicity (cfu/kg soil dry weight)**	**Max PEC_S (following 10 applications) CFU/kg of soil)**	**TER**
Eisenia fetida	56-day NOEC (survival, growth and reproduction	>1.1 x 10^{12} (nominal)	3.67 × 10^8	>3000
		>2.9 x 10^{12} (measured at study start))		>7900

The TER exceeds the trigger value of 5 specified in Annex VI to Directive 91/414/EEC when the toxicity is expressed as either nominal or measured concentration of MBI 600 in soil. It is concluded that Subtilex® does not present a significant risk to earthworm survival, growth or reproduction when used according to the proposed GAP.

Two non GLP studies have been conducted to determine the effects on non-target arthropods. The first examined effects on three non-target arthropods from three different arthropod orders (*Harmonia axyridis, Chrysoperla carnea* and *Phytoseiulus persimilis*) and the second examined effects on silkworm larvae. The studies demonstrated little or no effect of *Bacillus amyloliquefaciens* MBI600 on four non-target arthropod species tested.

2.6.5. Effects on soil micro-organisms

Bacillus amyloliquefaciens MBI600 is a naturally occurring, predominantly soil borne bacterium. It is a ubiquitous soil organism and occurs without geographical restriction. Introduced *B. subtilis* populations are subject to competition by the indigenous microflora (bacteria and fungi) and may also be affected by infectious agents like phages. As a result, high initial population numbers resulting from application of *Bacillus subtilis* will decline and reach a natural equilibrium. Any local effects that result from the use of Subtilex® according to the proposed GAP are likely to be transient as populations rapidly equilibrate in the soil. Microbial communities are extremely variable and environmental populations normally represent the most competitive species that have adapted to their niche and it is considered that introduction of *Bacillus amyloliquefaciens* MBI into natural soil will not impede the natural micro-flora.

Tests for toxicity of *Bacillus amyloliquefaciens* MBI600 to soil micro-organisms have not been conducted.

2.6.6. Effects on other non-target organisms (flora and fauna)

No data available. No further information required.

APPENDIX 1 – Standard terms and abbreviations

Part 1 - Technical Terms

A ampere
ACh acetylcholine
AChE acetylcholinesterase
ADI acceptable daily intake
ADP adenosine diphosphate
AE acid equivalent
AFID alkali flame-ionisation detector or detection
A/G albumin/globulin ratio
ai active ingredient
ALD_{50} approximate median lethal dose, 50%
ALT alanine aminotransferase (SGPT)
AOEL acceptable operator exposure level
AMD automatic multiple development
ANOVA analysis of variance
AP alkaline phosphatase
approx approximate
ARC anticipated residue contribution
ARfD acute reference dose
as active substance
AST aspartate aminotransferase (SGOT)
ASV air saturation value
ATP adenosine triphosphate

BCF bioconcentration factor
bfa body fluid assay
BOD biological oxygen demand
bp boiling point
BSAF biota-sediment accumulation factor
BSE bovine spongiform encephalopathie
BSP bromosulfophthalein
Bt bacillus thuringiensis
Bti bacillus thuringiensis israelensis
Btk bacillus thuringiensis kurstaki
Btt bacillus thuringiensis tenebrionis
BUN blood urea nitrogen
bw body weight

c centi- ($x\ 10^{-2}$)
°C degree Celsius (centigrade)
CA controlled atmosphere
CAD computer aided design
CADDY computer aided dossier and data supply (an electronic dossier interchange and archiving format)
cd candela
CDA controlled drop(let) application
cDNA complementary DNA
CEC cation exchange capacity
cf confer, compare to
CFU colony forming units
ChE cholinesterase
CI confidence interval
CL confidence limits
cm centimetre
CNS central nervous system
COD chemical oxygen demand
CPK creatinine phosphatase

cv	coefficient of variation
Cv	ceiling value
CXL	Codex Maximum Residue Limit (Codex MRL)
d	day
DES	diethylstilboestrol
DFR	dislodgeable foliar residue
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic Acid
dna	designated national authority
DO	dissolved oxygen
DOC	dissolved organic carbon
dpi	days post inoculation
DRES	dietary risk evaluation system
DT_{50}	period required for 50 percent dissipation (define method of estimation)
DT_{90}	period required for 90 percent dissipation (define method of estimation)
dw	dry weight
DWQG	drinking water quality guidelines
ε	decadic molar extinction coefficient
EC_{50}	median effective concentration
ECD	electron capture detector
ECU	European currency unit
ED_{50}	median effective dose
EDI	estimated daily intake
ELISA	enzyme linked immunosorbent assay
e-mail	electronic mail
EMDI	estimated maximum daily intake
EPMA	electron probe micro analysis
ERC	environmentally relevant concentration
ERL	extraneous residue limit
F	field
F_0	parental generation
F_1	filial generation, first
F_2	filial generation, second
FIA	fluorescence immunoassay
FID	flame ionisation detector
FOB	functional observation battery
fp	freezing point
FPD	flame photometric detector
FPLC	fast protein liquid chromatography
g	gram
G	glasshouse
GAP	good agricultural practice
GC	gas chromatography
GC-EC	gas chromatography with electron capture detector
GC-FID	gas chromatography with flame ionisation detector
GC-MS	gas chromatography-mass spectrometry
GC-MSD	gas chromatography with mass-selective detection
GEP	good experimental practice
GFP	good field practice
GGT	gamma glutamyl transferase
GI	gastro-intestinal
GIT	gastro-intestinal tract
GL	guideline level
GLC	gas liquid chromatography

GLP good laboratory practice
GM geometric mean
GMO genetically modified organism
GMM genetically modified micro-organism
GPC gel-permeation chromatography
GPPP good plant protection practice
GPS global positioning system
GSH glutathion
GV granulosevirus

h hour(s)
H Henry's Law constant (calculated as a unitless value) (see also K)
ha hectare
Hb haemoglobin
HCG human chorionic gonadotropin
Hct haematocrit
HDT highest dose tested
hL hectolitre
HEED high-energy electron diffraction
HID helium ionisation detector
HPAEC high performance anion exchange chromatography
HPLC high pressure liquid chromatography or high performance liquid chromatography
HPLC-MS high pressure liquid chromatography - mass spectrometry
HPPLC high pressure planar liquid chromatography
HPTLC high performance thin layer chromatography
HRGC high resolution gas chromatography
H_S Shannon-Weaver index
Ht haematocrit

I indoor
I_{50} inhibitory dose, 50%
IC_{50} median immobilisation concentration or median inhibitory concentration
ICM integrated crop management
ID ionisation detector
IEDI international estimated daily intake
IGR insect growth regulator
im intramuscular
inh inhalation
ip intraperitoneal
IPM integrated pest management
IR infrared
ISBN international standard book number
ISSN international standard serial number
iv intravenous
IVF in vitro fertilisation

k kilo
K Kelvin or Henry's Law constant (in atmospheres per cubic meter per mole, see also H)
K_{ads} adsorption constant
K_{des} apparent desorption coefficient
K_{oc} organic carbon adsorption coefficient
K_{om} organic matter adsorption coefficient
kg kilogram

L litre
LAN local area network
LASER light amplification by stimulated emission of radiation
LBC loosely bound capacity

LC liquid chromatography
LC-MS liquid chromatography- mass spectrometry
LC_{50} lethal concentration, median
LCA life cycle analysis
LC_{Lo} lethal concentration low
LC-MS-MS liquid chromatography with tandem mass spectrometry
LD_{50} lethal dose, median; dosis letalis media
LD_{Lo} lethal dose low
LDH lactate dehydrogenase
LOAEC lowest observable adverse effect concentration
LOAEL lowest observable adverse effect level
LOD limit of detection
LOEC lowest observable effect concentration
LOEL lowest observable effect level
LOQ limit of quantification (determination)
LPLC low pressure liquid chromatography
LSC liquid scintillation counting or counter
LSD least squared denominator multiple range test
LSS liquid scintillation spectrometry
LT lethal threshold

m metre
M molar
µm micrometer (micron)
MC moisture content
MCH mean corpuscular haemoglobin
MCHC mean corpuscular haemoglobin concentration
MCV mean corpuscular volume
MDL method detection limit
MFO mixed function oxidase
µg microgram
mg milligram
MHC moisture holding capacity
min minute(s)
mL millilitre
MLT median lethal time
MLD minimum lethal dose
mm millimetre
mo month(s)
mol Mole(s)
MOS margin of safety
mp melting point
MRE maximum residue expected
MRL maximum residue level or limit
mRNA messenger ribonucleic acid
MS mass spectrometry
MSDS material safety data sheet
MTD maximum tolerated dose

n normal (defining isomeric configuration) or number of observations
NAEL no adverse effect level
nd not detected
NEDI national estimated daily intake
NEL no effect level
NERL no effect residue level
ng nanogram
nm nanometer
NMR nuclear magnetic resonance

no	number
NOAEC	no observed adverse effect concentration
NOAEL	no observed adverse effect level
NOEC	no observed effect concentration
NOED	no observed effect dose
NOEL	no observed effect level
NOIS	notice of intent to suspend
NPD	nitrogen-phosphorus detector or detection
NPV	nuclear polyhedrosis virus
NR	not reported
NTE	neurotoxic target esterase
OC	organic carbon content
OCR	optical character recognition
ODP	ozone-depleting potential
ODS	ozone-depleting substances
OM	organic matter content
op	organophosphorous pesticide
Pa	Pascal
PAD	pulsed amperometric detection
2-PAM	2-pralidoxime
pc	paper chromatography
PC	personal computer
PCV	haematocrit (packed corpuscular volume)
PEC	predicted environmental concentration
PEC_A	predicted environmental concentration in air
PEC_S	predicted environmental concentration in soil
PEC_{SW}	predicted environmental concentration in surface water
PEC_{GW}	predicted environmental concentration in ground water
PED	plasma-emissions-detector
pH	pH-value
PHED	pesticide handler's exposure data
PHI	pre-harvest interval
PIC	prior informed consent
pic	phage inhibitory capacity
PIXE	proton induced X-ray emission
pKa	negative logarithm (to the base 10) of the dissociation constant)
PNEC	predicted no effect concentration
po	by mouth
P_{OW}	partition coefficient between n-octanol and water
POP	persistent organic pollutants
ppb	parts per billion (10^{-9})
PPE	personal protective equipment
ppm	parts per million (10^{-6})
ppp	plant protection product
ppq	parts per quadrillion (10^{-24})
ppt	parts per trillion (10^{-12})
PSP	phenolsulfophthalein
PrT	prothrombin time
PRL	practical residue limit
PT	prothrombin time
PTDI	provisional tolerable daily intake
PTT	partial thromboplastin time
QSAR	quantitative structure-activity relationship
r	correlation coefficient

r^2	coefficient of determination
RBC	red blood cell
REI	restricted entry interval
Rf	retardation factor
RfD	reference dose
RH	relative humidity
RL_{50}	median residual lifetime
RNA	ribonucleic acid
RP	reversed phase
rpm	rotations per minute
rRNA	ribosomal ribonucleic acid
RRT	relative retention time
RSD	relative standard deviation
s	second
SAC	strong adsorption capacity
SAP	serum alkaline phosphatase
SAR	structure/activity relationship
SBLC	shallow bed liquid chromatography
sc	subcutaneous
sce	sister chromatid exchange
SD	standard deviation
se	standard error
SEM	standard error of the mean
SEP	standard evaluation procedure
SF	safety factor
SFC	supercritical fluid chromatography
SFE	supercritical fluid extraction
SIMS	secondary ion mass spectroscopy
SOP	standard operating procedures
sp	species (only after a generic name)
SPE	solid phase extraction
SPF	specific pathogen free
spp	subspecies
sq	square
SSD	sulphur specific detector
SSMS	spark source mass spectrometry
STEL	short-term exposure limit
STMR	supervised trials median residue
t	tonne (metric ton)
$t_{½}$	half-life (define method of estimation)
T_3	tri-iodothyroxine
T_4	thyroxin
TADI	temporary acceptable daily intake
TBC	tightly bound capacity
TCD	thermal conductivity detector
TC_{Lo}	toxic concentration, low
TID	thermionic detector, alkali flame detector
TD_{Lo}	toxic dose low
TDR	time domain reflectrometry
TER	toxicity exposure ration
TER_I	toxicity exposure ration for initial exposure
TER_{ST}	toxicity exposure ration following repeated exposure
TER_{LT}	toxicity exposure ration following chronic exposure
tert	tertiary (in a chemical name)
TEP	typical end-use product
TGGE	temperature gradient gel electrophoresis

TIFF tag image file format
TLC thin layer chromatography
Tlm median tolerance limit
TLV threshold limit value
TMDI theoretical maximum daily intake
TMRC theoretical maximum residue contribution
TMRL temporary maximum residue limit
TOC total organic carbon
Tremcard Transport emergency card
tRNA transfer ribonucleic acid
TSH thyroid stimulating hormone (thyrotropin)
TWA time weighted average

UDS unscheduled DNA synthesis
UF uncertainty factor (safety factor)
ULV ultra low volume
UV ultraviolet

v/v volume ratio (volume per volume)

WBC white blood cell
wk week
wt weight
w/v weight per volume
ww wet weight
w/w weight per weight

XRFA X-ray fluorescence analysis

yr year

< less than
= less than or equal to
> greater than
= greater than or equal to

Part 2 - Organisations and Publications

ACPA American Crop Protection Association
ASTM American Society for Testing and Materials

BA Biological Abstracts (Philadelphia)
BART Beneficial Arthropod Registration Testing Group

CA Chemical Abstracts
CAB Centre for Agriculture and Biosciences International
CAC Codex Alimentarius Commission
CAS Chemical Abstracts Service
CCFAC Codex Committee on Food Additives and Contaminants
CCGP Codex Committee on General Principles
CCPR Codex Committee on Pesticide Residues
CCRVDF Codex Committee on Residues of Veterinary Drugs in Food
CE Council of Europe
CIPAC Collaborative International Pesticides Analytical Council Ltd
COREPER Comite des Representants Permanents

EC European Commission
ECB European Chemical Bureau
ECCA European Crop Care Association
ECDIN Environmental Chemicals Data and Information Network of the European Communities
ECDIS European Environmental Chemicals Data and Information System
ECE Economic Commission for Europe
ECETOC European Chemical Industry Ecology and Toxicology Centre
ECLO Emergency Centre for Locust Operations
ECMWF European Centre for Medium Range Weather Forecasting
ECPA European Crop Protection Association
EDEXIM European Database on Export and Import of Dangerous Chemicals
EHC (number) Environmental Health Criteria (number)
EINECS European Inventory of Existing Commercial Chemical Substances
ELINCS European List of New Chemical Substances
EMIC Environmental Mutagens Information Centre
EPA Environmental Protection Agency
EPO European Patent Office
EPPO European and Mediterranean Plant Protection Organization
ESCORT European Standard Characteristics of Beneficials Regulatory Testing
EU European Union
EUPHIDS European Pesticide Hazard Information and Decision Support System
EUROPOEM European Predictive Operator Exposure Model

FAO Food and Agriculture Organization of the UN
FOCUS Forum for the Co-ordination of Pesticide Fate Models and their Use
FRAC Fungicide Resistance Action Committee

GATT General Agreement on Tariffs and Trade
GAW Global Atmosphere Watch
GIFAP Groupement International des Associations Nationales de Fabricants de Produits Agrochimiques (now known as GCPF)
GCOS Global Climate Observing System
GCPF Global Crop Protection Federation (formerly known as GIFAP)
GEDD Global Environmental Data Directory
GEMS Global Environmental Monitoring System
GIEWS Global Information and Early Warning System for Food and Agriculture
GRIN Germplasm Resources Information Network
HRAC Herbicide Resistance Action Committee

IARC International Agency for Research on Cancer
IATS International Academy of Toxicological Science
IBT Industrial Bio-Test Laboratories
ICBB International Commission of Bee Botany
ICBP International Council for Bird Preservation
ICES International Council for the Exploration of the Seas
ICPBR International Commission for Plant-Bee Relationships
ILO International Labour Organization
IMO International Maritime Organisation
IOBC International Organisation for Biological Control of Noxious Animals and Plants
IPCS International Programme on Chemical Safety
IRAC Insecticide Resistance Action Committee
IRC International Rice Commission
ISCO International Soil Conservation Organization
ISO International Organization for Standardization
IUPAC International Union of Pure and Applied Chemistry

JECFA FAO/WHO Joint Expert Committee on Food Additives
JFCMP Joint FAO/WHO Food and Animal Feed Contamination Monitoring Programme
JMP Joint Meeting on Pesticides (WHO/FAO)
JMPR Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues (Joint Meeting on Pesticide Residues)

NATO North Atlantic Treaty Organisation
NAFTA North American Free Trade Agreement
NCI National Cancer Institute (USA)
NCTR National Centre for Toxicological Research (USA)
NGO non-governmental organisation
NTP National Toxicology Programme (USA)

OECD Organisation for Economic Co-operation and Development
OLIS On-line Information Service of OECD

PAN Pesticide Action Network

RNN Re-registration Notification Network
RTECS Registry of Toxic Effects of Chemical Substances (USA)

SCPH Standing Committee on Plant Health
SETAC Society of Environmental Toxicology and Chemistry
SI Systeme International d'Unites
SITC Standard International Trade Classification

TOXLINE Toxicology Information On-line

UN United Nations
UNEP United Nations Environment Programme

WCDP World Climate Data Programme
WCP World Climate Programme
WCRP World Climate Research Programme
WFP World Food Programme
WHO World Health Organization
WTO World Trade Organization
WWF World Wildlife Fund

Part 3- Preparation (formulation) types and codes

Code	Description	Definition
AB	Grain bait	Special forms of bait.
AE	Aerosol dispenser	A container-held preparation which is dispersed generally by a propellant as fine droplets/particles upon actuation of a valve.
AL	Other liquids to be applied undiluted	Self defining.
BB	Block blits	Special forms of bait.
BR	Briquette	Solid block designed for controlled release of active ingredient into water.
CB	Bait concentrate	A solid or liquid intended for dilution before use as a bait.
CG	Encapsulated granule	A granule with a protective or release controlling coating.
CS	Capsule suspension	A stable suspension of capsules in a fluid normally intended for dilution with water before use.
DC	Dispersible concentrate	A liquid homogeneous preparation to be applied as a solid dispersion after dilution in water.
DP	Dustable powder	A free-flowing powder suitable for dusting.
DS	Powder for dry seed treatment	A powder for application in the dry state directly to seed.
EC	Emulsifiable concentrate	A liquid, homogenous preparation to be applied as an emulsion after dilution in water.
ED	Electrochargeable liquid	Special liquid preparation for electrostatic (electrodynamic)spraying
EO	Emulsion. water in oil	A fluid. heterogeneous preparation consisting of a dispersion of fine globules of pesticide in water in a continuous organic liquid phase.
ES	Emulsion for seed treatment	A stable emulsion for application to the seed either directly or after dilution.
EW	Emulsion. Oil in water	A fluid, heterogeneous preparation consisting of a dispersion of fine globules of pesticide in an organic liquid in a continuous water phase.
FD	Smoke tin	Special form of smoke generator.
FG	Fine granule	A granule in the particle size range from 300 to 2500 :.
FK	Smoke candle	A smoke generator in the form of a candle.
FP	Smoke cartridge	Special form of smoke generator.
FR	Smoke rodlet	Special form of smoke generator.
FS	Flowable concentrate for seed treatment	A stable suspension for application to the seed either directly or seed treatment after dilution.
FT	Smoke tablet	Special form of smoke generator.
FU	Smoke generator	A combustible preparation generally solid, which upon ignition releases the active substances in the form of a smoke.
FW	Smoke pellet	Special form of smoke generator.
GA	Gas	A gas packed in pressure bottle or pressure tank.
GB	Granular bait	Special forms of bait.
GE	Gas generating product	A preparation which generates a gas by chemical reaction.
GG	Macrogranule	A granule in the particle size range from 2000 to 6000 :.
GP	Flo-dust	Very fine dustable powder for pneumatic application in glasshouses.
GR	Hot fogging concentrate	A free-flowing solid preparation of a defined granule size range ready for use.
GS	Cold fogging concentrate	Very viscous preparation based on oil or fat.
HN	Hot fogging concentrate	A preparation suitable for application by fogging equipment either directly or after dilution.
KN	Cold fogging concentrate	A preparation suitable for application by cold fogging equipment, either directly or after dilution.
LA	Lacquer	A solvent based film-forming preparation.
LS	Solution for seed treatment	A solution for application to the seed either directly or after dilution.

Code	Description	Definition
MG	Microgranule	A granule in the particle ske range from 100 to 600
OF	Oil miscible flowable (=oil active substances in a miscible suspension)	A stable suspension of concentrate fluid intended for dilution in an organic liquid before use.
OL	Oil miscible liquid	A liquid, homogenous preparation to be applied as a homogenous liquid after dilution in an organic liquid.
OP	Oil dispersible powder	A powder preparation to be applied as a suspension after dispersion in an organic liquid.
PA	Paste	A water based film forming preparation.
PB	Plate bait	Special forms of bait.
PC	Gel or paste concentrate	A solid preparation to be applied as a gel or a paste after dilution with water.
PR	Plant rodlet	A small rodlet, usually a few centimetres in length and a few millimetres in diameter containing active substance.
PS	Seed coated with a pesticide	Self defining.
RB	Bait (ready for use)	A preparation designed to attract and be eaten by the target species.
SB	Scrap bait	Special forms of bait.
SC	Suspension (= flowable concentrate)	A stable suspension of active substance(s) in a fluid intended for dilution with water before use
SE	Suspo-emulsion	A fluid. heterogeneous preparation consisting of a stable dispersion of active substance(s) in the form of solid particles.
SG	Water soluble granules	A preparation consisting of granules to be applied as a true solution of active substance after dissolution in water but many contain insoluble inert ingredients.
SL	Soluble concentrate	A liquid homogenous preparation to be applied as a true solution of active substance after dissolution in water but many contain insoluble inert ingredients.
SO	Spreading oil	A preparation designed to form a surface layer on application to water.
SP	Water soluble powder	A powder preparation to be applied as a true solution of the active substance after solution in water but which may contain insoluble inert ingredients.
SS	Water soluble powder for seed treatment	A powder to be dissolved in water before application to the seed.
SU	Ultra low volume (ULV) suspension	A suspension ready for use through ULV equipment.
TB	Tablet	Solid preparation in the form of small flat plates for dissolution in water.
TP	tracking powder	A rodenticidal contact preparation in powder form.
UL	Ultra low volume (ULV) liquid	A homogenous liquid ready for use through ULV equipment.
VP	Vapour releasing product	A preparation containing one or more volatile ingredients, the vapours of which are released into the air. Evaporation rate normally is controlled by using suitable preparations and/or dispensers.
WG	Water dispersible	A preparation granule consisting of granules to be applied after disintegration and dispersion in water.
WP	Wettable powder	A powder preparation to be applied as a suspension after dispersion in water.
WS	Water dispersible powder for slurry seed treatment	A powder to be dispersed at high concentration in water before application as a slurry to the seed.
XX	Others	

APPENDIX 2 – Specific terms and abbreviations

Appendix 2A – Specific terms and abbreviations

a	absolute organ weight
AAP	Algal Assay Procedure medium
aerob	aerobic test conditions
a-GT	alpha-glutamyl-transferase
ALAT	alanine aminotransferase
ALP	alkaline phosphatase
amu	atomic mass units
anaer	anaerobic test conditions
AR	applied radioactivity
ASAT	aspartate aminotransferase
ASTM	American Society for Testing and Materials
B	bacteria
biodeg	biodegradation
Chr. ab.	chromosome aberrations
CMC	carboxymethylcellulose
CoE	Council of Europe
crit.	criterion
d	decreased, but not statistically significantly
dc	statistically significantly decreased
DFI	Daily Food Intake
DMF	dimethylformamide
DO	Dissolved Oxygen
dr	dose-related
DWI	Daily Water Intake
E	total effect of mortality and fecundity/parasitic capacity, used in arthropod toxicity tests
E. coli	*Escherichia coli*
equal	used when the values given by the notifier are expressed in mg/kg bw/day.
equivalent	used when values given by the notifier are only expressed in mg/kg food, not in mg/kg bw/day, as species-dependent factor is used to translate these data to mg/kg bw/day.
ETE	Estimated Theoretical Exposure
GCP	good clinical practice
GIDH	glutamic-acid dehydrogenase
GOT	glutamic-oxalacetic transaminase
GPT	glutamic-pyruvic transaminase
HDL	high density lipoproteins
HPRT	hypoxanthine- guanine phosphoribosyl transferase
i	increased, but not statistically significantly
ic	statistically significantly increased
MC	moisture content in soil (v/v)
Mc	mammalian cells
MWHC	maximum water holding capacity (soils)
n/a	not applicable
n.d.	not detected
n.r.	not reported
ns	not significant
o.m.	organic matter
PEC	Predicted Environmental Concentration
PEG	polyethylene glycol

pF	moisture tension (soil) in [log $cm_{water\ column}$]
PIEC	Predicted Initial Environmental Concentration
pointmut.	pointmutations
r	relative organ weight
r.a.	radioactivity
res.	result
Ri	Reliability Index, referring to the intrinsic reliability of a test with respect to the quality of the study
S. typh.	*Salmonella typhimurium*
SPE	Solid Phase Extraction
Sub.	Substance
T	temperature
TWA	time weighted average
TWAEC	time weighted average environmental concentration
wat/sed	water/sediment systeem
w/w	weight per weight
-	negative
+	positive
-act.	without activation
+act.	with activation
%v/v	the percentage expressed by volume
%w/w	the percentage expressed by weight

Appendix 2B - Mycological terms

Anamorph	An anamorph fungus only reproduces with asexual spores
Basionym	This name of the fungus is based on the first description of the fungus
Biotroph	The organism is only able to grow on living material and is unable to grow on artificial medium
Ectotrophic	The place of action of the fungus is on the surface of the leaf or root. Growth within the leaf or root is excluded by this term
Epiphyte	A plant that grows on another plant, which it uses as a mechacal support, but not as a sourse of food
Hyphomycete	Fungus with a mycelial form, which bears conidia on separate hyphae or aggregations of hyphae.
Hyperparasite	Parasite, which parasitises other parasites and grow on these
Mitosporic	Sexual reproduction only via mitosis (asexual). Sexual spores (via meiosis have never been found
Mycoparasite	Parasite of fungi
Necotroph	The organism is only able to grow on on death substrate
Phyllosphere	Area immediately surrounding the leaves of a plant
Rhizosphere	Area immediately surrounding the roots of a plant
Saprofyte	Organism which grows on dead organic material
Smut	Phytopathogenic fungus belonging to the Ustilaginales, Basidiomycetes causing smut (brandschimmel)
Yeast	Single celled fungi, which reproduce by budding
	Growth form exhibited in some cases by primary filamentous fungi as a part of the life-cycle or under particular environmental conditions

APPENDIX 3 – Listing of end points

Identity, Biological Properties, Details of Uses, Further Information

Rapporteur Member State	Month and year	Active Ingredient
France	December 2014	*Bacillus amynoliquefaciens* strain MBI600

Chapter 1 - Identity, Biological properties, Details of Uses, Further Information

Active micro-organism	*Bacillus amyloliquefaciens* MBI 600
Function (e.g. control of fungi)	Fungicide

Identity of the micro-organism (Annex IIM 1)

Name of the organism	*Bacillus amyloliquefaciens* MBI 600
Taxonomy	**Species:** *Bacillus amyloliquefaciens* **Strain:** MBI 600 **Genus:** *Bacillus* **Family:** *Bacillaceae* **Division:** Bacteria
Species, subspecies, strain:	*Bacillus amyloliquefaciens* MBI 600
Identification	The identification is based on the 16S rRNA gene sequencing which is not sufficient for the discrimination at the strain level. The applicant is currently undertaking a genome sequencing project on MBI600, and this information will be available by December. **A new molecular method of identification for *Bacillus amyloliquefaciens MBI 600* at the strain level is required.**
Culture collection	Accession number: NCIMB 12376 Safe deposit agreement: 2009-SD-00011(ATCC) Safe deposit number: SD-1414

Biological properties of the micro-organism (Annex IIM 2)

Origin and natural occurrence	The bacterial strain is a naturally occurring, indigenous wild type. The strain was initially isolated in the UK. It is not known to be indigenous to the southern zone.
Target organism(s)	Grey mould, *Botrytis cinerea*
Mode of action	*Bacillus amyloliquefaciens* - disrupts the growth of the hyphae and prevents spore germination by contact - exhibits fungicidal properties via production of iturin A and surfactin, which are antagonistic to the fungal pathogen - colonizes developing shoot systems of plants, suppressing by competition (space and nutrients), disease organisms - induces systemic resistance response of the plant, indicated by enhanced peroxidase production.
Host specificity	None
Life cycle	4l discernible phases: - germination - outgrowth, - multiplication and - sporulation.
Infectivity, dispersal and colonisation ability	
Relationships to known plant, animal or human pathogens	No relationship to plant or animal pathogens. *Bacillus amyloliquefaciens* belongs to the *B. subtilis*-group which does not contain human pathogens. The *B. subtilis*-group is taxonomically clearly separated from the *B. cereus*-group. The *B. cereus*-group comprises different human pathogens such as *B. anthracis* and *B. cereus*.
Genetic stability	

Identity, Biological Properties, Details of Uses, Further Information

Rapporteur Member State	Month and year	Active Ingredient
France	December 2014	*Bacillus amynoliquefaciens* strain MBI600

Production of relevant metabolites/toxins	Strains belonging to the *Bacillus subtilis* group are able to produce a large array of metabolites. *B. amyloliquefaciens* MBI600 produces surfactin and iturin A, lipopeptides with surfactant activity. On the basis of available information there are no indications that *B. amyloliquefaciens* MBI600 has the potential to form toxins or metabolites of concern for human health.
Resistance/sensitivity to antibiotics used in human or veterinary medicine	*Bacillus amyloliquefaciens* is susceptible to a wide spectrum of antibiotics commonly used in human and veterinary medicine.

Identity, Biological Properties, Details of Uses, Further Information

Rapporteur Member State	Month and year	Active Ingredient
France	December 2014	*Bacillus amynoliquefaciens* strain MBI600

Summary of intended uses

Crop and /or situation (a)	Member state or Country	Product name	F G or I (b)	Pest or Group of pests controlled (c)	Formulation		Application				Application rate per treatment			PHI (days) (l)	Remarks (m)
					Type (d-f)	Minimum conc. of MPCA (i)	method kind (f-h)	growth stage & season (j)	number min max	interval between applications (min)	CFU MPCA/hl min max	water l/ha min max	CFU MPCA/ha min max		
Grapes	SEU	BUEXP1780S = SUBTILEX	F	*Botrytis* spp., Grey mould	WP	5.5 x 10^{10} cfu/g 50 g/kg	Spray tractor-mounted air assisted or knapsack sprayers	All	1 - 10	7 days	0.014 - 0.0055	400 - 1000	0.055 kg MPCA/ ha	-	Equivalent to 0.5 kg MPCP /ha

(a) For crops, the EU and Codex classifications (both) should be used; where relevant, the use situation should be described (*e.g.* fumigation of a structure)
(b) Outdoor or field use (F), glasshouse application (G) or indoor application (I)
(c) *e.g.* biting and suckling insects, soil born insects, foliar fungi, weeds
(d) *e.g.* wettable powder (WP), emulsifiable concentrate (EC), granule (GR)
(e) GCPF Codes - GIFAP Technical Monograph No 2, 1989
(f) All abbreviations used must be explained
(g) Method, *e.g.* high volume spraying, low volume spraying, spreading, dusting, drench
(h) Kind, *e.g.* overall, broadcast, aerial spraying, row, individual plant, between the plant - type of equipment used must be indicated
(i) Cfu=colony forming units and g/kg or g/l
(j) Growth stage at last treatment (BBCH Monograph, Growth Stages of Plants, 1997, Blackwell, ISBN 3-8263-3152-4), including where relevant, information on season at time of application
(k) Indicate the minimum and maximum number of application possible under practical conditions of use
(l) PHI - minimum pre-harvest interval
(m) Remarks may include: Extent of use/economic importance/restrictions

Identity, Biological Properties, Details of Uses, Further Information

Rapporteur Member State	Month and year	Active Ingredient
France	December 2014	*Bacillus amynoliquefaciens* strain MBI600

Further information

Production control	Confidential, see vol. 4 for more details
Proposal for classification and labelling	'Micro-organisms may have the potential to provoke sensitising reactions'

Analytical methods

Rapporteur Member State	Month and year	Active Ingredient
France	December 2014	*Bacillus amynoliquefaciens* strain MBI600

Chapter 2 - Analytical Methods

Analytical methods for the micro-organism (Annex IIM 4.2.8; 4.3; IIIM 5.3)

Manufactured micro-organism (principle of method)	Manufacturing process: Confidential- See volume 4 for more details Method for the determination of *B. amyloliquefaciens* MBI 600 in MPCA: The test item is plated on trypticase soy agar plates and the concentration calculated from plate counts as CFU/g.
Impurities and contaminating micro-organisms in manufactured material (principle of method)	Validated methods for the determination of microbial contaminants in MPCA are available and content are in accordance with OECD guidelines 65. **Validation methods for the determination of impurities higher than 1 g/kg are required.**
Plant protection product (principle of method)	Manufacturing process: Confidential- See volume 4 for more details. Method for the determination of *B. amyloliquefaciens* MBI 600 in MCPP: The test item is plated on trypticase soy agar plates and the concentration calculated from plate counts as CFU/g. Methods for the determination of microbial contaminants in MCPP: Validated methods for the determination of microbial contaminants in MCPP are available and content are in accordance with OECD guidelines 65.

Analytical methods for residues (viable and non-viable) in exposed compartments and organisms (Annex IIM 4.5)

of the active micro-organism (principle of method)	Not required
of relevant metabolites/toxins (principle of method)	Not required

Impact on Human and Animal Health

Rapporteur Member State	Month and year	Active Ingredient
France	December 2014	*Bacillus amynoliquefaciens* strain MBI600

Chapter 3 - Effects on Human Health

Effects on human health (Annex IIM 5; IIIM 7)

Medical data and direct observation, e.g. clinical cases (Annex IIM 5.2)	*Bacillus amyloliquefaciens* is suitable for Qualified Presumption of Safety (QPS) assessment. Case reports on infection or other incidents in humans related to *Bacillus amyloliquefaciens* or *Bacillus subtilis* are very scarce and mainly related to in immune compromised condition.
Medical surveillance on manufacturing plant personnel	No evidence of adverse health effects have been observed in any of the employees involved in the production, handling and application of *Bacillus amyloliquefaciens* strain MBI600.
Sensitisation/allergenicity observations, if appropriate	No reports of sensitization or allergenic response of workers. In the published literature a case of familial hypersensitivity pneumonitis induced by *Bacillus subtilis* has been described after exposure to wood dust. Based on general assumptions for micro-organisms, use of the warning phrase: "Microorganisms may have the potential to provoke sensitizing reactions."
Acute toxicity, pathogenicity and infectiveness	Toxicological studies revealed a low toxicity via the oral, dermal and intravenous routes and does not show any evidence of pathogenicity or infectivity. After intratracheal instillation of *Bacillus amyloliquefaciens* strain MBI600, deaths were seen shortly after administration, which is not consistent with an infection. Moreover, no trends of increase of body temperature at 24 hours were observed and no observable abnormalities were lesions were revealed at necropsy. This study did not show evidence of pathogenicity or infectivity, a pattern of clearance was established. Mortality in inhalation toxicity studies on microbial pesticides is not unusual, and these deaths seem to be the result of physical action rather than inherent toxicity of the substance. Furthermore, no mortality was observed in an intravenous study and in the inhalation toxicity study performed with the technical microorganism as currently marketed with a more realistic exposure pathway, no mortality was observed
Acute oral toxicity, pathogenicity and infectiveness	<u>Acute oral toxicity and infectivity/pathogenicity to rats</u> Clearance of live microorganism from all tissues analyzed was complete 21 days after the dosing. There was no mortality or evidence of toxicity pathogenicity or infectivity following oral administration of *Bacillus amyloliquefaciens* strain MBI600 at dose of 10^9 viable spores/animal and therefore the acute oral LD_{50} is $>10^9$ viable spores/animal. <u>Acute oral toxicity</u>

Impact on Human and Animal Health

Rapporteur Member State	Month and year	Active Ingredient
France	December 2014	*Bacillus amynoliquefaciens* strain MBI600

	No mortality was observed. The LD_{50} to female rats of *Bacillus amyloliquefaciens* strain MBI600 after a single oral dose is >5000 mg/kg bw corresponding to a $LD_{50} > 4.04\ 10^{11}$ spores/kg bw.
Acute inhalation toxicity, pathogenicity and infectiveness	<u>Acute pulmonary toxicity and infectivity/pathogenicity to rats</u> The acute pulmonary toxicity and infectivity/pathogenicity study performed in 1989 highlights a significant mortality in the treated group following a single dose of 10^8 viable spores of *Bacillus amyloliquefaciens* strain MBI600 by intratracheal instillation. Observed death occurred rapidly and there were no trends of increase of body temperature at 24 hours. This study does not show evidence of pathogenicity or infectivity, a pattern of clearance was established. Mortality in inhalation toxicity studies on microbial pesticides is not unusual, and these deaths seem to be the result of physical action rather than inherent toxicity of the substance. This conclusion is supported by the fact that no death was observed in acute intravenous toxicity study. <u>Acute inhalation toxicity study in rats</u> The inhalation toxicity study performed with the technical microorganism as currently marketed with a more realistic exposure pathway, no mortality was observed. The LC_{50} to rats of *Bacillus amyloliquefaciens* strain MBI600 after a 4h inhalation dose is >5.31 mg/L
Intravenous single dose	A clear pattern of clearance was established. There was no mortality or evidence of toxicity pathogenicity or infectivity following intravenous administration of *Bacillus amyloliquefaciens* strain MBI600 and therefore the acute intravenous LD_{50} is $>10^7$ viable spores/animal.
Genotoxicity (Annex IIM 5.3.5)	Supernatant of fermentation broth of *Bacillus amyloliquefaciens* strain MBI600 not mutagenic in an Ames test either in the presence or absence of metabolic activation in the strains tested. Based on literature data, *Bacillus amyloliquefaciens* not known as genotoxin producer.
Cell culture studies (Annex IIM, point 5.3.6)	No data, not considered necessary.
Information on short term toxicity and pathogenicity (Annex IIM, point 5.3.7)	No data, not considered necessary.
First aid measures, medical treatment (Annex IIM, point 5.2.5)	**Skin contact:** In case of contact with skin wash off immediately with soap and water. **Eye contact:** In the case of contact with eyes, rinse immediately with plenty of water for at least 15 minutes. **Ingestion:** Drink plenty of water. Do not induce vomiting. Seek medical advice. **Inhalation:** Move to fresh air and ventilate

	suspected area. **Notes to the physician** **Symptoms:** No specific symptoms are known. **Treatment:** No specific antidote. Initial treatment should be symptomatic and supportive.
Specific toxicity, pathogenicity and infectiveness studies (Annex IIM, point 5.5)	No data not required.

Exposure scenarios

Operators	Operator exposure estimates to the microorganism is not needed. Appropriate PPE considering potential sensitising properties of micro-organisms.
Workers	Worker exposure estimates to the microorganism is not needed.
Bystanders	Bystander exposure estimates to the microorganism is not needed.

Residues

Rapporteur Member State	Month and year	Active Ingredient
France	December 2014	*Bacillus amynoliquefaciens* strain MBI600

Chapter 4 – Residues

Residues in or on treated products, food and feed (Annex IIM 6; IIIM 8)

Viable residues	No risk for the consumer is expected from the organism itself i.e. it is not pathogenic.
Non-viable residues	*B. amyloliquefaciens* strain MBI600 does not produce any relevant metabolites or toxins

Rapporteur Member State	Month and year	Active Ingredient
France	December 2014	*Bacillus amynoliquefaciens* strain MBI600

Chapter 5 - Fate and Behaviour in the Environment (Annex IIM 7; IIIM 9)

Persistence and multiplication in soil	*B. amyloliquefasciens* is commonly observed in soil, especially in the rhizosphere. Based on available data, the abundance of *B. subtilis* varied from 3×10^4 CFU/g dry soil in desert soils to 7.6×10^4 CFU/g dry soil in a pine forest soil. Introduced spores are very resistant to abiotic environmental factors and do not compete with autochthon microorganisms for nutrients. They can survive in soil for a long period of time. A precondition for multiplication is germination of spores. Germination mainly depends on the availability of nutrients. Thus, sufficient nutrients and the ability to compete with autochthon microorganisms are necessary for forming of vegetative cells and for multiplication. This occurs mainly in the rhizosphere. Reduction of vegetative cells and of spores is mainly caused by grazing protozoa, bacterial predators, and bacteriophage infections. Following 10 applications of 0.5 kg Subtilex/ha (55 g *B. amyloliquefaciens* MBI600/ha, 2.75×10^{13} cfu/ha), the initial PECsoil (in 5cm) values are: 6.67 mg of Subtilex/kg soil, 0.73 mg of MPCA/kg soil, 3.67 x 10^8 cfu/kg soil
Persistence and multiplication in water	Introduced spores are unlikely to germinate and form vegetative cells in nutrient-poor surface water. As *B. amyloliquefaciens* MBI600 must compete with other microorganisms for available nutrients, germination and multiplication appears to be very limited. The total number of spores decreases over a specific period of time by grazing protozoa or probably by sediment-dwelling organisms. Following 10 applications of 0.5 kg Subtilex/ha (55 g *B. amyloliquefaciens* MBI600/ha, 2.75×10^{13} cfu/ha), the initial PEsw values at 3 m distance (drift of 8.02%) are: 133.7 µg of Subtilex/L, 14.70 µg of *B. amyloliquefaciens* MBI600/L, 7.35×10^6 cfu/L.
Persistence and multiplication in air	*B. amyloliquefaciens* strain MBI600 can disperse in air. However, contamination of air quickly reduced over time due to settling down of aerosols and spore killing by UV light.
Mobility	Risk of groundwater contamination for *B. amiloliquefacsiens* is considered as negligible.

Effects on non-target species

Rapporteur Member State	Month and year	Active Ingredient
France	December 2014	*Bacillus amynoliquefaciens* strain MBI600

Chapter 6 - Effects on Non-target Species (Annex IIM 8; IIIM 10)

Effects on birds and mammals

Colinus virginianus: LD_{50} > 4000 mg as/kg bw

Rat: LD_{50} > 5000 mg product/kg bw

No signs of toxicity, infectivity and pathogenicity were observed throughout the test period.

Acute Toxicity/exposure ratios for birds and mammals – screening step

Test substance	Crop, use pattern	Crop scenario, indicator species	Toxicity endpoint (mg a.s./kg bw)	DDD (mg a.s./kg bw)	TER	TER risk assessment trigger
Bacillus amyloliquefaciens MBI600	Vines	Small omnivorous bird	>4000	52.4	>76	10
Bacillus amyloliquefaciens MBI600	Vines	Small herbivorous mammal	>5000	75	>67	10

Effect on aquatic organisms

Test substance: MPCA

Oncorhynchus mykiss: LC50 (mortality) > 9.9 x 1010 CFU/L

(96 h semi-static; Maximum mean initial concentration)

Cyprinus carpio: NOEC (mortality) = 1.3 x 1011 CFU/L

(30 days; Maximum measured concentration)

Daphnia magna: NOEC (immobilisation) = 1.9 x 1010 CFU/L

(48 h semi-static; Maximum mean initial concentration)

Daphnia magna: NOEC (reproduction) = 2.7 x 107 CFU/L

(21 d ; measured initial concentration at the lowest concentration)

Pseudokirchneriella subcapitata: EyC50 > 5.8 x 1011 CFU/L

(72 h static; Maximum mean measured concentration)

No signs of infectiveness and pathogenicity

Acute Toxicity/exposure ratios for aquatic organisms

Species	Endpoint	**Toxicity** (mg as/L)	PEC_{SW}	TER	TER Trigger value
Oncorhyncus mykiss	96-hour LC_{50}	>100 mg MPCA/L > 9.9 x 10^{10} cfu/L	14.7 µg MPCA/L 7.35 x 10^6 cfu/L (10 applications)	> 6803 > 13469	100
Daphnia magna	48-hour EC_{50}	>100 mg MPCA/L > 1.9 x 10^{10}	14.7 µg MPCA/L 7.35 x 10^6 cfu/L (10 applications)	> 6803 > 2585	100

Effects on non-target species

Rapporteur Member State	Month and year	Active Ingredient
France	December 2014	*Bacillus amynoliquefaciens* strain MBI600

		cfu/L			
Daphnia magna	21-days EC_{50}	2.7 x 10^7 cfu/L	7.35 x 10^6 cfu/L (10 applications)	**3.7**	10
Daphnia magna	21-days EC_{50}	2.7 x 10^7 cfu/L	7.35 x 10^5 cfu/L (1 application)	37 *	10
Pseudokirchneriella subcapitata	72-hour EC_{50}	>100 mg MPCA/L (>1.1 x 10^{11} cfu/L) > 5.8 x 10^{11} cfu/L	14.7 µg MPCA/L 7.35 x 10^6 cfu/L (10 applications)	> 6803 > 79000	10

Effect on plants other than algae	No study available ; no study required.
Effects on bees	Acute oral toxicity: LD50 > 1.1 x 10^7 CFU/bee (estimated nominal concentration) Acute contact toxicity: LD50 > 100 µg product/bee equivalent to 4.4 x 10^8 CFU/bee (measured concentration) No evidence of toxicity, infectivity and pathogenicity.
Effects on other arthropods	No effect observed on *Harmonia axyridis*, *Phytoseiulus persimilis*, and *Chrysoperla carnea* Little to no effect on silkworm larvae No evidence of toxicity, infectivity and pathogenicity.
Effects on earthworms	*Eisenia fetida:* NOEC < 2.9 x 10^{12} CFU/kg soil (nominal concentration) No signs of toxicity, infectiveness and pathogenicity were observed
Effects on non-target soil micro-organisms	No study available Any local effects that result from the use of Subtilex® SC according to the proposed GAP are likely to be transient as populations rapidly equilibrate in the soil. Microbial communities are extremely variable and environmental populations normally represent the most competitive species that have adapted to their niche and it is considered that introduction of *Bacillus amyloliquefaciens* MBI600 into natural soil will not impede the natural micro-flora.
Additional studies	-

Level 3

Bacillus amyloliquefaciens strain MBI600

SUMMARY AND CONSIDERATION WITH RESPECT TO THE APPROVAL CRITERIA OF REGULATION (EC) No 1107/2009

IDENTIFICATION OF DATA GAPS, PROPOSED CONDITIONS, RISK MANAGEMENT MEASURES, ISSUES THAT COULD NOT BE FINALISED AND CRITICAL AREAS OF CONCERN

PROPOSED DECISION

3. Proposed decision with respect to the application

3.1. Background to the proposed decision

3.1.1. Proposal on acceptability against the decision making criteria – Article 4 and annex II of regulation (EC) No 1107/2009

3.1.1.1. Article 4				
		Yes	No	
i)	It is considered that Article 4 of Regulation (EC) No 1107/2009 is complied with. Specifically the RMS considers that authorisation in at least one Member State is expected to be possible for at least one plant protection product containing the active substance for at least one of the representative uses.	X		*Bacillus amyloliquefaciens* MBI600 has been considered in one representative formulation BUEXP1780S = SUBTILEX which is proposed for a use on grapes against Botrytis. Authorisation for the representative formulation is expected to be possible in at least one Member State for the representative use.
3.1.1.2. Submission of further information				
		Yes	No	
i)	It is considered that a complete dossier has been submitted	X		RMS considered that a complete dossier was submitted by the applicant but some information considered as confirmatory, are listed in the table 3.1.4
ii)	It is considered that in the absence of a full dossier the active substance may be approved even though certain information is still to be submitted because: (a) the data requirements have been amended or refined after the submission of the dossier; or (b) the information is considered to be confirmatory in nature, as required to increase confidence in the decision.			
3.1.1.3. Restrictions on approval				
		Yes	No	
	It is considered that in line with Article 6 of Regulation (EC) No 1107/2009 approval should be subject to conditions and restrictions.		X	
3.1.1.4. Criteria for the approval of an active substance				
Dossier				
		Yes	No	
	It is considered the dossier contains the information needed to establish, where relevant, Acceptable Daily Intake (ADI), Acceptable Operator Exposure Level (AOEL) and Acute Reference Dose (ARfD).	X		Not directly applicable to micro-organisms. The derivation of reference values for the microorganism is not considered necessary based on the lack of toxicity, infectivity or pathogenicity in the available studies.

	It is considered that the dossier contains the information necessary to carry out a risk assessment and for enforcement purposes (relevant for substances for which one or more representative uses includes use on feed or food crops or leads indirectly to residues in food or feed). In particular it is considered that the dossier: (a) permits any residue of concern to be defined; (b) reliably predicts the residues in food and feed, including succeeding crops (c) reliably predicts, where relevant, the corresponding residue level reflecting the effects of processing and/or mixing; (d) permits a maximum residue level to be defined and to be determined by appropriate methods in general use for the commodity and, where appropriate, for products of animal origin where the commodity or parts of it is fed to animals; (e) permits, where relevant, concentration or dilution factors due to processing and/or mixing to be defined.	X		The residue data submitted on the plant protection product are considered sufficient. A residue definition is not necessary and there is no need and no scientifically justified value to define an Acceptable Daily Intake (ADI). Therefore, calculation of the potential exposure of consumers in terms of the Theoretical Maximum Daily Intake (TMDI) and its relation to the ADI is not relevant, and conclusively a Maximum Residue Level (MRL) is not proposed. Thus, RMS proposes to include *Bacillus amyloliquefaciens* MBI 600 to the Annex IV of Reg. 396/2005.
	It is considered that the dossier submitted is sufficient to permit, where relevant, an estimate of the fate and distribution of the active substance in the environment, and its impact on non-target species.	X		*B. amyloliquefasciens* may survive in soil, but there is no risk for uncontrolled growth, since this widespread and naturally occurring soil Bacilli are subject to competition and antagonism in its natural habitat. Based on the available data, no significant ecotoxicological or environmental risk from the application of Subtilex® can occur according to Good Agricultural Practice.
Efficacy				
		Yes	No	
	It is considered that it has been established for one or more representative uses that the plant protection product, consequent on application consistent with good plant protection practice and having regard to realistic conditions of use is sufficiently effective.	X		To support the approval of *Bacillus amyloliquefaciens* MBI600 as active substance under regulation (EC) 1107/2009, efficacy data for the representative use against grey mould (*Botrytis cinerea*) on vine were presented for the product SUBTILEX (BUEXP1780S). 28 efficacy trials allow illustrating the efficacy of SUBTILEX and the interest of *Bacillus amyloliquefaciens* MBI600.
Relevance of metabolites				
		Yes	No	
	It is considered that the documentation submitted is sufficient to permit the establishment of the toxicological, ecotoxicological or environmental relevance of metabolites.	X		No metabolite of toxicological relevance suspected to be produced by *Bacillus amyloliquefaciens* MBI600

Composition				
		Yes	No	
	It is considered that the specification defines the minimum degree of purity, the identity and maximum content of impurities and, where relevant, of isomers/diastereo-isomers and additives, and the content of impurities of toxicological, ecotoxicological or environmental concern within acceptable limits.		X	- A new molecular method of identification for *Bacillus amyloliquefaciens MBI 600* at the strain level is required and should be available on December 2014 according to the applicant. - A new maximum certified value of the microbial active substance *Bacillus amyloliquefaciens* MBI 600 in accordance with batches of the analytical profile of MPCA should be provided. This value can be set at the maximum value founded in the batches (e.g: 1.4 x 10^{12} CFU/g). - Data on the characterization, the quantification, specification values for impurities in the five batches of technical MPCA were required in the confidential part (see volume 4). - The analysis certificates of the results indicated for the determination of contaminants in the formulation SUBTILEX are missing and are required.
	It is considered that the specification is in compliance with the relevant Food and Agriculture Organisation specification, where such specification exists.			
	It is considered for reasons of protection of human or animal health or the environment, stricter specifications than that provided for by the FAO specification should be adopted			
Methods of analysis				
		Yes	No	
	It is considered that the methods of analysis of the active substance, safener or synergist as manufactured and of determination of impurities of toxicological, ecotoxicological or environmental concern or which are present in quantities greater than 1 g/kg in the active substance, safener or synergist as manufactured, have been validated and shown to be sufficiently specific, correctly calibrated, accurate and precise.		X	A new maximum certified value of the microbial active substance *Bacillus amyloliquefaciens* MBI 600 in accordance with batches of the analytical profile of MPCA should be provided. This value can be set at the maximum value founded in the batches (1.4 x 10^{12} CFU/g). Data on the characterization, the quantification, specification values for impurities in the five batches of technical MPCA were required in the confidential part (see volume 4).
	It is considered that the methods of residue analysis for the active substance and relevant metabolites in plant, animal and environmental matrices and drinking water, as appropriate, shall have been validated	X		See Vol. B5 for more details.

	and shown to be sufficiently sensitive with respect to the levels of concern.			
	It is confirmed that the evaluation has been carried out in accordance with the uniform principles for evaluation and authorisation of plant protection products referred to in Article 29(6) of Regulation 1107/2009.	X		/
Impact on human health				
Impact on human health - ADI, AOEL, ARfD				
		Yes	No	
	It is confirmed that (where relevant) an ADI, AOEL and ARfD can be established with an appropriate safety margin of at least 100 taking into account the type and severity of effects and the vulnerability of specific groups of the population.	X		Not directly applicable to micro-organisms. Taking together the results of these experimental studies, the data from published literature, the QPS statute of *B.amyloliquefaciens*, the experience of safe production and application of *Bacillus amyloliquefaciens*–based plant protection products and the natural occurrence of this species, it is appropriate to state that no concern has been raised with regard to human health. The derivation of reference values for the microorganism is not considered necessary based on the lack of toxicity, infectivity or pathogenicity in the available studies.
Impact on human health – proposed genotoxicity classification				
		Yes	No	
	It is considered that, on the basis of assessment of higher tier genotoxicity testing carried out in accordance with the data requirements and other available data and information, including a review of the scientific literature, reviewed by the Authority, **the substance SHOULD BE classified or proposed for classification**, in accordance with the provisions of Regulation (EC) No 1272/2008, **as mutagen category 1A or 1B.**		X	Not applicable for micro-organisms.
Impact on human health – proposed carcinogenicity classification				
		Yes	No	
i)	It is considered that, on the basis of assessment of the carcinogenicity testing carried out in accordance with the data requirements for the active substances, safener or synergist and other available data and information, including a review of the scientific literature, reviewed by		X	Not applicable for micro-organisms.

	the Authority, **the substance SHOULD BE classified or proposed for classification**, in accordance with the provisions of Regulation (EC) No 1272/2008, **as carcinogen category 1A or 1B.**			
ii)	Linked to above classification proposal. It is considered that exposure of humans to the active substance, safener or synergist in a plant protection product, under realistic proposed conditions of use, is negligible, that is, the product is used in closed systems or in other conditions excluding contact with humans and where residues of the active substance, safener or synergist concerned on food and feed do not exceed the default value set in accordance with Article 18(1)(b) of Regulation (EC) No 396/2005.			
Impact on human health – proposed reproductive toxicity classification				
		Yes	No	
i)	It is considered that, on the basis of assessment of the reproductive toxicity testing carried out in accordance with the data requirements for the active substances, safeners or synergists and other available data and information, including a review of the scientific literature, reviewed by the Authority, **the substance SHOULD BE classified or proposed for classification**, in accordance with the provisions of Regulation (EC) No 1272/2008, **as toxic for reproduction category 1A or 1B.**		X	Not applicable for micro-organisms.
ii)	Linked to above classification proposal. It is considered that exposure of humans to the active substance, safener or synergist in a plant protection product, under realistic proposed conditions of use, is negligible, that is, the product is used in closed systems or in other conditions excluding contact with humans and where residues of the active substance, safener or synergist concerned on food and feed do not exceed the default value set in accordance with Article 18(1)(b) of Regulation (EC) No 396/2005.			
Impact on human health – proposed endocrine disrupting properties classification				
		Yes	No	
i)	It is considered that **the substance SHOULD BE classified or proposed for classification** in accordance with the provisions of Regulation (EC) No 1272/2008, as **carcinogenic category 2 and toxic for reproduction category 2 and on that basis shall be considered**		X	Not applicable for micro-organisms.

	to have endocrine disrupting properties			
ii)	It is considered that **the substance SHOULD BE classified or proposed for classification** in accordance with the provisions of Regulation (EC) No 1272/2008, as **toxic for reproduction category 2 and** in addition the RMS considers the substance **has toxic effects on the endocrine organs and on that basis shall be considered to have endocrine disrupting properties**		X	Not applicable for micro-organisms.
iii)	Linked to either i) or ii) immediately above. It is considered that exposure of humans to the active substance, safener or synergist in a plant protection product, under realistic proposed conditions of use, is negligible, that is, the product is used in closed systems or in other conditions excluding contact with humans and where residues of the active substance, safener or synergist concerned on food and feed do not exceed the default value set in accordance with Article 18(1)(b) of Regulation (EC) No 396/2005.			
Fate and behaviour in the environment				
Persistent organic pollutant (POP)				
		Yes	No	
	It is considered that the active substance **FULFILS** the criteria of a persistent organic pollutant (POP) as laid out in Regulation 1107/2009 Annex II Section 3.7.1.		X	Not applicable
Persistent, bioaccumulative and toxic substance (PBT)				
		Yes	No	
	It is considered that the active substance **FULFILS** the criteria of a persistent, bioaccumulative and toxic (PBT) substance as laid out in Regulation 1107/2009 Annex II Section 3.7.2.		X	Not applicable
Very persistent and very bioaccumulative substance (vPvB).				
		Yes	No	
	It is considered that the active substance **FULFILS** the criteria of a a very persistent and very bioaccumulative substance (vPvB) as laid out in Regulation 1107/2009 Annex II Section 3.7.3.		X	Not applicable
Ecotoxicology				
		Yes	No	

	It is considered that the risk assessment demonstrates risks to be acceptable in accordance with the criteria laid down in the uniform principles for evaluation and authorisation of plant protection products referred to in Article 29(6) under realistic proposed conditions of use of a plant protection product containing the active substance, safener or synergist. The RMS is content that the assessment takes into account the severity of effects, the uncertainty of the data, and the number of organism groups which the active substance, safener or synergist is expected to affect adversely by the intended use.	X		Based on the available data, no significant ecotoxicological or environmental risk from the application of Subtilex® can occur according to the representative Good Agricultural Practice.
	It is considered that, on the basis of the assessment of Community or internationally agreed test guidelines, the substance **HAS** endocrine disrupting properties that may cause adverse effects on non-target organisms.		X	Not applicable
	Linked to the consideration of the endocrine properties immediately above. It is considered that the exposure of non-target organisms to the active substance in a plant protection product under realistic proposed conditions of use is negligible.	X		Not applicable
	It is considered that it is established following an appropriate risk assessment on the basis of Community or internationally agreed test guidelines, that the use under the proposed conditions of use of plant protection products containing this active substance, safener or synergist: — will result in a negligible exposure of honeybees, or — has no unacceptable acute or chronic effects on colony survival and development, taking into account effects on honeybee larvae and honeybee behaviour.	X		Based on the available data, it is expected that exposure of honeybees to *B. amyloliquefaciens* MBI 600 following applications of Subtilex® according to the intended uses on grapes will not represent a risk to honeybees.
Residue definition				
		Yes	No	
	It is considered that, where relevant, a residue definition can be established for the purposes of risk assessment and for enforcement purposes.			No risk for the consumer is expected from the organism itself i.e. it is not pathogenic. Therefore no residue definition is proposed and it is proposed to include *Bacillus amyloliquefaciens* MBI 600 to the Annex IV of Reg. 396/2005
Fate and behaviour concerning groundwater				

		Yes	No	
	It is considered that it has been established for one or more representative uses, that consequently after application of the plant protection product consistent with realistic conditions on use, the predicted concentration of the active substance or of metabolites, degradation or reaction products in groundwater complies with the respective criteria of the uniform principles for evaluation and authorisation of plant protection products referred to in Article 29(6) of Regulation 1107/2009.	X		*B. amyloliquefaciens* is an ubiquitous soil microorganism groundwater contamination probably happens naturally. Endospores reaching groundwater environments do not germinate and grow due to insufficient nutrient availability. Dilution as a result of continuous water flux and predation by groundwater (micro-) organisms will cause a continuous decline of the spore populations. Thus, the groundwater contamination appears very unlikely.

3.1.2. Proposal – Candidate for substitution

Candidate for substitution				
		Yes	No	
	It is considered that the active substance shall be approved as a candidate for substitution		X	

3.1.3. Proposal – Low risk active substance

Low-risk active substances				
		Yes	No	
	It is considered that the active substance **shall be considered of low risk.** In particular it is considered that the substance **should NOT be classified or proposed for classification** in accordance with Regulation (EC) No 1272/2008 as at least one of the following: — carcinogenic,	X		The criteria on the left are not really adapted to microorganisms. However, considering the natural occurrence of the specie in the environment and its QPS (Qualified Presumption of Safety) status according to The Panel on Biological Hazards of EFSA[4], RMS considers that *Bacillus amyloliquefaciens* MBI 600 could be considered as a low risk active substance.

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	— mutagenic, — toxic to reproduction, — sensitising chemicals, — very toxic or toxic, — explosive, — corrosive. In addition it is considered that **the substance is NOT**: — persistent (half-life in soil more than 60 days), — has a bioconcentration factor higher than 100, — is deemed to be an endocrine disrupter, or — has neurotoxic or immunotoxic effects.			

3.1.4. List of studies to be generated, still ongoing or available but not peer reviewed

Data gap	**Relevance in relation to representative use(s)**	**Study status**		
		No confirmation that study available or on-going.	**Study on-going and anticipated date of completion**	**Study available but not peer-reviewed**
3.1.4.1. Identity of the active substance or formulation				
A new molecular method of identification for *Bacillus amyloliquefaciens MBI 600* at the strain level			X Dec. 2014 (information from the Applicant)	
A new maximum certified value of the microbial active substance *Bacillus amyloliquefaciens* MBI 600 in accordance with batches of the analytical profile of MPCA		X		

A specification value was proposed by the applicant for soluble impurities, and insoluble impurities were noticed in the technical MPCA Depending on the nature (relevant or significant) of these impurities, data on the characterization, quantification, and specification values should be provided		X		
The analysis certificates of the results indicated for the determination of contaminants in the formulation SUBTILEX.		X		
3.1.4.2. Physical and chemical properties of the active substance and physical, chemical and technical properties of the formulation				
The stability study at 35 °C for 12 weeks including content of the microbial active substance and microbial contaminants according to OECD issue paper, Oct. 2011.			X (started on Sept. 2014 – Information from the applicant)	
The stability study at ambient temperature for 2 years including content of the microbial active substance and microbial contaminants according to OECD issue paper, Oct. 2011		X		
3.1.4.3. Data on uses and efficacy				
No further data is required				
3.1.4.4. Data on handling, storage, transport, packaging and labelling				
No further data is required				
3.1.4.5. Methods of analysis				
No further data is required				
3.1.4.6. Toxicology and metabolism				
No further data is required				

3.1.4.7. Residue data				
No further data is required				
3.1.4.8. Environmental fate and behaviour				
More information on the soil mobility of *B. amyloliquefasciens* should be provided by the notifier to confirm the low risk of groundwater contamination by *B. amyloliquefasciens* cells.		X		
Notifier to provide to RMS a full copy of all cited articles.				X
3.1.4.9. Ecotoxicology				
No further data is required.				

3.1.5. Issues that could not be finalised

An issue is listed as an issue that could not be finalised where there is not enough information available to perform an assessment, even at the lowest tier level, for the representative uses in line with the Uniform Principles, as laid out in Commission Regulation (EU) No 546/2011, and where the issue is of such importance that it could, when finalised, become a concern (which would also be listed as a critical area of concern if it is of relevance to all representative uses).

Area of the risk assessment that could not be finalised on the basis of the available data	**Relevance in relation to representative use(s)**
	[specify if measure relates to a specific representative use/use scenario/product or to all uses/products]

3.1.6. Critical areas of concern

An issue is listed as a critical area of concern:
(a) where the substance does not satisfy the criteria set out in points 3.6.3, 3.6.4, 3.6.5 or 3.8.2 of Annex II of Regulation (EC) No 1107/2009 and the applicant has not provided detailed evidence that the active substance is necessary to control a serious danger to plant health which cannot be contained by other available means including non-chemical methods, taking into account risk mitigation measures to ensure that exposure of humans and the environment is minimised, or

(b) where there is enough information available to perform an assessment for the representative uses in line with the Uniform Principles, as laid out in Commission Regulation (EU) 546/2011, and where this assessment does not permit to conclude that for at least one of the representative uses it may be expected that a plant protection product containing the active substance will not have any harmful effect on human or animal health or on groundwater or any unacceptable influence on the environment.

An issue is also listed as a critical area of concern where the assessment at a higher tier level could not be finalised due to a lack of information, and where the assessment performed at the lower tier level does not permit to conclude that for at least one of the representative uses it may be expected that a plant protection product containing the active substance will not have any harmful effect on human or animal health or on groundwater or any unacceptable influence on the environment.

Critical area of concern identified	**Relevance in relation to representative use(s)**
	[specify if concern relates to all or specific representative use/use scenario/product or to all uses/products]

3.1.7. Overview table of the concerns identified for each representative use considered

(If a particular condition proposed to be taken into account to manage an identified risk, as listed in 3.3.1, has been evaluated as being effective, then 'risk identified' is not indicated in this table.)
All columns are grey as the material tested in the toxicological studies has not been demonstrated to be representative of the technical specification.

Representative use		Use "Grapes"
Operator risk	Risk identified	
	Assessment not finalised	
Worker risk	Risk identified	
	Assessment not finalised	
Bystander risk	Risk identified	
	Assessment not finalised	
Consumer risk	Risk identified	
	Assessment not finalised	
Risk to wild non target terrestrial vertebrates	Risk identified	
	Assessment not finalised	
Risk to wild non target terrestrial organisms other than vertebrates	Risk identified	
	Assessment not finalised	
Risk to aquatic organisms	Risk identified	
	Assessment not finalised	
Groundwater exposure active substance	Legal parametric value breached	
	Assessment not finalised	
Groundwater exposure metabolites	Legal parametric value breached	
	Parametric value of 10µg/L[(a)] breached	
	Assessment not finalised	
Comments/Remarks		

The superscript numbers in this table relate to the numbered points indicated within chapter 3.1.5 and 3.1.6. Where there is no superscript number, see level 2 for more explanation.

(a): Value for non relevant metabolites prescribed in SANCO/221/2000-rev 10-final, European Commission, 2003

3.1.8. Area(s) where expert consultation is considered necessary

It is recommended to organise a consultation of experts on the following parts of the assessment report:

Area(s) where expert consultation is considered necessary	**Justification**

3.1.9. Critical issues on which the Co RMS did not agree with the assessment by the RMS

Points on which the co-rapporteur Member State did not agree with the assessment by the rapporteur member state. Only the points relevant for the decision making process should be listed.

Not applicable: There is no Co-RMS

3.2. Proposed decision

It is proposed that:

***Bacillus amyloliquefaciens* strain MBI600 can be approved under Regulation (EC) No 1107/2009 as a low risk substance**

It is considered that the following is specified in Part A of the Commission Implementing Regulation for the approval of the active substance:

None

It is considered that the following be specified in Part B of the Commission Implementing Regulation as areas requiring particular attention from Member States when evaluating applications for product authorisation(s):

In the overall assessment Member States shall pay particular attention to the protection of operators and workers, taking into account that *Bacillus amyloliquefaciens* MBI600 is to be considered as a potential sensitizer. Conditions of use shall include general risk mitigation measures, where appropriate.
Strict maintenance of environmental conditions and quality control analysis during the manufacturing process shall be assured by the producer.

The applicant shall submit to the Commission, the Member States and the Authority confirmatory information as regard:

- *Bacillus amyloliquefaciens* MBI 600 must be identified at the strain level therefore a new molecular method of identification for *Bacillus amyloliquefaciens* MBI 600 at the strain level must be provided. However, according to the applicant this method should be available on December 2014 and could be considered during the peer review process.

- A specification value was proposed by the applicant for soluble impurities, and insoluble impurities were also noticed in the technical MPCA. Depending on the nature (relevant or significant) of these impurities, data on the characterization, quantification, and specification values should be provided if this cannot be finalised during the peer review process.

3.3. Rational for the conditions and restrictions to be associated with the approval or authorisation(s), as appropriate

3.3.1. Particular conditions proposed to be taken into account to manage the risks identified

Proposed condition/risk mitigation measure	Relevance in relation to representative use(s)

3.4. APPENDICES

GUIDANCE DOCUMENTS USED IN THIS ASSESSEMENT

Analytical methods
OECD Issue paper on microbial contaminant limits for microbial pest control products No.65. Oct. 2011.
SANCO 825-00/rev8.1: Guidance document on pesticide residue analytical methods SANCO 825-00/rev8.1

Toxicology Effects on Human Health
OECD ISSUE PAPER ON MICROBIAL CONTAMINANT LIMITS FOR MICROBIAL PEST CONTROL PRODUCTS. No 65. ENV/JM/MONO (2011)43
OCSPP HARMONIZED TEST GUIDELINES SERIES 885. GROUP C – TOXICOLOGY TEST GUIDELINES

Environmental fate and behavior and Effects on non-target organisms:

European Commission, 2002a. Guidance Document on Terrestrial Ecotoxicology Under Council Directive 91/414/EEC. SANCO/10329/2002 rev.2 final, 17 October 2002.

European Commission, 2002b. Guidance Document on Aquatic Ecotoxicology Under Council Directive 91/414/EEC. SANCO/3268/2001 rev 4 (final), 17 October 2002.

European Commission, 2001. Guidance Document on Aquatic Ecotoxicology in the frame of Directive 91/414/EEC. SANCO/3268/2001, 1 October 2001.

European Commission, 2002c. Guidance Document on Risk Assessment for Birds and Mammals Under Council Directive 91/414/EEC. SANCO/4145/2000.

European Food Safety Authority; Guidance Document on Risk Assessment for Birds & Mammals on request from EFSA. EFSA Journal 2009; 7(12):1438. doi:10.2903/j.efsa.2009.143

3.5. REFERENCE LIST

List [in the conventional format] any references specifically cited in Volume 1 (i.e references to underpinning documents such as PPR-Panel Opinions, EFSA conclusions, national documents etc.).

Environmental fate and behavior and Effects on non-target organisms:

European Food Safety Authority; Guidance Document on Risk Assessment for Birds & Mammals on request from EFSA. EFSA Journal 2009; 7(12):1438. doi:10.2903/j.efsa.2009.143

Draft Assessment Report

Bacillus amyloliquefaciens strain MBI600

Volume 2

Annex A

List of tests and studies submitted and of information available

Rapporteur Member State : France

Volume 1

Level 1: Statement of subject matter and purpose for which the monograph was prepared

Level 2: Reasoned statement of the overall conclusions drawn by the Rapporteur Member State

Appendix 1: Standard terms and abbreviations

Appendix 2: Specific terms and abbreviations

Appendix 3: List of endpoints

Level 3: Proposed decision with respect to the application for inclusion of the active substance in Annex I

Level 4: Further information to permit a decision to be made, or to support a review of the conditions and restrictions associated with the proposed inclusion in Annex 1

Volume 2

Annex A: List of the tests and studies submitted and of information available

Volume 3

Annex B: RMS summary, evaluation and assessment of the data and information

Annex B.1: Identity

Annex B.2: Biological, physical, chemical and technical properties

Annex B.3: Data application and further information.

Annex B.4: Proposals for classification and labelling

Annex B.5: Analytical methods

Annex B.6: Effects on human health

Annex B.7: Residues data

Annex B.8: Fate and behaviour in the environment

Annex B.9: Effects on non-target organisms

Annex B.10: Summary and evaluation of environmental impact

Appendix 1: Standard terms and abbreviations

Appendix 2: Specific terms and abbreviations

Volume 4

Annex C: Confidential information and summary and assessment of information relating to the collective submission of dossiers

Version History of Volume 2

Date	**Reason for revision**
December 2014	Initial DAR

Table of contents

A.1. Identity

Annex IIM Data and Information

Author(s)	Annex point / reference number	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed* Y/N	Owner **
ATCC	IIM 1.3.2/03	2009	Safe Deposit agreement IP Licensing Services, USA Becker Underwood No report No. Not GLP Unpublished **Supporting information, letter not fully summarised**	Y	BASF Agricultural Specialities Ltd
Brown A.	IIM 4.3.1/01	2008	Confidential business information of Becker Underwood, Unpublished report ➔ 4.3.1/04	Y	BASF Agricultural Specialities Ltd
Brown, A.	IIM 1.3.1/04	2012	Taxonomic re-classification of *Bacillus subtilis* strain MBI600 to *Bacillus amyloliquefaciens* strain MBI600 Becker Underwood No report No. Not GLP Unpublished **Supporting information, letter not fully summarised**	Y	BASF Agricultural Specialities Ltd
Brown, A.	IIM 1.3.2/02	2011	Confirmation of holding of *Bacillus subtilis* strain JB 3.6 Becker Underwood No report No. Not GLP Unpublished **Supporting information, letter not fully summarised**	Y	BASF Agricultural Specialities Ltd
Brown, A.	IIM 1.3.5/02	2011	Confirmation of holding of *Bacillus subtilis* strain JB 3.6 Becker Underwood No report No. Not GLP Unpublished ⇨ 1.3.2/02 **Supporting information, letter not fully summarised**	Y	BASF Agricultural Specialities Ltd
Bruch, M.K.	IIM 1.3.1/03	1990	Identification of microorganisms MicroBioTest Inc, USA Becker Underwood, report No. 186-102 GLP Unpublished **Supporting information, study not fully summarised**	Y	BASF Agricultural Specialities Ltd
Fukumoto, J.	IIM 1.3.1/01	1943	Studies on the production of bacterial amylase. I. Isolation of bacteria secreting potent amylases and their distribution (in Japanese) J. Agr. Chem. Soc. Japan 19: 487-503 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ****
Izu, S.	IIM 1.3.5/01	2007	Letter from supplier Becker Underwood No report No. Not GLP Unpublished **Supporting information, letter not fully summarised**	Y	BASF Agricultural Specialities Ltd
Kloepper, J.W.	IIM 1.3.1/05	2012	Identification of MBI600 by 16S rRNA gene sequencing using 8F and 1492R primers Auburn University, USA Becker Underwood No report No. Not GLP Unpublished **Supporting information, study not fully summarised**	Y	BASF Agricultural Specialities Ltd
Kloepper, J.W.	IIM 1.3.1/07	2012	No Title Department of Entomology and Plant Pathology, Auburn University, No Report No.		
Kloepper, J.W.	IIM 1.3.3/02	2007	Identification of MBI600 by 16S rRNA gene sequencing using 8F and 1492R primers Auburn University, USA Becker Underwood No report No. Not GLP Unpublished ⇨ 1.3.1/05 **Supporting information, study not fully summarised**	Y	BASF Agricultural Specialities Ltd
Naomi Yamashita	IIM 1.4.3/01	2008	Information on the manufacturing process, citation of which would allow conclusions concerning the manufacturing process.	Y	AHC Co., Ltd.
NCIB	IIM 1.3.2/01	1987	Accession number National Collection of Industrial Bacteria Torry Research Station, Scotland Becker Underwood No report No. Not GLP Unpublished **Supporting information, letter not fully summarised**	Y	BASF Agricultural Specialities Ltd
Priest, F.G.; Goodfellow, M.; Shute, L.A.; Berkeley, R.C.W.	IIM 1.3.1/02	1987	Bacillus amyloliquefaciens sp. nov., nom. rev. Int. J. Syst. Bacteriol., 1987, 37, 69-71 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Rossal, S.	IIM 1.3.6/01	2012	*Bacillus subtilis* isolate MBI 600 The University of Nottingham Becker Underwood No report No. Not GLP Unpublished **Supporting information, letter not fully summarised**	Y	BASF Agricultural Specialities Ltd
Whittaker, M	IIM 1.4.4/03	2014	Analysis certificates Bacillus amyloliquefaciens MBI 600 Technical Grade Ingredient batches BS1261, BS7111, BS7211, BS7311, BS9111	Y	APIS

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Whittaker, M.	IIM 4.3.1/01	2013	Detection and enumeration of *Bacillus amyloliquefaciens* and microbial contaminants in five production batches of MBI600 Technical Grade Active Ingredient APIS, UK Becker Underwood Report No. BUBA003 GLP Unpublished ⇨ Doc J 1.4.4/01	Y	BASF Agricultural Specialities Ltd.
Whittaker, M.	IIM 1.4.4/02	2014	Detection and enumeration of *Bacillus amyloliquefaciens*, yeasts and moulds in five production batches of MBI600 Microbial Pest Control Agent, Study : APIS-BASF-008, APIS (Applied Insect Science), GLP	Y	APIS
Yang, G.P.	IIM 1.3.1/06	2005	Identification of MBI600 by 16S rRNA gene sequencing Becker Underwood No report No. Not GLP Unpublished **Supporting information, study not fully summarised**	Y	BASF Agricultural Specialities Ltd

Annex IIIM Data and Information

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Whittaker, M.	IIIM 4.3.1/01	2014	Detection and enumeration of Bacillus amyloliquefaciens and microbial contaminants in five production batches of SUBTILEX Microbial Pest Control Product, Study No: APIS-BASF-006, APIS, GLP, Unpublished	Y	APIS

A.2. Biological, physical, chemical and technical properties

Annex IIM Data and Information

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Cavaglieri, L.; Orlando, J.; Rodríguez, M.I.; Chulze, S.; Etcheverry, M.	IIM, 2.1/02	2005	Biocontrol of *Bacillus subtilis* against *Fusarium verticillioides in vitro* and at the maize root level Research in Microbiology 2005, 156: 748-754. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Toure Y.; Ongena M.; Jacques P.; Guiro A.; Thonart P.	IIM, 2.1/03	2004	Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple Journal of applied microbiology 2004, 96:1151-1160. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Jacobsen, B.J.; Zidack, N.K.; Larson, B.J.	IIM, 2.1/04	2004	The role of *Bacillus*-based biological control agents in integrated pest management systems: plant diseases The American Phytopathological Society 2004, 94(11), 1272-1275 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Amner, W.; McCarthy, A.J.; Edwards, C.	IIM, 2.2/01	1991	Survival of plasmid-bearing strain of *Bacillus subtilis* introduced into compost Journal of General Microbiology 1991, 137, 1931-1937 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Welker, N.E.; Campbell, L.L.	IIM, 2.2/02	1967	Unrelatedness of *Bacillus amyloliquefaciens* and *Bacillus subtilis* Journal of Bacteriology 1967, 94(4): 1124-1130. Not GLP Published ⇨ 1.3.1/10 **Supporting information, paper not fully summarised**	N	Public
Katz, E; Demain, A.L.	IIM, 2.2/04	1977	The peptide antibiotics of *Bacillus*: chemistry, biogenesis and possible functions Bacteriological reviews 1977, 4(2):449-474 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Stein, T.	IIM, 2.2/05	2005	*Bacillus subtilis* antibiotics: structures, syntheses and specific functions Molecular Microbiology (2005) 56 (4), 845–857 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Montesinos, E.	IIM, 2.2/06	2007	Antimicrobial peptides and plant disease control FEMS Microbiology Letters 2007, 270: 1-11 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Hill, I.R.; Gray, T.R.G.	IIM, 2.2/07	1967	Application of the florescent-antibody-technique to an ecological study of bacteria in soil Journal of Bacteriology 1967, 93(6):1888-1896 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Zuber, P.; Nakano, M.M.; Marahiel, M.A.	IIM, 2.2/08	1993	Peptide antibiotics. Chapter 61, pp. 897-916 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Sansinenea, E.; Ortiz, A.	IIM, 2.2/09	2011	Secondary metabolites of soil *Bacillus* spp. Nordisk Biotechnol Lett (2011), 33, 1523-1538 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Hamdache, A.; Lamarti, A.; Aleu, J.; Collado, I.G.	IIM, 2.2/10	2011	Non-peptide metabolites from the genus *Bacillus*. Journal of Natural Products Published by the American Chemical Society and American Society of Pharmacognosy 14 March 2011 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Wulff, E.G.; Mguni, C.M.; Mansfeld-Giese, K.; Fels, J.; Lubeck, M.; Hockenhull, J.	IIM, 2.2/11	2002	Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris pv. Campestris* Plant Pathology (2002) 51 , 574–584 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Priest, F.G.:	IIM, 2.8-02	1989	Isolation and identification of aerobic endospore-forming Bacteria. *Bacillus*, Chapter 3 Colin R. Harwood (ed.) The University of Newcastle upon Tyne, UK Plenum Press 1989, 27-56 BMF2000-90	N	Public
EFSA	IIM, 5/09	2011	Scientific Opinion Technical guidance on the assessment of the toxigenic potential of *Bacillus* species use in human nutrition EFSA Journal 2011; 9(11):2445 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
EFSA	IIM, 5.1/01	2007	Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA Opinion of the Scientific Committee The EFSA Journal (2007) 587, 1-16 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Gupta, D.K.; Vyas, K.M.	IIM, 5.2.4/01	1989	Efficacy of *Bacillus subtilis* against mosquito Larvae (*Anophelis culicifacies*) Zeitschrift fuer Angewandte Zoologie 1989, 76(1) 85-91 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Fossum, K.; Herikstad, H.; Binde, M.; Pettersen K.E.	IIM, 5.2.4/02	1986	Isolations of *Bacillus subtilis* in connection with Bovine Mastitis Nordisk Veterinärmedicine, Vol. 38, 1986, 233-236 BMF2000-151 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Mikkola, R.; Andersson, M.A.; Grigoriev, P.; Teplova, V.V; Saris, N.E.L.; Rainey, F.A.; Salkinoja-Salonen, M.S.	IIM, 5.2.4/03	2004	*Bacillus amyloliquefaciens* strains isolated from moisture-damaged buildings produced surfactin and a substance toxic to mammalian cells. Arch Microbiol (2004) 181 : 314–323 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Apetroaie-Constantin, C.; Mikkola, R.; Andersson, M.A.; Teplova, V.; Suominen, I.; Johanssonand, T.; Salkinoja-Salonen, M.	IIM, 5.2.3/02	2009	*Bacillus subtilis* and *B. mojavensis* strains connected to food poisoning produce the heat stable toxin amylopsin Journal of Applied Microbiology 2009 ISSN 1364-5072 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
From, C.; Pukall, R.; Schumann, P.; Hormazábal, V.; Granum, P.E.	IIM, 5.2.3/03	2005	Toxin producing ability among *Bacillus* spp. outside the *Bacillus cereus* group Applied and Environmental Microbiology, 2005 71, 1178-1183 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
de Boer, A.S. and Diderichsen, B.	IIM, 5.2.3/06	1991	On the safety of *Bacillus subtilis and B. amyloliquefaciens*: a review Appl. Microbiol. Biotechnol., 36, 1991, 1-4 TOX2000-1212 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Mikkola, R.; Andersson, M.A.; Grigoriev, P.; Teplova, V.V; Saris, N.E.L.; Rainey, F.A.; Salkinoja-Salonen, M.S.	IIM, 5.2.4/03	2004	*Bacillus amyloliquefaciens* strains isolated from moisture-damaged buildings produced surfactin and a substance toxic to mammalian cells. Arch Microbiol (2004) 181 : 314–323 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Du Toit Schabort	IIM, 5.4/01	2011	Secondary compound analysis of *Bacillus subtilis* strain MBI600 Becker Underwood No report No. Not GLP Unpublished	Y	BASF Agricultural Specialities Ltd
Sansinenea, E.; Ortiz, A.	IIM, 5.4/02	2011	Secondary metabolites of soil *Bacillus* spp. Nordisk Biotechnol Lett (2011), 33, 1523-1538 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Hamdache, A.; Lamarti, A.; Aleu, J.; Collado, I.G.	IIM, 5.4/03	2011	Non-peptide metabolites from the genus *Bacillus* Journal of Natural Products Published by the American Chemical Society and American Society of Pharmacognosy 14 March 2011 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Wulff, E.G.; Mguni, C.M.; Mansfeld-Giese, K.; Fels, J.; Lubeck, M.; Hockenhull, J.	IIM, 5.4/04	2002	Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris pv. Campestris* Plant Pathology (2002) 51 , 574–584 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Welker, N.E.; Campbell, L.L.	IIM, 5/08	1967	Unrelatedness of *Bacillus amyloliquefaciens* and *Bacillus subtilis* Journal of Bacteriology 1967, 94(4): 1124-1130. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Kloepper, J.W.	IIM, 4.3.1/02	2012	No Title Auburn University, USA Becker Underwood No report No. Not GLP Unpublished	Y	BASF Agricultural Specialities Ltd
Green, L.A.	IIM,	2013	Antibiotic resistance testing against plant protection product: *Bacillus amyloliquefaciens* strain MBI600. Wickham Laboratories Limited. Unpublished report No. CN0007155a.DR		

Annex IIIM Data and Information

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Brown A.	IIM 4.1.3	2014	BASF confidential business information. Packaging to be used for BUEXP1780S End Use Product in Europe	Y	BASF Agricultural Specialities Ltd
Morgan, L.	IIIM 2.2	2013	Accelerated Storage Stability of BUEXP1780S for up to 12 weeks at 35°C stored in an aluminium laminate bag. Battelle UK Ltd. Becker Underwood Report No. LG/12/002/1 GLP Unpublished 2.1/01	Y	BASF Agricultural Specialities Ltd

A.3. Further Information

Annex IIM Data and Information

No studies were submitted

Annex IIIM Data and Information

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
G. Martin	IIIA 6.1 /01	2011	Mode of Action of *Bacillus subtilis* strain MBI600 against *Botrytis cinerea*. Becker Underwood, RD061108 Non-GEP, unpublished	Y	BASF Agricultural Specialities Limited
R. Morgan	IIIA 6.1 /02	2011	Inhibition of *Botrytis cinerea* spore germination by *Bacillus subtilis* strain MBI600. Becker Underwood, RD111104 Non-GEP, unpublished	Y	BASF Agricultural Specialities Limited
E. Ferré	IIIA 6.1 and IIIA 6.2/03	2011	Efficacy evaluation of Becker Underwood *Bacillus subtilis* strain MBI600 against *Botrytis cinerea* on grapevine. Anadiag S.A, 11 244 DR1 GEP, unpublished	Y	BASF Agricultural Specialities Limited
E. Ferré	IIIA 6.1 and IIIA 6.2/04	2011	Efficacy evaluation of Becker Underwood *Bacillus subtilis* strain MBI600 against *Botrytis cinerea* on grapevine. Anadiag S.A, 11 244 TL1 GEP, unpublished	Y	BASF Agricultural Specialities Limited
E. Ferré	IIIA 6.1 and IIIA 6.2/05	2011	Efficacy evaluation of Becker Underwood *Bacillus subtilis* strain MBI600 against *Botrytis cinerea* on grapevine. Anadiag S.A, 11 244 TL2 GEP, unpublished	Y	BASF Agricultural Specialities Limited
R. Scherrer	IIIA 6.1 and IIIA 6.2/06	2012	AN EVALUATION OF THE EFFICACY OF BUEXP1780S AGAINST *BOTRYTIS CINEREA* ON GRAPEVINE. Anadiag SAS, EU 12 180 AV1 GEP, unpublished	Y	BASF Agricultural Specialities Limited
R. Scherrer	IIIA 6.1 and IIIA 6.2/07	2012	AN EVALUATION OF THE EFFICACY OF BUEXP1780S AGAINST *BOTRYTIS CINEREA* ON GRAPEVINE. Anadiag SAS, EU 12 180 TL1 GEP, unpublished	Y	BASF Agricultural Specialities Limited

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
R. Scherrer	IIIA 6.1 and IIIA 6.2/08	2012	AN EVALUATION OF THE EFFICACY OF BUEXP1780S AGAINST *BOTRYTIS CINEREA* ON GRAPEVINE. Anadiag SAS, EU 12 180 TL2 GEP, unpublished	Y	BASF Agricultural Specialities Limited
O. Scrimshaw	IIIA 6.1 and IIIA 6.2/09	2012	R&DX121111 - BU Botrytis on vines Summer 2012, 1 site in France. Eurofins Agroscience Services, S12-00873-01 GEP, unpublished	Y	BASF Agricultural Specialities Limited
O. Scrimshaw	IIIA 6.1 and IIIA 6.2/10	2012	R&DX121111 - BU Botrytis on vines Summer 2012, 1 site in France. Eurofins Agroscience Services, S12-00873-05 GEP, unpublished	Y	BASF Agricultural Specialities Limited
E. Ferré	IIIA 6.1 and IIIA 6.2/11	2011	Efficacy evaluation of Becker Underwood *Baclillus Subtilis* strain MBI600 against *Botrytis cinerea* on table grapes. Anadiag Italia SRL, ITA 11106 BA1 GEP, unpublished	Y	BASF Agricultural Specialities Limited
E. Ferré	IIIA 6.1 and IIIA 6.2/12	2011	Efficacy evaluation of Becker Underwood *Baclillus Subtilis* strain MBI600 against *Botrytis cinerea* on table grapes. Anadiag Italia SRL, ITA 11106 BA2 GEP, unpublished	Y	BASF Agricultural Specialities Limited
E. Ferré	IIIA 6.1 and IIIA 6.2/13	2011	Efficacy evaluation of Becker Underwood *Baclillus Subtilis* strain MBI600 against *Botrytis cinerea* on table grapes. Anadiag Italia SRL, ITA 11107 TO1 GEP, unpublished	Y	BASF Agricultural Specialities Limited
R. Scherrer	IIIA 6.1 and IIIA 6.2/14	2012	Evaluate the efficacy of BUEXP1780S against against *Botrytis cinerea* on grapevine. Anadiag Italia SRL, ITA 12140 BA1 GEP, unpublished	Y	BASF Agricultural Specialities Limited
R. Scherrer	IIIA 6.1 and IIIA 6.2/15	2012	Evaluate the efficacy of BUEXP1780S against against *Botrytis cinerea* on grapevine. Anadiag Italia SRL, ITA 12140 BO1 GEP, unpublished	Y	BASF Agricultural Specialities Limited
R. Scherrer	IIIA 6.1 and IIIA 6.2/16	2012	Evaluate the efficacy of BUEXP1780S against against *Botrytis cinerea* on grapevine. Anadiag Italia SRL, ITA 12141 TO1 GEP, unpublished	Y	BASF Agricultural Specialities Limited
R. Scherrer	IIIA 6.1 and IIIA 6.2/17	2012	Evaluate the efficacy of BUEXP1780S against against *Botrytis cinerea* on grapevine. Anadiag Italia SRL, ITA 12141 TO2 GEP, unpublished	Y	BASF Agricultural Specialities Limited

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
R. Scherrer	IIIA 6.1 and IIIA 6.2/18	2012	AN EVALUATION OF THE EFFICACY OF BUEXP1780S AGAINST *BOTRYTIS CINEREA* ON GRAPEVINE. Anadiag Portugal, PO 12 096 PO1 GEP, unpublished	Y	BASF Agricultural Specialities Limited
R. Scherrer	IIIA 6.1 and IIIA 6.2/19	2012	AN EVALUATION OF THE EFFICACY OF BUEXP1780S AGAINST *BOTRYTIS CINEREA* ON GRAPEVINE. Anadiag Portugal, PO 12 096 PO2 GEP, unpublished	Y	BASF Agricultural Specialities Limited
O. Scrimshaw	IIIA 6.1 and IIIA 6.2/20	2012	R&DX121111 - BU Botrytis on vines Summer 2012, 1 site in Portugal. Eurofins Agroscience Services, S12-00873-03 GEP, unpublished	Y	BASF Agricultural Specialities Limited
O. Scrimshaw	IIIA 6.1 and IIIA 6.2/21	2012	R&DX121111 - BU Botrytis on vines Summer 2012, 1 site in Portugal. Eurofins Agroscience Services, S12-00873-04 GEP, unpublished	Y	BASF Agricultural Specialities Limited
E. Fernandez	IIIA 6.1 and IIIA 6.2/22	2012	FUNGICIDE EFFICACY AND SELECTIVITY OF THE FORMULATION BUEXP1780S (*Bacillus subtilis strain MBI600)* AGAINST GREY MOULD (*Botrytis cinerea*) ON GRAPEVINES IN SPAIN IN 2012. Promo-Vert Crop Services S.L., 12 F VI BU 01 GEP, unpublished	Y	BASF Agricultural Specialities Limited
E. Fernandez	IIIA 6.1 and IIIA 6.2/23	2012	FUNGICIDE EFFICACY AND SELECTIVITY OF THE FORMULATION BUEXP1780S (*Bacillus subtilis strain MBI600)* AGAINST GREY MOULD (*Botrytis cinerea*) ON GRAPEVINES IN SPAIN IN 2012. Promo-Vert Crop Services S.L., 12 F VI BU 02 GEP, unpublished	Y	BASF Agricultural Specialities Limited
O. Scrimshaw	IIIA 6.1 and IIIA 6.2/24	2012	R&DX121111 - BU Botrytis on vines Summer 2012, 1 site in Spain. Eurofins Agroscience Services, S12-00873-02 GEP, unpublished	Y	BASF Agricultural Specialities Limited
O. Scrimshaw	IIIA 6.1 and IIIA 6.2/25	2012	R&DX121111 - BU Botrytis on vines Summer 2012, 1 site in Spain. Eurofins Agroscience Services, S12-00873-06 GEP, unpublished	Y	BASF Agricultural Specialities Limited

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
E. Ferré	IIIA 6.1 and IIIA 6.2/26	2012	Efficacy evaluation of Becker Underwood *Bacillus subtilis* strain MBI600 against Botrytis cinerea on grapevine. Anadiag SA, 11 244 BM1 GEP, unpublished	Y	BASF Agricultural Specialities Limited
J.L. Paratte	IIIA 6.1 and IIIA 6.2/27	2012	EFFICACY OF *Bacillus Subtilis* strain MBI600 AGAINST GREY MOULD (DUE TO Botrytis cinerea) ON GRAPEVINE. Promo-Vert Crop, 12 F VI BU 03 GEP, unpublished	Y	BASF Agricultural Specialities Limited
C. Carnaille	IIIA 6.1 and IIIA 6.2/28	2012	EFFICACY OF *Bacillus Subtilis* strain MBI600 AGAINST GREY MOULD (DUE TO Botrytis cinerea) ON GRAPEVINE. Promo-Vert Crop, 12 F VI BU 04 GEP, unpublished	Y	BASF Agricultural Specialities Limited
R. Scherrer	IIIA 6.1 and IIIA 6.2/29	2012	AN EVALUATION OF THE EFFICACY OF BUEXP1780S AGAINST *BOTRYTIS CINEREA* ON GRAPEVINE. Anadiag SAS, EU 12 180 AN1 GEP, unpublished	Y	BASF Agricultural Specialities Limited
R. Scherrer	IIIA 6.1 and IIIA 6.2/30	2012	BUEXP1780S efficiency on vine grey mould. Inovitis, Inovitis 2012 GEP, unpublished	Y	BASF Agricultural Specialities Limited
G. Martin	IIIA 6.5/31	2011	Foliar persistence of BUEXP1780S on vine leaves. Becker Underwood, RD071101 Non-GEP, unpublished	Y	BASF Agricultural Specialities Limited

A.4. Analytical methods

Annex IIM Data and Information

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Anon	IIM, 4.3.1/03	2008	Kosan Analytical Test Method Spore Concentration and Bacterial Contamination Idemitsu Kosan, Japan Becker Underwood No report No. Not GLP Unpublished	Y	BASF Agricultural Specialities Ltd
Anon	IIM, 4.3.1/04	2008	Kosan Analytical Test Method Identity Test *Bacillus amyloliquefaciens* MBI600 spores Idemitsu Kosan, Japan Becker Underwood No report No. Not GLP Unpublished ⇨ 4.3.1/03	Y	BASF Agricultural Specialities Ltd
Kloepper, J.W.	IIM, 4.3.1/02	2012	No Title Auburn University, USA Becker Underwood No report No. Not GLP Unpublished	Y	BASF Agricultural Specialities Ltd
Whittaker, M.	IIM, 4.3.1/01	2013	Detection and enumeration of *Bacillus amyloliquefaciens* and microbial contaminants in five production batches of MBI600 Technical Grade Active Ingredient APIS, UK Becker Underwood Report No. BUBA003 GLP Unpublished ⇨ Doc J 1.4.4/01	Y	BASF Agricultural Specialities Ltd

Annex IIIM Data and Information

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Morgan, L.	IIIM 5.2.2.	2013	Accelerated Storage Stability of BUEXP1780S for up to 12 weeks at 35°C stored in an aluminium laminate bag. Battelle UK Ltd. Becker Underwood Report No. LG/12/002/1 GLP Unpublished ⇨ 2.1/01	Y	BASF Agricultural Specialities Ltd

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Whittaker, M.	IIIM 4.3.1/01	2014	Detection and enumeration of Bacillus amyloliquefaciens and microbial contaminants in five production batches of SUBTILEX Microbial Pest Control Product. APIS. Study: APIS-BASF-006, GLP Unpublished	Y	BASF Agricultural Specialities Ltd

A.5. Effects on human health

Annex IIM Data and Information

Author(s)	Annex point / reference number	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed* Y/N	Owner **
Abo-State, M.A.M.; Husseiny, H.M.; Helimish, F.A.; Zickry A.R.A.	IIM, 5.2.3/08	2012	Contamination of Eye Drops with *Bacillus* Species and Evaluation of Their Virulence Factors World Applied Sciences Journal 19 (6): 847-855, 2012 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Anon	IIM, 5.4/34	N/A	http://hazard.com/msds/tox/f/q84/q238.html Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Anon	IIM, 5.4/35	N/A	http://www.lookchem.com/Bacilysin/ Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Apetroaie-Constantin, C.; Mikkola, R.; Andersson, M.A.; Teplova, V.; Suominen, I.; Johanssonand, T.; Salkinoja-Salonen, M.	IIM, 5.2.3/02	2009	*Bacillus subtilis* and *B. mojavensis* strains connected to food poisoning produce the heat stable toxin amylopsin Journal of Applied Microbiology 2009 ISSN 1364-5072 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Aranda, F.J.; Teruel, J.A.; Ortiz, A.	IIM, 5.4/18	2005	Further aspects on the haemolytic activity of the antibiotic lipopeptide iturin A Biochimica et Biophysica Acta 1713 (2005) 51 – 56Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Arguelles-Arias, A.; Ongena, M.; Halimi, B.; Lara, Y.; Brans, A.; Joris, B.; Fickers, P.	IIM, 5.4/30	2009	*Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens Microb Cell Fact 2009 8:63–74 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Arima, K.; Kakinuma, A.; Tamura, G.	IIM, 5.4/12	1968	Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation Biochem Biophys Res Commun 1968 31:488–494 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

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Bérdy, J.; Boca Raton, F.L.	IIM, 5.4/16	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Bérdy, J.; Boca Raton, F.L.	IIM, 5.4/19	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Bérdy, J.; Boca Raton, F.L.	IIM, 5.4/21	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Bérdy, J.; Boca Raton, F.L.	IIM, 5.4/22	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Bérdy, J.; Boca Raton, F.L.	IIM, 5.4/23	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Bérdy, J.; Boca Raton, F.L.	IIM, 5.4/31	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Bérdy, J.; Boca Raton, F.L.	IIM, 5.4/32	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Bérdy, J.; Boca Raton, F.L.	IIM, 5.4/33	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Bruch, M.K.	IIM, 5/01	1990	Identification of microorganisms MicroBioTest Inc, USA Becker Underwood, report No. 186-102 Not GLP Unpublished **Supporting information, study not fully summarised**	Y	BASF Agricultural Specialities Ltd

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Caballero M.L.; Gomez M.; Gonzalez-Munoz, M.; Reinoso, L.; Rodrigues-Perez, R.; Moneo, I.	IIM, 5.2.2/01	2007	Occupational sensitisation to fungal enzymes used in animal feed industry Int Arch Allergy Immunol 2007; 144:231-239 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Carrillo, C.; Teruel, J.A.; Aranda, F.J.; Ortiz, A.	IIM, 5.4/08	2003	Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin Biochim Biophys Acta 2003 1611:91–97 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Claus, D.; Berkeley, R.C.W.	IIM, 5.2.3/04	1986	Genus *Bacillus* Cohn 1872, pp. 1105-1139. In: P.H.A. Sneath, *et al*. (eds.) Bergey's Manual of Systematic Bacteriology, Vol. 2. Williams and Wilkins Co., Baltimore, MD. Not GLP Published ⇨ 5/06 **Supporting information, paper not fully summarised**	N	Public
Claus, D.; Berkeley, R.C.W.	IIM, 5/06	1986	Genus *Bacillus* Cohn 1872, pp. 1105-1139. In: P.H.A. Sneath, *et al*. (eds.) Bergey's Manual of Systematic Bacteriology, Vol. 2. Williams and Wilkins Co., Baltimore, MD. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Cooper, D.G.; MacDonald, C.R.; Duff, S.J.B.; Kosaric, N.	IIM, 5.4/10	1981	Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions Appl Environ Microbiol 1981 42:408–412 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
de Boer, A.S. and Diderichsen, B.	IIM, 5.2.3/06	1991	On the safety of *Bacillus subtilis and B. amyloliquefaciens*: a review Appl. Microbiol. Biotechnol., 36, 1991, 1-4 TOX2000-1212 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
[redacted]	IIM, 5.3.3/01	2011	Acute inhalation toxicity study in rats [redacted] Report No. 15628-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Du Toit Schabort	IIM, 5.4/01	2011	Secondary compound analysis of *Bacillus subtilis* strain MBI600 Becker Underwood No report No. Not GLP Unpublished	Y	BASF Agricultural Specialities Ltd
EFSA	IIM, 5.1/01	2007	Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA Opinion of the Scientific Committee The EFSA Journal (2007) 587, 1-16 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
EFSA	IIM, 5.2.3/05	2011	Scientific Opinion Technical guidance on the assessment of the toxigenic potential of *Bacillus* species use in human nutrition EFSA Journal 2011; 9(11):2445 Not GLP Published ⇨ 5/09 **Supporting information, paper not fully summarised**	N	Public
EFSA	IIM, 5.3.5/01	2007	Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA Opinion of the Scientific Committee The EFSA Journal (2007) 587, 1-16 Not GLP Published ⇨ 5.1/01 **Supporting information, paper not fully summarised**	N	Public
EFSA	IIM, 5.6/01	2007	Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA Opinion of the Scientific Committee The EFSA Journal (2007) 587, 1-16 Not GLP Published ⇨ 5.1/01 **Supporting information, paper not fully summarised**	N	Public
EFSA	IIM, 5/09	2011	Scientific Opinion Technical guidance on the assessment of the toxigenic potential of *Bacillus* species use in human nutrition EFSA Journal 2011; 9(11):2445 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Fossum, K.; Herikstad, H.; Binde, M.; Pettersen K.E.	IIM, 5.2.4/02	1986	Isolations of *Bacillus subtilis* in connection with Bovine Mastitis Nordisk Veterinärmedicine, Vol. 38, 1986, 233-236 BMF2000-151 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
From, C.; Pukall, R.; Schumann, P.; Hormazábal, V.; Granum, P.E.	IIM, 5.2.3/03	2005	Toxin producing ability among *Bacillus* spp. outside the *Bacillus cereus* group Applied and Environmental Microbiology, 2005 71, 1178-1183 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Gabriel, M.A.; Jabara, H.; Al-Khalidi, U.A.S.	IIM, 5.4/29	1971	Metabolism of Acetoin in Mammalian Liver Slices and Extracts Biochem. J. (1971) 124, 793-800 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Green, B.J.; Beezhold, D.H.	IIM, 5.2.2/03	2011	Industrial fungal enzymes: an occupational allergen perspective Journal of allergy Vol. 2011, article ID 628574 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Gupta, D.K.; Vyas, K.M.	IIM, 5.2.4/01	1989	Efficacy of *Bacillus subtilis* against mosquito Larvae (*Anophelis culicifacies*) Zeitschrift fuer Angewandte Zoologie 1989, 76(1) 85-91 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Hamdache, A.; Lamarti, A.; Aleu, J.; Collado, I.G.	IIM, 5.4/03	2011	Non-peptide metabolites from the genus *Bacillus* Journal of Natural Products Published by the American Chemical Society and American Society of Pharmacognosy 14 March 2011 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Hashimoto, J.	IIM, 5.3.5/02	2012	Bacterial reverse mutation test of the supernatant of the fermentation broth of *Bacillus subtilis* MBI600 UBE Scientific Analysis Laboratory Inc Becker Underwood Report No. USA-R-12224 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIM, 5.3.2/01	1989a	Acute oral toxicity and infectivity/pathogenicity to rats of MBI600 [redacted] Report No. 89396D/AGC 1/0/AC GLP Unpublished	Y	BASF Agricultural Specialities Ltd

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
[redacted]	IIM, 5.3.3/02	1989b	Acute pulmonary toxicity and infectivity/pathogenicity to rats of MBI600 [redacted] Report No. 89397D/AGC 1/2/EC GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIM, 5.3.4/01	1989c	Acute intravenous toxicity and infectivity/pathogenicity to rats of MBI600 [redacted] Report No. 89398D/AGC 1/3/AC GLP Unpublished	Y	BASF Agricultural Specialities Ltd
Idris, E. E. S.; Bochow, H.; Ross, H.; Borriss, R. J.	IIM, 5.4/27	2004	Use of *Bacillus subtilis* as biocontrol agent. VI phytohormone like action of culture filtrates FZB24, FZB42, FZB45 and *Bacillus subtilis* FZB37 Plant Dis. Prot. 2004, 111, 583–597 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Idris, E. E. S.; Iglesias, D.; Talon, M.; Borriss, R.	IIM, 5.4/28	2007	Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42 Mol. Plant-Microbe Interact. 2007, 20, 619–626 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Ihde, D.C.; Armstrong D.	IIM, 5.2.3/07	1973	Clinical spectrum of infection due to *Bacillus* species Amer. J. Med., Vol. 55, 1973, 839-845 BMF2000-152 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Izu, S.	IIM, 5.2.1/01	2012	Exposure to, and adverse health effects from *Bacillus subtilis*, strain MBI 600 Becker Underwood No report No. Not GLP Unpublished **Supporting information, paper not fully summarised**	Y	BASF Agricultural Specialities Ltd
Johnson, C.L.; Bernstein, I.L.; Gallagher, J.S.; Bonventre, P.F.; Brooks, S.M.	IIM, 5.2.2/02	1980	Familial hypersensitivity pneumonitis induced by *Bacillus subtilis* American Review of Respiratory Disease 1980; 122:339-348 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Joshi, R.; McSpadden Gardener, B.B.	IIM, 5/04	2006	Identification and characterisation of novel genetic markers associated with biological control activities in *Bacillus subtilis* Phytopathology 96 (2): 145-154. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Kalinovskaya, N.I.; Kuznetsova, T.A.; Ivanova, E.P.; Romanenko, L.A.; Voinov, V.G.; Huth, F.; Laatsch, H.	IIM, 5.4/06	2002	Characterization of Surfactin-like Cyclic Depsipeptides Synthesized by *Bacillus pumilus* from Ascidian *Halocynthia aurantium* Mar. Biotechnol. 2002 4, 179–188 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Kameda, Y.; Matsui, K.; Hisato, K.; Yamada, T.; Sagai, H.	IIM, 5.4/14	1972	Antitumor activity of *Bacillus natto*. III. Isolation and characterization of a cytolytic substance on Ehrlich ascites carcinoma cells in the culture medium of *Bacillus natto* KMD 1126 Chem Pharm Bull (Tokyo) 1972 20: 1551-1553 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Kloepper, J.W.	IIM, 5/02	2012	Identification of MBI600 by 16S rRNA gene sequencing using 8F and 1492R primers Auburn University, USA Becker Underwood No report No. Not GLP Unpublished **Supporting information, study not fully summarised**	Y	BASF Agricultural Specialities Ltd
[redacted]	IIM, 5.3.2/02	2011a	Acute oral toxicity study (UDP) in rats [redacted] Report No. 15626-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIM, 5.5.1/02	2011b	Acute dermal toxicity study in rats [redacted] Report No. 15627-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIM, 5.5.1/03	2011c	Acute dermal irritation study in rabbits [redacted] Report No. 15630-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
[redacted]	IIM, 5.5.1/05	2011d	Acute eye irritation study in rabbits [redacted] Report No. 15629-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIM, 5.3.1/02	1989	Delayed contact hypersensitivity in the guinea pig with MBI600 [redacted] Report No. 89429D/AGC 2/SS GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIM, 5.5.1/01	1989	Acute dermal toxicity to rabbits of MBI600 [redacted] Report No. 89270D/AGC 1/1/AC GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIM, 5.5.1/04	1989	Primary eye irritation and infectivity of MBI600 [redacted] Report No. 89399D/AGC 1/4/SE GLP Unpublished	Y	BASF Agricultural Specialities Ltd
Logan, N.A.	IIM, 5/05	1988	*Bacillus* species of medical and veterinary importance J. Med. Microbiol. 25:157-165. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Maget-Dana, R.; Peypoux, F.	IIM, 5.4/17	1994	Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties Toxicology 1994 87:151–174 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Maget-Dana, R.; Thimon, L.; Peypoux, F.; Ptak, M.	IIM, 5.4/15	1992	Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A Biochimie 1992 74: 1047-1051 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Mikkola, R.; Andersson, M.A.; Grigoriev, P.; Teplova, V.V; Saris, N.E.L.; Rainey, F.A.; Salkinoja-Salonen, M.S.	IIM, 5.2.4/03	2004	*Bacillus amyloliquefaciens* strains isolated from moisture-damaged buildings produced surfactin and a substance toxic to mammalian cells. Arch Microbiol (2004) 181 : 314–323 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Mikkola, R.; Andersson, M.A.; Teplova, V.; Grigoriev, P.; Kuehn, T.; Loss, S.; Tsitko, I.; Apetroaie, C.; Saris, N.E.L.; Veijalainen, P.; Salkinoja-Salonen, M.S.	IIM, 5.2.3/01	2007	Amylosin from *Bacillus amyloliquefaciens*, a K+ and Na+ channel-forming toxic peptide containing a polyene structure Toxicon, 2007 49, 1158-1171 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Moldenhauer, J.; Goetz, D. C. G.; Albert, Ch. R.; Bischof, S. K.; Schneider, K.; Suessmuth, R. D.; Engeser, M.; Gross, H.; Bringmann, G.; Piel, J.	IIM, 5.4/26	2010	The final steps of Bacillaene Biosynthesis in *Bacillus amyloliquefaciens* FZB42: direct evidence for β,γ dehydration by a trans-acyltransferase polyketide synthase Angew. Chem., Int. Ed. 2010, 49, 1465–1467 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Patel, P. S.; Huang, S.; Fisher, S.; Pirnik, D.; Aklonis, C.; Dean, L.; Meyers, E.; Fernandes, P.; Mayerl, F. J.	IIM, 5.4/25	1995	Bacillaene, a novel inhibitor of prokaryotic protein synthesis produced by *Bacillus subtilis*: production, taxonomy, isolation, physico-chemical characterisation and biological activity Antibiot. 1995, 48, 997–1003 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Peypoux, F.; Bonmatin, J.M.; Wallach, J.	IIM, 5.4/07	1999	Recent trends in the biochemistry of surfactin Appl Microbiol Biotechnol 1999 51: 553–563 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
PRAPeR	IIM, 5.3.1/01	2009	Report of PRAPeR expert meeting M2. Peer Review Programme under Directive 91/414/EEC. Meeting to discuss the approach for assessment of plant protection products containing micro-organisms as the active substance Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Priest, F.G.; Goodfellow, M.; Shute, L.A.; Berkeley, R.C.W.	IIM, 5/07	1987	*Bacillus amyloliquefaciens* sp. nov., nom. rev. Int. J. Syst. Bacteriol., 1987, 37, 69-71 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Sansinenea, E.; Ortiz, A.	IIM, 5.4/02	2011	Secondary metabolites of soil *Bacillus* spp. Nordisk Biotechnol Lett (2011), 33, 1523-1538 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Shaligram, N.S.; Singhal, R.S.	IIM, 5.4/11	2010	Surfactin–a review on biosynthesis, fermentation, purification and applications Food Technol Biotechnol 2010 48:119–134 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Stein, T.	IIM, 5.4/05	2005	*Bacillus subtilis* antibiotics: structures, syntheses and specific functions Molecular Microbiology (2005) 56 (4), 845–857 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Tsukagoshi, N.; Tamura, G.; Arima, K.	IIM, 5.4/13	1970	A novel protoplast-bursting factor (surfactin) obtained from *Bacillus subtilis* IAM 1213. I. The effects of surfactin on *Bacilllus megaterium* KM Biochim Biophys Acta 1970 196: 204-210 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Vollenbroich, D.; Ozel, M.; Vater, J.; Kamp, R.M.; Pauli, G.	IIM, 5.4/09	1997	Mechanism of inactivation of enveloped viruses by the biosurfactant surfactin from *Bacillus subtilis* Biologicals 1997 25:289–297 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Welker, N.E.; Campbell, L.L.	IIM, 5/08	1967	Unrelatedness of *Bacillus amyloliquefaciens* and *Bacillus subtilis* Journal of Bacteriology 1967, 94(4): 1124-1130. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Wulff, E.G.; Mguni, C.M.; Mansfeld-Giese, K.; Fels, J.; Lubeck, M.; Hockenhull, J.	IIM, 5.4/04	2002	Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris pv. Campestris* Plant Pathology (2002) 51 , 574–584 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Yang, G.P.	IIM, 5/03	2005	Identification of MBI600 by 16S rRNA gene sequencing Becker Underwood No report No. Not GLP Unpublished **Supporting information, study not fully summarised**	Y	BASF Agricultural Specialities Ltd
Yuan, J.; Li, B.; Zhang, N.; Waseem, R.; Shen, Q.; Huang, Q.	IIM, 5.4/20	2012	Production of Bacillomycin- and Macrolactin-Type Antibiotics by *Bacillus amyloliquefaciens* NJN-6 for Suppressing Soilborne Plant Pathogens Journal of Agricultural and Food Chemistry. Published by the American Chemical Society, 4 March 2012 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Zimmerman, S. B.; Schwartz, C. D.; Monaghan, R. L.; Pelak, B. A.; Weissberger, B.; Gilfillan, E. C.; Mochales, S.; Hernandez, S.; Currie, S. A.; Tejera, E.; Stapley, E. O. J.	IIM, 5.4/24	1987	Difficidin and oxydifficidin: novel broad spectrum antibacterial antibiotics produced by *Bacillus subtilis*. I. Production, taxonomy and antibacterial activity Antibiot. 1987, 40, 1677–1681 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Annex IIIM Data and Information

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
[redacted]	IIIM1 7.1.3/01	2011	Acute inhalation toxicity study in rats [redacted] Report No. 15389-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIIM1 7.1.1/01	2011a	Acute oral toxicity (UDP) in rats [redacted] Report No. 15387-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIIM1 7.1.2/01	2011b	Acute dermal toxicity study in rats [redacted] Report No. 15388-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
[redacted]	IIIM1 7.1.4/01	2011c	Acute dermal irritation study in rabbits [redacted] Report No. 15391-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIIM1 7.1.5/01	2011d	Acute eye irritation study in rabbits [redacted] Report No. 15390-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIIM1 7.1.6/01	2003	Skin sensitization study in guinea pigs [redacted] Report No. 7831-03 GLP Unpublished	Y	BASF Agricultural Specialities Ltd

A.6. Residue data

Annex IIM Data and Information

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
EFSA	IIM, 6.1/01	2007	Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA Opinion of the Scientific Committee The EFSA Journal (2007) 587, 1-16 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Campbell, R.	IIM 6.2/01	1989	Biocontrol on leaf surfaces Biological control of microbial plant pathogens, Cambridge University Press, Cambridge, Department of Botany, University of Bristol, Chapter 3, 1989, 66-94 BMF2000-100 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
Martin, G.	IIM6.3/01	2011	Foliar persistence of BUEXP1780S on vine leaves Becker Underwood R&D071101 Not GLP Unpublished	Y	BASF Agricultural Specialities Ltd

A.7. Fate and behaviour in the environment

Annex IIM Data and Information

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Alexander, M.	IIM 7/01	1977	Introduction to Soil Microbiology. John Wiley and Sons, Inc., New York. Not GLP Published	N	Public
Asaka, O., Ano, T. and Shoda, M.,	IIM 7.1.1/02	1996	Persistence of *Bacillus subtilis* RB14 and its derivative strains in soil with respect to the lpa-14 gene. Journal of Fermentation and Bioengineering 81, 1–6. Not GLP Published	N	Public
Bennett, A.J., Leifert, C. and Whipps, J.M.	IIM 7.1.1/03	2003	Survival of the biocontrol agents *Coniothyrium minitans* and *Bacillus subtilis* MBI600 introduced into pasteurised, sterilised and non-sterile soils. Soil Biology & Biochemistry 35: 1565 – 1573. Not GLP Published	N	Public
Bochow H and Gantcheva K		1995	Soil introductions of *Bacillus subtilis* as biocontrol agent and its population and activity dynamic. *Acta - Horti*. 382: 164–172 Not GLP Published	N	Public
Casida LE		1988	Response in Soil *of Cupriavidus necator* and Other Copper-Resistant Bacterial Predators of Bacteria to Addition of Water, Soluble Nutrients, Various Bacterial Species, or Bacillus thuringiensis Spores and Crystals. *Applied and environmental microbiology* 54: 2161–2166 Not GLP Published	N	Public

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
England LS, Lee H and Trevors JT		1993	Bacterial survival in soil: Effect of clays and protozoa. *Soil Biology and Biochemistry*, 525–531 Not GLP Published	N	Public
Krebs B, Höding B and Kübart S		1998	Use of *Bacillus subtilis* as biocontrol agent. I. Activities and characterization of *Bacillus subtilis* strains. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* 105: 181–197 Not GLP Published	N	Public
Leff LG, McArthur J V. and Shimkets LJ		1998	Persistence and dissemination of introduced bacteria in freshwater microcosms. *Microbial Ecology* 36: 202–211 Not GLP Published	N	Public
Liang, L.N., Sinclair, J.L., Mallory, L.M. and Alexander, M.	IIM 7.1.2/02	1982	Fate in model ecosystems of microbial species of potential use in genetic engineering. *Appl. Environ. Microbiol*. 44, 708-14. Not GLP Published.	N	Public
Mahaffee WF and Backman PA		1993	Effects of seeds factors on spermosphere and rhizosphere colonisation of cotton by *Bacillus subtilis* GB03. *Phytopathology* 83: 1120–1125 Not GLP Published	N	Public
Milus EA and Rothrock CS		1993	Rhizosphere colonization of wheat by selected soil bacteria over diverse environments. *Canadian Journal of Microbiology*. NRC Research Press 39(3): 335–341 Not GLP Published	N	Public

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Moir A		1992	Spore germination Doi, R.H. & Mc Gloughlin, M (eds) Biology of Bacilli. Application to Industry, Chapter 2, 22-28 Not GLP Published	N	Public
Morgan R. and Brown A.	IIM 2.8.2	2011	Effect of temperature on the growth rate of BLUEXP1780, Serenade and *Botrytis cinerea* strains, report R&D0411001 Not GLP Not published	?	BASF Agricultural Specialities Ltd
Nicholson WL		2002	Roles of Bacillus endospores in the environment. *Cellular and Molecular Life Sciences*, 410–416 Not GLP Published	N	Public
Pandey A, Palni LM and Bisht D		2001	Dominant fungi in the rhizosphere of established tea bushes and their interaction with the dominant bacteria under in situ conditions. *Microbiological research* 156: 377–382 Not GLP Published	N	Public
Pantastico-Caldas M, Duncan K, Istock C and Bell J		1992	Population dynamics of bacteriophage and *Bacillus subtilis* in soil. *Ecology* 73: 1888–1902 Not GLP Published	N	Public
Reva ON, Dixelius C, Meijer J and Priest FG		2004	Taxonomic characterization and plant colonizing abilities of some bacteria related to *Bacillus amyloliquefaciens* and *Bacillus subtilis*. *FEMS Microbiology Ecology* 48: 249–259 Not GLP Published	N	Public

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Siala A, Hill IR and Gray TRG		1974a	Populations of Spore-forming Bacteria in an Acid Forest Soil, with Special Reference to *Bacillus subtilis*. *Journal of General Microbiology* 81(1): 183–190 Not GLP Published	N	Public
Siala A and Gray TRG		1974b	Growth of *Bacillus subtilis* and Spore Germination in Soil Observed by a Fluorescent-antibody Technique. *Journal of General Microbiology* 81: 191–198 Not GLP Published	N	Public
Sinclair JL and Alexander M		1984	Role of resistance to starvation in bacterial survival in sewage and lake water. *Applied and environmental microbiology* 48: 410–415.*Not GLP Not GLP Published	N	Public
Tokuda Y, Ano T and Shoda M		1993	Survival of *Bacillus subtilis* NB22, an antifungal-antibiotic iturin producer, and its transformant in soil-systems. *Journal of Fermentation and Bioengineering* 75(2): 107–111 Not GLP Published	N	Public
Van Elsas J, Dijkstra A, Govaert J and Vanveen J	IIM 7.1.1/01	1986	Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. *FEMS Microbiology Ecology* 38(3): 151–160 Not GLP Published	N	Public
Weekers PH, Bodelier PL, Wijen JP and Vogels GD		1993	Effects of Grazing by the Free-Living Soil Amoebae Acanthamoeba castellanii, Acanthamoeba polyphaga, and Hartmannella vermiformis on Various Bacteria. *Applied and environmental microbiology* 59: 2317–2319 Not GLP Published	N	Public

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******
Zimmer J, Issoufou I, Schmieddeknecht G and Bocow H		1998	Poupulations dynamik, phytoeffektivität und antagonistische Wirksamkeit von *Bacillus subtilis* als nutzbakterium, Mitt a.d. Biol. Bundesanst. H. 357 Not GLP Published	N	Public

Annex IIIM Data and Information

Author(s)	**Annex point / reference number**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed*** **Y/N**	**Owner ******

A.8. Effects on non-target organisms

Annex IIM Data and Information

Author(s)	Annex point / reference number	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed* Y/N	Owner **
[redacted]	IIM 8.1/01	1993	*Bacillus subtilis* Strain MBI 600: An avian oral pathogenicity and toxicity study in the bobwhite. [redacted] 301-102 GLP, unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIM 8.2/01	2013(a)	*Bacillus amyloliquefaciens* strain MBI600 Rainbow Trout (*Oncorhynchus mykiss*) 96-Hour Acute Toxicity Test [redacted] 17142-13 GLP, unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIM 8.2/02	1997	Toxicity study of MBI-600 on carp (*Cyprinus carpio*) [redacted] STS (2)-97004 Non-GLP, unpublished	Y	BASF Agricultural Specialities Ltd
Mikulas, J.	IIM 8.3/01	2013(b)	*Bacillus amyloliquefaciens* strain MBI600 *Daphnia magna* 48-Hour Acute Toxicity Test STILLMEADOW, Inc. 16672-12 GLP, unpublished	Y	BASF Agricultural Specialities Ltd
Fujii, Y.	IIM 8.3/02	1997	Reproductive toxicity on *Daphnia*. Japan Food Research Laboratories 597040201-001 Non-GLP, unpublished	Y	BASF Agricultural Specialities Ltd
Mikulas, J.	IIM 8.4/01	2013(c)	*Bacillus amyloliquefaciens* strain MBI600 *Pseudokirchneriella subcapitata* 72-Hour Algal InhibitionTest STILLMEADOW, Inc. 17141-13 GLP, unpublished	Y	BASF Agricultural Specialities Ltd
Mori, K.	IIM 8.7/01	1997(a)	Studies on the effect of microbial pesticides on environmental organisms: Effect of IK-1080 WP on honeybee Research Institute, Japan Plant Protection Association. Non-GLP, unpublished	Y	BASF Agricultural Specialities Ltd
Younger, C.	IIM 8.7/02	2012	*Bacillus amyloliquefaciens* strain MBI600 Honey Bee Acute Contact Toxicity Limit Test STILLMEADOW, Inc. 16674-12 GLP- Unpublished	Y	BASF Agricultural Specialities Ltd
Mori, K.	IIM 8.8/01	1997 (b)	Studies on the effect of microbial pesticides on environmental organisms: Effect of IK-1080 WP on off-target insects Research Institute, Japan Plant Protection Association. Non-GLP, unpublished	Y	BASF Agricultural Specialities Ltd

Author(s)	Annex point / reference number	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed* Y/N	Owner **
Wada, Y.	IIM 8.8/02	1996	Studies on the Effect of Microbial Pesticides on Environmental Organisms: Effect of IK-1080 WP on Silkworm Larvae Research Institute, Japan Plant Protection Association. Non-GLP, unpublished	Y	BASF Agricultural Specialities Ltd
Whittaker, M.	IIM 8.9.1/01	2013	Effects of *Bacillus amyloliquefaciens* MBI600 on reproduction and growth of the earthworm *Eisenia fetida* in an artificial soil substrate. Laboratory study No. BUBA001. GLP, unpublished	Y	BASF Agricultural Specialities Ltd

Annex IIIM Data and Information

Author(s)	Annex point / reference number	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed* Y/N	Owner **
[redacted]	IIIM1 7.1.3/01	2011	Acute inhalation toxicity study in rats [redacted] Report No. 15389-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIIM1 7.1.1/01	2011a	Acute oral toxicity (UDP) in rats [redacted] Report No. 15387-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIIM1 7.1.2/01	2011b	Acute dermal toxicity study in rats [redacted] Report No. 15388-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIIM1 7.1.4/01	2011c	Acute dermal irritation study in rabbits [redacted] Report No. 15391-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIIM1 7.1.5/01	2011d	Acute eye irritation study in rabbits [redacted] Report No. 15390-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
[redacted]	IIIM1 7.1.6/01	2003	Skin sensitization study in guinea pigs [redacted] Report No. 7831-03 GLP Unpublished	Y	BASF Agricultural Specialities Ltd

Draft Assessment Report

Bacillus amyloliquefaciens strain MBI600

Volume 3
Annex B.1
Identity

Rapporteur Member State: France

Version History of Volume 3 B1

Date	**Reason for revision**
Decembre 2014	Initial DAR

Table of contents

B.1. Identity

B.1.1. Identity of the Micro-organism (Annex IIM 1)

B.1.1.1. Applicant (Annex IIM 1.1)

Applicant: BASF Agricultural Specialities Ltd (Formerly Becker Underwood Ltd.)
Harwood Industrial Estate
Harwood Road
Littlehampton
BN17 7AU
United Kingdom

Contact Point: [redacted]

Representative: [redacted]

Contact Point: [redacted]

B.1.1.2. Producer (Annex IIM 1.2)

Confidential information-See Annex C.

B.1.1.3. Name and species description, strain characterisation (Annex IIM 1.3)

B.1.1.3.1. Accession number in culture collection

The bacterial strain is a naturally occurring, indigenous wild type. The strain was initially isolated in the UK. It is not known to be indigenous to the southern zone.

The strain is deposited in the National Collection of Industrial, Marine and Food Bacteria Ltd (NCIMB), Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA, Scotland.

Accession number: NCIMB 12376 (II M 1.3.2/02 Brown, A., 2011)

The strain is further deposited in the USA in the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, USA.

Safe deposit agreement: 2009-SD-00011 (II M 1.3.2/03 ATCC, 2009)
Safe deposit number: SD-1414

B.1.1.3.2. Scientific name and taxonomic grouping, *i.e.* family, genus, species, strain, serotype, pathovar or any other denomination relevant to the micro-organism

Taxonomic name and strain

Species:	*Bacillus amyloliquefaciens*
First description:	The first description of the species is from J. Fukumoto (1943) (IIM, 1.3.1/01-Fukumoto, J. 1943). In 1987, a group of scientists, including Fergus G. Priest of Heriot_Watt University, established it as a separate species to *Bacillus subtilis* (IIM, 1.3.1/02, Priest, F.G and al., 1987).
Strain:	MBI600
Genus:	*Bacillus*
Family:	*Bacillaceae*
Division:	Bacteria

B.1.1.3.3. Test procedures and criteria used for identification

The organism was originally identified by Yang (II 1.3.1/05, Yang G.P., 2005) as *Bacillus subtilis* and then subsequently by Auburn University (II 1.3.1/05 Kloepper, 2012), USA as *Bacillus amyloliquefaciens*.

The identification was based on 16S rRNA gene sequencing. Amplification of the 1.5kb 16S rRNA from MBI600 can be carried out with PCR primers fD1 and rD1. Internal trial has characterised the genetic code for this region. Identification as *Bacillus amyloliquefaciens* was based on the best match by comparison of the gene sequence using GenBank, available on the website www.ncbi.nlm.nih.gov/blast/.

Articles and relevant study reports provided are summarized below. Studies (Joshi, R. and *al*., 2006; Longan, N.A, 1988; Claus, D. and *al.*, 1986; Welker, N.E. and *al*., 1967; EFSA, 2011 and Rossal, S., 2012) but there are no further relevant information included in these studies.

Report: II 1.3.1/06, Yang, G.P. (2005) Identification of MBI600 by 16S rRNA gene sequencing Becker Underwood; No report No., Unpublished, Sponsor: Becker Underwood.

Guideline: None
GLP: No

Description of the method
1.5 kb 16S rRNA from MBI600 was amplified by PCR (PCR product purification kit) using fD1and rD1and sequenced. Two pieces of DNA sequences were obtained with one from 5'end and the other from 3' end and were merged into one complete sequence by finding out the overlapping sequence. Homology searching using the complete 1.5 kb 16S rRNA sequence to search for the best match in the web site: https://www.ncbi.nlm.nih.gov/blast/. The identification of MBI600 was based on the best match.

Sequence of fD1: 5'-AGA GTT TGA TCC TGG CTC AG-3'
Sequence of rD1: 5'-AAG GAG GTG ATC CAG CC-3'

Results:
16S rRNA of MBI 600

5′TGCCGACGTCTACTACAGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGC
GGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAA
CCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCG
GCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCA
AGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGC
CCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGA
GCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAAC
AAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTA
AAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGG
TCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGT
GAAATGCGTAGAGATGTGGA**GGAACACCA**GTGGCGAAGGCGACTCTCTGGTCTGTAACTG
ACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATT
AAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCT
TGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGG
TGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAAC
CGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGT
GCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCT
GTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATC
GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACC
ACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAAGG
TGGGACAGATGATTGGGCAGTGAAGGTACAGTA-3′

The top 20 best matches to MBI600 16 rRNA: sequences producing significant alignments:

		Score (bits)	E val
gi|47118163|gb|AY601723.1|	Bacillus sp. WL-3 16S ribosomal ...	2855	0.0
gi|61744004|gb|AY962472.1|	Bacillus subtilis strain LBM1024...	2847	0.0
gi|56383045|emb|AJ809499.1|	Bacillus sp. TMW 2.480 partial ...	2847	0.0
gi|49659885|gb|AY649000.1|	Bacillus sp. YNUCC0002 16S ribos...	2847	0.0
gi|59624757|gb|AY905693.1|	Bacillus subtilis strain CICC102...	2847	0.0
gi|4519613|dbj|AB017592.1|	Bacillus sp. gene for 16S rRNA, ...	2847	0.0
gi|61744005|gb|AY962473.1|	Bacillus subtilis strain P40 16S...	2839	0.0
gi|15705859|gb|AF411118.1|	Bacillus sp. Bch1 16S ribosomal ...	2839	0.0
gi|47834651|gb|AY553097.1|	Bacillus subtilis strain MO4 16S...	2839	0.0
gi|4519612|dbj|AB017591.1|	Bacillus sp. gene for 16S rRNA, ...	2839	0.0
gi|46560670|gb|AY587809.1|	Bacterium Te22R 16S ribosomal RN...	2837	0.0
gi|57864229|dbj|AB199317.1|	Bacillus sp. Q-12 gene for 16S ...	2835	0.0
gi|4519611|dbj|AB017590.1|	Bacillus sp. gene for 16S rRNA, ...	2835	0.0
gi|4519610|dbj|AB017589.1|	Bacillus sp. gene for 16S rRNA, ...	2835	0.0
gi|4519609|dbj|AB017588.1|	Bacillus sp. gene for 16S rRNA, ...	2833	0.0
gi|55274012|gb|AY775778.1|	Bacillus subtilis strain BFAS 16...	2831	0.0
gi|60280619|gb|AY920508.1|	Bacillus subtilis strain BOH2 16...	2831	0.0
gi|32468687|emb|Z99104.2|BSUB0001	Bacillus subtilis complet...	2831	0.0
gi|46391587|gb|AY583216.1|	Bacillus subtilis strain BS-S3 1...	2831	0.0
gi|57919052|gb|AY867793.1|	Bacillus subtilis strain 4-7 16S...	2831	0.0

RMS's conclusion:
The sequencing of 16S rRNA of MBI 600 demonstrates only that the bacterium is a *Bacillus subtilis*.

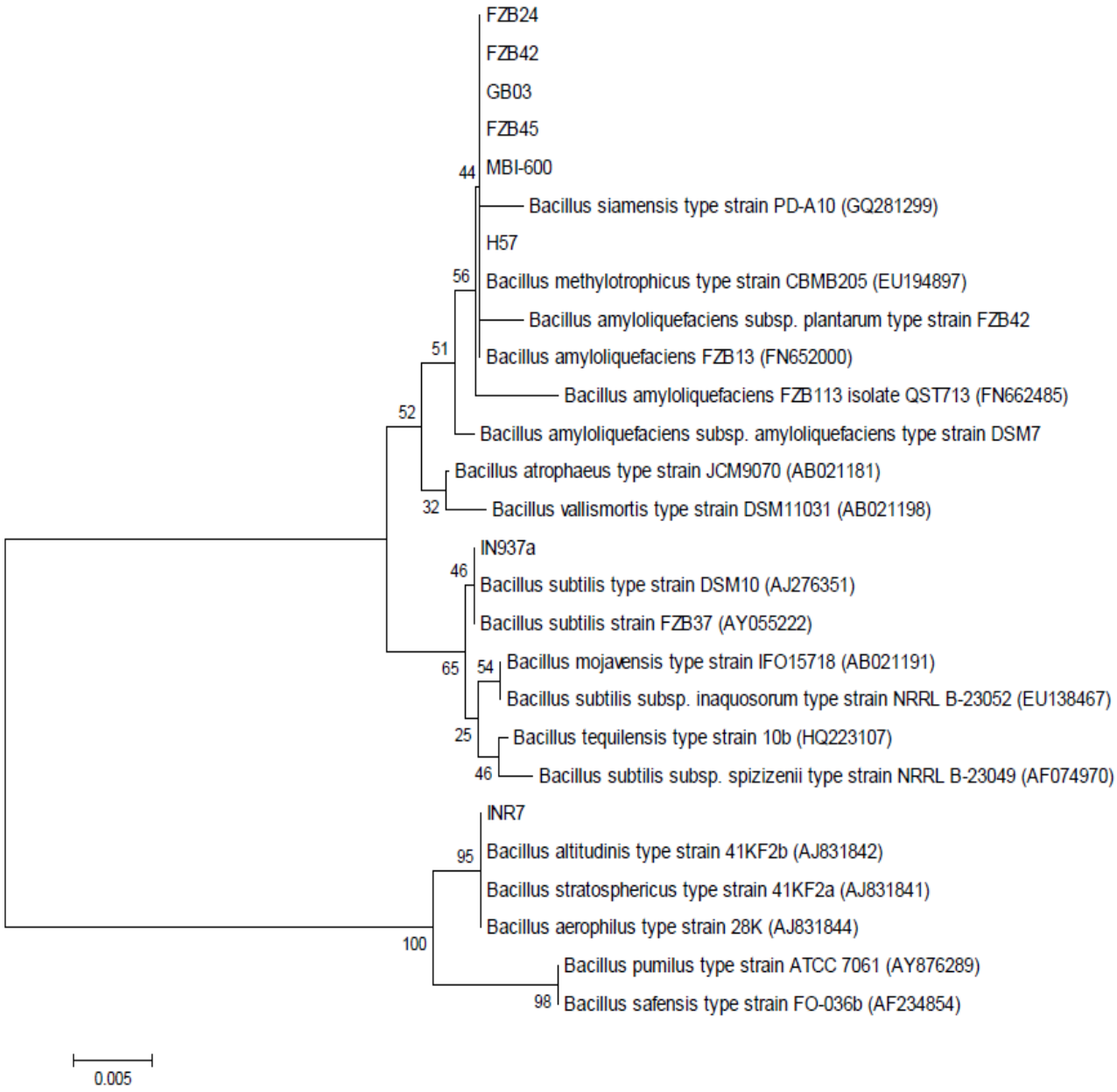
Report: 1.3.1/07, Kloepper, J.W. (2012) Department of Entomology and Plant Pathology, Auburn University, No Report No.
Guideline: None
GLP: No

Principle of the Method
Molecular analysis was performed on the MPCA using 16S rRNA gene sequencing.

Description of the method
Definite identification of strain MBI600 as *Bacillus amyloliquefaciens* was achieved by 16S rRNA gene sequencing. The method for 16S rRNA gene sequencing starts with pure cultures of test strains. DNA is extracted by heat shocking bacterial cells at 94°C for 7 minutes. The DNA is then amplified by polymerase chain reaction using Eubacterial primers 8F and 1492R and Go-Green PCR master mix (Lucigen Corp., Wisconsin). The polymerase chain reaction ramps up and down between 57°C and 95°C for 31 cycles. Presence of PCR amplicons is confirmed by gel electrophoresis before being sequenced by Lucigen Corporation (Wisconsin). Contiguous sequences of DNA fragment data is compiled using Chromas Pro (Technelysium pty Ltd.) software. Contiguous files are then compared to type-strains of bacterial species using GenBank (www.ncbi.nlm.nih.gov/BLAST) or Ribosomal Database Project II (www.rdp.cme.msu.edu) websites.

A phylogenetic tree was created to reveal the association of the 16S ribosomal subunit gene of MBI600 and other closely-related species, including some strains in current commercially available competitor products. The phylogenetic tree was constructed based on partial 16S rRNA nucleotide sequences. Neighbour-Joining analysis was performed with MEGA (5.05), using the Tamura algorithm with options of using both transversional and transitional changes and complete deletion of gaps. Bootstrap values were calculated by 1,000 replicates of the analysis. Strain and accession numbers are indicated. Bar 0.005 substitutions per nucleotide position. Numbers indicate bootstrap values[1].

[1] *Tamura, K., Dudley, J., Nei, M., and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24:1596-1599.*



RMS's conclusion:
Strain MBI600 was identified as *Bacillus amyloliquefaciens*.

Report: II 1.3.1/02, Priest, F.G.; Goodfellow, M.; Shute, L.A.; Berkeley, R.C.W. (1987) *Bacillus amyloliquefaciens* sp. nov., nom. rev. Int. J. Syst. Bacteriol., 1987, 37, 69-71
Not GLP, Published

Guideline: None
GLP: No

Bacillus amyloliquefaciens produces α-amylase and protease. It is phenotypically so similar to *B. subtilis.* Deoxyribonucleic acids (DNAs) from strains of *B.amyloliquefacien* have consistently been found to share less than 25, 13, and 5% homology with DNAs from strains of *B. subtilis, B. licheniformis,* and *B. pumilus,* respectively, under optimal conditions (60 to 65°C). DNA homology is no high enough for these two groups of organisms to be considered a single species. Species was separated by using pyrolysis gas-liquid chromatography, pyrolysis mass spectrometry or basing on enzyme electrophoresis profiles (using probabilistic identification methods based on API tests), on inability of most strains to hydrolyze DNA and pectin, the failure

of the organisms to produce acid from inulin and on the ability of formation long chains of cells for *B. amyloliquefacien*. Data are summarised in the table below.

Characteristics of diagnostic value in distinguishing B. amyloliquefaciens from B. subtilis and other closely related Bacillus species

Characteristic	% Of strains positive			
	B. amyloliquefaciens[a]	*B. subtilis*	*B. licheniformis*	*B. pumilus*
API tests[b]				
Acid produced from:				
Galactose	44	30	100	100
Sorbitol	88	88	95	17
Inulin[c]	11	83	68	1
Arginine dihydrolase	0	0	95	0
α-L-Arabinosidase	0	75	100	100
N-benzoyl-L-leucine aminopeptidase	0	0	100	12
α-D-Glucosidase	12	100	100	0
β-D-Glucosidase	0	87	75	100
L-Pyrrolidone aminopeptidase	100	100	100	0
L-Tryptophan aminopeptidase	100	0	100	87
Degradation tests				
DNA	33	93	100	100
$NO_3 \rightarrow NO_2$	78	100	100	0
Starch	100	100	100	0
Pectin	0	27	95	85
Physiological tests				
Anaerobic growth	0	0	100	0
Phosphatase	100	67	100	15
Propionate utilization	0	0	100	0
Chain formation[c]	84	22	80	5
Guanine-plus-cytosine content (mol%)	44–46	42–48	43–47	42–47

[a] The type strain conforms to the pattern of characteristics of the majority of the strains.

In resume characteristics of *B. amyloliquefaciens*:

- Appearance: cells often form chains and are motile, with peritrichous flagella; oval spores (0.6 to 0.8 by 1.0 to 1.4 pm) are central or paracentral in sporangia which are not swollen.
- Optical condition for developpement: 30 to 40°C. No growth occurs below 15°C or above 50°C.
- Characteristics of degradation: Casein, elastin, gelatin, starch, tributyrin, Tween 20, Tween 40, and Tween 60 are degraded but adenine, cellulose; guanine, hypoxanthine, pectin, testosterone, tyrosine, and xanthine are not. Acetoin and phosphatase are produced, nitrate is reduced to nitrite, esculin and arbutin are hydrolyzed, citrate is used as a sole carbon source, and growth occurs in the presence of 5% (w/v) NaCl, and for most strains 10% NaCl; but neither allantoin nor urea is hydrolyzed.
- Characteristics of production: Acid is produced from cellobiose, fructose, glucose, glycerol, lactose, maltose, mannose, mannitol, raffinose, salicin, sorbitol, sucrose, and trehalose.
- Content G+C: 44.35 % mol, not significatively different from the other species.

RMS's conclusion:
According to information provided in the article Priest, F.G and *al.* (1987), it can be concluded that there are significant differences between the different species *B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis*, and *B. pumilus* which indicates that *B. amyloliquefaciens* is not to be included in the other species.

Report: II 1.3.1/03, Brunch M.K. (1990), Identification of microorganisms GB07; Performing laboratory: Microbiotest, Chantilly, Virginia 22021, Laboratory project ID: 186-102, Unpublished.

Guideline: 40 CFR Guideline 151
GLP: Yes

The aim of the study is to identify of the microorganism GB07 (DS719) received from the Sponsor. The strain GB07 is synonymous to *Bacillus subtilis* strain JB 3.6, MBI600 and AP-301 (IIM, 1.3.2/01 NCIB, 1987; IIM, 1.3.2/02, Brown, A., 2011).

Description of the method

Examination of the cultural characteristics was made from TSA streak plates. Stained smears, as well as wet mounts of cultures were examined under phase microscopy.
API strips were prepared as directed by the manufacturer and examined at 48 hours according to API instructions for identification of Bacillus species. Results were verified with API confirmation. Incubation was standardised at 35±2°C.

Results:

Morphological characteristics			
***Bacillus subtilis* (Information from Bergy's manual)**		**GB07(DS719)**	
0.7-0.8 µm by 2-3 µm in length		?	
Sporangium not swollen		not swollen although marginal	
Shape of spore is ellipsoidal		ellipsoidal	
Spore location is central		Central, cell is small and fatter than 718	
Colony characteristics of *B. subtilis* can be highly irregular. Usually non pigmented.		Colonies large flat, irregular edge and spread out, dull-wrinkled surface do not disperse easily.	
Voges Proskauer	+	Voges Proskauer	+
Hydrolysis Casein Gelatin Starch	 + + +	Hydrolysis Casein Gelatin Starch	 ND + +
Use Citrate Propionate	 + -	Use Citrate Propionate	
Degradation of Tyrosine Phenylalanine Egg yolk lecithinase Nitrate reduced to nitrite	 - - - +	Degradation of Tyrosine Phenylalanine Egg yolk lecithinase Nitrate reduced to nitrite	 ND
Fermentation of indole growth at 55 °C	- -	Fermentation of indole growth at 50 °C	- 11-80 %
Oxydase β-galactosidase lipase	I I I	Oxydase β-galactosidase lipase	
Hydrolysis of Esculin	 +	Hydrolysis of Esculin	 +

ND: No data available

In addition, percent positive reactions of *B. subtilis* from API Rapid CH and from API 20 E were provided but not provided for and of the strain GB07.

RMS's conclusion:
According to the results the strain GB07 can be considered as *Bacillus subtilis*.

General conclusion for identification from RMS
Articles and report studies provided demonstrate that *Bacillus amyloliquefaciens MBI 600* was previously identified as *Bacillus subtilis MBI 600.*
Identification on the basis of 16S rRNA gene sequencing is not sufficient for the discrimination at the strain level. The applicant is currently undertaking a genome sequencing project on MBI600, and this information will be available by December.
A new molecular method of identification for *Bacillus amyloliquefaciens MBI 600* at the strain level is required.

B.1.1.3.4. Common name or alternative and superseded names and code names used during the development

Trade names:	None
Common names:	*Bacillus amyloliquefaciens* strain MBI600
Developmental code names:	*Bacillus amyloliquefaciens* MBI600, MBI 600, AP-301, GB 07, NCIMB 12376, JB3.6, ATTC SD-1414 or IK-1080WP. The organism was originally identified as *Bacillus subtilis* strain MBI600 and this name is thus used in many studies (II 1.3.1/04, Brown A. (2012),.

Recent studies with more modern techniques have identified the organism as *Bacillus amyloliquefaciens* (II 1.3.1/05 Kloepper, J.W. 2012), see B.1.1.3.3..

B.1.1.3.5. Relationship to known pathogens

The Panel on Biological Hazards of EFSA[2] has considered that *Bacillus amyloliquefaciens* is suitable for Qualified Presumption of Safety (QPS) assessment for biological agents intentionally added to food and feed with the qualification of absence of toxigenic activity since 2007. Following the annual updates of QPS list, EFSA has confirmed the recommendation of *Bacillus amyloliquefaciens* for the QPS list based on a literature review for the preceding years (EFSA QPS updates in 2010, 2011, 2012 and 2013).

The genus Bacillus is composed of two major groups (*B. subtilis* and *B. cereus* groups, respectively) and several minor groups.
Other species of the Genus *Bacillus* are known as toxin forming pathogens of vertebrates and arthropods:

- *Bacillus anthracis* causes anthrax in humans and animals.
- *Bacillus cereus* causes gastroenteritis (via food) and opportunistic infections.
- *Bacillus thuringiensis*, *Bacillus larvae*, *Bacillus lentimorbus*, *Bacillus popilliae* and some strains of *Bacillus sphaericus* act as insect pathogens.

Bacillus amyloliquefaciens is not related to the human pathogen species, *B. anthracis* and *B.cereus*, both belonging to *B.cereus* group.
Bacillus amyloliquefaciens belongs to *B. subtilis* - group which is quite homogenous. Different species of the *B. subtilis* group, especially *B. subtilis* and *B. amyloliquefaciens* have been used for many years in biotechnology for the production of enzymes, surfactants, probiotics, or antibiotics. Biocontrol uses comprise control of plant pathogenic fungi or bacteria on leaves and fruits, in the soil, or as postharvest treatment.

B.1.1.4. Specification of the material used for manufacturing of formulated products (Annex IIM 1.4)

B.1.1.4.1. Content of the micro-organism

The content of pure micro-organism in *Bacillus amyloliquefaciens* strain MBI600 technical is 80 % w/w (5.1 x 10^{11} CFU/g) mean and ranges from 78.2 (5.0 x 10^{11} CFU/g) to 86.2 % w/w (5.5 x 10^{11} CFU/g).

B.1.1.4.2. Identity and content of impurities, additives, contaminating micro-organisms

Confidential information-See Annex C.

B.1.1.4.3. Analytical profile of batches

Confidential information-See Annex C.

[2] *EFSA Journal (2007) 587, 1-16 Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA Opinion of the Scientific Committee.*

B.1.2. Identity of the plant protection product (Annex IIIM 1)

The representative formulation is Subtilex®..

B.1.2.1. Applicant (Annex IIIM 1.1)

Company: BASF Agricultural Specialities Ltd
Harwood Industrial Estate
Harwood Road
Littlehampton
BN17 7AU
UK
Contact: [redacted]
Phone: [redacted]
Fax:
E-Mail: [redacted]

Representative
Company: [redacted]
Contact:
Phone:
E-Mail:

B.1.2.2. Manufacturer of the preparation and the micro-organism (Annex IIIB 1.2)

Company: BASF Agricultural Specialities Ltd
Harwood Industrial Estate
Harwood Road
Littlehampton
BN17 7AU
UK
Contact: [redacted]
Phone:
Fax:
E-Mail: [redacted]m

Representative
Company: [redacted]
Contact:
Phone:
E-Mail:

Plant location: Confidential information-See Annex C.

B.1.2.3. Trade name or proposed trade name, and manufacturer's development code number of the preparation if appropriate (Annex IIIM 1.3)

The representative formulation is Subtilex®. It is a Wettable Powder (WP) containing 11 % (w/w) (1.21×10^{11} CFU/g) of the technical microbial pest control at a minimum purity of 80 % (5.0×10^{11} CFU/g).

Trade name: BUEXP1780S.

B.1.2.4. Detailed quantitative and qualitative information on the composition of the preparation (Annex IIIM 1.7)

Confidential information-See Annex C.

B.1.2.5. Physical state and nature of preparation (Annex IIIM 1.5)

Subtilex® is a Wettable Powder (WP).

B.1.2.6. Function (Annex IIIM 1.6)

Fungicide, fungistat

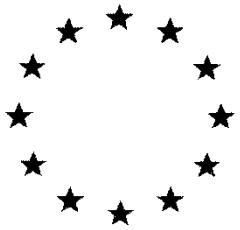
B.1.3. References relied on

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
Annex II Data and Information					
IIM, 1.3.1/01	Fukumoto, J.	1943	Studies on the production of bacterial amylase. I. Isolation of bacteria secreting potent amylases and their distribution (in Japanese) J. Agr. Chem. Soc. Japan 19: 487-503 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 1.3.1/02	Priest, F.G.; Goodfellow, M.; Shute, L.A.; Berkeley, R.C.W.	1987	Bacillus amyloliquefaciens sp. nov., nom. rev. Int. J. Syst. Bacteriol., 1987, 37, 69-71 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 1.3.1/03	Bruch, M.K.	1990	Identification of microorganisms MicroBioTest Inc, USA Becker Underwood, report No. 186-102 GLP Unpublished **Supporting information, study not fully summarised**	Y	BASF Agricultural Specialities Ltd
IIM, 1.3.1/04	Brown, A.	2012a	Taxonomic re-classification of *Bacillus subtilis* strain MBI600 to *Bacillus amyloliquefaciens* strain MBI600 Becker Underwood No report No. Not GLP Unpublished **Supporting information, letter not fully summarised**	Y	BASF Agricultural Specialities Ltd

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIM, 1.3.1/05	Kloepper, J.W.	2012	Identification of MBI600 by 16S rRNA gene sequencing using 8F and 1492R primers Auburn University, USA Becker Underwood No report No. Not GLP Unpublished **Supporting information, study not fully summarised**	Y	BASF Agricultural Specialities Ltd
IIM, 1.3.1/06	Yang, G.P.	2005	Identification of MBI600 by 16S rRNA gene sequencing Becker Underwood No report No. Not GLP Unpublished **Supporting information, study not fully summarised**	Y	BASF Agricultural Specialities Ltd
IIM, 1.3.1/07	Kloepper, J.W.	2012	No Title Department of Entomology and Plant Pathology, Auburn University, No Report No.		
IIM, 1.3.2/01	NCIB	1987	Accession number National Collection of Industrial Bacteria Torry Research Station, Scotland Becker Underwood No report No. Not GLP Unpublished **Supporting information, letter not fully summarised**	Y	BASF Agricultural Specialities Ltd
IIM, 1.3.2/02	Brown, A.	2011	Confirmation of holding of *Bacillus subtilis* strain JB 3.6 Becker Underwood No report No. Not GLP Unpublished **Supporting information, letter not fully summarised**	Y	BASF Agricultural Specialities Ltd
IIM, 1.3.2/03	ATCC	2009	Safe Deposit agreement IP Licensing Services, USA Becker Underwood No report No. Not GLP Unpublished **Supporting information, letter not fully summarised**	Y	BASF Agricultural Specialities Ltd
IIM, 1.3.3/02	Kloepper, J.W.	2007	Identification of MBI600 by 16S rRNA gene sequencing using 8F and 1492R primers Auburn University, USA Becker Underwood No report No. Not GLP Unpublished ⇨ 1.3.1/05 **Supporting information, study not fully summarised**	Y	BASF Agricultural Specialities Ltd

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIM, 1.3.5/01	Izu, S.	2007	Letter from supplier Becker Underwood No report No. Not GLP Unpublished **Supporting information, letter not fully summarised**	Y	BASF Agricultural Specialities Ltd
IIM, 1.3.5/02	Brown, A.	2011	Confirmation of holding of *Bacillus subtilis* strain JB 3.6 Becker Underwood No report No. Not GLP Unpublished ⇨ 1.3.2/02 **Supporting information, letter not fully summarised**	Y	BASF Agricultural Specialities Ltd
IIM, 1.3.6/01	Rossal, S.	2012	*Bacillus subtilis* isolate MBI 600 The University of Nottingham Becker Underwood No report No. Not GLP Unpublished **Supporting information, letter not fully summarised**	Y	BASF Agricultural Specialities Ltd

Draft Assessment Report



Bacillus amyloliquefaciens strain MBI600

Volume 3

Annex B.2

Biological, physical, chemical and technical properties

Rapporteur Member State : France

Volume 1

Level 1: Statement of subject matter and purpose for which the monograph was prepared

Level 2: Reasoned statement of the overall conclusions drawn by the Rapporteur Member State

Appendix 1: Standard terms and abbreviations

Appendix 2: Specific terms and abbreviations

Appendix 3: List of endpoints

Level 3: Proposed decision with respect to the application for inclusion of the active substance in Annex I

Level 4: Further information to permit a decision to be made, or to support a review of the conditions and restrictions associated with the proposed inclusion in Annex 1

Volume 2

Annex A: List of the tests and studies submitted and of information available

Volume 3

Annex B: RMS summary, evaluation and assessment of the data and information

Annex B.1: Identity

Annex B.2: Biological, physical, chemical and technical properties

Annex B.3: Data application and further information.

Annex B.4: Proposals for classification and labelling

Annex B.5: Analytical methods

Annex B.6: Effects on human health

Annex B.7: Residues data

Annex B.8: Fate and behaviour in the environment

Annex B.9: Effects on non-target organisms

Annex B.10: Summary and evaluation of environmental impact

Appendix 1: Standard terms and abbreviations

Appendix 2: Specific terms and abbreviations

Volume 4

Annex C: Confidential information and summary and assessment of information relating to the collective submission of dossiers

Version History of Volume 3 B2

Date	**Reason for revision**
December 2014	Initial DAR

Table of contents

B.2. Biological, physical, chemical and technical properties

B.2.1. Biological properties of the micro-organism

B.2.1.1. History of the micro-organism and its uses. Natural occurrence and geographical distribution (Annex IIM 2.1 and 2.2)

B.2.1.1.1. Historical background

Origin of the isolate
The bacterial strain is a naturally occurring, indigenous wild type and was isolated from leaf washings from leaves of broad bean plants (*Vicia faba*). The crop of broad beans was located at what is now the School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, UK.

Historical information on testing and use of the strain
Bacillus amyloliquefaciens strain MBI600 is a new active substance in Europe. It is currently approved for use in the USA, Canada, Japan, New Zealand and Turkey.

History of use of closely related strains or species
Bacillus species as a group offer several advantages over other bacteria for protection against root pathogens because of their ability to form endospores, and because of the broad-spectrum of activity of their antibiotics (Cavaglieri *et al*., 2005). Their work is exemplified by the closely related species *Bacillus subtilis* for controlling *Fusarium verticillioides* in maize. *B. amyloliquefaciens* is also known to produce a wide range of very similar antibiotics.

B. amyloliquefaciens is a ubiquitous soil bacterium, certain strains of which have applications against soil-borne and post-harvest pathogenic fungi. As a soil-dwelling saprophytic micro-organism *B. amyloliquefaciens* contributes to the mineralisation of organic molecules which are broken down by secreted proteases and amylases. Some of these enzymes are responsible for the occurrence of soft-rot disease caused by *B. amyloliquefaciens* in several crops and are commercially exploited.

B. amyloliquefaciens strain GA1 (formerly *B. subtilis* GA1) displays high *in vitro* inhibitory activity toward growth of multiple fungal and oomycete plant pathogens (Toure *et al*., 2004). When used as seed treatment, strain GA1 was shown to alleviate seedling diseases through direct antibiosis against soil-borne pathogens. The strain was also shown to reduce post-harvest infection of apples caused by *Botrytis cinerea*, the causative agent of grey mould disease. These data suggest the secretion of multiple antibiotics and demonstrate the potential application of *B. amyloliquefaciens* GA1 as a biocontrol agent.

Jacobsen *et al*. (2004) have considered the use of *Bacillus*-based biocontrol agents (BCAs) in integrated pest management (IPM) including resistant cultivars, fungicides or bactericides, or other BCAs to obtain more efficacious and consistent levels of disease control. Control of Cercospora leaf spot (*Fusarium oxysporum*) on sugarbeet, *Pythium torulosum* on tomatoes, Phytophthora root rot on soybean and black spot (*Pseudocercospora purpurea*) on avocados were reported. It is considered that the most common combination of *Bacillus*-based BCAs with fungicides is as seed treatments, the BCA providing control via colonization of the rhizosphere long after the fungicides have degraded, and also increasing plant growth, vigour and yields.

The related *B. subtilis* strain QST 713 is already included on Annex I to 91/414/EC for use as a fungicide and is marketed as Serenade. The EU review considered uses in viticulture, orchards (pome and stone fruit) and lettuce to control scab, fire blight, *Monilia*, *Oidium*, *Botrytis cinerea* and *Bremia lactucae*.

B.2.1.1.2. Origin and natural occurrence

Bacillus amyloliquefaciens is an ubiquitous micro-organism, which is most commonly found in soil environments and on plant undergrowth, but is also found naturally occurring in aquatic environments, plants, animals and their faeces. The strain *B. amyloliquefaciens* strain MBI600 was initially isolated in the UK. It is not

known to be indigenous to the southern zone. Based on available data, the abundance of *B. subtilis* varied from 3×10^4 CFU/g dry soil in desert soils to 7.6×10^4 CFU/g dry soil in a pine forest soil.

Introduced endospores are very resistant to abiotic environmental factors and do not compete with autochthon microorganisms for nutrients. They can survive in soil for a long period of time. A precondition for multiplication is germination of endospores. Germination mainly depends on the availability of nutrients. Thus, sufficient nutrients and the ability to compete with autochthon microorganisms are necessary for forming of vegetative cells and for multiplication. This occurs mainly in the rhizosphere. Reduction of vegetative cells and of endospores is mainly caused by grazing protozoa, bacterial predators, and bacteriophage infections.

B. amyloliquefaciens optimally grows in the mesophilic temperature range (37^{o}C) (Amner *et al*. 1991). Generally *B. amyloliquefaciens* reproduces under aerobic conditions, although in the presence of glucose and nitrate anaerobic growth occurs (Welker and Campbell, 1967). *B. amyloliquefaciens* is notably found in low nutrient soils and dominates the restricted micro-flora of soils with low carbon content. When carbon, nitrogen and phosphorus-nutrient levels fall below the bacterium's optimal threshold, it produces spores. It has been demonstrated that *B. amyloliquefaciens* and the closely related *Bacillus subtilis* concurrently produce antibiotics, cyclic lipo-peptides and spores (Toure *et al*. 2004; Katz and Demain, 1977; Stein, 2005; Montesinos, 2007; Hill and Gray, 1967, Zuber et al., 1993, Sansinenea and Ortiz 2011, Hamdache *et al*. 2011, Wulff *et al*. 2002). Antibiotic production increases *B. amyloliquefaciens*' chance of survival, since the organism produces spores and a toxin that might kill surrounding gram positive microbes that compete for the same nutrients.

B. amyloliquefaciens supports plant growth; it often plays a role in replenishing soil nutrients by supplying the terrestrial carbon cycle and the nitrogen cycle. *B. amyloliquefaciens* forms rough biofilms, which are beneficial since they allow for the control of plant pathogen infections. *B. amyloliquefaciens* biofilm communities form a mutualistic interaction with plant rhizome systems: the plant benefits because *B. amyloliquefaciens* provides pre-emptive colonization, preventing other pathogens from infecting the plant; *B. amyloliquefaciens* benefits by deriving nutrients and surface area for biofilm formation from the plant's root structure.

B.2.1.2. Information on target organism(s) (Annex IIM 2.3)

B.2.1.2.1. Description of target organism(s)

Bacillus amyloliquefaciens is to be used against fungal plant pathogens, exemplified in this submission by *Botrytis spp*.

B.2.1.2.2. Mode of action

Bacillus amyloliquefaciens strain MBI600 is fungicidal and fungistatic, with multiple modes of action.

Bacillus amyloliquefaciens strain MBI600 works by preventing further fungal growth rather than by killing the hyphae already present. Following contact with the fungal pathogen at the leaf surface it disrupts hyphal growth and prevents spore germination. In addition, *Bacillus amyloliquefaciens* exhibits fungicidal properties via production of iturin A and surfactin whilst in the endospore phase, which are antagonistic to fungal pathogens, resulting in an exclusion zone around the application site. It also exhibits bactericidal properties via the production of various antibiotics.
Bacillus amyloliquefaciens strain MBI600 colonises the roots and developing shoot systems of plants, suppressing by competition disease organisms.
In addition to antagonism, nutrient competition is involved in the mode of action. Furthermore, *Bacillus amyloliquefaciens* induces the systemic resistance response of the plant, indicated by enhanced peroxidase production.

It should be noted that *Bacillus amyloliquefaciens* species strain MBI600 promotes the growth of plants[1] via the production of plant growth stimulating compounds and increased mineral solubilisation. *Bacillus amyloliquefaciens* strain MBI600 produces siderophores, which increase the iron availability to plants associated

[1] *Kumar, V.K.K.; Reddy, M.S.; Kloepper, J.W.; Yellareddygari, S.KR; Lawrence, K.S.; Zhou, X.G.; Sudini, H.; Miller, M.E.; Podile, R.A.; Reddy, S.E.C.; Niranhana, S.R.; Nayaka, C.S.: Plant growth-promoting activities of Bacillus subtilis MBI 600 (Integral®) and its compatability with commonly used fungicides in rice sheath blight management. International Journal of Microbiology Research, 2011, 3(2): 120-130.*

with the bacteria and also promotes nodule formation via the cytokinin signalling pathway in various legume crops. *Bacillus amyloliquefaciens* strain MBI600 was found to produce volatile organic compounds such as 2,3-butanediol and acetoin that stimulated plant growth.

B.2.1.3. Host specificity range and effects on species other than the target harmful organism (Annex IIM 2.4)

Bacillus amyloliquefaciens strain MBI600 is not dependent upon a host, but rather the availability of decomposable organic matter. *Bacillus amyloliquefaciens* strain MBI600 is active against a number of plant pathogenic fungi, including *Botrytis spp*., powdery mildew, *Rhizoctonia solani, Fusarium oxysporum, F. solani, Pythium irregular, Pythium dimorphum and Phytophthora cinnamomi* and does not cause any permanent changes in the natural microflora (Kumar *et al.* 2011, Liang *et al*. 1982[2], Podile, 1994[3]), although there may be a temporary reduction in soil fungi following application. The endospore is the prevalent stage of *Bacillus amyloliquefaciens* in all environmental compartments. *Bacillus amyloliquefaciens* is not geographically restricted.

B.2.1.4. Development stages/life cycle of the micro-organism (Annex IIM 2.5)

All spore-formers, including members of the Genus *Bacillus*, undergo a cycle consisting of several discernible phases: germination, outgrowth, multiplication and sporulation. The germination is the conversion of the quiescent and dormant spore to a metabolising cell capable of outgrowth. The primary cell formed at the end of outgrowth can, under some conditions (such as insufficient supply of nutrients) divides asymmetrically and proceed directly to sporulation. Under favourable conditions the primary cell can divide symmetrically and proceed through many divisions before sporulating (Slepecky, 1992[4]). A specific review on spore germination is given by Moir (1992[5]).

The endospore plays a dominant role in the biology and the life-cycle of *Bacillus* species, including *Bacillus amyloliquefaciens* (Priest, 1993[6]). It is a dormant structure which enables the micro-organism to survive when environmental conditions become unfavourable for vegetative growth, and also permits air-borne dispersal (Priest, 1993). The global distribution of *Bacillus* species may largely be derived from the endospore-forming capability. The endospore is the most heat tolerant bacterial life form, enduring temperatures in excess of 80°C or even 100°C (Schlegel 1985[7]). The endospore is not an obligate stage in the life-cycle; vegetative growth by cell-division may be predominant or even the norm, unless for instance a lack of nutrients occurs (Priest, 1993). Studies on the population dynamics of the closely related *Bacillus subtilis* strongly suggest that it generally occurs in the endospore form in soil (van Elsas *et al*., 1986[8]; Asaka *et al*., 1996[9]).

In a dry state, endospores can remain viable for several years. After 50 years of storage in dry soil, 10% of the spores retain their capability to germinate (Schlegel, 1985).

---

[2] *Liang, L.N.; Sinclair, J.L.; Mallory, L.M.; Alexander, M.: Fate in model ecosystems of microbial species of potential use in genetic engineering. Applied and Environmental Microbiology 1982, 44(3): 708-714.*

[3] *Podile, A.R.: Survival of Bacillus subtilis AF 1 in the bacterized peanut rhizosphere and its influence on native microflora and seedling growth. World Journal of Microbiology 1994, 10:700-703*

[4] *Slepecky, R.A.: What is a Bacillus?. Biology of Bacilli (Chapter 1) Doi, R. H. and Mc Gloughlin, M. (eds.) Application to Industry Buttenworth-Heinemann, Boston, 1992, 1-21 BMF2000-58.*

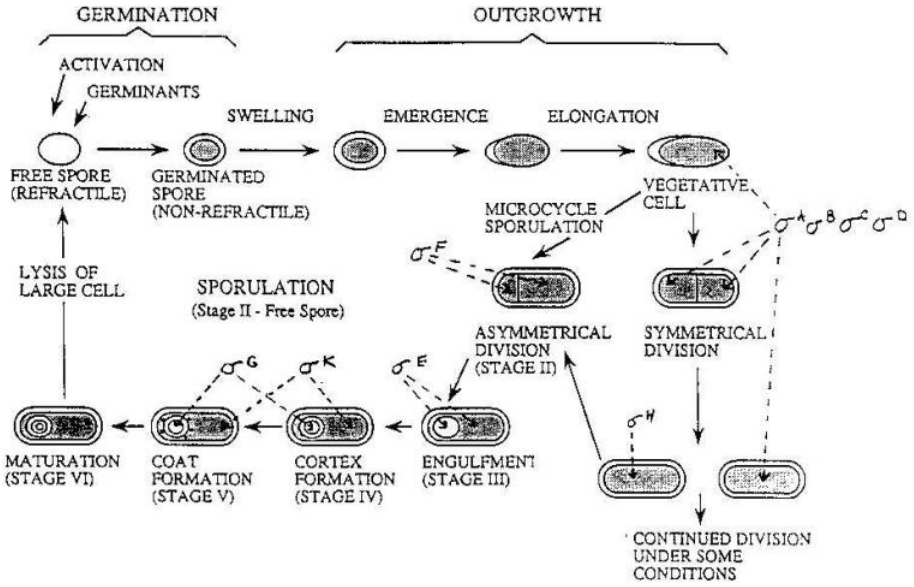
[5] *Moir, A. Spore germination (Chapter 2) Doi, R. H. and Mc Gloughlin, M. (eds.) Application to Industry Buttenworth-Heinemann, Boston, 1992, 23-38 BMF2000-58.*

[6] *Priest, F.G.: Systematics and Ecology of Bacillus. Bacillus subtilis and other gram-positive bacteria. American Society of Microbiology, Washington D.C. (ed.), 1993, 3-16 BMF2000-57*

[7] *Schlegel, H.G.: Allgemeine Mikrobiologie Georg Thieme Verlag Stuttgart New York, 1985, 70-411 BMF2000-55*

[8] *van Elsas, J.D., Dijkstra, A.F., Govaert, J.M. and van Veen, J.A.: Survival of Pseudomonas fluorescens and Bacillus subtilis introduced into two soils of different texture in field microplots. FEMS Microbiology Ecology, 38, 1986, 151-160 BOD2000-1365*

[9] *Asaka, O., Ano, T. and Shoda, M.: Persistence of Bacillus subtilis RB14 and its derivative strains in soil with respect to the LPA-14 gene. J. of Fermentation and Bioengineering, Vol. 81, 1996, 1-6 BMF2000-92*



B.2.1.5. Infectiveness, dispersal and colonisation ability (Annex IIM 2.5)

Generally *Bacillus amyloliquefaciens* reproduces under aerobic conditions, although in the presence of glucose and nitrate anaerobic growth occurs. *B. amyloliquefaciens*, together with closely related species such as *B. subtilis*, is primarily a soil inhabitant, particularly in low-nutrient soils (Priest, 1993; not provided) and dominates the restricted micro-flora of soils with low organic content (Priest, 1989).

The reported predominance of *Bacillus* species in such soils may indicate low demands for nutrition, or more probably the successful survival strategy afforded by sporulation. *Bacillus amyloliquefaciens* is considered to occur predominantly in the resting stage (endospore), unless fresh organic matter has been supplied to the soil. In either case, application of organic matter, e.g. manure, will support growth of existing *Bacillus amyloliquefaciens* populations.

The influence of pH and temperature on growth of *B. amyloliquefaciens* was tested by Pruitt (1990[10]), Amner *et al*. (1991[11]; not provided) and Welker and Campbell (1967[12]; not provided). The pH-range for growth was found to be pH 4.5 to 9.5-10.0. The temperature range for growth of *B. amyloliquefaciens* is 10 to 50°C. *B. amyloliquefaciens* was found to reproduce under aerobic and saline conditions. Pruitt (1990) observed no growth under anaerobic conditions, whereas Welker and Campbell (1967) observed poor growth.

In conclusion the results indicate that *B. amyloliquefaciens* will survive under a broad spectrum of environmental conditions.

B.2.1.6. Relationships to known plant or animal or human pathogens (Annex IIM 2.7)

The Panel on Biological Hazards of EFSA[13] has considered that *Bacillus amyloliquefaciens* is suitable for Qualified Presumption of Safety (QPS) assessment for biological agents intentionally added to food and feed with the qualification of absence of toxigenic activity.
The genus Bacillus is composed of two major groups (*B. subtilis* and *B. cereus* groups, respectively) and several minor groups.

[10] Pruitt, J. Bacterial characterisation. Becker Underwood (1990)
[11] Amner, W. *et al*. Survival of plasmid-bearing strain of *Bacillus subtilis* introduced into compost. Journal of General Microbiology 1991, 137, 1931-1937
[12] Welker, N.E. & Campbell, L.L. Unrelatedness of *Bacillus amyloliquefaciens* and *Bacillus subtilis.* Journal of Bacteriology 1967, 94(4): 1124-1130
[13] *EFSA Journal (2007) 587, 1-16 Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA Opinion of the Scientific Committee.*

Other species of the Genus *Bacillus* are known as toxin forming pathogens of vertebrates and arthropods:

- *Bacillus anthracis* causes anthrax in humans and animals.
- *Bacillus cereus* causes gastroenteritis (via food) and opportunistic infections.
- *Bacillus thuringiensis*, *Bacillus larvae*, *Bacillus lentimorbus*, *Bacillus popilliae* and some strains of *Bacillus sphaericus* act as insect pathogens.

Bacillus amyloliquefaciens is not related to the human pathogen species, *B. anthracis* and *B.cereus*, both belonging to *B.cereus* group.
Bacillus amyloliquefaciens belongs to *B. subtilis* - group which is quite homogenous. Different species of the *B. subtilis* group, especially *B. subtilis* and *B. amyloliquefaciens* have been used for many years in biotechnology for the production of enzymes, surfactants, probiotics, or antibiotics. Biocontrol uses comprise control of plant pathogenic fungi or bacteria on leaves and fruits, in the soil, or as postharvest treatment.
There is no difficulty in distinguishing between *Bacillus amyloliquefaciens* and the toxin-producing strains of *Bacillus.* Purity checks of the technical product of *Bacillus amyloliquefaciens* strain MBI600 are continuously performed to exclude the presence of the above mentioned species. Purity control is also applied to the manufacturing process of the formulated product, with samples determined to be 100 % pure *Bacillus amyloliquefaciens* strain MBI600 and not to contain detectable levels of human pathogens or contaminant micro-organisms.

Animals/humans:
The general population already is continually exposed to *Bacillus amyloliquefaciens* since it is a ubiquitous microorganism which primarily inhabits the soil environment and plant residues. *Bacillus amyloliquefaciens* is not pathogenic or highly toxic as demonstrated by the submitted toxicological studies and has been shown to clear the body following oral ingestion. The apparent lack of naturally induced interspecies gene transfer and consequently the improbability of a potential shifting of resistance genes into human pathogens supports the safety of *Bacillus amyloliquefaciens.*

No publications were found on *Bacillus amyloliquefaciens* acting as a pathogen. Gupta and Vyas (1989) reported insect and mammalian-pathogenicity of some of the *Bacillus subtilis* strains isolated from diseased mosquito larvae, causing mortality due to invasive infection (with subsequent decomposition of larvae) after ingestion by larvae or after intraperitoneal injection in mice, respectively. *Bacillus subtilis* has been implicated in bovine mastitis (Fossum *et al*., 1986), although the findings were inconclusive. Since *Bacillus subtilis* is so ubiquitous in the environment contamination cannot be ruled out. Antibodies against proteinase of *Bacillus subtilis* were found in some samples, meaning that it could not be ruled out as the causative agent in bovine mastitis. However in other work *Listeria monocytogenes* and *Staphylococcus aureus* are strongly linked with bovine mastisis (Kamau *et al*., 1990).

In very rare cases, *Bacillus* species can cause foodborne diseases. This is due to the production of heat stable surfactins. *Bacillus amyloliquefaciens* has been shown to produce the toxic peptide amylosin (Mikkola *et al*., 2007), which has been implicated in foodborne poisoning (Apetroaie-Constantin *et al*., 2009). However, it is considered that the strain involved may have been misidentified (From *et al*., 2005) since *Bacillus cereus* is a well-established cause of food poisoning and it is considered likely, given the extremely low incidence of food poisoning reported and attributed to *Bacillus amyloliquefaciens*, that *Bacillus cereus* has been misidentified as *Bacillus amyloliquefaciens*.

EFSA has reviewed the toxigenic potential of *Bacillus* species and has concluded that it is unlikely that any *Bacillus cereus*-like enterotoxins are produced by any species other than the *Bacillus cereus* group. Any toxigenic potential in other species is far more likely to arise from the production of surfactins.

Bacillus amyloliquefaciens does not appear in the literature as an infectious organism, but a few cases of *Bacillus subtilis* have been found, associated with drug users and severely debilitated patients (de Boer and Diderichsen, 1991). Immuno-compromised individuals inoculated with high numbers of the micro-organism may be susceptible to an infection. According to de Boer and Diedrichsen (1991), *Bacillus subtilis* is even consumed in large quantities in the Japanese food “Natto”. It is therefore considered an opportunistic micro-organism with no pathogenic potential to humans. *Bacillus subtilis* is virtually ubiquitous and it is therefore inevitable that it may sometimes be found in association with other micro-organisms in infected humans, however only patients treated with immunosuppressive drugs appear to be susceptible to infection with this otherwise harmless micro-organism.

Mikkola *et al*. (2004) reported that isolated strains of *Bacillus amyloliquefaciens* produced a substance that inhibited motility of boar spermatozoa and killed feline lung cells. The substance was partially characterized as being 1,197 Da, moderately hydrophobic and containing leucine, proline, serine, aspartic acid, glutamic acid and tyrosine, in addition to chromophore(s) absorbing at 365 nm. This appears to be the only report of *Bacillus amyloliquefaciens* producing a heat-stable, non-protein substance that is toxic to mammalian cells.

Bacillus amyloliquefaciens strain MBI600 did not exert pathogenic or toxic effects on mammals, as proved in relevant toxicological studies (refer to section 3). In addition, no hazardous effects of *Bacillus amyloliquefaciens* strain MBI600 were observed in testing non-target organisms (invertebrates, arthropods, fish and birds) that might be exposed to it under conditions of use.

In conclusion, adverse impacts and risks of field application of *Bacillus amyloliquefaciens* strain MBI600 for exposed animals are not expected.

Reference: Gupta, D.K.; Vyas, K.M. (1989)
Efficacy of *Bacillus subtilis* against mosquito Larvae (*Anophelis culicifacies*)
Zeitschrift fuer Angewandte Zoologie 1989, 76(1) 85-91

Abstract: Four strains of Bacillus subtilis viz. A, B, D, and H were isolated from diseased Anopheles larvae collected from Sagar (M.P.). All the four strains of B. subtilis were found to cause more or less toxic effects on different instar larvae of Anopheles culicifacies. The comparative analysis of LC(59) values, however, indicated that B. subtilis strain 'B' was most effective for causing the highest percentage of mortality amongst the treated larvae. All the strains were also tested for their mammalian safety on mice. It was found that B. subtilis strains 'B' and 'D' appeared to be quite safe as none of them caused any pathological symptoms of mortality in test animals.

Reference: Fossum, K.; Herikstad, H.; Binde, M.; Pettersen K.E. (1986)
Isolations of *Bacillus subtilis* in connection with Bovine Mastitis
Nordisk Veterinärmedicine, Vol. 38, 1986, 233-236 BMF2000-151

Abstract: Bacillus subtilis has only seldom been associated with pathological conditions in mammals. As the organism is considered to be ubiquitous in the environment, care has to be taken not to put too much emphasis on the pathogenicity of the organism, even in cases where it is isolated in pure culture. Bacillus subtilis was isolated from 17 cases of bovine mastitis in which it was considered to be the etiological factor.

Reference: Mikkola, R.; Andersson, M.A.; Teplova, V.; Grigoriev, P.; Kuehn, T.; Loss, S.; Tsitko, I.; Apetroaie, C.; Saris, N.E.L.; Veijalainen, P.; Salkinoja-Salonen, M.S. (2007)
Amylosin from *Bacillus amyloliquefaciens*, a K+ and Na+ channel-forming toxic peptide containing a polyene structure.Toxicon, 2007 49, 1158-1171

Abstract: Bacillus amyloliquefaciens strains isolated from the indoor environment of moisture-damaged buildings produce a 1197 Da toxin, named amylosin. Nuclear magnetic resonance (NMR) data showed that amylosin contains a chromophoric polyene structure and the amino acids leucine/isoleucine, proline, aspartic acid/asparagine, glutamic acid/glutamine and tyrosine. A quantitation method for amylosin was developed using commercially available amphotericin B as a reference compound and a known concentration of amylosin determined by NMR with the electronic reference to access in vivo concentration (ERETIC) method. Purified amylosin inhibited motility of boar sperm cells at an exposure concentration of 135 nM and hyperpolarized their cell membrane and depolarized their mitochondria at exposure to concentration of 33-67 nM for 10 min. In a 3-d exposure time only 27 nM of amylosin was needed to provoke the same toxicity functions. Amylosin was cytotoxic to feline lung cells at concentrations of <170 nM. Purified amylosin provoked adenosine 5'-triphosphate (ATP)-independent cation influx into isolated rat liver mitochondria (RLM), inducing swelling of the mitochondria at concentrations of 200 nM K(+) or >250 nM Na(+) medium. In the K(+)- or Na(+)-containing medium, amylosin uncoupled RLM, causing oxidation of pyridine nucleotides (PN), loss of the mitochondrial membrane potential, and suppressed ATP synthesis. Purified amylosin produced cation channels in black-lipid membranes (BLMs) with a selectivity K(+)>Na(+) at a concentration of 26 nM, i.e. the same concentration at which amylosin was toxic to boar sperm cells. The amylosin cation channels were cholesterol- and ATP-independent and more effective with K(+) than with Na(+). We propose that the toxicity of amylosin may be due its ionophoric properties, representing the first K(+)/Na(+) channel-forming substance reported from B. amyloliquefaciens.

Reference: Apetroaie-Constantin, C.; Mikkola, R.; Andersson, M.A.; Teplova, V.; Suominen, I.; Johanssonand, T.; Salkinoja-Salonen, M. (2009)
***Bacillus subtilis* and *B. mojavensis* strains connected to food poisoning produce the heat stable toxin amylopsin**
Journal of Applied Microbiology 2009 ISSN 1364-5072

Abstract: Aim: To screen and characterize toxic, heat-stable substances produced by food borne strains from Bacillus subtilis group.
Methods and Results: Using the boar sperm motility inhibition assay, six isolates from two outbreaks, out of the 94 isolates from 26 foods, were found to produce ethanol-soluble heat-stable substances that were toxic to sperm cells by depleting the mitochondrial membrane potentials. The toxic isolates were identified as Bacillus subtilis and B mojavensis. Colon carcinoma cells (Caco-2) were used to model the contact with the human digestive tract. The extract of B. subtilis F 2564⁄96 depolarized the mitochondria in intact Caco-2 cells similarly as in sperm cells. The substance responsible for these effects was purified using HPLC and identified by electron spray ionization ion trap mass spectrometry analysis as amylosin. The temperature requirement for amylosin production was 21–37_C for B. subtilis and 11–21_C for B. mojavensis. Bothspecies produced amylosin in air as well as in 7–8% CO2 with 8–9% O2.
Conclusions: Food borne illness related strains of B. subtilis and B. mojavensis, produced the heat-stable toxin amylosin.
Significance and Impact of the Study: This is the first report that suggests a role for the heat-stable, ion-channel forming toxin amylosin, as a virulence factor in food borne Bacillus.

Reference: From, C.; Pukall, R.; Schumann, P.; Hormazábal, V.; Granum, P.E. (2005)
Toxin producing ability among *Bacillus* spp. outside the *Bacillus cereus* group
Applied and Environmental Microbiology, 2005 71, 1178-1183

Abstract: A total of 333 *Bacillus* spp. isolated from foods, water, and food plants were examined for the production of possible enterotoxins and emetic toxins using a cytotoxicity assay on Vero cells, the boar spermatozoa motility assay, and a liquid chromatography-mass spectrometry method. Eight strains produced detectable toxins; six strains were cytotoxic, three strains produced putative emetic toxins (different in size from cereulide), and one strain produced both cytotoxin(s) and putative emetic toxin(s). The toxin-producing strains could be assigned to four different species, *B. subtilis*, *B. mojavensis*, *B. pumilus*, or *B. fusiformis*, by using a polyphasic approach including biochemical, chemotaxonomic, and DNA-based analyses. Four of the strains produced cytotoxins that were concentrated by ammonium sulfate followed by dialysis, and two strains produced cytotoxins that were not concentrated by such a treatment. Two cultures maintained full cytotoxic activity, two cultures reduced their activity, and two cultures lost their activity after boiling. The two most cytotoxic strains (both *B. mojavensis*) were tested for toxin production at different temperatures. One of these strains produced cytotoxin at growth temperatures ranging from 25 to 42°C, and no reduction in activity was observed even after 24 h of growth at 42°C. The strains that produced putative emetic toxins were tested for the influence of time and temperature on the toxin production. It was shown that they produced putative emetic toxin faster or just as fast at 30 as at 22°C. None of the cytotoxic strains produced *B. cereus*-like enterotoxins as tested by PCR or by immunological methods.

Reference: de Boer, A.S. and Diderichsen, B. (1991)
On the safety of *Bacillus subtilis and B. amyloliquefaciens*: a review
Appl. Microbiol. Biotechnol., 36, 1991, 1-4 TOX2000-1212

Conclusion No case demonstrating invasive properties of *Bacillus subtilis and B. amyloliquefaciens* has been dexribed butin a few case, *B. subtilis* has been found associated with drug abusers or severely debilitated patients. Thus there is no evidence of any pathogenic potential of *B. subtilis* to humans in general. of *B. subtilis* has been associated with some cases of food poisoning which in part may be due to misclassification of *B. cereus*. Thus there are few example of *B. subtilis* strain as confirmed causes of food poisoning. We conclude that *B. subtilis* is a safe host for the production of harmless products.

Reference: Mikkola, R.; Andersson, M.A.; Grigoriev, P.; Teplova, V.V; Saris, N.E.L.; Rainey, F.A.; Salkinoja-Salonen, M.S (2004)
***Bacillus amyloliquefaciens* strains isolated from moisture-damaged buildings produced**

surfactin and a substance toxic to mammalian cells.
Arch Microbiol (2004) 181 : 314–323

Abstract: Fungicidic Bacillus amyloliquefaciens strains isolated from the indoor environment of moisture-damaged buildings contained heat-stable, methanol-soluble substances that inhibited motility of boar spermatozoa within 15 min of exposure and killed feline lung cells in high dilution in 1 day. Boar sperm cells lost motility, cellular ATP, and NADH upon contact to the bacterial extract (0.2 microg dry wt/ml). Two bioactive substances were purified from biomass of the fungicidal isolates. One partially characterized substance, 1,197 Da, was moderately hydrophobic and contained leucine, proline, serine, aspartic acid, glutamic acid and tyrosine, in addition to chromophore(s) absorbing at 365 nm. In boar sperm and human neural cells (Paju), the compound depolarized the transmembrane potentials of mitochondria (Delta Psi(m)) and the plasma membrane (Delta Psi(p)) after a 20-min exposure and formed cation-selective channels in lipid membranes, with a selectivity K(+):Na(+):Ca(2+) of 26:15:3.5. The other substance was identified as a plasma-membrane-damaging lipopeptide surfactin. Plate-grown biomass of indoor Bacillus amyloliquefaciens contained ca. 7% of dry weight of the two substances, 1,197 Da and surfactin, in a ratio of 1:6 (w:w). The in vitro observed simultaneous collapse of both cytosolic and mitochondrial ATP in the affected mammalian cell, induced by the 1,197-Da cation channel, suggests potential health risks for occupants of buildings contaminated with such toxins.

B.2.1.7. Genetic stability and factors affecting it (Annex IIM 2.10)

Taken together, available knowledge indicates that gene transfer within *Bacillus amyloliquefaciens* or between *Bacillus amyloliquefaciens* and related species under natural conditions may be a rare event, but cannot be fully ruled out (Welker and Campbell 1967). In the case of strain MBI600 this does not present a problem because fermentation is started with pure cultures. A transfer to MBI600 of genes governing undesirable properties can therefore be ruled out. Regarding a possible gene transfer after application it must be considered that *Bacillus amyloliquefaciens* strain MBI600 does not have undesirable traits, e.g. pathogenicity to humans, animals or plants. Furthermore, antibiotic resistance studies have been performed showing that *Bacillus amyloliquefaciens* MBI600 is not suspected to harbour multi-resistance genes.
Please, see also point B.8.1.1 in Volume 3 B9 for more details.

B.2.1.8. Information on the production of metabolites (especially toxins) (Annex IIM 2.6)

A screening test on metabolites produced by *B.amyloliquefaciens* MBI600 has been performed and is presented below.

Report: Du Toit Schabort (2011) Secondary compound analysis of *Bacillus subtilis* strain MBI600. Becker Underwood. No report No.
Guideline: None
GLP: No

Materials and methods:
A metabolite screen was performed on *Bacillus amyloliquefaciens* strain MBI600 from BASF Agricultural Specialities. Cultures were grown for four days in 4 L bioreactor shake flasks containing 80 g/L soy flour and 67 g/L maltodextrin. The crude supernatant was evaluated through a series of HPLC chromatograms to determine which cyclic lipopeptides were produced. Samples were prepared by centrifuging the supernatant, making a 50:50 mixture with n-butanol and vortex mixing, centrifuging, and then removing the butanol layer to analyse using a gradient from 100% water to 100% methanol.

Samples of supernatant with different levels of the various compounds had their anti-fungal properties determined to identify which of the secondary compounds produced by the organism were responsible for disease control. This was done by pipetting 50µl of supernatant solution into small holes made in petri dishes filled will potato dextrose agar (PDA). A small sample of *Botrytis cinerea* (originally isolated from a tomato plant in South Africa) was then placed next to the supernatant and the growth of the disease monitored.

Findings:
Cultures grown on both media plates or in shake flasks produced the same secondary compound profile. Metabolites were identified as iturin A and surfactin. A further peak was tentatively identified as fengycin or plipastatin.

Samples from flasks with cultures containing high levels of surfactin or iturin A were collected and then assessed for their bioactivity against pathogenic fungi. Significant anti-fungal effects were seen in petri dish assessments.

Conclusions:
Bacillus amyloliquefaciens strain MBI600 produces both iturin A and surfactin and these compounds are able to control fungal diseases *in vitro*.
The production of these compounds is one of the contributing modes of action that result in this organism being able to control plant disease.

In the literature, *Bacillus amyloliquefaciens* has been shown to produce many antibiotics, antifungals and siderophores including surfactin, iturin (bacillomycin D, F, L and FC, and iturin A and C, mycosubtilin), macrolactin, azalomycin F, the auxin indole-3-acetic acid (IAA), 3-hydroxybutan-2-one (acetoin), butane-2,3-diol, bacilysin, fengycin, amphomycin, acivicin, arthrobactin, difficidin, oxidifficidin, bacillaene, diHydroBacillaene, valinomycin, enterobactin and nocardamin (Sansinenea and Ortiz 2011, Hamdache *et al*. 2011, Wulff *et al*. 2002, Stein 2005, Toure *et al*., 2004). Welker and Campbell (1967) also report the production of α-amylase. None of these compounds are a concern for human health or the environment.

Reference: Sansinenea, E.; Ortiz, A. (2011)
Secondary metabolites of soil *Bacillus* spp.
Nordisk Biotechnol Lett (2011), 33, 1523-1538

Abstract: Bacillus species produce secondary metabolites that are the object of natural product chemistry studies. The wide structural variability of these compounds has attracted the curiosity of chemists and their biological activities have inspired the pharmaceutical industry to search for lead structures in microbial extracts. Screening of microbial extracts reveals the large structural diversity of natural compounds with broad biological activities, such as antimicrobial, antiviral, immunosuppressive, and antitumor activities, that enable the bacterium to survive in its natural environment. These findings widen the potential industrial importance of Bacillus spp., particularly of B. thuringiensis, beyond insecticidal usage and may help explain the role of Bacillus spp. in the soil ecosystem.

Reference: Hamdache, A.; Lamarti, A.; Aleu, J.; Collado, I.G. (2011)
Non-peptide metabolites from the genus *Bacillus*. Journal of Natural Products
Published by the American Chemical Society and American Society of Pharmacognosy 14 March 2011

Abstract: *Bacillus* species produce a number of non-peptide metabolites that display a broad spectrum of activity and structurally diverse bioactive chemical structures. Biosynthetic, biological, and structural studies of these metabolites isolated from *Bacillus* species are reviewed. This contribution also includes a detailed study of the activity of the metabolites described, especially their role in biological control mechanisms.

Reference: Wulff, E.G.; Mguni, C.M.; Mansfeld-Giese, K.; Fels, J.; Lubeck, M.; Hockenhull, J. (2002)
Biochemical and molecular characterization of *Bacillus amyloliquefaciens, B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris pv. Campestris*
Plant Pathology (2002) 51 , 574–584

Abstract: Fifty-one *Bacillus* isolates were characterized by fatty acid methyl ester (FAME) analysis; universal primer polymerase chain reaction (UP-PCR) fingerprinting; production of secondary metabolites and antagonistic activity against *Xanthomonas campestris* pv. *Campestris* (causal agent of black rot in cabbage) *in vitro* and *in vivo*. Based on FAME analysis and/or PCR fingerprinting, the isolates were clustered into three different groups, named as *Bacillus amyloliquefaciens,B. subtilis* and *B. pumilus*. Seed treatment with *Bacillus* spp. generally reduced germination of seeds and incidence of black rot, but no relationship was found between the results of *in vitro* and *in vivo* experiments. The *B. amyloliquefaciens* group contained isolates that were generally the most effective at reducing attack of black rot *in vivo*. The metabolic profiles of these isolates suggested that they produced surfactin, iturin, bacillomycine and/or azalomycin F. Isolates belonging to the *B. subtilis* group were mostly able to synthesize surfactin and arthrobactin. Surfactin, amphomycin, arthrobactin and valinomycin were generally found in culture extracts of isolates belonging to the *B. pumilus* group. No effect on growth of the pathogen was detected when the activity of filtered culture

extracts and selected metabolites produced by the three different *Bacillus* species was tested *in vitro* against *X.c*. pv.*campestris*. However, inhibition was seen when bacterial liquid cultures were used. When the ability to colonize cabbage endophytically was examined for seven selected isolates with different antagonistic potential against black rot, it was found that the ability was related to the species and not to the antagonistic activity of the isolates.

Reference: Toure Y.; Ongena M.; Jacques P.; Guiro A.; Thonart P. (2004)
Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple
Journal of applied microbiology 2004, 96:1151-1160.

Abstract: AIM:
Test of Bacillus subtilis strain GA1 for its potential to control grey mould disease of apple caused by Botrytis cinerea.
METHODS AND RESULTS:
GA1 was first tested for its ability to antagonize in vitro the growth of a wide variety of plant pathogenic fungi responsible for diseases of economical importance. The potential of strain GA1 to reduce post-harvest infection caused by B. cinerea was tested on apples by treating artificially wounded fruits with endospore suspensions. Strain GA1 was very effective at reducing disease incidence during the first 5 days following pathogen inoculation and a 80% protection level was maintained over the next 10 days. Treatment of fruits with an extract of GA1 culture supernatant also exerted a strong preventive effect on the development of grey mould. Further analysis of this extract revealed that strain GA1 produces a wide variety of antifungal lipopeptide isomers from the iturin, fengycin and surfactin families. A strong evidence for the involvement of such compounds in disease reduction arose from the recovery of fengycins from protected fruit sites colonized by bacterial cells.
CONCLUSIONS:
The results presented here demonstrate that, despite unfavourable pH, B. subtilis endospores inoculated on apple pulp can readily germinate allowing significant cell populations to establish and efficient in vivo synthesis of lipopeptides which could be related to grey mould reduction.
SIGNIFICANCE AND IMPACT OF THE STUDY:
This work enables for the first time to correlate the strong protective effect of a particular B. subtilis strain against grey mould with in situ production of fengycins in infected sites of apple fruits.

Reference: Welker, N.E.; Campbell, L.L. (1967)
Unrelatedness of *Bacillus amyloliquefaciens* and *Bacillus subtilis*
Journal of Bacteriology 1967, 94(4): 1124-1130.

Abstract: Eight strains of highly amylolytic, sporeforming bacilli (hereafter reffered to as *Bacillus amyloliquefaciens*) were compared with respect to their taxonomic relationship to *B. subtilis*. The physiological-biochemical properties of these two groups of organism showed that *B. amyloliquefacien*s differed from *B. subtilis* by their ability to grow in 10% NaCl, characteristic growth on potato plugs, increased production of α-amylase, and their ability to ferment lactose with the production of acid. The base composition of the deoxyribonucleic acid (DNA) of the *B. subtilis* strains consistently fell in the range of 41.5 and 43.5% guanine +cytosine (G+C), whereas that of the *B. amyloliquefaciens* stains was in the 43.5 to 44.9% G+C range. Hybrid formation between *B. subtilis* W23 and *B. amyloliquefaciens* F DNA revealed only a 14.7 to 15.4% DNA homology between the two species. Transducing phage, SP-10, was able to propagate on *B. subitilis* W23 and *B. Amyloliquefaciens* N, and would transduce on *B. Subtilis* 168 (indole-) and *B. Amyloliquefaciens* N-10 (arginine-) to prototrophy with a frequency of 3.9 x 10-4 and 2.4 x 10-5 transductants per plaque-forming unit, respectively. Attemps to tranduce between the two species were unsuccessful. These data show that *B. Amyloliquefaciens* is a valid species and should not be classified as a starin or variety of *B. Subtilis*.

RMS comment:
The strategy of search literature is described in the part B.2.1.8.
Applicant provided a literature research on metabolites produced by *Bacillus amyloliquefaciens* in agreement with information presented in different report[14][15] submitted to EFSA and in other Draft assessment reports of *Bacillus subtilis*.

[14] *C. Martel et al. Bibliographic review on the potential microorganisms, microbial products and enzymes to induce respiratory sensitization- Scinetif/Technical report submitted to EFSA*
[15] *S. Mudgal et al. Scientific support, literature review and data collection and analysis for risk assessment on microbial organisms used as active substance in plant protection products –Lot 1 Environmental Risk characterisation- EFSA supporting publication 2013:EN-518*

The mode of action of *Bacillus amyloliquefaciens strain* MBI600 against plant pathogenic fungi is partly based on the production and secretion of metabolites that disrupts hyphal growth and prevents spore germination (see part B2.1.2.2). It makes sense to find surfactin and iturin in the broth fermentation of *Bacillus amyloliquefaciens strain* MBI600.
The potential effects of metabolite on the human health are presented in B6.1.1.2.5 Part.

B.2.1.9. Antibiotics and other anti-microbial agents (Annex IIM 2.12)

Two antibiotic resistance studies were performed on *Bacillus amyloliquefaciens* MBI600.

Report: Green, L.A. (2013) Antibiotic resistance testing against plant protection product: *Bacillus amyloliquefaciens* strain MBI600. Wickham Laboratories Limited. Unpublished report No. CN0007155a.DR.
Guideline: None
GLP: No (but is GMP compliant)

Materials and methods:
The test microorganism *Bacillus amyloliquefaciens* strain MBI600 was tested against the various common antibiotics listed below:

- Cefotaxime (cephalosporins group)
- Ciprofloxacin (quinolones and fluoroquinolones)
- Clindamycin (lincosaminde group)
- Imipenem (carbapenems group)
- Kanamycin (aminoglycoside group)
- Linezolid (oxazolidinone group)
- Metronidazole (nitroimidazole group)
- Penicillin G (penicillin group) benzylpenicillin
- Streptomycin (aminoglycoside group)
- Teicoplanin (glycopeptide group)

An agar plate was inoculated with the microorganism suspension and an Etest strip placed onto the surface of the seeded plate. The plates were incubated at 35-37^{o}C for 24 to 48hrs. The zone of inhibition that surrounded the Etest strip was assessed to determine the point at which it crosses the evaluator scale giving the Minimum Inhibitory Concentration (MIC) value. The test was performed in triplicate.

Etest® is a method for antimicrobial resistance testing in microbiology laboratories and consists of a predefined gradient of antibiotic concentrations on a plastic strip and is used to determine the MIC of antibiotics, antifungal agents and antimycobacterial agents.

Findings:

Bacillus amyloliquefaciens strain MBI600 showed sensitivity to ciprofloxacin, clindamycin, imipenem, linezolid, penicillin G, streptomycin and teicoplanin. However, the MIC for clindamycin and streptomycin were above cut-off values of EFSA guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance.[16]The microorganism was resistant to cefotaxime, kanamycin and metronidazole.

Report: Kloepper (2012) Antibiotic sensitivity of *Bacillus amyloliquefaciens* strain MBI600. Auburn University. No report No.
Guideline: None
GLP: No

Materials and methods:

[16] *EFSA Journal 2012;10(6):2740*

The test microorganism *Bacillus amyloliquefaciens* strain MBI600 was tested against the various common antibiotics listed below:

- Amoxicillin
- Ampicillin
- Chloramphenicol
- Erythromycin
- Gentamycin
- Oxacillin
- Rifampicin
- Tetracycline
- Tobramycin

In addition 18 diverse compounds with anti-microbial activity were also tested for resistance:

- Fusidic Acid
- D-Serine
- Troleandomycin
- Rifamycin SV
- Minocycline
- Lincomycin
- Guanidine Hydrochloride
- Niaproof 4
- Vancomycin
- Tetrazolium Violet
- Tetrazolium Blue
- Nalidixic Acid
- Lithium Chloride
- Potassium Tellurite
- Aztreonam
- Sodium Butyrate
- Sodium Bromate

Findings:

Bacillus amyloliquefaciens strain MBI600 showed sensitivity to chloramphenicol, erythromycin, gentamycin, oxacillin, rifampicin, and tobramycinn and tolerance (or resistance) to amoxicillin, ampicillin, and tetracycline. *Bacillus amyloliquefaciens* strain MBI600 was found to be controlled by two aminoglycocides gentamycin and tobramycin. It was shown that strain MBI600 is very sensitive to those antibiotics (MIC 0.33 µg/ml and 0.3 µg/ml respectively) and therefore whatever the mechanism for resistance reported in the study by Green to kanamycin, it does not cover the entire aminoglycocide antibiotic group.

RMS comments:

Applicant has performed two antibiotic resistance assays with an important list of tested antibiotics.
Bacillus amyloliquefaciens strain MBI600 is susceptible to a wide spectrum of antibiotics commonly used in human and veterinary medicine.
According to the EFSA opinion[2], "*it was reported in the genome of several Bacillus species cfr-like genes, including Bacillus amyloliquefaciens, suggesting that Bacillales are a natural residence of cfr-like genes*". It can be concluded from these studies that *Bacillus amyloliquefaciens* strain MBI600 (sensitive to chloramphenicol and linezolid) does not possess the cfr gene since the presence of this gene in the genome provides resistance to several classes of antibiotics including phenicols (chloramphenicol) and oxazolidinone (linezolid).

B.2.2. Physical, chemical and technical properties of the plant protection product (Annex IIM 2)

The representative formulation is Subtilex® (BUEXP 1780S). It is a Wettable Powder (WP)
Concentration uses:
Minimum: 0.05 % (w/v)
Maximum: 0.125 % (w/v)

Test or study & Annex point	**Method used / deviations**	**Test material purity and specification**	**Findings**	**GLP Y/N**	**Reference**	**Acceptability / comments**

<table>
<tr><th>Test or study & Annex point</th><th>Method used / deviations</th><th>Test material purity and specification</th><th>Findings</th><th>GLP Y/N</th><th>Reference</th><th>Acceptability / comments</th></tr>
<tr><td>B.2.2.1. Appearance (colour and odour) (Annex IIIM 2.1)</td><td>EPA OPPTS 830.6302, 830.6303, 830.6304</td><td>Bacillus amyloliquefaciens strain MBI600 WP (BUEXP 1780S) Batch No. 54389 >5.5 x 10^{10} cfu/g</td><td>Light brown free-flowing homogeneous powder with a faint yeasty odour.</td><td>Y</td><td>Morgan, L. (2013)</td><td>Acceptable</td></tr>
<tr><td colspan="7">B.2.2.2. Storage stability and shelf-life (Annex IIIM 2.2)</td></tr>
<tr><td>B.2.2.2.1. Effects of light, temperature and humidity on technical characteristics of the plant protection product</td><td>Regulation 1107/2009/EC and Regulation 545/2011/EC</td><td>Bacillus amyloliquefaciens strain MBI600 WP (BUEXP 1780S) Batch No. 54389 >5.5 x 10^{10} cfu/g</td><td>Analysis was performed on preparation packaged in its commercial package (white laminated PET/metallised Polyester/PE barrier film).
<table>
<tr><th></th><th>T0</th><th>After 12 weeks at 35°C</th></tr>
<tr><td>Appearance (method :EPA OPPTS 830.6302, 830.6303, 830.6304)</td><td colspan="2">Light brown free-flowing homogeneous powder with a faint yeasty odour.</td></tr>
<tr><td>Packaging (aluminium laminate bag (19cm ×26 cm)</td><td colspan="2">No deformation, swelling, cracking, crazing, odour, leakage, staining or visual contamination on storage. Packaging remained intact.</td></tr>
<tr><td>Bacillus amyloliquefaciens strain MBI600 (CFU/g) (Method: SJ-</td><td>8.13 x 10^{10}</td><td>3.30 x 10^{10}</td></tr>
</table></td><td>Y</td><td>Morgan, L. (2013)</td><td>Acceptable
The preparation is stable for at least 12 months at 35 °C as physical state: viable Bacillus amyloliquefaciens strain MBI600 content, pH, wettability after swirling, suspensibility (with continuous agitation) and wet sieve test checked after storage are comparable to initial characteristics.

The content of MPCA after 12 weeks at 35° is below the minimum certified value. The Notifier explains that the study will be- re-run in September 2014 due to a problem with the sampling method. These new data should be provided as soon</td></tr>
</table>

Test or study & Annex point	Method used / deviations	Test material purity and specification	Findings			GLP Y/N	Reference	Acceptability / comments
			SOP-8202-BBRC)					**as possible using a validated method.** **Data on microbial contaminants according to OECD issue paper, Oct. 2011 were not provided before and after storage (12 weeks at 35 °C) and are required as soon as possible using validated methods.** **Study of storage during 2 years at ambient temperature in the commercial packaging is required as soon as possible using validated methods.** **The study should include microbial contaminants according OECD guidelines 65 (Oct. 2011) using validated methods.**
			pH (1% dilution in deionised water) (CIPAC MT 75.3)	8.7	8.1			
			Wettability (sec) (CIPAC MT 53.3)					
			No swirling	599	142			
			Swirling	16	19			
			Persistent foaming (mL) (CIPAC MT 47.2) 10 sec 1 min 3 min 12 min	 <1 mL 0 mL - -	 4 mL <1 mL <1 mL <1 mL			
			Suspensibility (CIPAC MT 184)					
			0.05% in Water D	50 %	14 %			
			0.125% in Water D	54 %	33 %			
			Wet sieve test (CIPAC MT 185) (75 µm sieve)	1.11%	2.06%			
			Microbial contaminants					
			S. aureus					
			E. coli					
			Total					

Test or study & Annex point	**Method used / deviations**	**Test material purity and specification**	**Findings**	**GLP Y/N**	**Reference**	**Acceptability / comments**
			coliforms *Salmonella* *Vibrio* *Shigella* Anaerobes *Listeria monocytogenes* Yeast and mould			

Test or study & Annex point	Method used / deviations	Test material purity and specification	Findings	GLP Y/N	Reference	Acceptability / comments
B.2.2.2.2. Other factors affecting stability	/	/	/	/	/	/
B.2.2.3. Explosivity and oxidising properties (Annex IIIM 2.3)	Statement	-	The active substance and co-formulants of Subtilex® are naturally occurring products with no explosive or oxidizing properties. Please refer to Volume C for the detailed composition of the product.	-	-	Acceptable The formulation has no explosive and no oxidising properties.
B.2.2.4. Flash point and other indications of flammability or spontaneous ignition (Annex IIIM 2.3.2)						
B.2.2.4.1. Flash point, Flammability	Statement	-	The active substance and co-formulants of Subtilex® are naturally occurring products with no flammable properties. Please refer to Volume C for the detailed composition of the product.			Acceptable The preparation is not highly flammable at ambient temperature.
B.2.2.4.2. Auto-flammability	Statement	-	The active substance and co-formulants of Subtilex® are naturally occurring products with no flammable properties. Please refer to Volume C for the detailed composition of the product.			Acceptable The preparation is not auto-flammable at ambient temperature.
B.2.2.5. Acidity/alkalinity and if necessary pH value (Annex IIIM 2.3.3)	CIPAC MT 75.3	*Bacillus amyloliquefaciens* strain MBI600 WP (BUEXP 1780S) Batch No. 54389 $>5.5 \times 10^{10}$ cfu/g	Acidity and alkalinity was not required as pH >4 and <10. The pH at 1 % dilution in deionised water= 8.7 at 22°C.	Y	Morgan, L. (2013)	Acceptable
B.2.2.6. Viscosity and surface tension (Annex IIIM 2.3.4)	/	/	Not required for solid preparation	/	/	Acceptable

<table>
<tr><th>Test or study & Annex point</th><th>Method used / deviations</th><th>Test material purity and specification</th><th>Findings</th><th>GLP Y/N</th><th>Reference</th><th>Acceptability / comments</th></tr>
<tr><td colspan="7">B.2.2.7. Technical characteristics of the plant protection product (Annex IIIM 2.4)</td></tr>
<tr><td>B.2.2.7.1. Wettability</td><td>CIPAC MT 53.3</td><td>Bacillus amyloliquefaciens strain MBI600 WP (BUEXP 1780S) Batch No. 54389 >5.5 x 10^{10} cfu/g</td><td>599 secs (no swirling)
16 secs (swirling)</td><td>Y</td><td>Morgan, L. (2013)</td><td>Acceptable
The preparation is considered as wettable after swirling. The formulation should be used under continuous agitation.</td></tr>
<tr><td>B.2.2.7.2. Persistent foaming</td><td>CIPAC MT 47.2</td><td>Bacillus amyloliquefaciens strain MBI600 WP (BUEXP 1780S) Batch No. 54389 >5.5 x 10^{10} cfu/g</td><td>Persistent foaming of a test item diluted in water D:<table><tr><th>Time</th><th>Foam (mL) at 0.05% (w/v)</th><th>Foam (mL) at 0.125 % (w/v)</th></tr><tr><td>10 s</td><td>< 1</td><td>< 1</td></tr><tr><td>1 min</td><td>0</td><td>0</td></tr><tr><td>3 min</td><td></td><td>-</td></tr><tr><td>12 min</td><td></td><td>-</td></tr></table></td><td>Y</td><td>Morgan, L. (2013)</td><td>Acceptable</td></tr>
<tr><td>B.2.2.7.3. Suspensibility and suspension stability</td><td>CIPAC MT 184</td><td>Bacillus amyloliquefaciens strain MBI600 WP (BUEXP 1780S) Batch No. 54389 >5.5 x 10^{10} cfu/g</td><td>At 0.05 % dilution in CIPAC water D: 50 %
At 0.125 % dilution in CIPAC water D: 54 %</td><td>Y</td><td>Morgan, L. (2013)</td><td>Acceptable
The suspensibility is outside the acceptable limits, nevertheless, according to the Good Agriculture Practices as the application is performed in continuous agitation, no more data required.</td></tr>
<tr><td>B.2.2.7.4. Dry sieve test and wet sieve</td><td>CIPAC MT 185</td><td>Bacillus amyloliquefacie</td><td>1.11 % retention on >75µm</td><td>Y</td><td>Morgan, L. (2013)</td><td>Acceptable</td></tr>
</table>

Test or study & Annex point	Method used / deviations	Test material purity and specification	Findings	GLP Y/N	Reference	Acceptability / comments
test		*ns* strain MBI600 WP (BUEXP 1780S) Batch No. 54389 >5.5 x 10^{10} cfu/g				
B.2.2.7.5. Particle size distribution (dustable and wettable powders, granules), content of dust/fines (granules), attrition and friability (granules)	CIPAC MT 187	*Bacillus amyloliquefaciens* strain MBI600 WP (BUEXP 1780S) Batch No. 54389 >5.5 x 10^{10} cfu/g	Initial D_{10} (µm) – 0.8 D_{50} (µm) – 6.0 D_{90} (µm) – 38.9	Y	Morgan, L. (2013)	Acceptable More than 1 % of particles are <50 µm.
B.2.2.7.6. Emulsifiability, re-emulsifiablilty and emulsion stability B.2.2.7.7.	-	-	Not required for WP formulation.	-	-	
B.2.2.7.8. Flowability, pourability (rinsibility) and dustability	-	-	Not required for WP formulation.	-		
B.2.2.8. Physical, chemical and biological compatibility with other products including plant protection products with which its use is to be authorized (Annex IIIB 2.8)						
B.2.2.8.1. Physical compatibility	-	-	-	-	-	The preparation is not intended to be mixed with other products. Studies will be
B.2.2.8.2. Chemical	-	-	-	-	-	

Test or study & Annex point	Method used / deviations	Test material purity and specification	Findings	GLP Y/N	Reference	Acceptability / comments
compatibility						required when a dossier of authorisation for mixture of Subtilex® with others formulations will be deposited.
B.2.2.8.3. Biological compatibility	-	-	-	-	-	
B.2.2.9. Adherence and distribution to seeds (Annex IIIM 2.6)	-	-	The preparation is not to be used as a seed treatment.	-	-	Acceptable
B.2.2.10. Other properties						
Bulk density	CIPAC MT 33	*Bacillus amyloliquefaciens* strain MBI600 WP (BUEXP 1780S) Batch No. 54389 >5.5 x 10^{10} cfu/g	1.04 g/mL	Y	Morgan, L. (2013)	Acceptable

B.2.2.11. Summary and evaluation of data submitted in B.2.2.1 - B.2.2.9 (Annex IIIM 2.7)

The product Subtilex® is a WP (wettable powder) formulation containing 110 g/kg (5.5×10^{10} CFU/g) of technical MPCA *Bacillus amyloliquefaciens* MBI600. The formulation is Light brown free-flowing homogeneous powder with a faint yeasty odour. It is not explosive, has no oxidising properties and is not flammable. It has slightly alkaline pH value around 8 for a 1% dilution. As the wettability is outside the acceptable limit before swirling, the formulation should used under a continuous agitation. The content of MPCA after 12 weeks at 35°C is below the minimum certified value. The Notifier explains that the study will be- re-run in September 2014 due to a problem with the sampling method. **These new data should be provided as soon as possible using a validated method.**

Data on microbial contaminants according to OECD issue paper, Oct. 2011 before and after storage (12 weeks at 35 °C) were not provided and are required as soon as possible using validated methods.

Study of storage during 2 years at ambient temperature in the commercial packaging is required as soon as possible using validated method. The study should include microbial contaminants according OECD guidelines 65 (Oct. 2011).

B.2.3. References relied on

Section 1, (Annex IIA, Point 1)

Annex point/ reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N	Owner**
Annex II Data and information					
IIM, 2.1/02	Cavaglieri, L.; Orlando, J.; Rodríguez, M.I.; Chulze, S.; Etcheverry, M.	2005	Biocontrol of *Bacillus subtilis* against *Fusarium verticillioides in vitro* and at the maize root level Research in Microbiology 2005, 156: 748-754. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 2.1/03	Toure Y.; Ongena M.; Jacques P.; Guiro A.; Thonart P.	2004	Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple Journal of applied microbiology 2004, 96:1151-1160. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 2.1/04	Jacobsen, B.J.; Zidack, N.K.; Larson, B.J.	2004	The role of *Bacillus*-based biological control agents in integrated pest management systems: plant diseases The American Phytopathological Society 2004, 94(11), 1272-1275 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 2.2/01	Amner, W.; McCarthy, A.J.; Edwards, C.	1991	Survival of plasmid-bearing strain of *Bacillus subtilis* introduced into compost Journal of General Microbiology 1991, 137, 1931-1937 Not GLP Published	N	Public

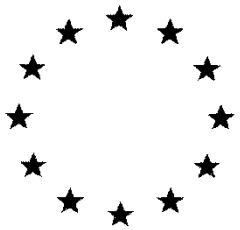
Annex point/ reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant), Published or not**	**Data Protection Claimed** **Y/N**	**Owner****
			Supporting information, paper not fully summarised		
IIM, 2.2/02	Welker, N.E.; Campbell, L.L.	1967	Unrelatedness of *Bacillus amyloliquefaciens* and *Bacillus subtilis* Journal of Bacteriology 1967, 94(4): 1124-1130. Not GLP Published ⇨ 1.3.1/10 **Supporting information, paper not fully summarised**	N	Public
IIM, 2.2/04	Katz, E; Demain, A.L.	1977	The peptide antibiotics of *Bacillus*: chemistry, biogenesis and possible functions Bacteriological reviews 1977, 4(2):449-474 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 2.2/05	Stein, T.	2005	*Bacillus subtilis* antibiotics: structures, syntheses and specific functions Molecular Microbiology (2005) 56 (4), 845–857 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 2.2/06	Montesinos, E.	2007	Antimicrobial peptides and plant disease control FEMS Microbiology Letters 2007, 270: 1-11 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 2.2/07	Hill, I.R.; Gray, T.R.G.	1967	Application of the florescent-antibody-technique to an ecological study of bacteria in soil Journal of Bacteriology 1967, 93(6):1888-1896 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 2.2/08	Zuber, P.; Nakano, M.M.; Marahiel, M.A.	1993	Peptide antibiotics. Chapter 61, pp. 897-916 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 2.2/09	Sansinenea, E.; Ortiz, A.	2011	Secondary metabolites of soil *Bacillus* spp. Nordisk Biotechnol Lett (2011), 33, 1523-1538 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 2.2/10	Hamdache, A.; Lamarti, A.; Aleu, J.; Collado, I.G.	2011	Non-peptide metabolites from the genus *Bacillus*. Journal of Natural Products Published by the American Chemical Society and American Society of Pharmacognosy 14 March 2011 Not GLP Published **Supporting information, paper not fully**	N	Public

Annex point/ reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant), Published or not**	**Data Protection Claimed** **Y/N**	**Owner****
			summarised		
IIM, 2.2/11	Wulff, E.G.; Mguni, C.M.; Mansfeld-Giese, K.; Fels, J.; Lubeck, M.; Hockenhull, J.	2002	Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris pv. Campestris* Plant Pathology (2002) 51 , 574–584 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 2.8-02	Priest, F.G.:	1989	Isolation and identification of aerobic endospore-forming Bacteria. *Bacillus*, Chapter 3 Colin R. Harwood (ed.) The University of Newcastle upon Tyne, UK Plenum Press 1989, 27-56 BMF2000-90	N	Public
IIM, 5/09	EFSA	2011	Scientific Opinion Technical guidance on the assessment of the toxigenic potential of *Bacillus* species use in human nutrition EFSA Journal 2011; 9(11):2445 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.1/01	EFSA	2007	Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA Opinion of the Scientific Committee The EFSA Journal (2007) 587, 1-16 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.4/01	Gupta, D.K.; Vyas, K.M.	1989	Efficacy of *Bacillus subtilis* against mosquito Larvae (*Anophelis culicifacies*) Zeitschrift fuer Angewandte Zoologie 1989, 76(1) 85-91 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.4/02	Fossum, K.; Herikstad, H.; Binde, M.; Pettersen K.E.	1986	Isolations of *Bacillus subtilis* in connection with Bovine Mastitis Nordisk Veterinärmedicine, Vol. 38, 1986, 233-236 BMF2000-151 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.4/03	Mikkola, R.; Andersson, M.A.; Grigoriev, P.; Teplova, V.V; Saris, N.E.L.; Rainey, F.A.; Salkinoja-Salonen, M.S.	2004	*Bacillus amyloliquefaciens* strains isolated from moisture-damaged buildings produced surfactin and a substance toxic to mammalian cells. Arch Microbiol (2004) 181 : 314–323 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.3/02	Apetroaie-Constantin, C.; Mikkola, R.;	2009	*Bacillus subtilis* and *B. mojavensis* strains connected to food poisoning produce the heat stable toxin amylopsin	N	Public

Annex point/ reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant), Published or not**	**Data Protection Claimed** **Y/N**	**Owner****
	Andersson, M.A.; Teplova, V.; Suominen, I.; Johanssonand, T.; Salkinoja-Salonen, M.		Journal of Applied Microbiology 2009 ISSN 1364-5072 Not GLP Published **Supporting information, paper not fully summarised**		
IIM, 5.2.3/03	From, C.; Pukall, R.; Schumann, P.; Hormazábal, V.; Granum, P.E.	2005	Toxin producing ability among *Bacillus* spp. outside the *Bacillus cereus* group Applied and Environmental Microbiology, 2005 71, 1178-1183 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.3/06	de Boer, A.S. and Diderichsen, B.	1991	On the safety of *Bacillus subtilis and B. amyloliquefaciens*: a review Appl. Microbiol. Biotechnol., 36, 1991, 1-4 TOX2000-1212 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.4/03	Mikkola, R.; Andersson, M.A.; Grigoriev, P.; Teplova, V.V; Saris, N.E.L.; Rainey, F.A.; Salkinoja-Salonen, M.S.	2004	*Bacillus amyloliquefaciens* strains isolated from moisture-damaged buildings produced surfactin and a substance toxic to mammalian cells. Arch Microbiol (2004) 181 : 314–323 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/01	Du Toit Schabort	2011	Secondary compound analysis of *Bacillus subtilis* strain MBI600 Becker Underwood No report No. Not GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM, 5.4/02	Sansinenea, E.; Ortiz, A.	2011	Secondary metabolites of soil *Bacillus* spp. Nordisk Biotechnol Lett (2011), 33, 1523-1538 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/03	Hamdache, A.; Lamarti, A.; Aleu, J.; Collado, I.G.	2011	Non-peptide metabolites from the genus *Bacillus* Journal of Natural Products Published by the American Chemical Society and American Society of Pharmacognosy 14 March 2011 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/04	Wulff, E.G.; Mguni, C.M.; Mansfeld-Giese, K.; Fels, J.; Lubeck, M.; Hockenhull, J.	2002	Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris pv. Campestris* Plant Pathology (2002) 51 , 574–584 Not GLP Published	N	Public

Annex point/ reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant),** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner****
			Supporting information, paper not fully summarised		
IIM, 5/08	Welker, N.E.; Campbell, L.L.	1967	Unrelatedness of *Bacillus amyloliquefaciens* and *Bacillus subtilis* Journal of Bacteriology 1967, 94(4): 1124-1130. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 4.3.1/02	Kloepper, J.W.	2012	No Title Auburn University, USA Becker Underwood No report No. Not GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM,	Green, L.A.	2013	Antibiotic resistance testing against plant protection product: *Bacillus amyloliquefaciens* strain MBI600. Wickham Laboratories Limited. Unpublished report No. CN0007155a.DR		
Annex III Data and Information					
IIM 4.1.3	Brown A.	2014	BASF confidential business information. Packaging to be used for BUEXP1780S End Use Product in Europe	Y	BASF Agricultural Specialities Ltd
IIIM 2.2	Morgan, L.	2013	Accelerated Storage Stability of BUEXP1780S for up to 12 weeks at 35°C stored in an aluminium laminate bag. Battelle UK Ltd. Becker Underwood Report No. LG/12/002/1 GLP Unpublished 2.1/01	Y	BASF Agricultural Specialities Ltd

Draft Assessment Report



Bacillus amyloliquefaciens strain MBI600

Volume 3

Annex B.3

Data application and further information

Rapporteur Member State : France

Version History of Volume 3 B3

Date	**Reason for revision**
December 2014	Initial DAR

Table of contents

B.3. Data on application and further information

B.3.1. Further information on the micro-organism (Annex IIB 3)

B.3.1.1. Function (Annex IIM 3.1)

Fungicide, fungistat

B.3.1.2. Field of use envisaged (Annex IIM 3.3)

B. amyloliquefaciens strain MBI600 is intended to be used in viticulture.

B.3.1.3. Crops or products protected or treated (Annex IIM 3.3)

B. amyloliquefaciens strain MBI600 is intended to be used on vines to protect grapes.

B.3.1.4. Method of production and quality control (Annex IIM 1.4.3)

See Volume 4, C.1.2.7

B.3.1.5. Information on the occurrence or possible occurrence of the development of resistance of the target organism(s) (Annex IIM 3.6)

In glasshouse trials with Astilbe micro-plants under condition which favour fungal attack it was found that resistance in the fungal pathogen *Botryotinia fuckeliana* developed within 10 consecutive crop generations. Results of *in vitro* assays suggested that the fungal strains had become resistant to two antibiotics known to be the principal control mechanism of the *Bacillus subtilis* isolate (Li and Leifert, 1994[1]).

In the case of *Bacillus amyloliquefaciens* strain MBI600, the mode of action has been demonstrated to rely on a broader base than single site action, since it includes diverse mechanisms not easily overcome by pathogens. The risk of development of resistance is therefore classified as low.

B.3.1.6. Methods to prevent loss of virulence of seed stock of the micro-organism (Annex IIB 4.1)

See Volume 4, C.1.2.

B.3.1.7. Recommended methods and precautions concerning handling, storage, transport or fire (Annex IIM 3.7)

Handling
PPE is required during mixing, loading or application due to the sensitisation potential of micro-organisms. The following PPE is recommended if there is a risk of skin or eye contact with the product:

Hand protection:	Chemical resistant (e.g. nitrile rubber) gloves
Eye protection:	Safety glasses/face shield
Skin and body protection:	Impermeable clothing
Hygiene measures:	Handle in accordance with good industrial hygiene and safety practice.

Storage
The product must be stowed away from food, drink and animal feeding stuffs and without access for children. The product should be stored under conditions which prevent entering any body of water and any misuse by non-authorised persons.

[1] *Li, H.; Leifert, C.: Development of resistance in Botryotinia fuckeliana (de Bary) Whetzel against the biological control agent Bacillus subtilis CL27. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz 1994, 101:414-418*

The product should be stored in compliance with GCPF (former GIFAP) Guidelines for Safe Warehousing of crop protection products and GIFAP Guidelines for Safe Handling of Pesticides during their Formulation, Packing, Storage and Transport.

The recommended storage temperature is between 4°C and 25°C.

Transport
Subtilex® is not classified hazardous under transport regulations.

Transport should be in compliance with GIFAP Guidelines for the safe transport of pesticides and Guidelines for Safe Handling of Pesticides during their Formulation, Packing, Storage and Transport.

Fire

Suitable extinguishing media:	Water spray, foam, dry powder, carbon dioxide (CO_2)
Specific hazards during fire fighting:	Explosive dust-air mixtures may form
Special protective equipment:	No specific equipment is required
Additional precautions:	If possible, avoid discharge of fire-extinguishing water into drains or water courses.

Waste minimisation
To minimise surplus product waste, users are recommended to buy not more than is consumed within the shelf life period of the product and to store the product before use according to storage recommendation (cool and dry). Always calculate the amount required and buy the appropriate container sizes.

B.3.1.8. Procedures for destruction or decontamination (Annex IIM 3.8)

Spill containment
Prevent entry into drains, waters or soil. Dampen the dust, scoop or sweep up and place into sealable containers. Use a damp cloth to clean floors and other objects after removal of powder and also place in sealable container. Dispose of all waste and contaminated clothing in the same manner as waste chemicals (i.e. via an authorised disposal facility).

Since *Bacillus amyloliquefaciens* is not considered to have any adverse environmental impact due to its ubiquitous natural occurrence. An accidental release of product indoor or outdoor does not require special treatment.

Decontamination
The procedures described above are sufficient for decontamination. Solid surfaces may be further cleaned by washing with detergents.

B.3.1.9. Measures in case of an accident (Annex IIM 3.12)

Protection of emergency workers
Wear full protective clothing and NIOSH or other regulation-approved self-contained breathing apparatus with full face piece operated in the pressure demand or other positive pressure mode. Keep unnecessary people away. Use as little water as possible. Dike area of fire, to prevent material run-off.

Decontaminate emergency personnel with soap and water before leaving the fire area. Avoid breathing dusts, vapours and fumes from burning materials. Control run-off water.

Protection of bystanders
Bystanders should not be allowed to remain in the vicinity of accidental release or fire. In case bystanders cannot be removed to a safe location, they should be placed up-wind from the accidental release or fire, and provided with full protective equipment.

First aid

Skin contact:	In case of contact with skin wash off immediately with soap and water.
Eye contact:	In the case of contact with eyes, rinse immediately with plenty of water for at least 15 minutes.
Ingestion:	Drink plenty of water. Do not induce vomiting. Seek medical advice.
Inhalation:	Move to fresh air.

Notes to the physician

Symptoms:	No specific symptoms are known.
Treatment:	No specific antidote. Initial treatment should be symptomatic and supportive.

B.3.2. Data on application (Annex IIIM 3)

B.3.2.1. Field of use envisaged (Annex IIIM 1.6.1)

It is intended for use as fungicide to protect preventatively grapevine against *Botrytis spp*.

B.3.2.2. Mode of action (Annex IIIM 3.1)

Strains of *Bacillus amyloliquefaciens* are known to be antagonistic towards a range of fungal plant pathogens. However, the mechanism for control of pathogens and the modes of action involved are not clearly understood for each host/pathogen system.

The mode of action of *Bacillus amyloliquefaciens* species MBI600 is fungicidal and fungistatic; it works by preventing further growth rather than killing the hyphae already present. *Bacillus amyloliquefaciens* disrupts the growth of the hyphae and prevents spore germination, following contact with the fungal pathogen at the leaf surface. In addition, *Bacillus amyloliquefaciens* exhibits fungicidal properties via production of iturin A and surfactin, which are antagonistic to the fungal pathogen, resulting in an exclusion zone around the application site. It also exhibits bactericidal properties via the production of various antibiotics.

Besides antagonism, nutrient competition is involved in the mode of action, and *Bacillus amyloliquefaciens* also induces the systemic resistance response of the plant, indicated by enhanced peroxidase production.

According to Campell's review[2] on biocontrol agents the germination of *Botrytis* spores on the leaf surface is inhibited in the presence of various bacteria and yeasts by nutritional competition, and the author also explains the phenomenon of induced systemic resistance towards pathogens: an initial contact with a non-pathogenic species may elicit a resistance response of the plant via a systemically acting chemical signal.

Marten *et al*. (1998) explain the antagonistic effect of application of the closely related species *Bacillus subtilis* against phytopathogens (*Rhizoctonia solani* and *Fusarium oxysporum*) with several involved mechanisms, including protease-activity (causing lysis) and competition due to production of siderophores.

Competition for nutrients (especially carbon) occurs among the saprophytic members of the microflora within the natural habitat, the soil, and in the rhizosphere. Successfully competing bacteria inhibit fungal spore germination (fungistasis) and therefore competition is believed to present one potential mode of action in the suppression of fungal plant diseases such as *Fusarium* wilts (Alabouvette and Lemanceau, 1998[3]). Together with other factors competition promotes vital growth and development of the roots (Kilian *et al*., 1998, BMF 2000-86).

[2] *Campbell, R.: Biocontrol on leaf surfaces. Biological control of microbial plant pathogens, Cambridge University Press, Cambridge, Department of Botany, University of Bristol, Chapter 3, 1989, 66-94 BMF2000-100*
[3] *Alabouvette, C.; Lemanceau, P.: Joint action of microbials for disease control (Article 8). Methods in Biotechnology, Vol. 5 Biopesticides: Use and Delivery, 1998, 117-135*

B.3.2.3. Details of intended use (Annex IIM 3.1)

Table B.3.2.3.a Summary of intended uses of SUBTILEX

Crop and /or situation (a)	Member state or Country	Product name	F G or I (b)	Pest or Group of pests controlled (c)	Formulation		Application				Application rate per treatment			PHI (days) (l)	Remarks (m)
					Type (d-f)	Conc. of MPCA (i)	method kind (f-h)	growth stage & season (j)	number min max	interval between applications (min)	CFU MPCA/hl min max	water l/ha min max	CFU MPCA/ha min max		
Grapes	SEU	BUEXP1780S = SUBTILEX	F	*Botrytis* spp., Grey mould	WP	5.5 x 10^{10} spores/g 110 g/kg	Spray tractor-mounted air assisted or knapsack sprayers	All	1 - 10	7 days	0.014 - 0.0055	400 - 1000	0.055 kg MPCA/ ha	-	Equivalent to 0.5 kg MPCP /ha

Explanation of a -m
a: The EU classification for crops (90/642/EEC).
b: Outdoor or field use (F), glasshouse application (G) or indoor application (I)
c: e.g. biting and sucking insects, soil born insects, foliar fungi, weeds
d: e.g. wettable powder (WP), emulsifiable concentrate (EC), granule (GR), water soluble powder (SP)
e: GCPF Codes - GIFAP Technical Monograph No 2, 1989
f: all abbreviations used must be explained
g: Method, e.g. high volume spraying, low volume spraying, spreading, dusting, drench,
h: Kind, e.g. overall, broadcast, aerial spraying, row, individual plant, between the plants - type of equipment used must be indicated.
i: g/kg, g/l or appropriate term for micro-organisms
j: Growth stage at last treatment (BBCH Monograph, Growth stage of plants, 1997, Blackwell, ISBN 3-8263-3152-4)
k: The minimum and maximum number of application possible under practical conditions of use must be provided
l: PHI - minimum pre-harvest interval
m: Remarks may include: Extent of use/economic importance/restrictions

B.3.2.4. Application rate (Annex IIIM 3.3 and 3.4)

Crop	Method of application	Rate of application per unit treated (as preparation)	Rate of application per unit treated (as active substance)
Grapes	Foliar spray	0.5 kg MPCP /ha	0.055 kg MPCA/ ha

B.3.2.5. Content of micro-organism in material used (e.g., in the diluted spray, baits or treated seed) (Annex IIIM 3.3)

Crop	Method of application	Material used (e.g. diluted spray, baits, treated seed)	Content of microorganism in material used
Grapes	Foliar spray	Diluted spray	0.014 - 0.0055 CFU MPCA/hL

B.3.2.6. Method of application (Annex IIIM 3.5)

Crop	Method of application	Type of equipment used	Type and volume of diluent per unit of area or volume
Grapes	Foliar spray	Spray tractor-mounted air assisted or knapsack sprayers	400 – 1000 water L/ha

B.3.2.7. Number and timing of applications (Annex IIIM 3.6)

Crop	Method of application	Maximum number of applications	Timing of application
Grapes	Foliar spray	10	7 days

B.3.2.8. Necessary waiting periods or other precautions to avoid phytopathogenic effects on succedding crops (Annex IIIM 3.7)

The product is based on a micro-organism that is already commonly found in the soil and on plant surfaces. *B. Amyloliquefaciens* strain MBI 600 is recognized to be harmless for treated plants and succeeding crops. For the present dossier, application is intended in vine only, which is a permanent crop where succeeding crops are not concerned. There are, therefore, no minimum waiting periods between the last application and sowing or planting of succeeding crops, nor limitations in the choice of these.

B.3.2.9. Proposed instructions for use (Annex IIIM 3.8)

Text of the label submitted by the applicant for the product SUBTILEX:

Subtilex

MAPP XXXXX

A wettable powder (WP) formulation containing 11.0 % w/w (>5.5 x 10^{10} cfu/g) *Bacillus amyloliquefaciens* strain MBI600.

For the preventative control of *Botrytis* spp. and powdery mildew on grapevines.

The (COSHH) Control of Substances Hazardous to Health Regulations may apply to the use of this product at work.

IMPORTANT INFORMATION

COMPLIANCE WITH THE FOLLOWING CONDITIONAS OF USE AND ALL SAFETY

PRECAUTIONS MARKED # IS A LEGAL REQUIREMENT

FOR USE ONLY AS AN AGRICULTURAL FUNGICIDE

Crop	Maximum individual dose (kg/ha)	Maximum number of applications	Maximum total dose (kg/ha)	Latest time of application
Grapes	0.5	10 per year	5	Up to harvest

Other specific restrictions: A minimum of 7 days must be observed between applications.

READ ALL OTHER SAFETY PRECAUTIONS AND DIRECTIONS FOR USE BEFORE USE

Net Contents: 0.5 kg

BASF Agricultural Specialities Ltd
Harwood Industrial Estate
Harwood Road
Littlehampton
BN17 7AU
UK

Tel. +44 (0)1903 732323

Batch No. XXXX

Date of manufacture. XXXX

SAFETY PRECAUTIONS

Contains *Bacillus amyloliquefaciens*. Micro-organisms may have the potential to provoke sensitising reactions.

Operator protection
Engineering control of operator exposure must be used where reasonably practicable in addition to the following personal protective equipment:

WEAR SUITABLE PROTECTIVE CLOTHING (COVERALLS, APRON), SUITABLE PROTECTIVE GLOVES AND FACE PROTECTION (FACESHIELD) when handling the concentrate and during application.

However, engineering controls may replace personal protective equipment if a COSHH assessment shows they provide an equal or higher standard of protection.

WASH ALL PROTECTIVE CLOTHING thoroughly after use, especially the insides of gloves.
TAKE OFF IMMEDIATELY all contaminated clothing.
DO NOT BREATH DUST OR SPRAY.
AVOID ALL CONTACT WITH SKIN AND EYES.
AFTER CONTACT WITH SKIN, WASH IMMEDIATELY with soap and water.
WHEN USING DO NOT EAT, DRINK OR SMOKE.

Environmental Protection
DO NOT CONTAMINATE SURFACE WATERS OR DITCHES with chemical or used container.

Storage and Disposal
KEEP AWAY FROM FOOD, DRINK AND ANIMAL FEEDING STUFFS.
KEEP OUT OF REACH OF CHILDREN.
EMPTY CONTAINER COMPLETELY and dispose of safely.

DIRECTIONS FOR USE

IMPORTANT: This information is approved as part of the product label. All instructions within this section must be read carefully in order to obtain safe and successful use of this product.

RESTRICTIONS:
Do not apply during rain or if rain is expected.
A maximum of 10 applications can be made.
Applications must be made at least 7 days apart.
The last application can be made up to harvest.

DISEASES CONTROLLED:
Subtliex® may be used on all commercial varieties of grapes for preventative control of *Botrytis* spp. and powdery mildew.

CROP SPECIFIC INFORMATION:

Grapevine

Maximum individual dose:	0.5 kg/ha
Maximum number of applications per season:	10
Water volume:	400-1,000 L/ha Use the higher rate for larger or denser vines.

Spray quality:	Tractor-mounted air blast sprayer with hydraulic nozzles or using hand held equipment (hydraulic or air blast knapsack sprayer).
Timing of application:	For best results apply before infestation. Subtilex® provides preventative control. Repeat application frequently.

MIXING:
Before use ensure that all application equipment is clean. Add half the required volume of water and start agitation. Add the required quantity of Subtilex®. Fill the tank to the required volume whilst maintaining agitation. Continuous agitation must be maintained until spraying is complete.

All application equipment should be cleaned thoroughly with water prior to storage.

FOLLOWING CROPS:
Not applicable as grapes are a perennial crop. However there are no restrictions in following crops.

COMPATIBILITY:
No tank mixes are recommended.

CLEANING INSTRUCTIONS:
Cleaning of spray equipment should be performed by triple-rinsing with water (3 times 10% of the tank volume). The equipment can afterwards be used in any crops without damage. No addition of detergents is necessary.

Gloves can be washed with a dilute detergent solution and rinsed with water. Any contaminated clothing should be laundered with a dilute detergent solution and thoroughly rinsed with clean water.

CLEAN-UP OF SPILLS
Prevent entry into drains, waters or soil. Dampen the dust, scoop or sweep up and place into sealable containers. Use a damp cloth to clean floors and other objects after removal of powder and also place in sealable container. Dispose of all waste and contaminated clothing in the same manner as waste chemicals (i.e. via an authorised disposal facility).

B.3.2.10. Effectiveness

To support approval of *B. amyloliquefaciens* strain MBI600 as active substance under regulation (EC) 1107/2009, efficacy data for one representative use was presented, namely for *Botrytis cinerea* on grapevine.
28 efficacy trials were realized in 2011 and 2012 in France (12), UK (2), Italy (7), Portugal (4) and Spain (3) to have some information on the efficacy of the representative product SUBTILEX for the inscription of the micro-organism against *Botrytis cinerea* on grapevine.

In the trials Subtilex was applied at the claimed dose rate of 0.5 kg/ha corresponding with 2.75 $x10^{13}$ cfu. of *Bacillus amyloliquefaciens* strain MBI600 per hectare as well as at dose rates of 0.25 kg/ha and 1.0 kg/ha. As reference, products based on *Bacillus subtilis* strain QST713 (Serenade Biofungicide and Serenade Max) and on iprodion (Rovral Aquaflo) were used.

Trials have been conducted according to the following EPPO guidelines No PP 1/152(3), PP 1/181(3), PP 1/135(3) and PP 1/17(3) and following the CEB method number 37.

3 to 8 applications were performed by foliar spray. Treatments were applied at intervals varying from 4-68 days at a spray volume of 200-1000 L/ha. The large intervals were used when the disease was not visually present in the crop in the beginning of the trial period. At presence of first symptoms and/or closer to harvest, application intervals were reduced to 4-10 days.
During the trial period the efficacy of the different products was determined by assessing 50-100 bunches per plot.

In 23 trials, no clear dose effect was observed between the 3 tested doses 0.25, 0.5 and 1 kg/ha of the product SUBTILEX. Efficacies were very variable:

- at the claimed dose of 0.5 kg/ha from 2.6 to 100% in terms of pest incidence and from 3.9 to 100% in terms of pest severity
- at 0.25 kg/ha from 2.1 to 84% in terms of pest incidence and from 4.4 to 100% in terms of pest severity
- at 1 kg/ha from 0.6 to 100% in terms of pest incidence and from 0 to 100% in terms of pest severity

Incidence
SUBTILEX permitted to obtain a similar level of control than the product of reference Serenade max except in 1 trial out of 25 were SUBTILEX at 0.5 kg/ha gave a statistically better level of control than Serenade max.
In the 3 validated trials where iprodion was included as reference, 2 trials gave statistically similar levels of efficacy with SUBTILEX than with iprodion and 1 trial gave statistically better results of efficacy with iprodion.

Severity
SUBTILEX permitted to obtain a similar level of control than the product of reference Serenade max except in 3 trials out of 25 were SUBTILEX at 0.5 kg/ha gave a statistically better level of control than Serenade max.
In the 3 validated trials where iprodion was included as reference, similar levels of efficacy with SUBTILEX than with iprodion were observed statistically even if in tendency, iprodion gave better results in 2 trials.

Based on the 28 trial data, it can be concluded that 0.5 kg/ha of SUBTILEX has an interest to control grey mould on bunches of grapes generally comparable to the control given by the reference Serenade Max at 2.0-4.0 kg/ha. Compared to the chemical fungicide based on iprodion (0.15 l/hl Rovral Aquaflo), the control given by 0.5 kg/ha SUBTILEX was less important.

B.3.2.11. Information on the development of resistance

In the case of *Bacillus amyloliquefaciens* strain MBI600, the mode of action has been demonstrated to rely on a broader base than single site action, since it includes diverse mechanisms not easily overcome by pathogens. The risk of development of resistance is therefore classified as low.

B.3.2.12. Adverse effects on treated crops

In efficacy trials performed in France, Italy, Spain and Portugal on grapevines, no phytotoxic symptoms were observed. So SUBTILEX is not expected to have any phytotoxic effects on the host crop.
Considering the selectivity of SUBTILEX, no negative impact is expected on the yield and quality of harvest.

B.3.2.13. Observations on other undesirable or unintended side-effects

Considering the ubiquity of *Bacillus amyloliquefaciens* in the environment and the selectivity of SUBTILEX on grapevine, no negative impact is expected on succeeding and adjacent crops and on parts of plants used for propagating purposes.

B.3.3. Further information on the plant protection product (Annex IIIM 4)

B.3.3.1. Packaging and compatibility of the preparation with proposed packaging materials (Annex IIIM 4.1)

Subtilex® is marketed in matt silver 0.5 kg re-sealable plastic covered foil pouches of size 190 x 265 x 90 mm (Laminated PET/Metalised PET/Poly barrier film). The pouches are heat sealed, but also contain a re-sealable inner seal. The pouches are stored in cardboard boxes.
For the compatibility with the formulation see B.2.2.2.

B.3.3.2. Procedures for cleaning application equipment (Annex IIIM 4.3 and 4.4)

B.3.3.2.1. Effectiveness of the cleaning procedures

Application equipment
Cleaning of spray equipment should be performed by triple-rinsing with water (3 times 10% of the tank volume). The equipment can afterwards be used in any crops without damage. No addition of detergents is necessary.

B.3.3.3. Re-entry periods, necessary waiting periods or other precautions to protect man, livestock and the environment (Annex IIIM 4.5)

B.3.3.3.1. Pre-harvest intervals, re-entry or withholding periods to minimise residues in crops, plants, plant products, treated areas or spaces

Pre-harvest interval (in days) for each relevant crop

Crop	Application				
	Formulation (type & content of a.s.)	No.	Rate kg a.s./ ha	Spray conc. kg a.s./hl	Proposed pre-harvest interval (days)

B.3.3.3.2. Information on any specific agricultural, plant health or environmental conditions under which the preparation may or may not be used

B.3.3.4. Recommended methods and precautions concerning: handling, storage, transport or fire (Annex IIIM 4.7)

B.3.3.5. Measures in the case of an accident (Annex IIIB 4.9)

Handling
PPE is required during mixing, loading or application due to the sensitisation potential of micro-organisms. The following PPE is recommended if there is a risk of skin or eye contact with the product:

Hand protection:	Chemical resistant (e.g. nitrile rubber) gloves
Eye protection:	Safety glasses/face shield
Skin and body protection:	Impermeable clothing
Hygiene measures:	Handle in accordance with good industrial hygiene and safety practice.

Storage
The product must be stowed away from food, drink and animal feeding stuffs and without access for children. The product should be stored under conditions which prevent entering any body of water and any misuse by non-authorised persons.

The product should be stored in compliance with GCPF (former GIFAP) Guidelines for Safe Warehousing of crop protection products and GIFAP Guidelines for Safe Handling of Pesticides during their Formulation, Packing, Storage and Transport.

For the recommended storage temperature see B.2.2.

Transport
Subtilex® is not classified hazardous under transport regulations.

Transport should be in compliance with GIFAP Guidelines for the safe transport of pesticides and Guidelines for Safe Handling of Pesticides during their Formulation, Packing, Storage and Transport.

Fire

Suitable extinguishing media:	Water spray, foam, dry powder, carbon dioxide (CO_2)
Specific hazards during fire fighting:	Explosive dust-air mixtures may form
Special protective equipment:	No specific equipment is required
Additional precautions:	If possible, avoid discharge of fire-extinguishing water into drains or water courses.

Waste minimisation
To minimise surplus product waste, users are recommended to buy not more than is consumed within the shelf life period of the product and to store the product before use according to storage recommendation (cool and dry). Always calculate the amount required and buy the appropriate container sizes.

B.3.3.6. Procedures for destruction or decontamination of the plant protection product and its packaging (Annex IIIM 4.10)

B.3.3.6.1. Controlled incineration

Leftover quantities of the product must be disposed of in a commercial incinerator according to local regulations. Incineration must be done under controlled conditions according to the directives 94/67/EC and 2000/76/EC:

- Residence time greater than 2 seconds
- Presence of more than 6 % of oxygen
- Temperature above 850 °C

Contaminated packaging has to be thoroughly rinsed with plenty of water, then burned in a commercial incinerator. In field applications the washing solutions have to be added to the spray mixture, otherwise they have to be burned in a commercial incinerator.

B.3.3.6.2. Others

No methods other than controlled incineration are recommended for disposal.

B.3.4. References relied on

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
Annex II Data and Information					
Annex III Data and Information					
IIIA 6.1 /01	G. Martin	2011	Mode of Action of *Bacillus subtilis* strain MBI600 against *Botrytis cinerea*. Becker Underwood, RD061108 Non-GEP, unpublished	Y	Becker Underwood
IIIA 6.1 /02	R. Morgan	2011	Inhibition of *Botrytis cinerea* spore germination by *Bacillus subtilis* strain MBI600. Becker Underwood, RD111104 Non-GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/03	E. Ferré	2011	Efficacy evaluation of Becker Underwood *Bacillus* *subtilis* strain MBI600 against *Botrytis cinerea* on grapevine. Anadiag S.A, 11 244 DR1 GEP, unpublished	Y	Becker Underwood

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIIA 6.1 and IIIA 6.2/04	E. Ferré	2011	Efficacy evaluation of Becker Underwood *Bacillus subtilis* strain MBI600 against *Botrytis cinerea* on grapevine. Anadiag S.A, 11 244 TL1 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/05	E. Ferré	2011	Efficacy evaluation of Becker Underwood *Bacillus subtilis* strain MBI600 against *Botrytis cinerea* on grapevine. Anadiag S.A, 11 244 TL2 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/06	R. Scherrer	2012	AN EVALUATION OF THE EFFICACY OF BUEXP1780S AGAINST *BOTRYTIS CINEREA* ON GRAPEVINE. Anadiag SAS, EU 12 180 AV1 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/07	R. Scherrer	2012	AN EVALUATION OF THE EFFICACY OF BUEXP1780S AGAINST *BOTRYTIS CINEREA* ON GRAPEVINE. Anadiag SAS, EU 12 180 TL1 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/08	R. Scherrer	2012	AN EVALUATION OF THE EFFICACY OF BUEXP1780S AGAINST *BOTRYTIS CINEREA* ON GRAPEVINE. Anadiag SAS, EU 12 180 TL2 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/09	O. Scrimshaw	2012	R&DX121111 - BU Botrytis on vines Summer 2012, 1 site in France. Eurofins Agroscience Services, S12-00873-01 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/10	O. Scrimshaw	2012	R&DX121111 - BU Botrytis on vines Summer 2012, 1 site in France. Eurofins Agroscience Services, S12-00873-05 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/11	E. Ferré	2011	Efficacy evaluation of Becker Underwood *Baclillus Subtilis* strain MBI600 against *Botrytis cinerea* on table grapes. Anadiag Italia SRL, ITA 11106 BA1 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/12	E. Ferré	2011	Efficacy evaluation of Becker Underwood *Baclillus Subtilis* strain MBI600 against *Botrytis cinerea* on table grapes. Anadiag Italia SRL, ITA 11106 BA2 GEP, unpublished	Y	Becker Underwood

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIIA 6.1 and IIIA 6.2/13	E. Ferré	2011	Efficacy evaluation of Becker Underwood *Baclillus Subtilis* strain MBI600 against *Botrytis cinerea* on table grapes. Anadiag Italia SRL, ITA 11107 TO1 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/14	R. Scherrer	2012	Evaluate the efficacy of BUEXP1780S against against *Botrytis cinerea* on grapevine. Anadiag Italia SRL, ITA 12140 BA1 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/15	R. Scherrer	2012	Evaluate the efficacy of BUEXP1780S against against *Botrytis cinerea* on grapevine. Anadiag Italia SRL, ITA 12140 BO1 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/16	R. Scherrer	2012	Evaluate the efficacy of BUEXP1780S against against *Botrytis cinerea* on grapevine. Anadiag Italia SRL, ITA 12141 TO1 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/17	R. Scherrer	2012	Evaluate the efficacy of BUEXP1780S against against *Botrytis cinerea* on grapevine. Anadiag Italia SRL, ITA 12141 TO2 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/18	R. Scherrer	2012	AN EVALUATION OF THE EFFICACY OF BUEXP1780S AGAINST *BOTRYTIS CINEREA* ON GRAPEVINE. Anadiag Portugal, PO 12 096 PO1 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/19	R. Scherrer	2012	AN EVALUATION OF THE EFFICACY OF BUEXP1780S AGAINST *BOTRYTIS CINEREA* ON GRAPEVINE. Anadiag Portugal, PO 12 096 PO2 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/20	O. Scrimshaw	2012	R&DX121111 - BU Botrytis on vines Summer 2012, 1 site in Portugal. Eurofins Agroscience Services, S12-00873-03 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/21	O. Scrimshaw	2012	R&DX121111 - BU Botrytis on vines Summer 2012, 1 site in Portugal. Eurofins Agroscience Services, S12-00873-04 GEP, unpublished	Y	Becker Underwood

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIIA 6.1 and IIIA 6.2/22	E. Fernandez	2012	FUNGICIDE EFFICACY AND SELECTIVITY OF THE FORMULATION BUEXP1780S (*Bacillus subtilis strain MBI600)* AGAINST GREY MOULD (*Botrytis cinerea*) ON GRAPEVINES IN SPAIN IN 2012. Promo-Vert Crop Services S.L., 12 F VI BU 01 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/23	E. Fernandez	2012	FUNGICIDE EFFICACY AND SELECTIVITY OF THE FORMULATION BUEXP1780S (*Bacillus subtilis strain MBI600)* AGAINST GREY MOULD (*Botrytis cinerea*) ON GRAPEVINES IN SPAIN IN 2012. Promo-Vert Crop Services S.L., 12 F VI BU 02 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/24	O. Scrimshaw	2012	R&DX121111 - BU Botrytis on vines Summer 2012, 1 site in Spain. Eurofins Agroscience Services, S12-00873-02 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/25	O. Scrimshaw	2012	R&DX121111 - BU Botrytis on vines Summer 2012, 1 site in Spain. Eurofins Agroscience Services, S12-00873-06 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/26	E. Ferré	2012	Efficacy evaluation of Becker Underwood *Bacillus subtilis* strain MBI600 against Botrytis cinerea on grapevine. Anadiag SA, 11 244 BM1 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/27	J.L. Paratte	2012	EFFICACY OF *Bacillus Subtilis* strain MBI600 AGAINST GREY MOULD (DUE TO Botrytis cinerea) ON GRAPEVINE. Promo-Vert Crop, 12 F VI BU 03 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/28	C. Carnaille	2012	EFFICACY OF *Bacillus Subtilis* strain MBI600 AGAINST GREY MOULD (DUE TO Botrytis cinerea) ON GRAPEVINE. Promo-Vert Crop, 12 F VI BU 04 GEP, unpublished	Y	Becker Underwood
IIIA 6.1 and IIIA 6.2/29	R. Scherrer	2012	AN EVALUATION OF THE EFFICACY OF BUEXP1780S AGAINST *BOTRYTIS CINEREA* ON GRAPEVINE. Anadiag SAS, EU 12 180 AN1 GEP, unpublished	Y	Becker Underwood

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIIA 6.1 and IIIA 6.2/30	R. Scherrer	2012	BUEXP1780S efficiency on vine grey mould. Inovitis, Inovitis 2012 GEP, unpublished	Y	Becker Underwood
IIIA 6.5/31	G. Martin	2011	Foliar persistence of BUEXP1780S on vine leaves. Becker Underwood, RD071101 Non-GEP, unpublished	Y	Becker Underwood

Draft Assessment Report

Bacillus amyloliquefaciens strain MBI600

Volume 3
Annex B.4
Proposals for classification and labelling

Rapporteur Member State : France

Volume 1

Level 1: Statement of subject matter and purpose for which the monograph was prepared

Level 2: Reasoned statement of the overall conclusions drawn by the Rapporteur Member State

Appendix 1: Standard terms and abbreviations

Appendix 2: Specific terms and abbreviations

Appendix 3: List of endpoints

Level 3: Proposed decision with respect to the application for inclusion of the active substance in Annex I

Level 4: Further information to permit a decision to be made, or to support a review of the conditions and restrictions associated with the proposed inclusion in Annex 1

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Annex A: List of the tests and studies submitted and of information available

Volume 3

Annex B: RMS summary, evaluation and assessment of the data and information

Annex B.1: Identity

Annex B.2: Biological, physical, chemical and technical properties

Annex B.3: Data application and further information.

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Annex B.6: Effects on human health

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Volume 4

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Version History of Volume 3 B4

Date	**Reason for revision**
December 2014	Initial DAR

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B.4. Proposals for classification and labelling

B.4.1. Proposals for classification and labelling of the MPCA

Classification and labelling of chemical substances based on the criteria according to Regulation (EC) No 1272/2008 and Directive 67/548/EEC and are not applicable to micro-organisms (Pesticide Risk Assessment Peer Review, Pre-meeting for microorganisms, February 2007).
In the EU the experts agreed on the possible sensitising potential after dermal and/or inhalation exposure of micro-organisms (PRAPER Expert Meeting on Micro-organisms M03, June 2009) and a hazard statement has to be applied mandatory unless acceptable experimental studies revealed no hazard potential:
As the available methods for testing dermal sensitisation are not suitable for testing micro-organisms and there are no validated test methods for sensitization by inhalation, all micro-organisms should be regarded as potential sensitisers (Regulation (EU) No 283/2013).

***Bacillus amyloliquefaciens* strain MBI600:**

Labelling
In conclusion, for ***Bacillus amyloliquefaciens strain* MBI 600**, the following hazard statement is proposed:
'Micro-organisms may have the potential to provoke sensitising reactions'.

B.4.2. Proposals for classification and labelling of the MPCP

Preparation Subtilex®:

Classification

In accordance with Directives 67/548/EEC and 1999/45/EC the following classification is proposed for the preparation:

Hazard symbol:	None
Indication of danger:	None
Risk phrases:	None

According to Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16th December 2008, the following classification is proposed for the preparation:

Hazard class:	None
Signal word:	None
Hazard statements:	None

Labelling
'Contains *Bacillus amyloliquefaciens* strain MBI600, micro-organisms may have the potential to provoke sensitising reactions'.

This labelling phrase implies that PPE have to be worn when handling the product or applying the product :

- Gloves (nitrile, EN 374-3)
- Working coveralls
- Disposable filtering facepiece respirator to at least EN149 FFP3 or equivalent

B.4.3. References relied on

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
Annex II Data and Information					
Annex III Data and Information					

Draft Assessment Report

Bacillus amyloliquefaciens strain MBI600

Volume 3

Annex B.5

Analytical methods

Rapporteur Member State : France

Version History of Volume 3 B5

Date	**Reason for revision**
December 2014	Initial DAR

Table of contents

B.5. Analytical methods (Annex IIM 5)

B.5.1. Methods for the analysis of the micro-organism as manufactured and for the analysis of the preparation (Annex IIM 4.3)

B.5.1.1. Methods for the analysis of the micro-organism as manufactured

B.5.1.1.1. Methods for the identification of the micro-organism

See Volume 3, B.1.1.3.3.

B.5.1.1.2. Methods for providing information on possible variability of seed stock/active micro-organism

Mother stock cultures are maintained by the manufacturer as described in the Volume 4, C.1.1. .

B.5.1.1.3. Methods to differentiate a mutant of the micro-organism from the parent wild strain

Each fermentation run is started with initial seed stock culture, which is maintained frozen in vials. Thus mutations in the original parent strain MBI600 are precluded. In addition the above described QC methods are employed and the colony growth and appearance are closely monitored.

B.5.1.1.4. Methods for the establishment of purity of see d stock from which batches are produced and methods to control that purity

See Volume 4, C.1.2.

B.5.1.1.5. Methods to determine the content of the micro-organism in the manufactured material used for the production of formulated products and methods to show that contaminating micro-organisms are controlled to an acceptable level

Method 1:

Report: 4.3.1/03 Anon (2008) Kosan Analytical Test Method Spore Concentration and Bacterial Contamination, Idemitsu Kosan, No Report No.
Guideline: None
GLP: No

Principle of the Method

Samples of the MPCA powder are plated for overnight incubation prior to counting to determine the number of viable colonies. All enumerations of the active substance in MBI600 were conducted by plating serial dilutions of a known quantity of spore powder on a suitable agar medium. Only viable spores will grow on the agar, resulting in discreet colonies. However, a single colony may have grown from a single spore, or from a cluster of spores that were insufficiently separated during sample preparation. Consequently the convention is to state active substance content in terms of colony forming units (cfu) per gram.

Description of the method

Powder preparation
Weigh and aseptically transfer 0.99-1.01 grams of unknown or test powder to mixing with 100 ml of sterile water in Pyrex bottle. After 15 minutes shake vigorously and resume platform mixing to complete a 30 minute primary mixing cycle. Sonicate for 15 minutes, shaking half way through. Stir for another 60 minutes.

Assay dilution preparation
To 7 sterile tubes dispense 4.5 ml of peptone broth and number 10^{-3} through 10^{-9}. To lower the chance of background contamination, do this 30 minutes before the end of the powder prep mixing. Place the 10^{-8} dilution tube in the 70°C heating block for pre-heating. Label 5 nutrient agar plates for each test powder (run number/date/dilution). The plating dilution is 10^{-10}. The plates should have been incubated for three days at 30°C prior to use to insure they are free of contamination and dry.

Powder solution dilution
After the powder mixing cycle is completed, transfer 0.5 ml of solution serially through the dilution series. Post solution delivery, vortex the dilution blank. At the 10^{-8} tube delivery, return the tube to the 70°C heating block for 30 minutes of incubation. After the 10^{-8} tube has completed the 70°C incubation, complete the dilution series to the 10^{-9} tube.

Plating
Using a 1.0 ml pipette deliver 0.1 ml to each of the 5 nutrient agar plates for the particular test sample. Using a disposable spreading bar, spread the dilution sample on the plate, then place in an incubator at 37°C. This will be counted after an overnight period.

Enumeration
The plates are counted after overnight incubation (16-20 hours). They are counted again on the second day. Only plates having more than 30 and less than 300 colonies are counted. The resulting number is averaged and multiplied by 10^{10} CFU/g.

Validation data:
No validation data were provided.

Conclusion
A method Anon (2008) for the determination of *Bacillus amyloliquefaciens* strain MBI600 is provided but no validation data are available. Nevertheless, as validation data were provided for method described below, no more data required.

Method 2:

Report: 4.3.1/01: Whittaker, M. (2013) Detection and enumeration of *Bacillus amyloliquefaciens* and microbial contaminants in five production batches of MBI600 Technical Grade Active Ingredient. Test facility: APIS, UK., Sponsor: BASF Agricultural Specialities, UK, Unpublished report No. BUBA003,
Guideline: OECD ENV/JM/MONO(2011) 43
GLP: Yes

Report: 4.3.1/01: Whittaker, M. (2014) Detection and enumeration of *Bacillus amyloliquefaciens* and microbial contaminants in five production batches of SUBTILEX Microbial Pest Control Product. APIS. Tudy: APIS-BASF-006, Unpublished
Guideline: OECD ENV/JM/MONO(2011) 43
GLP: Yes

Principle of the Method
The test item is plated on microbiological media and the concentration calculated from plate counts as CFU/g.

Description of the method
The test item was handled aseptically. One gram of each batch was weighed out accurately (to 1.0 mg) and added to 99.0 mL of sterile (autoclaved) phosphate buffered saline (PBS) in a 250 ml sterile Nalgene jar which placed onto a magnetic stirrer for 10 minutes. This provided a 1 x 10^{-2} dilution. The jar was transferred to an oven set at 70°C for 30 minutes and after placed in an ice bath. The jar was transferred for sonication during 10 minutes at 50% output power. The suspension was allowed to rest for 10 minutes before sonication resumed for a further 10 minutes.

1.0 ml of the suspension was dispensed into 9.0 ml of buffer, yielding a 1 x 10^{-3} dilution. The tube was vortex mixed for 10 seconds and the process repeated to provide 1 x 10^{-4} through to 1 x 10^{-10} dilutions, vortex mixing for 10 seconds between each.

3 ml aliquots of each dilution from 1 x 10^{-6} through to 1 x 10^{-10} were dispensed into glass tubes and placed in an ultrasonic bath for 3 minutes, after which 100 µl from the appropriate dilution tubes was plated in triplicate on trypticase soy agar plates. Separately, 100 µl of the dilution buffer was plated in triplicate to ensure the efficiency of the autoclaving process.
Plates were incubated at 37°C ± 2°C and read after 24 hours. The concentration of active MPCA ingredient in test item was calculated from plate counts as CFU/g. The triplicate plate count from a dilution producing ~30-300 colonies/plate was averaged for each batch and the mean of this calculation for all batches was used to determine an average count for all 5 batches cumulatively. Standard and relative standard deviations were also calculated.

Validation data:

Repeatability
Mean of five batches analyses: 9.4 × 10^{11} CFU/g
%RSD: 37.9
For details of results see Volume C, C.1.2.7.

Conclusions:
The method is suitable for enumeration of *Bacillus amyloliquefaciens* strain MBI600.

General conclusion for the determination of *Bacillus amyloliquefaciens* strain MBI600 in MPCA
A method Whittaker, M. (2013, 2014) for the determination of *Bacillus amyloliquefaciens* strain MBI600 in MPCA is available and is considered sufficient.

B.5.1.1.6. Methods for the determination of relevant impurities in the manufactured material

Complementary data are still missing. See Volume 4, C.1.2.1.

B.5.1.1.7. Methods to control the absence and to quantify (with appropriate limits of determination) the possible presence of any human and mammalian pathogen

Staphylococcus aureus (4.3.1/01Whittaker M. 2013)

A 1 x 10^{-1} stock suspension of each batch was made by placing 25 g of the test item in sterile flask with 225 ml of sterile phosphate buffered saline and shaking gently.

As it is highly selective and differential and allows for decreased ambiguity between *S. aureus* and other non-pathogenic staphylococcal species, Mannitol Salt agar was employed for this test. The MSA medium was prepared according to manufacturer's instructions, then cooled to approximately 47°C using a water bath. The appropriate quantity of the medium was poured into sterile Petri dishes and allowed to solidify. Before use, plates were incubated at 35-39°C to dry until any condensate droplets disappeared from the surface of the medium.
1.0 ml of the initial (1 x 10^{-1}) suspension was inoculated onto the surface of each of nine agar plates and spread as quickly as possible using a sterile plate spreader. A further three plates were inoculated with a reference culture of *Staphylococcus aureus* to act as a positive control. The plates were allowed to dry with lids on for about 15 minutes at laboratory temperature before being inverted and incubated at 35-39°C for 24 hours ± 2 hours. After this time plates were removed and assessed for growth, with typical colonies being yellow on a yellow background and atypical colonies being pink on a pink background.

Escherichia coli and other coliforms (4.3.1/01Whittaker M. 2013)
A preliminary screen was conducted with a Colilert-24 test kit using a reference culture of *E. coli* as a positive control. The contents of a Colilert test vial and 1.0 g of test substance were added to 100 ml of deionised water in a sterile non-fluorescing vessel, capped, shaken and then incubated at 35 ± 1.0°C for 24 hours, after which vessels were examined under UV light. Yellow fluorescence indicates a positive result.

The presence of *E. coli* was assessed by plating 1.0 ml of the 1 x 10^{-1} stock suspension onto each of nine TBX plates, prepared according to the manufacturer's instructions. A further three plates were inoculated with a reference culture of *E. coli* to act as a positive control. The plates were allowed to dry with lids on for about 15 minutes at laboratory temperature before being inverted and incubated at 37 ± 2°C for 48 hours. After this time plates were removed and assessed for growth, with positive colonies being blue-green.

For other coliforms, 1.0 ml of the 1 x 10^{-1} stock suspension was plated onto the selective and differential media HiChrome Coliform Agar, prepared according to the manufacturer's instructions. A further three plates were inoculated with a reference culture of *E. coli* to act as a positive control. The plates were allowed to dry with lids on for about 15 minutes at laboratory temperature before being inverted and incubated at 37 ± 2°C for 48 hours. After this time plates were removed and assessed for growth, with *E. coli* forming dark blue/violet colonies and other coliforms forming salmon/red colonies.

Salmonella (4.3.1/01Whittaker M. 2013)
Two approaches were used to detect *Salmonella* contamination. In the first, 1.0 ml of the initial (1 x 10^{-1}) suspension was inoculated onto the surface of each of nine SSA plates and spread as quickly as possible over the surface using a sterile plate spreader. A further three plates were inoculated with a reference culture of *Salmonella typhimurium* to act as a positive control. The plates were allowed to dry with lids on for about 15 minutes at laboratory temperature before being inverted and incubated at 35-39°C for 48 hours. After this time plates were removed and assessed for growth, with typical colonies being colourless with black centres.

In the second approach, 1.0 ml of the initial (1 x 10^{-1}) suspension was inoculated onto the surface of each of nine HEA plates and spread as quickly as possible over the surface using a sterile plate spreader. A further three plates were inoculated with a reference culture of *Salmonella typhimurium* to act as a positive control. The plates

were allowed to dry with lids on for about 15 minutes at laboratory temperature before being inverted and incubated at 35-39°C for 24 hours. After this time plates were removed and assessed for growth, with typical colonies being blue-green, sometimes with black centres.

Vibrio (4.3.1/01Whittaker M. 201; 1.4.4/02 Whittaker M. 2014)
The selective and differential medium HiChrome Vibrio agar was used, prepared according to the manufacturer's instructions. Stock culture flasks were incubated at 35 ± 2°C for 7 ± 1 hours, after which a 3 mm loop from the surface pellicle of the culture was transferred to the surface of nine dried plates and streaked in a manner that would yield isolated colonies. No positive control was included due to inherent health risk to lab personnel. Plates were incubated for 18 - 24 hours at 35° ± 2°C. After this time plates were removed and assessed for growth, with typical colonies being blue-green to purple.

Furthermore, three plates of HiChrome Vibrio agar medium were streaked with the reference culture *Vibrio alginolyticus* ATCC 17749 and incubated at approximately 37°C for approximately 24 hours before being read (positive control).

Shigella (4.3.1/01Whittaker M. 2013; 1.4.4/02 Whittaker M. 2014)
25 g of test item was weighed into 225 ml of *Shigella* broth, prepared according to the manufacturer's instructions, to which 0.5 µg/ml novobiocin had been added following autoclaving. The suspension was held for 10 ± 2 minutes at room temperature and shaken periodically. The supernatant was poured into a sterile 500 ml Erlenmeyer flask and the pH adjusted to 7.0 ± 0.2 with sterile 1 N NaOH.

The flasks were incubated at 44 ± 2°C and 5% CO_2 for 20 ± 2 hours, after which the enrichment culture suspension was agitated and streaked onto nine SSA and nine HEA plates. No positive control was included due to inherent health risk to lab personnel. Plates were incubated for 20 ± 2 hours at 35 ± 2°C. After this time plates were removed and assessed for growth, with typical colonies being transparent on both media.

Furthermore, three plates of HEA medium were streaked with the reference culture *Shigella boydii* ATCC 9207 and incubated at approximately 37°C for approximately 22 hours before being read (positive control).

Anaerobic bacteria (4.3.1/01Whittaker M. 2013)
1.0 ml of the initial (1×10^{-1}) suspension was inoculated onto the surface of each of nine pre-poured TSA plates and spread as quickly as possible over the surface using a sterile plate spreader. A further three plates were inoculated with a reference culture of the obligate anaerobe *Bacteroides fragilis* in thioglycollate broth culture to act as a positive control. The plates were immediately transferred to an anaerobic gas bag and incubated at 28 ± 2°C for 72 hours.

Yeast and moulds (1.4.4/02 Whittaker M. 2014)
1.0 mL of the enumeration suspension was inoculated onto each of nine yeast and mould Agar plates (YMA). A further six plates were inoculated with reference cultures of *Candida albicans* (three plates) and Aspergillus brasoliensis (three plates). Plates were incubated at approximately 26°C for approximately 48 hours and then observed for growth.

Validation data:
The validity of the test was confirmed via the use of positive controls and via the repeatability of test for each plate (See volume C. C1.2.2.2.).
No method and no validation data were provided for *Listeria monocytogenes.* These data are not required as according to the OECD issue paper on microbial contaminants the screening for *Listeria monocytogenes* is optional, especially where screening for other indicators of hygienic production consistently demonstrate acceptably low levels of contamination. As none of the human pathogens indicated in the OECD issue paper on microbial contaminants have been detected in the 5-batch analysis of MBI600, a screen for *Listeria monocytogenes* was not considered necessary.

Conclusions:
Provided methods are suitable for screening for microbial contaminants in *Bacillus amyloliquefaciens* strain MBI600.

B.5.1.1.8. Methods to determine storage stability, shelf-life of the micro-organism, if appropriate

This OECD data point is not an EC data requirement.

B.5.2. Methods for the analysis of the preparation (Annex IIIM 5)

B.5.2.1. Methods for the identification and the determination of the content of the micro-organism(s) in the preparation

Report: 4.3.1/01: Whittaker, M. (2014) Detection and enumeration of *Bacillus amyloliquefaciens* and microbial contaminants in five production batches of SUBTILEX Microbial Pest Control Product. APIS. Tudy: APIS-BASF-006, Unpublished
Guideline: OECD ENV/JM/MONO(2011) 43
GLP: Yes

Principle of the Method

The test item was handled aseptically. Approximately one gram of each batch was weighed out accurately (to 1 mg) and added to 99.0 ml of sterile (autoclaved) phosphate buffered saline (PBS) in a 250 ml sterile Nalgene jar. A magnetic stir bar was placed into the jar, the jar was capped and placed onto a magnetic stirrer for 10 minutes. This provided a 1 x 10-2 dilution.
After stirring the jar was transferred to an oven set at 70°C for 30 minutes, after which the jar was placed in an ice bath. Whilst in the ice bath the jar was transferred to the soundproof box of an MSE Soniprep 150 300W Ultrasonic Processor with a 19 mm diameter probe, and the probe inserted into the suspension to a depth of approximately 1.5 mm. The sonicator was run continuously for 10 minutes at 40% output power. The suspension was allowed to rest for 10 minutes before sonication resumed for a further 10 minutes. The jar was then removed from the sonicator and placed on the magnetic stirrer for 10 minutes. 1.0 ml of the suspension was dispensed into 9.0 ml of PBS, yielding a 1 x 10^{-3} dilution. The tube was vortex mixed for 10 seconds and the process repeated to provide 1 x 10^{-4} through to 1 x 10^{-10} dilutions, vortex mixing for 10 seconds between each.
3 ml aliquots of the 1 x 10-6 through to the 1 x 10^{-10} dilution were dispensed into glass tubes and placed in an ultrasonic bath for 3 minutes, after which 100 µl of each dilution was plated in triplicate on Trypticase Soy Agar plates. Plates were incubated at 37°C +/- 2°C and read after 18-24 hours.

Validation data:

Repeatability
Mean of five batches analyses: 8.8 × 10^{10} CFU/g
%RSD: 7.5
For details of results see Volume C, C.1.3.3.

Conclusions:
A method Whittaker, M. (2014) for the determination of *Bacillus amyloliquefaciens* strain MBI600 in the representative formulation SUBTILIEX is available and is considered validated.

B.5.2.2. Methods to establish regular control of the preparation to show that it does not contain other organisms than the indicated ones and to establish uniformity

Report: IIIM1 5.1.3/01 Morgan, L. (2013) Accelerated Storage Stability of BUEXP1780S for up to 12 weeks at 35°C stored in an aluminium laminate bag. Method: SJ-SOP-8202-BBRC; Test facility: Battelle UK Ltd., UK; Sponsor: Becker Underwood Ltd, UK Unpublished report No. LG/12/002/1
Guideline: Regulation 1107/2009
Regulation 545/2011
GLP: Yes

Principle of the Method
Samples of the MPCP powder are plated for overnight incubation prior to counting to determine the number of viable colonies.

Description of the method
Dispense dry powder product into dilution buffer to provide a 10^{-2} dilution. Add sterile glass beads to the test sample. Switch on the heating block and standardize to 70±2°C.Place the test sample in a slanted position (30-45 degrees inclination) on a rotary shaker and agitate on the rotary shaker. Following 10-fold dilution steps, transfer 1.0 ml of the test sample into 9.0 ml of dilution buffer blank contained in the disposable polystyrene round-bottom tubes. This provides 10^{-3} dilution. Vortex the 10^{-3} diluted sample and continue the 10-fold dilution series to obtain 10^{-7} and 10^{-8} test sample dilutions. Dispense 3 ml each of the 10^{-8} and 10^{-7} dilutions into sterile screw-cap Pyrex glass tubes. (These tubes should fit snugly into the heating block). Place the tubes into the heating block and heat shock. Dispense 0.1 ml of 10^{-8} dilution heat shocked sample onto the surface of a pre-dried (equilibrated to room temp for a minimum of 2 hours) Trypticase Soy agar plate. Dispense another 0.1 ml on a second and third agar plate. Spread plate. Similarly, plate the 10^{-7} dilution in triplicate. Incubate the plates at 37±2°C for 18 to 24 hours. Count and record number of colonies for each dilution (30-300 colonies) and calculate the number of spores in the original undiluted sample by multiplying the average colony count times the dilution factor.

Validation data:
Validation data of the method Morgan, L. (2013) for the determination of *Bacillus amyloliquefaciens* MBI600 in the formulation Subtilex® are not available; nevertheless, as sufficient validation data are available in the study Whittaker, M. (2014), no more data required.

B.5.2.3. Methods to identify any contaminating micro-organisms of the preparation

Report: 4.3.1/01: Whittaker, M. (2014) Detection and enumeration of *Bacillus amyloliquefaciens* and microbial contaminants in five production batches of SUBTILEX Microbial Pest Control Product. APIS. Study: APIS-BASF-006, Unpublished
Guideline: OECD ENV/JM/MONO(2011) 43
GLP: Yes

Staphylococcus aureus
1.0mL of the stock suspension was inoculated onto each of nine CHROMAgar Staph aureus (CASA) plates. A further three plates were inoculated with a reference culture of *Staphylococcus aureus*. Plates were incubated at approximately 37°C for approximately 24 hours and then observed for growth.

Escherichia coli and other coliforms
A preliminary screen was conducted with a Colilert-24 test kit using a reference culture of E. coli as a positive control. The contents of a Colilert test vial and 1.0 g of test substance were added to 100 ml of deionised water in a sterile non-fluorescing vessel, capped, shaken and then incubated at 35 +/- 1.0°C for 24 hours, after which vessels were examined under UV light. Yellow fluorescence indicates a positive result.
The presence of E. coli was assessed by plating 1.0 ml of the stock suspension onto each of nine CHROMAgar E. coli (CAEC) plates. A further three plates were inoculated with a reference culture of E. coli. Plates were incubated at approximately 37°C for approximately 24 hours and then observed for growth.

Salmonella
The method used is the same as described in B.5.1.1.7.

Vibrio
Approximately 25 g of each batch of test item was weighed into a sterile flask containing 225 ml of alkaline peptone water. The flask was thoroughly mixed for approximately 2 minutes and then incubated at 37°C for 7 hours. A 3 mm loop from the surface pellicle was streaked onto each of nine CHROMAgar Vibrio (CAVIB) plates. A further three plates were inoculated with a reference culture

of *Vibrio alginolyticus*. Plates were incubated at approximately 37°C for approximately 24 hours and then observed for growth.

Shigella
The method used is the same as described in B.5.1.1.7.

Enterobacter
1.0 ml of the stock suspension was inoculated onto each of nine Violet Red Bile Glucose Agar (VRBGA) plates. A further three plates were inoculated with a reference culture of *Enterobacter cloacae*. Plates were incubated at approximately 37°C for approximately 24 hours and then observed for growth.

Anaerobic bacteria
The method used is the same as described in B.5.1.1.7.

Listeria monocytogenese
1.0 mL of the stock suspension was inoculated onto each of nine Listeria Selective Agar (LSA) plates. A further three plates were inoculated with a reference culture of *Listeria monocytogenes*. Plates were incubated at 37°C for approximately 48 hours and then observed for growth.
The incubation temperature stated in the study plan was 30°C. However, public domain data show that the optimum growth temperature for *Listeria* species is between 30°C and 37°C, and as all positive controls showed strong growth this deviation had no effect on the outcome of the study.

Yeast and moulds
The method used is the same as described in B.5.1.1.7.

Validation data:
The validity of the test was confirmed via the use of positive controls and via the repeatability of test for each plate (See volume C. C1.3.3.).
No method and no validation data were provided for *Listeria monocytogenes*. These data are not required as according to the OECD issue paper on microbial contaminants the screening for *Listeria monocytogenes* is optional, especially where screening for other indicators of hygienic production consistently demonstrate acceptably low levels of contamination. As none of the human pathogens indicated in the OECD issue paper on microbial contaminants have been detected in the 5-batch analysis of the formulation, a screen for *Listeria monocytogenes* was not considered necessary.

Conclusions:
Provided methods are suitable for screening for microbial contaminants in the formulation SUBTILEX

B.5.2.4. Methods used to determine the storage stability and shelf life of the preparation

See B.5.2.2..

B.5.3. Methods to determine and quantify residues (viable or non-viable) of the micro-organism as manufactured and for the analysis of the preparation (Annex IIM 4.5 and IIIM 5.4)

B.5.3.1. Methods to determine and quantify residues (viable or non-viable) of the micro-organism

B.5.3.1.1. The active micro-organism(s) on and/or in crop, in foodstuffs and feeding stuffs, in animal and human body tissues and fluids, in soil, in water (including drinking water, ground water and surface water) and in air where relevant

No residue definition is applicable for *Bacillus amyloliquefaciens* strain MBI600 or its metabolites. Therefore no post-registration monitoring methods are needed.

B.5.3.1.2. Relevant metabolites (especially toxins) on and/or in crop, in foodstuffs and feeding stuffs, in animal and human body tissues and fluids, in soil, in water (including drinking water, ground water and surface water) and in air where relevant

No relevant non-microbial impurities are contained in the product as *Bacillus amyloliquefaciens* strain MBI600 is not able to produce any toxin.

B.5.3.1.3. Methods to determine and quantify residues (viable or non-viable) of the micro-organism for the analysis of the preparation

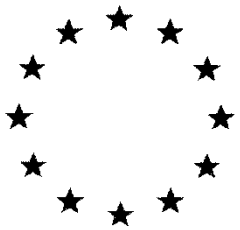
Not relevant. Method is not required.

B.5.4. References relied on

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
Annex II Data and Information					
IIM, 4.3.1/01	Whittaker, M.	2013	Detection and enumeration of *Bacillus amyloliquefaciens* and microbial contaminants in five production batches of MBI600 Technical Grade Active Ingredient APIS, UK Becker Underwood Report No. BUBA003 GLP Unpublished ⇨ Doc J 1.4.4/01	Y	BASF Agricultural Specialities Ltd
IIM, 4.3.1/02	Kloepper, J.W.	2012	No Title Auburn University, USA Becker Underwood No report No. Not GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM, 4.3.1/03	Anon	2008	Kosan Analytical Test Method Spore Concentration and Bacterial Contamination Idemitsu Kosan, Japan Becker Underwood No report No. Not GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM, 4.3.1/04	Anon	2008	Kosan Analytical Test Method Identity Test *Bacillus amyloliquefaciens* MBI600 spores Idemitsu Kosan, Japan Becker Underwood No report No. Not GLP Unpublished ⇨ 4.3.1/03	Y	BASF Agricultural Specialities Ltd
Annex III Data and Information					

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIIM 5.2.2.	Morgan, L.	2013	Accelerated Storage Stability of BUEXP1780S for up to 12 weeks at 35°C stored in an aluminium laminate bag. Battelle UK Ltd. Becker Underwood Report No. LG/12/002/1 GLP Unpublished ⇨ 2.1/01	Y	BASF Agricultural Specialities Ltd
IIIM 4.3.1/01	Whittaker, M.	2014	Detection and enumeration of Bacillus amyloliquefaciens and microbial contaminants in five production batches of SUBTILEX Microbial Pest Control Product. APIS. Study: APIS-BASF-006, GLP Unpublished	Y	BASF Agricultural Specialities Ltd

Draft Assessment Report



Bacillus amyloliquefaciens strain MBI600

Volume 3

Annex B.6

Effects on human health

Rapporteur Member State : France

Volume 1

Level 1: Statement of subject matter and purpose for which the monograph was prepared

Level 2: Reasoned statement of the overall conclusions drawn by the Rapporteur Member State

Appendix 1: Standard terms and abbreviations

Appendix 2: Specific terms and abbreviations

Appendix 3: List of endpoints

Level 3: Proposed decision with respect to the application for inclusion of the active substance in Annex I

Level 4: Further information to permit a decision to be made, or to support a review of the conditions and restrictions associated with the proposed inclusion in Annex 1

Volume 2

Annex A: List of the tests and studies submitted and of information available

Volume 3

Annex B: RMS summary, evaluation and assessment of the data and information

Annex B.1: Identity

Annex B.2: Biological, physical, chemical and technical properties

Annex B.3: Data application and further information.

Annex B.4: Proposals for classification and labelling

Annex B.5: Analytical methods

Annex B.6: Effects on human health

Annex B.7: Residues data

Annex B.8: Fate and behaviour in the environment

Annex B.9: Effects on non-target organisms

Annex B.10: Summary and evaluation of environmental impact

Appendix 1: Standard terms and abbreviations

Appendix 2: Specific terms and abbreviations

Volume 4

Annex C: Confidential information and summary and assessment of information relating to the collective submission of dossiers

Version History of Volume 3 B6

Date	**Reason for revision**
December 2014	Initial DAR

Table of contents

B.6. Effects on human health (Annex IIM 5)

B.6.1. Tier I – the active micro-organism

B.6.1.1. Basic information (Annex IIM 5.1)

Bacillus amyloliquefaciens is a ubiquitous soil bacterium, certain strains of which have applications against soil-borne and post-harvest pathogenic fungi. Strain MBI600 colonises the roots and developing shoot systems of plants, suppressing by competition disease organisms such as *Botrytis, Fusarium, Rhizoctonia*, and *Alternaria* as well as those organisms causing powdery mildew and anthracnose.

Work carried out at Auburn University, using more accurate methods, has resulted in the re-classification of *Bacillus subtilis* strain MBI600 as *Bacillus amyloliquefaciens* strain MBI600. The organism has not changed and therefore all proprietary studies performed and reported with the strain previously identified as *Bacillus subtilis* strain MBI600 have actually been carried out with the organism that is now identified as *Bacillus amyloliquefaciens* strain MBI600. In some cases *Bacillus subtilis* literature data are referred to in this report. For the reasons stated above, it is considered that these data are equally applicable to *Bacillus amyloliquefaciens*, which is part of the *Bacillus subtilis* group.

Bacillus amyloliquefaciens MBI600 was isolated from broad bean in United Kingdom. *Bacillus amyloliquefaciens* MBI600 is neither a mutant nor a genetically modified strain.
Bacillus amyloliquefaciens MBI600 has been used in plant protection products since 1984 in USA and since 2007 in Canada.
Different strains of the species are approved for in Articles 5 and 6 of Directive 91/414/EEC1 concerning the placing of plant protection products on the market. Further uses include feed and food additives and biotechnological application i.e. enzyme production.

The Panel on Biological Hazards of EFSA[1] has considered that *Bacillus amyloliquefaciens* is suitable for Qualified Presumption of Safety (QPS) assessment for biological agents intentionally added to food and feed with the qualification of absence of toxigenic activity. Following the annual updates of QPS list, EFSA has confirmed the recommendation of *Bacillus amyloliquefaciens* for the QPS list based on a literature review for the preceding years (EFSA QPS updates in 2010, 2011, 2012 and 2013). Moreover, for all QPS bacterial taxonomic units, the strains should not harbour any acquired antimicrobial resistance genes to clinically relevant antibiotic. Two studies of antibiotic resistance were performed on *Bacillus amyloliquefaciens* MBI600. Results were detailed in the Volume 3 B2.1.9. *Bacillus amyloliquefaciens* strain MBI600 is susceptible to a wide spectrum of antibiotics commonly used in human and veterinary medicine.

Bacillus amyloliquefaciens is not listed in the directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work.

A search of the published literature has been conducted using CAB direct and ChemSpider.
CAB Direct is the most thorough and extensive source of reference in the applied life sciences, incorporating the leading bibliographic databases CAB Abstracts and Global Health. Its coverage of the applied life sciences includes agriculture, environment, veterinary sciences, applied economics, food science and nutrition.
ChemSpider is a free chemical structure database providing fast access to over 29 million structures, properties and associated information. By integrating and linking compounds from more than 440 data sources, ChemSpider enables researchers to discover the most comprehensive view of freely available chemical data from a single online search. It is owned by the Royal Society of Chemistry.

The following relevance criteria were applied to database searches conducted under each data point:

- Publication date should be between 2003 and 2013
- Key words must appear in the title field
- Must be of clear relevance to the corresponding data point.

[1] *EFSA Journal (2007) 587, 1-16 Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA Opinion of the Scientific Committee.*

Due to the low number of results returned even for broad search terms, the relevance criteria are not restrictive.

The completed search term used for the section 3was presented in follow document:

Document 1: Doc K IIA O

RMS commentary: while requested to the applicant, the review of the scientific open literature provided does not strictly follow the EFSA guidance on Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009 (EFSA Journal 2011;9(2):209)2.
The relevance criteria have not been fully described for each topic, the studies selected have not been assessed for their reliability and none the summaries have been reported.
The methodology performed to select data published before 2003 and that to cover grey literature has not been described.
However an extended review has been performed and those limitations do not invalidate the overall conclusions.

B.6.1.1.1. Medical data (Annex IIM 5.2)

A case of familial pneumonitis induced by *Bacillus subtilis* has been described by Johnson *et al*. (1980): six members of one family developed symptom consistent with hypersensitivity pneumonitis after exposure to wood dust generated during the remodelling of a bathroom in their house. A detailed microbiologic investigation of the house and surrounding areas resulted in the isolation of *Bacillus* species. All symptomatic members of the family demonstrated positive bronchoprovocation responses to vegetative cell extracts of *Bacillus subtilis*.

Reference: Johnson, C.L.; Bernstein, I.L.; Gallagher, J.S.; Bonventre, P.F.; Brooks, S.M. (1980)
Familial hypersensitivity pneumonitis induced by *Bacillus subtilis*
American Review of Respiratory Disease 1980; 122:339-348

Abstract: Six members of one family developed symptoms consistent with hypersensitivity pneumonitis after exposure to wood dust generated during the remodeling of a bathroom in their house. A detailed microbiologic investigation of the house and surrounding areas resulted in the isolation of 2-Bacillus species. The vegetative cell and spore extracts of these organisms were used for extensive in vivo and in vitro laboratory tests. All symtomatic members of the family demonstrated positive bronchoprovocation responses to vegetative cell extracts of B. subtilis. Two patients with clinical disease exhibited immediate positive skin tests to similar extracts. Positive lymphoproliferative responses to the vegetative cell extract of B. subtilis were observed in 4 of 5 symptomatic patients and in 1 additional patient tested with the spore form of B. subtilis. It is postulated that the abrupt appearance of this family epidemic depended on the special circumstance of continued exposure to high concentrations of B. subtilis organisms within the household. B. subtilis should be added to the list of antigens causing hypersensitivity penumonitis. Prompt recognition and elimination of this new causal agent could prevent irreversible lung damage in susceptible patients.

B.6.1.1.2. Medical surveillance on manufacturing plant personnel

No evidence of adverse health effects have been observed in any of the employees involved in the production, handling and application of *Bacillus amyloliquefaciens* strain MBI600. However, microbial products are generally expected to be skin sensitisers, due to the high protein content. It is therefore important that employees, workers and operators handling *Bacillus amyloliquefaciens* strain MBI600 wear appropriate protective equipment.

B.6.1.1.3. Sensitisation/allergenicity observations, if appropriate

No evidence of adverse health effects have been observed in any of the employees involved in the production, handling and application of *Bacillus amyloliquefaciens* strain MBI600.
Moreover, in Caballero *et al*. (2007), 86 workers who were exposed to fungal enzymes in 2 animal feed factories were tested for their sensitisation response to *Bacillus amyloliquefaciens* by skin prick tests. *Bacillus amyloliquefaciens* did not cause sensitisation in any workers.

Reference: Caballero M.L.; Gomez M.; Gonzalez-Munoz, M.; Reinoso, L.; Rodrigues-Perez, R.; Moneo, I. (2007)
Occupational sensitisation to fungal enzymes used in animal feed industry
Int Arch Allergy Immunol 2007; 144:231-239

Abstract:
BACKGROUND:
Industrial enzymes cause the increasing prevalence of occupational hypersensitivity. Our objective was to study workers occupationally exposed to fungal enzymes in 2 animal feed factories to determine if the sensitization originated in the enzymes or was caused by the microorganism used to produce the enzymes.
METHODS:
Eighty-six consenting workers were studied by skin prick tests with extracts from the enzymatic products handled in their factories. Positive workers were then studied by IgE immunoblotting and basophil activation was measured by flow cytometry.
RESULTS:
Eight of the 86 workers analysed (9%) tested positive and were more frequently sensitized to phytase from Trichoderma and Peniophora. Glucanase and alpha-amylase from Bacillus amyloliquefaciens did not cause sensitization in any worker. No cross-reactions were observed between Trichoderma and Peniophora sp. phytases. Workers were sensitized to the product that they handled.
CONCLUSIONS:
Fungal enzymes cause occupational hypersensitivity in animal feed industries. Immunoblotting and basophil activation are useful to evaluate the effects of handling enzymes as part of the medical surveillance of enzyme-exposed workers. We describe Peniophora sp. 6-phytase as a new allergen and enzymes from Trichoderma as strong sensitizers.

RMS's comment:
This study was also mentioned in the "*Bibliographic review on the potential of microorganisms, microbial products and enzymes to induce respiratory*" sensitization submitted to EFSA (Martel, 2010), therefore the default warning sentence "Micro-organisms may have the potential to provoke sensitising reactions" has to be used for microbial bio-control agents.

Reference Martel, C., Nielsen, G.D., Mari, A., Licht, T.R., Poulsen, L.K.(2010)
Bibliographic review on the potential of microorganisms, microbial products and enzymes to induce respiratory sensitization
EFSA Eur. Food Saf. Auth., CFP/EFSA/FEEDAP/2009, 1-95

Abstract The immune system has evolved to protect individuals from microbial pathogens as well as larger parasites. However, the immune system can sometimes react inappropriately to innocuous antigens, triggering allergic reactions. The potential of microorganisms, microbial products and enzymes to induce respiratory sensitization when used as food and feed additives was investigated in this report. A short review of the state-of-the-art methods to predict allergenicity was also conducted. Our results indicate that there is currently no established model to predict the allergenicity of a molecule. Although *in-silico* models can be useful to predict cross-reactivity between allergens, they do not take into account phenomenons like the context of presentation of the antigen to the immune system. There is no realiable, predictive in-vitro or in-vivo model of allergenicity. Cases of occupational allergy to both fungi and bacteria have been documented, but allergic reactions to microorganisms purposely introduced in the work environment seem to concern only a limited number of fungi. Enzymes were more a matter of concern, with 17 out of 71 enzymes investigated in this report being linked to respiratory allergies. Because these risks are well known, enzyme exposures are strictly controlled both by regulatory authorities and companies. The patterns of prevalence of allergic reactions to enzyme indicate that they are more common at the level of enzyme manufacturers and large-scale users than in the general population.

Results

Cultures of microorganisms allowed in foods and feeds

B.6.1.1.4. Direct observation, e.g. clinical cases

In very rare cases, *Bacillus* species can cause food borne diseases. This is due to the production of heat stable surfactins. *Bacillus amyloliquefaciens* has been shown to produce the toxic peptide amylosin (Mikkola *et al*., 2007), which has been implicated in food borne poisoning (Apetroaie-Constantin *et al*., 2009). However, it is considered that the strain involved may have been misidentified (From *et al*., 2005) since *Bacillus cereus* is a well-established cause of food poisoning (Claus and Berkeley, 1986) and it is considered likely, given the extremely low incidence of food poisoning reported and attributed to *Bacillus amyloliquefaciens*, *Bacillus cereus* may have been misidentified as *Bacillus amyloliquefaciens*.

EFSA has reviewed the toxigenic potential of *Bacillus* species and has concluded that it is unlikely that any *Bacillus cereus*-like enterotoxins are produced by any species other than the *Bacillus cereus* group. Any toxigenic potential in other species is far more likely to arise from the production of surfactins.

Bacillus amyloliquefaciens does not appear in the literature as an infectious organism, but a few cases of *Bacillus subtilis* have been found, associated with drug users and severely debilitated patients (de Boer and Diderichsen, 1991). *Bacillus subtilis* is even consumed in large quantities in the Japanese food “Natto”. Infections attributed to *Bacillus subtilis* include bacteraemia, endocarditis, pneumonia, and septicaemia. However, these infections were found in patients in compromised immune states.
There must be immunosuppression of the host followed by inoculation in high numbers before infection with *Bacillus subtilis* can occur. Clinical cases have been investigated by Ihde and Armstrong (1973) over a 6-year period. In twelve patients *Bacillus* species were determined to be present. They report that disseminated bacterial infections by *Bacillus subtilis* and other bacteria developed in two patients with acute leukaemia who were under intense chemotherapy and finally died of their infections. *Bacillus subtilis* isolates from the remaining ten patients were locally restricted to surgical wound or tumour drainages and did not appear to affect wound healing. Other pathogenic bacteria were sometimes present in such culture material as well. The authors conclude that the presence of *Bacillus* species seems to indicate the infection of a wound or tumour mass. With the exception of the two immune-compromised patients no colonization of other organs or tissues took place. Due to the ubiquitous distribution of *Bacillus subtilis* it is inevitable that sometimes it may be found in association with other bacteria in infected humans.
Bacillus subtilis has been linked to eye infections. However, out of 75 samples of eye drops tested in Egypt 42.6% were found to be contaminated with *Bacillus* spp. indicating that it is extremely unlikely to cause an eye infection (Abo-State *et al*. 2012). Out of 57 *Bacillus* isolates, 41 (71.9%) isolates produced different levels of hemolysins. Two *Bacillus* isolates (MAM-9 and MAM-40) produced large quantity of phospholipace C around their colonies in addition to hemolysin production. These two isolates were also lethal to mice.

The general population is already exposed to *Bacillus amyloliquefaciens* since it is a ubiquitous microorganism primarily inhabiting the soil environment and plant residues. *Bacillus amyloliquefaciens* is not pathogenic or highly toxic as demonstrated by the submitted toxicological studies and has been shown to clear the body following oral ingestion.

Gupta and Vyas (1989) reported insect and mammalian-pathogenicity of some of the *Bacillus subtilis* strains isolated from diseased mosquito larvae (*Bacillus subtilis* viz. A, B, D, and H) , causing mortality due to invasive infection (with subsequent decomposition of larvae) after ingestion by larvae. All the strains were also tested for their mammalian safety on mice. It was found that *Bacillus subtilis* strains 'B' and 'D' appeared to be quite safe as none of them caused any pathological symptoms of mortality in test animals.
Bacillus subtilis has been implicated in bovine mastitis (Fossum *et al*., 1986), however the findings were inconclusive. Since *Bacillus subtilis* is so ubiquitous in the environment contamination cannot be ruled out. Antibodies against proteinase of *Bacillus subtilis* were found in some samples, meaning that it could not be ruled out as the causative agent in bovine mastitis. Mikkola *et al*. (2004) reported *Bacillus amyloliquefaciens* strains isolated from the indoor environment of moisture-damaged buildings contained heat-stable, methanol-soluble substances that inhibited motility of boar spermatozoa within 15 min of exposure and killed feline lung cells in high dilution in 1 day.

Reference: Green, B.J.; Beezhold, D.H. (2011)
Industrial fungal enzymes: an occupational allergen perspective. Journal of allergy Vol. 2011, article ID 628574

Abstract: Occupational exposure to high-molecular-weight allergens is a risk factor for the development and pathogenesis of IgE-mediated respiratory disease. In some occupational environments, workers are at an increased risk of exposure to fungal enzymes used in industrial production. Fungal enzymes have been associated with adverse health effects in the work place, in particular in baking occupations. Exposure-response relationships have been demonstrated, and atopic workers directly handling fungal enzymes are at an increased risk for IgE-mediated disease and occupational asthma. The utilization of new and emerging fungal enzymes in industrial production will present new occupational exposures. The production of antibody-based immunoassays is necessary for the assessment of occupational exposure and the development of threshold limit values. Allergen avoidance strategies including personal protective equipment, engineering controls, protein encapsulation, and reduction of airborne enzyme concentrations are required to mitigate occupational exposure to fungal enzymes.

Reference: Mikkola, R.; Andersson, M.A.; Teplova, V.; Grigoriev, P.; Kuehn, T.; Loss, S.; Tsitko, I.; Apetroaie, C.; Saris, N.E.L.; Veijalainen, P.; Salkinoja-Salonen, M.S. (2007)
Amylosin from *Bacillus amyloliquefaciens*, a K+ and Na+ channel-forming toxic peptide containing a polyene structure.Toxicon, 2007 49, 1158-1171

Abstract: Bacillus amyloliquefaciens strains isolated from the indoor environment of moisture-damaged buildings produce a 1197 Da toxin, named amylosin. Nuclear magnetic resonance (NMR) data showed that amylosin contains a chromophoric polyene structure and the amino acids leucine/isoleucine, proline, aspartic acid/asparagine, glutamic acid/glutamine and tyrosine. A quantitation method for amylosin was developed using commercially available amphotericin B as a reference compound and a known concentration of amylosin determined by NMR with the electronic reference to access in vivo concentration (ERETIC) method. Purified amylosin inhibited motility of boar sperm cells at an exposure concentration of 135 nM and hyperpolarized their cell membrane and depolarized their mitochondria at exposure to concentration of 33-67 nM for 10 min. In a 3-d exposure time only 27 nM of amylosin was needed to provoke the same toxicity functions. Amylosin was cytotoxic to feline lung cells at concentrations of <170 nM. Purified amylosin provoked adenosine 5'-triphosphate (ATP)-independent cation influx into isolated rat liver mitochondria (RLM), inducing swelling of the mitochondria at concentrations of 200 nM K(+) or >250 nM Na(+) medium. In the K(+)- or Na(+)-containing medium, amylosin uncoupled RLM, causing oxidation of pyridine nucleotides (PN), loss of the mitochondrial membrane potential, and suppressed ATP synthesis. Purified amylosin produced cation channels in black-lipid membranes (BLMs) with a selectivity K(+)>Na(+) at a concentration of 26 nM, i.e. the same concentration at which amylosin was toxic to boar sperm cells. The amylosin cation channels were cholesterol- and ATP-independent and more effective with K(+) than with Na(+). We propose that the toxicity of amylosin may be due its ionophoric properties, representing the first K(+)/Na(+) channel-forming substance reported from B. amyloliquefaciens.

Reference: Apetroaie-Constantin, C.; Mikkola, R.; Andersson, M.A.; Teplova, V.; Suominen, I.; Johanssonand, T.; Salkinoja-Salonen, M. (2009)
***Bacillus subtilis* and *B. mojavensis* strains connected to food poisoning produce the heat**

stable toxin amylopsin
Journal of Applied Microbiology 2009 ISSN 1364-5072

Abstract: Aim: To screen and characterize toxic, heat-stable substances produced by food borne strains from Bacillus subtilis group.
Methods and Results: Using the boar sperm motility inhibition assay, six isolates from two outbreaks, out of the 94 isolates from 26 foods, were found to produce ethanol-soluble heat-stable substances that were toxic to sperm cells by depleting the mitochondrial membrane potentials. The toxic isolates were identified as Bacillus subtilis and B mojavensis. Colon carcinoma cells (Caco-2) were used to model the contact with the human digestive tract. The extract of B. subtilis F 2564/96 depolarized the mitochondria in intact Caco-2 cells similarly as in sperm cells. The substance responsible for these effects was purified using HPLC and identified by electron spray ionization ion trap mass spectrometry analysis as amylosin. The temperature requirement for amylopsin production was 21–37_C for B. subtilis and 11–21_C for B. mojavensis. Both species produced amylosin in air as well as in 7–8% CO2 with 8–9% O2.
Conclusions: Food borne illness related strains of B. subtilis and B. mojavensis, produced the heat-stable toxin amylosin. Significance and Impact of the Study: This is the first report that suggests a role for the heat-stable, ion-channel forming toxin amylosin, as a virulence factor in food borne Bacillus.

Reference: From, C.; Pukall, R.; Schumann, P.; Hormazábal, V.; Granum, P.E. (2005)
Toxin producing ability among *Bacillus* spp. outside the *Bacillus cereus* group
Applied and Environmental Microbiology, 2005 71, 1178-1183

Abstract: A total of 333 *Bacillus* spp. isolated from foods, water, and food plants were examined for the production of possible enterotoxins and emetic toxins using a cytotoxicity assay on Vero cells, the boar spermatozoa motility assay, and a liquid chromatography-mass spectrometry method. Eight strains produced detectable toxins; six strains were cytotoxic, three strains produced putative emetic toxins (different in size from cereulide), and one strain produced both cytotoxin(s) and putative emetic toxin(s). The toxin-producing strains could be assigned to four different species, *B. subtilis*, *B. mojavensis*, *B. pumilus*, or *B. fusiformis*, by using a polyphasic approach including biochemical, chemotaxonomic, and DNA-based analyses. Four of the strains produced cytotoxins that were concentrated by ammonium sulfate followed by dialysis, and two strains produced cytotoxins that were not concentrated by such a treatment. Two cultures maintained full cytotoxic activity, two cultures reduced their activity, and two cultures lost their activity after boiling. The two most cytotoxic strains (both *B. mojavensis*) were tested for toxin production at different temperatures. One of these strains produced cytotoxin at growth temperatures ranging from 25 to 42°C, and no reduction in activity was observed even after 24 h of growth at 42°C. The strains that produced putative emetic toxins were tested for the influence of time and temperature on the toxin production. It was shown that they produced putative emetic toxin faster or just as fast at 30 as at 22°C. None of the cytotoxic strains produced *B. cereus*-like enterotoxins as tested by PCR or by immunological methods.

Reference: de Boer, A.S. and Diderichsen, B. (1991)
On the safety of *Bacillus subtilis and B. amyloliquefaciens*: a review
Appl. Microbiol. Biotechnol., 36, 1991, 1-4 TOX2000-1212

Conclusion No case demonstrating invasive properties of *Bacillus subtilis and B. amyloliquefaciens* has been dexribed butin a few case, *B. subtilis* has been found associated with drug abusers or severely debilitated patients. Thus there is no evidence of any pathogenic potential of *B. subtilis* to humans in general. of *B. subtilis* has been associated with some cases of food poisoning which in part may be due to misclassification of *B. cereus*. Thus there are few example of *B. subtilis* strain as confirmed causes of food poisoning. We conclude that *B. subtilis* is a safe host for the production of harmless products.

Reference: Ihde, D.C.; Armstrong D. (1973)
Clinical spectrum of infection due to *Bacillus* species
Amer. J. Med., Vol. 55, 1973, 839-845 BMF2000-152

Abstract: Bacillus species other than B anthracis were isolated from culture material and seen on gram-stained smear in 12 patients over a 6 year period in a hospital for patients with neoplastic diseases. In some cases Bacillus species were recovered repeatedly from an individual patient, occasionally from more than one site. Pulmonary and disseminated infection developed in two patients with acute leukemia who were under intensive chemotherapy, and they died following rupture of a brain abscess into the ventricular system. In both patients,

Bacillus species were recovered from cultures of blood and sputum antemortem, and in the heart's blood after death, and seen in histopathologic sections of the lung and brain. These cases were similar to those few previously reported cases in that the usually nonpathogenic Bacillus disseminated in an immunologically compromised host. Nearly all the remaining isolates were from drainage from recent surgical wounds. Rarely was specific treatment given for the Bacillus, and they seemed to exert no definite influence on the patient's clinical course, although occasionally the character of a wound drainage altered after the Bacillus was no longer recovered on culture.

Reference: Abo-State, M.A.M.; Husseiny, H.M.; Helimish, F.A.; Zickry A.R.A. (2012)
Contamination of Eye Drops with *Bacillus* Species and Evaluation of Their Virulence Factors
World Applied Sciences Journal 19 (6): 847-855, 2012

Abstract: Seventy five eye drops samples were collected from different pharmacies in Cairo, Egypt. Out of 75 testedsamples, 58 (77.3%) are contaminated with bacterial growth when cultured on MYP medium. The bacterial count ranging from 1.0 CFU/ml to 6.0x102 CFU/ml. The microscopic examination indicated that 57 out of 133 isolates are Gram positive, spore forming bacilli. From 58 positive samples 32 samples were found to be contaminated with *Bacillus* spp. (55.1%). So, the contaminated samples with *Bacillus* spp. Represent 42.6% of the total eye drops samples. Out of 57 *Bacillus* isolates, 41 (71.9%) isolates produced different levels of hemolysins. Two *Bacillus* isolates (MAM-9 and MAM-40) produced large quantity of phospholipace C around their colonies in addition to hemolysin production. These two isolates were also lethal to mice. *Bacillus* spp are resistant to gamma radiation, ceftazidime, cefuroxime and colistin. Ten kGy reduced the viable count of *Bacillus* strain MAM-40 by 4.17 log cycles.

Reference: Gupta, D.K.; Vyas, K.M. (1989)
Efficacy of *Bacillus subtilis* against mosquito Larvae (*Anophelis culicifacies*)
Zeitschrift fuer Angewandte Zoologie 1989, 76(1) 85-91

Abstract: Four strains of Bacillus subtilis viz. A, B, D, and H were isolated from diseased Anopheles larvae collected from Sagar (M.P.). All the four strains of B. subtilis were found to cause more or less toxic effects on different instar larvae of Anopheles culicifacies. The comparative analysis of LC(59) values, however, indicated that B. subtilis strain 'B' was most effective for causing the highest percentage of mortality amongst the treated larvae. All the strains were also tested for their mammalian safety on mice. It was found that B. subtilis strains 'B' and 'D' appeared to be quite safe as none of them caused any pathological symptoms of mortality in test animals.

Reference: Fossum, K.; Herikstad, H.; Binde, M.; Pettersen K.E. (1986)
Isolations of *Bacillus subtilis* in connection with Bovine Mastitis
Nordisk Veterinärmedicine, Vol. 38, 1986, 233-236 BMF2000-151

Abstract: Bacillus subtilis has only seldom been associated with pathological conditions in mammals. As the organism is considered to be ubiquitous in the environment, care has to be taken not to put too much emphasis on the pathogenicity of the organism, even in cases where it is isolated in pure culture. *Bacillus subtilis* was isolated from 17 cases of bovine mastitis in which it was considered to be the etiological factor.

Reference: Mikkola, R.; Andersson, M.A.; Grigoriev, P.; Teplova, V.V; Saris, N.E.L.; Rainey, F.A.; Salkinoja-Salonen, M.S (2004)
***Bacillus amyloliquefaciens* strains isolated from moisture-damaged buildings produced surfactin and a substance toxic to mammalian cells.**
Arch Microbiol (2004) 181 : 314–323

Abstract: Fungicidic Bacillus amyloliquefaciens strains isolated from the indoor environment of moisture-damaged buildings contained heat-stable, methanol-soluble substances that inhibited motility of boar spermatozoa within 15 min of exposure and killed feline lung cells in high dilution in 1 day. Boar sperm cells lost motility, cellular ATP, and NADH upon contact to the bacterial extract (0.2 microg dry wt/ml). Two bioactive substances were purified from biomass of the fungicidal isolates. One partially characterized substance, 1,197 Da, was moderately hydrophobic and contained leucine, proline, serine, aspartic acid, glutamic acid and tyrosine, in addition to chromophore(s) absorbing at 365 nm. In boar sperm and human neural cells (Paju), the compound depolarized the transmembrane potentials of mitochondria (Delta Psi(m)) and the plasma membrane (Delta Psi(p)) after a 20-min exposure and formed cation-selective channels in lipid membranes, with a selectivity

K(+):Na(+):Ca(2+) of 26:15:3.5. The other substance was identified as a plasma-membrane-damaging lipopeptide surfactin. Plate-grown biomass of indoor Bacillus amyloliquefaciens contained ca. 7% of dry weight of the two substances, 1,197 Da and surfactin, in a ratio of 1:6 (w:w). The in vitro observed simultaneous collapse of both cytosolic and mitochondrial ATP in the affected mammalian cell, induced by the 1,197-Da cation channel, suggests potential health risks for occupants of buildings contaminated with such toxins.

RMS's comment:
This literature research is in line with the previous cases of *Bacillus* clinical infections in human discussed in the QPS Opinions (EFSA, 2007; 2008; 2009) and with a report entitled: " *The need to revise the Technical Guidance on the assessment of the toxigenic potential of Bacillus species used in animal nutrition*" of Feedap panel 18/06/2013,
Bacillus amyloliquefaciens is recommended for the QPS list, although it has been implicated in rare human infections, especially opportunistic infections since 2007. Following the annual updates of QPS list, EFSA has confirmed the recommendation of *Bacillus amyloliquefaciens* for the QPS list based on a literature review for the preceding years (EFSA QPS updates in 2010, 2011, 2012 and 2013).

Moreover, the US Environmental Protection Agency (US EPA) assessed the risk of the *Bacillus subtilis* strains (QST713, GB03, MBI 600) and *Bacillus subtilis* var. *amyloliquefaciens* FZB24 used in plant protection products. Matthews (2010, 2039918) concluded that these strains do not pose any risk to humans when applied as plant protection products.

B.6.1.2. Basic studies (Annex IIB 5.3)

Two sets of studies were provided by the applicant:

- Studies performed in 1989 by [redacted]: these studies were run with fermentation broth. The broth was designed to provide optimal conditions for *Bacillus amyloliquefaciens* MBI600 to produce metabolites. The studies therefore tested the toxicity of spores, other life-stages and a representative metabolite profile which the organism would produce under optimal conditions.
- Studies performed in 2011 and 2012 on *Bacillus amyloliquefaciens* strain MBI600 Technical. Tested batches were representative of current commercial lots.

B.6.1.2.1. Sensitisation

Sensitisation studies are not considered suitable for microbial products (Report from PRAPeR M2 (16 – 18 February 2009)). However, a dermal sensitisation study is available and is summarised here for completeness.

Reference:	[redacted] (1989) Delayed contact hypersensitivity in the guinea pig with MBI600. [redacted] Unpublished report No. 89429D/AGC 2/SS
Guideline / GLP:	OECD 406, EEC / Yes
Deviation:	20 test and 10 control animals were used.
Acceptability:	Not validated for micro-organisms based products.
Test substance / purity:	*Bacillus subtilis* strain MBI600 [later confirmed to be *Bacillus amyloliquefaciens* strain MBI600] (NCIB 12376) spore/metabolite suspension..
Species / Strain:	Guinea pig / Hartley Dunkin

The item was tested by [redacted]:
"Small amount of MBI 600 was streaked out and incubated at 32°C for 72 hours. Two colonial types were observed." However the study author stated that *Bacillus subtilis (*later confirmed to be *Bacillus amyloliquefaciens* strain MBI600) is known to show dimorphic colonies.
This statement has been done for all set of studies performed in 1989.

Materials and methods:
A skin sensitisation study was conducted on *Bacillus amyloliquefaciens* strain MBI600 according to the Magnusson and Kligman (M&K) method.

The treatment regime involved induction of sensitization by intradermal injection on Day 0, induction of sensitization by topical administration on Day 7 and challenge by topical administration on Day 21. The test levels for dermal and intradermal inductions and challenge were selected following preliminary irritancy testing.

Intradermal Induction:
The day before the start of the study guinea pigs had the hair removed from an area on their scapula region (approximately 4 cm × 2 cm). On test Day 0, three pairs of intradermal injections (0.1 ml/site) were administered within the boundary of the clipped region as follows:

Test group (20 animals)
1. 1:1 (v/v) mixture of FCA with water.
2. 5 % test item solution in water.
3. 5 % test item solution in water formulated in a 1:1 (v/v) mixture of FCA and water.

Control group (10 animals)
1. 1:1 (v/v) mixture of FCA with water.
2. Water.
3. Water formulated in a 1:1 (v/v) mixture of FCA and water.

Epidermal Induction
On Day 7, the same area was clipped free of hair and topical administration was performed as follows:

Test group (20 females)
A 2 x 4 cm patch of Whatman No. 3 filter paper was saturated with Bacillus amyloliquefaciens strain MBI600, as supplied and held in place by an occlusive dressing for 48 hours.

Control group (10 females)
A 2 x 4 cm patch of Whatman No. 3 filter paper was saturated with water and held in place by an occlusive dressing for 48 hours.

Challenge
Test group (20 animals) and control group (10 animals)
0.2 ml of Bacillus amyloliquefaciens strain MBI600 was applied to the anterior side of the left flank and held in place by an occlusive dressing for 24 hours.

0.2 ml of *Bacillus amyloliquefaciens* strain MBI600 in water (1:1) was applied to the posterior side of the left flank and held in place by an occlusive dressing for 24 hours.

The dermal reactions were scored according to Draize and were noted at approximately 24, 48 and 72 hours after patch removal.

Results: Well defined to moderate erythema and slight to moderate oedema was observed in all test animals 24 hours after application. Responses generally persisted up to the end of the observation period, with necrosis and severe oedema observed in some test animals.

Localised reactions to slight erythema and oedema were observed in 4 control animals during the observation period. Dermal reactions seen in all twenty test animals were significantly more marked than those seen in the controls.

Conclusion: *Bacillus amyloliquefaciens* strain MBI600 elicited a 100% sensitisation response under the conditions of this study.

According to Commission Regulation (EU) No 283/2013, the available methods for testing dermal sensitisation are not suitable for testing micro-organisms.

The warning phrase for the sensitisation potential of micro-organisms: "Micro-organisms may have the potential to provoke sensitising reactions" is proposed for *Bacillus amyloliquefaciens* strain MBI600.

B.6.1.2.2. Acute toxicity, pathogenicity and infectiveness

B.6.1.2.2.1. Acute oral toxicity, pathogenicity and infectiveness

Reference:	[redacted] (1989a) Acute oral toxicity and infectivity/pathogenicity to rats of MBI600. [redacted] Unpublished report No. 89396D/AGC 1/0/AC
Guideline / GLP:	EPA FIFRA 152A-10 / Yes
Deviation:	None
Acceptability:	**Yes**
Test substance / purity:	*Bacillus subtilis* strain MBI600 [later confirmed to be *Bacillus amyloliquefaciens* strain MBI600] (NCIB 12376) spore/metabolite suspension
Species / Strain:	Rats / Crl:CD®
Doses	10^9 viable spores /animal

Materials and methods:
Equal numbers of male and female Crl:CD® rats were assigned to test and control groups. Dose solutions were freshly prepared prior to dosing using distilled water as a vehicle. Animals were fasted overnight prior to dosing and for 4 hours post dosing. On Day 1 all rats received by oral gavage 10.0 ml/kg bw of a 1.0% solution of *Bacillus amyloliquefaciens* strain MBI600, providing at least 10^9 viable spores per animal (Groups A-D). A control group (Group E) received a 1.0% solution of autoclaved *Bacillus amyloliquefaciens* strain MBI600, checked for the absence of viable spores. A further control group (Group F) was not dosed. The treatment regime is shown below:

Table B6.1.2.2-1 Number of rats and observation per group

Group	No. Rats		Observation period
	♀	♂	
A	2	2	24h after dosing
B	2	2	Day 8
C	2	2	Day 15
D	5	5	Day 22
E	5	5	Day 22
F	5	5	Day 22

Following dosing, animals were observed for signs of toxicity and mortality soon after dosing and then at 5 hourly intervals on Day 1, then twice daily during the observation period for clinical signs, and mortality. Body temperature was measured prior to dosing and 2, 4 and 24 hours post dosing. Samples of urine and faeces were collected from rats in Group D on Days 2, 3, 4 and 22. Individual body weights were recorded on days 1, 8, 15 and 22. The body weight of rats in Group A was recorded on Day 2, immediately prior to sacrifice.

Following the observation period, rats were sacrificed by cervical dislocation and subjected to gross pathological examination, consisting of external examination and opening of the cranial, abdominal and thoracic cavities. The presence of bacterial spores was checked in the faeces, urine, intestinal contents, organs and tissues (brain heart, lungs, liver, kidneys, spleen and mesenteric lymph nodes).

<u>Detection of the presence of bacterial spores in organs/tissues</u>

Blood samples were spread directly onto the surface of Tryptone Soya Agar plates.
The organ samples were sliced in half, one half was used directly inoculated the surface of a Tryptone Soya Agar plate. The plates were incubated and examined for the presence of colonies of bacterial spores.

The other halves of the organs and remaining blood samples were transferred to sterile containers and stored at -18°C. Quantification of viable counts of bacterial spores was determined from those samples showing the presence of organisms following the direct inoculation onto agar plate.

Quantification of viable bacterial spores
Approximately 1 g of organ/tissues was macerated in 9 ml of phosphate buffered saline. Serial ten-fold dilutions were prepared in the same medium and 1 ml aliquots were added to molten Tryptone Soya Agar and pour-plates prepared. Following incubation the number of typical colonies of bacterial spores was calculated and the number of organisms per gram of tissues was calculated.

Results:
Mortality
No mortality was observed in any of the rats.

Clinical signs
Pilo-erection was observed in all rats dosed with *Bacillus amyloliquefaciens* strain MBI600 and the autoclaved control within 10 minutes of dosing, recovery was complete by the end of Day 1. No other clinical signs were noted.

Body temperature
Body temperature measurements showed no trends indicative of a pyrogenic response to oral administration of the test substance or the autoclaved test substance.

Bodyweight
Slightly low body weight gains were recorded for 2 males in Group D on Day 15. On Day 22 slightly low body weight gains were recorded for 2 males and all females in Group D and one female in Group E. However animals in group D were deprived of food and water overnight prior to weighing. All other animals achieved expected bodyweight gains throughout the study.

Necropsy
Gross necropsy revealed no observable abnormalities.

Dose quantification
The stock solution of *Bacillus amyloliquefaciens* strain MBI600 was estimated to contain 10^{10} cfu per ml. No microbial contamination was found.

Presence of *Bacillus amyloliquefaciens* strain MBI600 in faeces, urine and intestinal contents
All animals showed high faecal counts on Day 2, with counts $>10^7$ spores/g in 8 rats and $>10^6$ spores/g in the other two rats. On Day 3 counts were reduced to $>10^6$ spores/g in 7 rats and on Day 4 counts were further reduced to $>10^5$ spores/g in 1 rat, $>10^4$/g in eight rats and $>10^3$ spores/g in 1 rat. There was no evidence of intestinal infection.

Table B6.1.2.2-2 Count of *Bacillus amyloliquefaciens* MBI600 in in the intestinal tract of rats in group A, B, C and D

Group	Time after dosing	Rat n°	Number of viable organisms (cfu/g)			
			Stomach	Small intestine 1st loop	Small intestine 7th loop	Cecum
A	24 hours	1♂	3.96×10^2	<10	<10	1.38×10^4
		2♂	<10	<10	<10	4.93×10^6
		22♀	3.70×10^3	1.16×10^4	1.68×10^5	1.80×10^6
		23♀	1.92×10^3	5.18×10^4	<10	3.21×10^5
B	7 days	3♂	<10	<10	<10	1.5×10^2
		4♂	<10	<10	<10	57
		24♀	<10	<10	<10	<10
		25♀	<10	<10	<10	<10

C	14 days	5♂	<10	<10	<10	<10
		6♂	<10	<10	<10	<10
		26♀	<10	<10	<10	<10
		27♀	<10	<10	<10	<10
D	21 days	7♂	<10	<10	<10	<10
		8♂	<10	<10	<10	<10
		9♂	<10	<10	<10	<10
		10♂	<10	<10	<10	<10
		11♂	<10	<10	<10	<10
		28♀	<10	<10	<10	<10
		29♀	<10	<10	<10	<10
		30♀	<10	<10	<10	<10
		31♀	<10	<10	<10	<10
		32♀	<10	<10	<10	<10

Relatively low numbers of spores were detected in the urine. Spores were detected in half the rats on Day 2, but in one rat on Day 3. Due to the difficulty in collecting urine without some risk of faecal contamination, it is not possible to conclude that the spores found in the urine had reached the urine via the bloodstream and kidneys. There was no evidence of renal infection.

On Day 2 spores were recovered from the stomach (3/4 rats), first loop small intestine (2/4 rats), seventh loop small intestine (1/4 rats) and in the cecum contents of all rats, with counts ranging from $>10^4$ - $>10^6$ spores/g. Thereafter, no spores were recovered, except for small numbers in the cecum contents (2/4 rats) on Day 8. There was no evidence of systemic infection.

No spores were detected 21 days after the dosing in faeces, urine and intestinal contents.

Presence of *Bacillus amyloliquefaciens* strain MBI600 in blood and organs

Spores were detected in only 2 samples – the lymph node of one rat and the blood of another on Day 8. Subsequent viable counts confirmed the presence in the blood (59 spores/ml) but none were detected in the lymph nodes. Technical difficulties exist in removing organs aseptically and without some risk of contamination and therefore undue emphasis should not be placed on this isolated result.

Table 6.1.2.2-3 Detection of *Bacillus amyloliquefaciens* MBI600 in organs and tissues of rats in group A, B, C and D

Group	Time after dosing	Rat n°	Absence (-), presence (+) and number of colonies							
			Brain	Heart	Lung	liver	Kidney	Spleen	Lumph nodes	Blood
A	24 hours	1♂	-	-	-	-	-	-	-	-
		2♂	-	-	-	-	-	-	-	-
		22♀	-	-	-	-	-	-	-	-
		23♀	-	-	-	-	-	-	-	-
B	7 days	3♂	-	-	-	-	-	-	-	-
		4♂	-	-	-	-	-	-	+	-
		24♀	-	-	-	-	-	-	-	+
		25♀	-	-	-	-	-	-	-	-
C	14 days	5♂	-	-	-	-	-	-	-	-
		6♂	-	-	-	-	-	-	-	-
		26♀	-	-	-	-	-	-	-	-
		27♀	-	-	-	-	-	-	-	-
D	21 days	7♂	-	-	-	-	-	-	-	-
		8♂	-	-	-	-	-	-	-	-
		9♂	-	-	-	-	-	-	-	-
		10♂	-	-	-	-	-	-	-	-
		11♂	-	-	-	-	-	-	-	-
		28♀	-	-	-	-	-	-	-	-
		29♀	-	-	-	-	-	-	-	-
		30♀	-	-	-	-	-	-	-	-
		31♀	-	-	-	-	-	-	-	-
		32♀	-	-	-	-	-	-	-	-

Table 6.1.2.2-4 Count *Bacillus amyloliquefaciens* MBI600 from organs and tissues in which the organism was detected by smear plates

Animal number	Tissue	Viable count, cfu/g or ml
4♂	Lymph node	None detected (with a limit of detection of 10 viable organism per gram)
24♀	Blood	59

Conclusions:
Large numbers of spores survived passage through the intestinal tract, but were rapidly eliminated within 7 days. Clearance of live microorganism from all tissues analysed was complete 21 days after the dosing. *Bacillus amyloliquefaciens* strain MBI600 showed no evidence of pathogenicity or infectivity when administered via the oral route.

There was no mortality or evidence of toxicity following oral administration of *Bacillus amyloliquefaciens* strain MBI600 at dose of 10^9 viable spores/animal and therefore the acute oral LD_{50} is $>10^9$ viable spores/animal.

Reference: [redacted] (2011a) Acute oral toxicity study (UDP) in rats. [redacted] Unpublished report No. 15626-11

Guideline / GLP: OCSPP 870.1100, OECD 425 / yes

Deviation: Relative humidity was at times outside of the protocol range. This was not considered to affect the results of the study.

Acceptability: **Yes**

Test substance / purity: *Bacillus subtilis* strain MBI600 Technical [later confirmed to be *Bacillus amyloliquefaciens* strain MBI600]

	Lot No.: BS7211 Purity: 8.07 x 10^{11} spores/g
Species / Strain:	Albino rat/Sprague-Dawley
Doses	5000 mg/kg bw

Materials and methods:
An acute oral toxicity study was conducted on rats using the up and down procedure.

Animals were fasted overnight for 16 hours prior to dosing. Dose solutions were freshly prepared prior to dosing using de-ionised water as a vehicle. All rats received a dose volume of 12.5 ml/kg bw of 40% *Bacillus amyloliquefaciens* strain MBI600 by gavage.

A single female rat received a dose of 5000 mg/kg bw. No mortality was observed at this dose, therefore a second group of 2 females was administered the same dose level. Again, there was no mortality at this dose level; therefore no further groups were treated.

Following dosing, animals were observed for signs of toxicity and mortality within 30 minutes, and twice more on the day of dosing, then once daily during the 14-day observation period. Body weights were recorded on the day of dosing (Day 0) and on Days 7 and 14.

Following the 14-day observation period, rats were sacrificed by carbon dioxide asphyxiation and subjected to gross pathological examination, consisting of external examination and opening of the abdominal, pelvic and thoracic cavities.

Results:
Mortality
No mortality was observed in any of the rats.

Clinical signs
All animals appeared normal for the duration of the study.

Bodyweight
All animals achieved expected bodyweight gains throughout the study.

Necropsy
Gross necropsy conducted at termination of the study revealed no observable abnormalities.

Conclusions:
Based on the above results, the median lethal dose (LD_{50}) to female rats of *Bacillus amyloliquefaciens* strain MBI600 after a single oral dose is >5000 mg/kg bw corresponding to a LD_{50} > 4.04 10^{11} spores/kg bw.

B.6.1.2.2.2. Acute inhalation toxicity, pathogenicity and infectiveness

Reference:	[redacted] (1989b) Acute pulmonary toxicity and infectivity/pathogenicity to rats of MBI600. [redacted] Unpublished report No. 89397D/AGC 1/2/EC
Guideline / GLP:	EPA FIFRA 152A-12 / Yes
Deviation:	Relative humidity was at times outside of the protocol range. This was not considered to affect the results of the study.
Acceptability:	**Yes**
Test substance / purity:	*Bacillus subtilis* strain MBI600 Technical [later confirmed to be *Bacillus amyloliquefaciens* strain MBI600] Lot No.: BS7211

	Purity: 8.07 x 10^{11} spores/g
Species / Strain:	Rats / Crl:CD®
Dose:	~ 3.5 x 10^{8} viable spores /animal

Materials and methods:
Thirteen male and 13 female CD rats were treated with 10% *Bacillus amyloliquefaciens* strain MBI600 at 1.2 ml/kg bw, providing at least 10^{8} viable spores per rat. Each rat was anaesthetised with ether. A miniature laryngoscope with a fibre optic light was used to open the mouth and a 5 cm length of translucent tubing was inserted into the trachea such that it was possible to identify the phase of respiration. The test suspension was introduced into the endotracheal tube using a 1ml syringe and a hypodermic needle. Small aliquots were injected at the beginning of several inspirations until the entire dose-volume had been instilled.

Animals were observed for signs of toxicity and mortality frequently on the day of exposure. Subsequently, the rats were observed twice daily. Body weights were recorded on Days 1, 8, 15 and 22 or at death. Rats were sacrificed at staged intervals:

Table 6.1.2.2-5 The treatment regime and constitution of the groups

Group	No. of rats		Sacrifice
	♂	♀	
A (group treated with 10.0% MBI 600 at 1.2 ml/kg body weight)	2	2	1 hour after dosing
B (group treated with 10.0% MBI 600 at 1.2 ml/kg body weight)	2	2	Day 2 (24 hours after dosing)
C (group treated with 10.0% MBI 600 at 1.2 ml/kg body weight)	2	2	Day 8
D (group treated with 10.0% MBI 600 at 1.2 ml/kg body weight)	2	2	Day 15
E (group treated with 10.0% MBI 600 at 1.2 ml/kg body weight)	5	5	Day 22
F (group treated with autoclaved solution at 1.2 ml/kg body weight)	5	5	Day 22
G (undosed controls)	5	5	Day 22

Samples of faeces and urine were collected from all rats in groups E. Each rat was placed in a combined faeces/urine collecting cage without access to food or water. Samples were taken throughout a period of 15-18 hours on Day 2 and Day 22.

Following the observation period, rats were sacrificed by an overdose of ether and subjected to gross pathological examination, consisting of external examination and opening of the cranial, abdominal and thoracic cavities. Prior to sacrifice, a 1 ml sample of blood was taken from the orbital sinus of each rat. Samples of brain, heart, lungs, liver, kidneys, spleen and mesenteric lymph nodes were taken from all rats. Samples of the contents of the cecum were collected.

<u>Detection of the presence of bacterial spores in organs/tissues</u>
Blood samples were spread directly onto the surface of Tryptone Soya Agar plates.
The organ samples, with the exception of the lungs, were sliced in half, one half was used directly inoculated the surface of a Tryptone Soya Agar plate. The plates were incubated and examined for the presence of colonies of bacterial spores.

The other halves of the organs and remaining blood samples were transferred to sterile containers and stored at -18°C. Quantification of viable counts of bacterial spores was determined from those samples showing the presence of organisms following the direct inoculation onto agar plate.

Quantification of viable bacterial spores
Approximately 1 g of organ/tissues and pooled lings was macerated in 9 ml of phosphate buffered saline. Serial ten-fold dilutions were prepared in the same medium and 1 ml aliquots were added to molten Tryptone Soya Agar and pour-plates prepared. Following incubation the number of typical colonies of bacterial spores was calculated and the number of organisms per gram of tissues was calculated.

Results:

Mortality
There were deaths following a single dose of *Bacillus amyloliquefaciens* strain MBI600 by intratracheal instillation, containing at least 10^8 viable spores per animal, in group D (2/4 animals) and E (5/10 animals). Deaths occurred from Day 2 and Day 4. Bodyweight losses were recorded for all rats that died. Autopsy of rats that died revealed no macroscopic abnormalities. No mortality was observed in groups F and G.

Clinical signs
Collapse, pallor of the extremities and gasping was observed in the majority of rats dosed with *Bacillus amyloliquefaciens* strain MBI600 and the autoclaved control within 10 minutes of dosing. Collapse and gasping were no longer observed 1 hour after dosing.

Pilo-erection, abnormal body carriage (hunched posture), abnormal gait (waddling) and lethargy were observed in all rats dosed with *Bacillus amyloliquefaciens* strain MBI600 and the autoclaved control, with the exception of Group A. Recovery, as judged by external appearance and behaviour was apparent 2 hours after dosing and complete by Day 2.

The above signs were considered to be due to the effects of the ether anaesthesia.

Pilo-erection, abnormal body carriage (hunched posture), abnormal gait (waddling) and pallor of the extremities was observed in one male (Group E) on Day 5. Recovery was apparent by Day 6 and complete by Day 8.

Pilo-erection, abnormal body carriage (hunched posture), pallor of the extremities and increased respiration was observed in one female (Group E) on Day 3. Recovery was apparent by Day 5 and complete by Day 7.

Body temperature
A drop in body temperature (3-4°C) was recorded for all rats dosed with *Bacillus amyloliquefaciens* strain MBI600 and the autoclaved control. Recovery of body temperature was apparent at 24 hours. The drop in body temperature was considered to be due to the ether anaesthesia. There were no trends indicative of a pyrogenic response to intratracheal instillation of the test substance or the autoclaved test substance.

Table 6.1.2.2-6 Summary of increment of mean temperature (°C) from pre-dose measurement until

Group	2 hours after dosing	4 hours after dosing	24 hours after dosing
A, B, C, D and E ♂ (*Bacillus amyloliquefaciens* strain MBI600)	-3.9	-1.6	-0.6
F♂ (Autoclaved control)	-2.7	-1.1	-0.2
G♂ (untreated)	+0.2	+0.7	0
A, B, C, D and E ♀ (*Bacillus amyloliquefaciens* strain MBI600)	-3.9	-3.7	-1.5
F♀ (Autoclaved control)	-4.6	-1.8	-1.0
G♀ (untreated)	-0.1	-0.3	-0.5

Bodyweight

Bodyweight losses were recorded for one male and two females (Groups C and E) on Day 8. One female dosed with the autoclaved control (Group F) on Day 15. Three dosed females (Group E) on Day 22 (following deprivation of food and water).

Low bodyweight gains were recorded for one male (Group D) and two females (Group E), four females dosed with the autoclaved control (Group F) and 2 untreated females (Group G) on Day 8. On Day 15 low bodyweight gains were observed for one female (Group E), 2 females dosed with the autoclaved control (Group F) and one male and one female in the untreated group (Group G). Two males (Group E), 2 males and two females dosed with the autoclaved control (Group F) and two males in the untreated group (Group G) demonstrated low bodyweight gain on Day 22.

Necropsy
Gross necropsy revealed no observable abnormalities.

Dose quantification
The stock solution of *Bacillus amyloliquefaciens* strain MBI600 was estimated to contain 10^{10} cfu per ml. No microbial contamination was found.

Presence of *Bacillus amyloliquefaciens* strain MBI600 in organs and body fluids
One hour after dosing all rats gave spore counts $>10^6$ per pair of lungs. After 24 hours, all four sacrificed rats had $>10^5$ per pair of lungs. On day 7, three rats had $>10^6$ spores per pair of lungs and the other had $>10^5$ spores per pair of lungs. Of the two surviving rats, on Day 14 one had $>10^6$ spores per pair of lungs and the other had $>10^5$ spore per pair of lungs. After 21 days, all nine surviving rats gave spore counts $>10^4$ per pair of lungs.

Table 6.1.2.2-7 Quantification of *Bacillus amyloliquefaciens* strain MBI600 in rats lungs following intratracheal dosing

Group	Time after dosing	Rat Number	Viable count as colony forming units per pair of lungs
A	1 hr	89♂ 90♂ 112♀ 113♀	8.23×10^6 1.99×10^6 4.87×10^6 2.48×10^6
B	24 hr	91♂ 92♂ 114♀ 115♀	5.93×10^5 5.40×10^5 3.53×10^5 4.33×10^5
C	7 Days	93♂ 94♂ 116♀ 117♀	1.06×10^6 1.41×10^6 6.27×10^5 5.61×10^6
D	14 Days	95♂ 96♂ 118♀ 119♀	RD 1.04×10^6 RD 4.17×10^5
E	21 Days	97♂ 98♂ 99♂ 100♂ 101♂ 120♀ 121♀ 122♀ 123♀ 124♀	2.89×10^4 RD 3.63×10^4 1.79×10^4 9.80×10^4 6.33×10^4 7.00×10^4 2.60×10^4 2.52×10^4 5.57×10^4

RD: Rat died prior to sacrifice

One hour after dosing no spore was found in the blood, but small quantities were isolated from the organs in some, but not all of the four rats.
The most significant organ was the brain, from which >10^2 spores per gram were isolated from 3 of the four rats. After 24 hours small numbers of spores were detected in various organs, with most consistent isolation from the spleen from which >10^2 spores per gram were isolated from 3 of the four rats. After 7 days spores were isolated only from the heart of one rat, the livers of 2 rats and the spleen of three rats. After 15 and 21 days, no spores were isolated from the blood or organs of any rats.

Table B6.1.2.2-8 Absence or presence and numbers of B. subtilis on smear plates from blood and organs of rats following intratracheal dosing

Group	Time after dosing	Rat No.	Absence (-), presence (+) and numbers of colonies						
			Brain	Heart	Liver	Kidney	Spleen	Lymph nodes	Blood
A	1 hr	89♂	-	-	-	-	+	-	-
		90♂	+	+	-	+	-	-	-
		112♀	+	-	+	+	+	+	-
		91♂	+	+	+	+	-	+	-
B	24 hr	92♂	-	+	-	-	-	+	+
		114♀	+	-	-	+	+	-	-
		115♀	-	-	+	+	+	-	-
		93♂	+	+	+	-	+	+	-
C	7 Days	94♂	-	-	+	-	+	-	-
		116♀	-	+	-	-	-	-	+
		117♀	-	-	+	-	+	+	-
		89♂	-	+	+	-	+	-	-
D	14 days	95♂	RD	RD	RD	RD	RD	-	RD
		96♂	-	-	-	-	-	-	-
		118♀	RD	RD	RD	RD	RD	RD	RD
		119♀	-	-	-	-	-	-	-
E	21 days	97♂	-	-	-	-	-	-	-
		98♂	RD	RD	RD	RD	RD	RD	RD
		99♂	-	-	-	-	-	-	-
		100♂	-	-	-	-	-	-	-
		101♂	-	-	-	-	-	-	-
		120♀	-	-	-	-	-	-	-
		121♀	-	-	-	-	-	-	-
		122♀	-	-	-	-	-	-	-
		123♀	-	-	-	-	-	-	-
		124♀	-	-	-	-	-	-	-

RD: Rat died prior to sacrifice

Table B6.1.2.2-9 Viable counts of *Bacillus amyloliquefaciens* strain MBI600 in blood and organs of rats in which the organism was detected by smear plates

Group	Time after dosing	Rat No.	Colony forming units per g or per ml						
			Brain	Heart	Liver	Kidney	Spleen	Lymph nodes	Blood
A	1 hr	89♂	-	-	-	-	<10	-	-
		90♂	2.8 x 10^2	62	-	<10	-	-	-
		112♀	3.50x 10^2	-	<10	<10	<10	1.67 x 10^3	-
		113♀	1.09 x 10^2	3.81 x 10^2	<10	<10	-	<10	-
B	24 hr	91♂	-	<10	-	-	-	<10	<10
		92♂	<10	-	-	<10	3.20 x 10^2	-	-
		114♀	-	-	53	12	4.41 x 10^2	-	-

		115♀	1.29 x 10^2	<10	71	-	3.78 x 10^2	<10	-
C	7 Days	93♂	-	-	<10	-	65	-	-
		94♂	-	<10	-	-	-	-	<10
		116♀	-	-	31	-	3.87 x 10^2	<10	-
		117♀	-	20	1.24 x 10^2	-	1.65 x 10^3	-	-

No spores were present in the cecum contents at 1 hour after dosing, but after 24 hours spores were present in the cecum contents of all animals at a level of $>10^5$ spores per gram and in the faeces at $>10^2$ to $>10^4$ spores per gram. Spores were also isolated from the urine at $>10^2$ to $>10^3$ spores per ml. Samples of cecum contents taken 7, 15 and 21 days after dosing showed persistent levels of $>10^2$ to $>10^3$ spores per gram. Faecal samples taken 21 days after dosing showed closely similar counts, but by the time none of the urine samples showed the presence of viable spores

Table B6.1.2.2-10 Quantification of *Bacillus amyloliquefaciens* strain MBI600 in rats- cecum contents following intratracheal dosing

Group	Time after dosing	Rat No.	Colony forming units per gram
A	1 hour	89♂	<10
		90♂	<10
		112♀	<10
		91♂	<10
B	24 hour	92♂	>1.00 X 10^5
		114♀	>1.00 x 10^5
		115♀	7.32 x 10^5
		93♂	7.31 x 10^5
C	7 days	94♂	1.11 x 10^3
		116♀	4.78 x 10^3
		117♀	7.40 x 10^3
		89♂	1.75 x 10^3
D	14 days	95♂	RD
		96♂	9.34 x 10^2
		118♀	RD
		119♀	7.57 x 10^3
E	21 days	97♂	2.60 x 10^3
		98♂	RD
		99♂	1.04 x 10^3
		100♂	3.40 x 10^3
		101♂	1.87 x 10^3
		120♀	9.18 x 10^2
		121♀	4.99 x 10^2
		122♀	2.05 x 10^3
		123♀	1.00 x 10^3
		124♀	1.91 x 10^3

RD: Rat died prior to sacrifice

Conclusion

There were some early deaths in the rats dosed with *Bacillus amyloliquefaciens* strain MBI600 compared to the autoclaved material. No pyrogenic response was seen and at post-mortem, all organs including the lymph nodes appeared normal.

Viable spore counts showed *Bacillus amyloliquefaciens* spores were capable of surviving in the lungs for 3 weeks, but there was no evidence of pathogenicity or infectivity. Spores were consistently isolated from the cecum contents and faeces, demonstrating evidence of a pattern of established clearance.
No evidence of systemic, enteric, renal or pulmonary infectivity was seen at dose of 3.5 x 10^8 viable spores /animal.

Reference:	[redacted] (2011) Acute inhalation toxicity study in rats. [redacted] Unpublished report No. 15628-11
Guideline / GLP:	OCSPP 870.1300, OECD 403 / Yes
Deviation:	Relative humidity was at times outside of the protocol range. This was not considered to affect the results of the study.
Acceptability:	**Yes**
Test substance / purity:	*Bacillus subtilis* strain MBI600 Technical [later confirmed to be *Bacillus amyloliquefaciens* strain MBI600] Lot No.: BS7211 Purity: 8.07×10^{11} spores/g
Species / Strain:	Rat/Sprague-Dawley
Doses	5.31 mg/L

Materials and methods:
Five male and 5 female albino rats were exposed via the inhalation route to *Bacillus amyloliquefaciens* strain MBI600 in air for 4 hours. Rats were exposed to a nose only concentration of 5.31 mg/L and the air flow rate was maintained at 8.6 L/min. The concentration of test substance in the breathing zone of the animals was determined gravimetrically twice per hour and nominally at the end of exposure. The particle size was monitored twice during the exposure using a cascade particle impactor.

Animals were observed for signs of toxicity and mortality frequently on the day of exposure. Subsequently, the rats were observed at least once daily during the 14-day observation period for mortality and clinical signs. Individual body weights were recorded on days 0, 7 and 14.

Following the 14-day observation period, rats were sacrificed by intraperitoneal injection of Fatal Plus® and subjected to gross pathological examination, consisting of external examination and opening of the abdominal and thoracic cavities.

Results:
Mortality
No mortality was observed in any of the rats.

Clinical signs
Clinical signs included decreased activity, piloerection and test substance on the face. All animals appeared normal by Day 3.

Bodyweight
All animals achieved expected bodyweight gains throughout the study.

Necropsy
Gross necropsy conducted at termination of the study revealed no observable abnormalities.

Conclusions:
Based on the above results, the median lethal concentration (LC_{50}) to rats of *Bacillus amyloliquefaciens* strain MBI600 after a 4h inhalation dose is >5.31 mg/L.

General comment on inhalation toxicity:
The acute pulmonary toxicity and infectivity/pathogenicity study performed in 1989 highlights a significant mortality in the treated group following a single dose of 10^8 viable spores of *Bacillus amyloliquefaciens* strain MBI600 by intratracheal instillation.
Observed death occurred rapidly and there were no trends of increase of body temperature at 24 hours. This study does not show evidence of pathogenicity or infectivity, a pattern of clearance was established. Mortality in

inhalation toxicity studies on microbial pesticides is not unusual, and these deaths seem to be the result of physical action rather than inherent toxicity of the substance.
This conclusion is supported by the fact that no death was observed in acute intravenous toxicity study (see part B.6.1.2.2.3).
Moreover, in the inhalation toxicity study performed with the technical microorganism as currently marketed with a more realistic exposure pathway, no mortality was observed.

B.6.1.2.2.3. Intravenous single dose

Reference:	[redacted] (1989c) Acute intravenous toxicity and infectivity/pathogenicity to rats of MBI600. [redacted] Unpublished report No. 89398D/AGC 1/3/AC
Guideline / GLP:	EPA FIFRA 152A-13 / Yes
Deviation:	None
Acceptability:	**Yes**
Test substance / purity:	*Bacillus subtilis* strain MBI600 [later confirmed to be *Bacillus amyloliquefaciens* strain MBI600] (NCIB 12376) spore/metabolite suspension
Species / Strain:	Rats / Crl:CD®
Doses	~ 4.5 x 10^7 viable spores /animal

Materials and methods:
Equal numbers of male and female Crl:CD® rats were assigned to test and control groups. Dose solutions were freshly prepared prior to dosing using physiological saline as a vehicle. On Day 1 all rats received, via injection into a lateral tail vein, 3.0 ml/kg bw of a 1.0% solution of *Bacillus amyloliquefaciens* strain MBI600, providing at least 10^7 viable spores per animal (Groups A-E). A control group (Group F) received a 1.0% solution of autoclaved *Bacillus amyloliquefaciens* strain MBI600, checked for the absence of viable spores. A further control group (Group G) was not dosed.
The treatment regime is shown below:

Table 6.1.2.2-11 The treatment regime and constitution of the groups

Group	No. Rats		Observation period
	♂	♀	
A	2	2	1h after dosing
B	2	2	24h after dosing
C	2	2	Day 8
D	2	2	Day 15
E	5	5	Day 22
F	5	5	Day 22
G	5	5	Day 22

Following dosing, animals were observed for signs of toxicity and mortality at soon after dosing and then at 5 hourly intervals on Day 1, then twice daily during the observation period for clinical signs, and mortality. Body temperature was measured prior to dosing and 2, 4 and 24 hours post dosing. Samples of urine and faeces were collected from rats in Group E on Days 2 and 22. Individual body weights were recorded on days 1, 8, 15 and 22. The body weight of rats in Groups A and B were recorded on Days 1 and 2 respectively, immediately prior to sacrifice.

Following the observation period, rats were sacrificed by overdose of ether inhalation and subjected to gross pathological examination, consisting of external examination and opening of the cranial, abdominal and thoracic cavities. The presence of bacterial spores was checked in the faeces, urine, intestinal contents, organs and tissues.

Detection of the presence of bacterial spores in organs/tissues
Blood samples were spread directly onto the surface of Tryptone Soya Agar plates.

The organ samples were sliced in half, one half was used directly inoculated the surface of a Tryptone Soya Agar plate. The plates were incubated and examined for the presence of colonies of bacterial spores.

The other halves of the organs and remaining blood samples were transferred to sterile containers and stored at -18°C. Quantification of viable counts of bacterial spores was determined from those samples showing the presence of organisms following the direct inoculation onto agar plate.

Quantification of viable bacterial spores
Approximately 1 g of organ/tissues was macerated in 9 ml of phosphate buffered saline. Serial ten-fold dilutions were prepared in the same medium and 1 ml aliquots were added to molten Tryptone Soya Agar and pour-plates prepared. Following incubation the number of typical colonies of bacterial spores was calculated and the number of organisms per gram of tissues was calculated.

Results:
Mortality
No mortality was observed in any of the rats.

Clinical signs
Pilo-erection was observed in all rats dosed with *Bacillus amyloliquefaciens* strain MBI600 and the autoclaved control, recovery was complete by the end of Day 1 for the autoclaved control and Day 2 for the test animals. No other clinical signs were noted.

Body temperature
Body temperature measurements showed no trends indicative of a pyrogenic response to intravenous administration of the test substance or the autoclaved test substance.

Bodyweight
Slightly low body weight gains were recorded for 1 male and 2 females in Group E on Day 22. However animals in Group E were deprived of food and water overnight, prior to weighing. All other animals achieved expected bodyweight gains throughout the study.

Necropsy
Gross necropsy revealed no observable abnormalities.

Dose quantification
The stock solution of *Bacillus amyloliquefaciens* strain MBI600 was estimated to contain 10^{10} cfu per ml. No microbial contamination was found.

Presence of *Bacillus amyloliquefaciens* strain MBI600 in faeces, urine and cecum contents
None of the cecum samples showed the presence of *Bacillus amyloliquefaciens* strain MBI600.

All animals showed high faecal counts on Day 2, with counts $>10^3$ or $>10^4$ spores/g. By Day 22, none of the faecal samples contained any spores. There was no evidence of intestinal infection

Relatively low numbers of spores were detected in the urine on Day 2. By Day 22, none of the faecal samples contained any spores. There was no evidence of renal infection.

Presence of *Bacillus amyloliquefaciens* strain MBI600 in blood and organs
1h after dosing, relatively low numbers of spores (up to $>10^2$ spores/ml) were found in the blood of 3 rats. Spores were also isolated in the brain (2/4 rats), lymph node (1/4 rats), heart (4/4 rats), lungs (4/4 rats) and kidneys (4/4 rats) in numbers ranging from $>10^2$->10^4 spores/g. Over the sampling period, the number and frequency of detection declined until at Day 22 no spores were recovered, other than in the liver and spleen, all of which gave counts $<10^4$ spores/g.

Table B6.1.2.2-12. The presence of *Bacillus amyloliquefaciens* strain MBI600 in blood and body organs following intravenous dosing

Group	Time after dosing	Rat No.	Absence (-) , presence (+) and numbers of colonies							
			Brain	Heart	Lung	Liver	Kidney	Spleen	Lymph nodes	Blood
A	1hr	43♂	-	+	+	+	+	+	-	+
		44♂	+	+	+	+	+	+	+	+
		66♀	-	+	+	+	+	+	-	-
		67♀	+	+	+	+	+	+	-	+
B	24 hr	45♂	+	+	+	+	+	+	-	-
		46♂	+	+	+	+	+	+	+	+
		68♀	+	+	+	+	+	+	-	+
		69♀	-	+	+	+	-	+	-	-
C	7 days	47♂	-	+	+	+	+	+	-	-
		48♂	-	+	+	+	+	+	+	-
		70♀	-	-	+	+	+	+	+	+
		71♀	-	-	+	+	+	+	+	-
D	14days	49♂	-	-	+	+	-	+	-	-
		50♂	-	-	-	+	-	+	-	-
		72♀	-	+	+	+	-	+	-	-
		73♀	-	-	+	+	-	+	-	-
E	21 days	51♂	-	-	-	+	-	+	-	-
		52♂	-	-	-	-	-	-	-	-
		53♂	-	-	-	-	-	-	-	-
		54♂		-	-	-	-	-	-	-
		55♂	-	-	-	+	-	+	-	-
		74♀	-	-	-	+	-	+	-	-
		75♀	-	-	-	+	-	+	-	-
		76♀	-	-	-	+	-	+	-	-
		77♀	-	-	-	-	-	-	-	–
		78♀	-	-	-	+	-	+	-	-

Table B6.1.2.2-13 Counts of *Bacillus amyloliquefaciens* strain MBI600 in blood and body organs following intravenous dosing

Group	Time after dosing	Rat No	Viable count, cfu/ml or g							
			Brain	Heart	Lung	Liver	Kidney	Spleen	Lymph nodes	Blood
A	1 hr	43♂	-	4.38×10^2	$4.66\ 10^4$	2.64×10^5	1.62×10^3	5.47×10^5	-	$1.59\ 10^2$
		44♂	1.82×10^4	6.96×10^4	3.43×10^4	5.49×10^5	1.78×10^3	7.05×10^5	1.60×10^3	83
		66♀		1.86×10^4	3.70×10^4	9.86×10^5	7.47×10^4	4.31×10^5		-
		67♀	88	1.25×10^3	4.03×10^4	2.84×10^5	3.54×10^4	4.69×10^5		6.75×10^2
B	24 hr	45♂	1.54×10^2	6.51×10^2	6.93×10^3	4.63×10^2	2.48×10^3	8.68×10^4		-
		46♂	1.06×10^4	2.46×10^4	8.61×10^3	2.89×10^5	1.52×10^3	1.13×10^5	<10	1.62×10^2
		68♀	89	2.73×10^2	6.05×10^3	2.07×10^5	1.42×10^3	2.45×10^5	-	<10
		69♀		<10	1.67×10^4	1.93×10^3	-	2.33×10^3		-
		47♂		<10	8.60×10^2	4.43×10^4	1.35×10^2	5.80×10^4		-

C	7 Days	48♂ 70♀ 71♀		<10 - -	5.96x 10^2 2.58x 10^3 NS	4.37x10^4 1.04x10^5 1.52x10^5	99 <10 <10	9.24 x 10^4 3.96 x 10^4 2.62 x 10^4	5.0x 10^3 <10 <10	- 2.70x 10^3 -
D	14 days	49♂ 50♂ 72♀ 73♀		- - <10 -	<10 - 1.37x 10^2 <10	2.92x10^3 1.18x10^3 8.71x10^3 8.30x10^3	- - - -	1.26 x 10^4 9.66 x 10^3 3.48 x 10^4 2.22 x 10^4	- - -	- - - -
E	21 days	51♂ 52♂ 74♀ 75♀ 76♀ 78♀		- - - - -	- - - - - -	4.56x 10^3 4.42x10^3 5.69x10^3 2.50x10^3 NS 2.58x10^3	- - - - - -	2.86x10^3 3.02 x 10^3 2.52 x 10^3 2.97 x 10^3 3.23 x 10^3 4.00 x 10^3	- 	- - - - - -

NS No sample available
* Mean of two plates only for spleen

Table B6.1.2.2-14 Counts of B. subtilis in faeces and urine of rats of Group E 24 hours and 21 days following intravenous dosing

Rat No.	Colony forming units per gram or per ml			
	24 hr. after dosing		21 days after dosing	
	Faeces	Urine	Faeces	Urine
51♂	1.96x 10^3	1.13 x 10^2	<10	<10
52♂	9.67x 10^3	27	<10	<10
53♂	8.83x 10'	57	<10	<10
54♂	1.53x 10'	1.6 x 10^2	<10	<10
55♂	<10	80	<10	<10
74♀	5.79 x 10'	90	<10	<10
75♀	3.27x 10^3	53	<10	<10
76♀	5.68x 10^3	30	<10	<10
77♀	7.64 x 10^3	1.3 x 10^2	<10	<10
78♀	2.29x 10^3	37	<10	<10

Conclusions:
It was clear from the results of the blood and organ counts that the spores remained in a viable state, in reduced numbers, in the liver and spleen 3 weeks after dosing. But by this time they had disappeared from the other organs and were not circulating in the blood. A clear pattern of clearance was established. *Bacillus amyloliquefaciens* strain MBI600 showed no evidence of pathogenicity or infectivity when administered via the intravenous route.

There was no mortality or evidence of toxicity following intravenous administration of *Bacillus amyloliquefaciens* strain MBI600 and therefore the acute intravenous LD_{50} is $>10^7$ viable spores/animal.

B.6.1.2.3. Genotoxicity testing/toxicity on metabolites

B.6.1.2.3.1. *In vitro* studies

EFSA has considered *Bacillus amyloliquefaciens* within its assessment of *Bacillus* species and has concluded an absence of emetic food poisoning toxins with surfactant activity and an absence of enterotoxic activity. Based on this assessment *Bacillus amyloliquefaciens* is included in their list of microorganisms which warrant a Qualified Presumption of Safety (QPS). The Ames test presented below confirms that the fermentation broth of *Bacillus amyloliquefaciens* strain MBI600 is not genotoxic under the conditions of this study.

Reference:	Hashimoto, J. (2012) Bacterial reverse mutation test of the supernatant of the fermentation broth of *Bacillus subtilis* MBI600. UBE Scientific Analysis Laboratory Inc. Unpublished report No. USA-R-12224
Guideline / GLP:	OECD 471 / Yes
Deviation:	-
Acceptability:	**Yes**
Test substance / purity:	Supernatant of the fermentation broth of *Bacillus subtilis* strain MBI600 [later confirmed to be *Bacillus amyloliquefaciens* strain MBI600] (NCIB 12376) Lot No.: BS1261B Purity: 100%

Materials and methods:

Test item:

Bacillus subtilis strain MBI600 was fermented in liquid media with fermentor. The fermentation broth was centrifuged to obtain the supernatant. Filtration of the supernatant was filtrated using a pore size 0.2 µm filter to obtain the eluate.

Cytotoxicity test

A preliminary test was run using *Salmonella typhimurium* strains TA100, TA1535, TA98, TA1537 and Escherichia *coli* strain WP2*uvrA*, to determine the cytotoxic concentration in the presence and absence of S9 mix. Cytotoxicity to the tester strains was tested at six dose levels of 4.88, 19.5, 78.1, 313, 1250 and 5000 µg/plate in both the presence and absence of S9 mix. Two positive controls – requiring or not requiring S9 mix were also run to confirm the validity of the test.

Trial I

Based on the results of the cytotoxicity study, the fermentation broth of *Bacillus subtilis* strain MBI600 was investigated using the same five histidine deficient mutant tester strains of *Salmonella typhimurium*. Strains were tested at 156, 313, 625, 1250, 2500 and 5000 µg/plate (factor 2) in the presence and absence of 10 % v/v S9 mix. Triplicate plates were maintained for the test and positive controls.

Trial II

A second trial was conducted to confirm the negative results of Trial I. The same test conditions were employed.

Results:

Cytotoxicity test

Six doses of the test material ranging from 4.88 to 5000 µg/plate ±S9 were evaluated. No inhibition of the bacterial lawn and reduction in the number of colonies was observed at 5000 µg/plate in both the presence and absence of S9 mix. Hence 5000 µg/plate of the fermentation broth of *Bacillus subtilis* strain MBI600 was selected as the highest concentration for the mutagenicity study for all tester strains.

Trial I

There was no positive mutagenic effect in strains TA100, TA1535, TA1537, TA98 and WP2*uvrA* in the presence or absence of metabolic activation (10% v/v S9 mix).

Trial II

There was no positive mutagenic effect in strains TA100, TA1535, TA1537, TA98 and WP2*uvrA* in the presence or absence of metabolic activation (10% v/v S9 mix).

Conclusions:

Based on the above results, the fermentation broth of *Bacillus subtilis* strain MBI600 was not mutagenic in this bacterial test system either in the presence or absence of metabolic activation in the strains tested. The sensitivity of the plate-incorporation procedure to detect mutagenesis was adequately demonstrated by the results obtained with the positive controls.

RMS comment:

The conclusion of this test is available for the supernatant only.
However, there is no evidence coming from the literature and experimental work provided by the applicant that any genotoxic substance was produced by *Bacillus amyloliquefaciens* MBI600 in the environment or in feed or food.

B.6.1.2.3.2. *Toxicity studies on metabolites (IIM 5.4)*

The notifier has provided a former review of the open literature on metabolites. Further information has been requested on the search strategy.

Reference:	Du Toit Schabort (2011) Secondary compound of *Bacillus subtilis* MBI600. Unpublished report. Date 23 Augus 2011.
Guideline / GLP:	No
Deviation:	-
Acceptability:	**Yes**
Test substance / purity:	*Bacillus amyloliquefaciens* grown for four days.

A metabolite screen was performed on *Bacillus amyloliquefaciens* strain MBI600. Metabolites were identified as iturin A and surfactin. A further peak was tentatively identified as fengycin or plipastatin. No metabolites in addition to those identified in the literature were noted.

RMS's comment: *Bacillus amyloliquefaciens* strain MBI600 cultures were grown for four days in 4L bioreactor shake flasks containing 80g/L soy flour and 67g/L maltrodextrin. This culture under laboratory conditions was considered to be comparable with the technical product due to the similarity of substrate.

In the literature *Bacillus amyloliquefaciens* is shown to produce many antibiotics, antifungals and siderophores including surfactin, iturin (bacillomycin D, F, L and FC, and iturin A and C, mycosubtilin), macrolactin, azalomycin F, the auxin indole-3-acetic acid (IAA), 3-hydroxybutan-2-one (acetoin), butane-2,3-diol, bacilysin, fengycin, amphomycin, acivicin, arthrobactin, difficidin, oxidifficidin, bacillaene, diHydroBacillaene, rhodutorola acid, stenothricin, valinomycin, enterobactin and nocardamin (Sansinenea and Ortiz 2011, Hamdache *et al*. 2011, Wulff *et al*. 2002, Stein 2005).

Surfactin, iturin, bacillomycine and/or azalomycin were the principal secondary metabolites of *Bacillus amyloliquefaciens* (Wulff *et al*. 2002).

Reference:	Sansinenea, E.; Ortiz, A.: Secondary metabolites of soil Bacillus spp. Nordisk Biotechnol Lett (2011), 33, 1523-1538World Applied Sciences Journal 19 (6): 847-855, 2012
Abstract:	Bacillus species produce secondary metabolites that are the object of natural product chemistry studies. The wide structural variability of these compounds has attracted the curiosity of chemists and their biological activities have inspiredthe pharmaceutical industry to search for lead structures in microbial extracts. Screening of microbial extracts reveals the large structural diversity of natural compounds with broad biological activities, such as antimicrobial, antiviral, immunosuppressive, and antitumor activities, that enable the bacterium to survive in its natural environment. These findings widen the potential industrial importance of Bacillus spp., particularly of B. thuringiensis, beyond insecticidal usage and may help explain the role of Bacillus spp. in the soil ecosystem.
Reference:	Hamdache, A.; Lamarti, A.; Aleu, J.; Collado, I.G.: Non-peptide metabolites from the genus Bacillus. Journal of Natural Products Published by the American Chemical Society and American Society of Pharmacognosy 14 March 2011
Abstract:	Bacillus species produce a number of non-peptide metabolites that display a broad spectrum of activity and structurally diverse bioactive chemical structures. Biosynthetic, biological, and structural studies of these metabolites isolated from Bacillus species are reviewed. This

contribution also includes a detailed study of the activity of the metabolites described, specially their role in biological control mechanisms.

Reference: Wulff, E.G.; Mguni, C.M.; Mansfeld-Giese, K.; Fels, J.; Lubeck, M.; Hockenhull, J.: Biochemical and molecular characterization of Bacillus amyloliquefaciens, B. subtilis and B. pumilus isolates with distinct antagonistic potential against Xanthomonas campestris pv. Campestris. Plant Pathology (2002) 51 , 574–584

Abstract: Fifty-one *Bacillus* isolates were characterized by fatty acid methyl ester (FAME) analysis; universal primer polymerase chain reaction (UP-PCR) fingerprinting; production of secondary metabolites and antagonistic activity against *Xanthomonas campestris* pv. *Campestris* (causal agent of black rot in cabbage) *in vitro* and *in vivo*. Based on FAME analysis and/or PCR fingerprinting, the isolates were clustered into three different groups, named as *Bacillus amyloliquefaciens B.subtilis* and *B.pumilus*. Seed treatment with *Bacillus* spp. generally reduced germination of seeds and incidence of black rot, but no relationship was found between the results of *in vitro* and *in vivo* experiments. The *B.amyloliquefaciens* group contained isolates that were generally the most effective at reducing attack of black rot *in viv*. The metabolic profiles of these isolates suggested that they produced surfactin, iturin, bacillomycine and/or azalomycin F. Isolates belonging to the *B. subtilis* group were mostly able to synthesize surfactin and arthrobactin. Surfactin, amphomycin, arthrobactin and valinomycin were generally found in culture extracts of isolates belonging to the *B. pumilus* group. No effect on growth of the pathogen was detected when the activity of filtered culture extracts and selected metabolites produced by the three different *Bacillus* species was tested *in vitro* against *X.c*. pv.*campestris*. However, inhibition was seen when bacterial liquid cultures were used. When the ability to colonize cabbage endophytically was examined for seven selected isolates with different antagonistic potential against black rot, it was found that the ability was related to the species and not to the antagonistic activity of the isolates.

Reference: Stein, T.: Bacillus subtilis antibiotics: structures, syntheses and specific functions. Molecular Microbiology (2005) 56 (4), 845–857

Abstract: The endospore-forming rhizobacterium
Bacillus subtilis – the model system for Gram-positive organisms, is able to produce more than two dozen antibiotic with an amazing variety of structures. The produced anti-microbial active compounds include predominantly peptides that are either ribosomally synthesized and post-translationally modified (lantibiotics and lantibiotic-like peptides) or non-ribosomally generated, as well as a couple of non-peptidic compounds such a polyketides, an aminosugar, and a phospholipid. Here I summarize the structures of all known *B. subtilis* antibiotics, their biochemistry and genetic analysis of their biosyntheses. An updated summary of well-studied antibiotic regulation pathways is given. Furthermore, current findings are resumed that show roles for distinct *B. subtilis* antibiotics beyond the 'pure' anti-microbial action: Non ribosomally produced lipopeptides are involved in biofilm and swarming development, lantibiotics function as pheromones in quorum-sensing, and a 'killing factor' effectuates programmed cell death in sister cells. A discussion of how these antibiotics may contribute to the survival of *B. subtilis* in its natural environment is given.

The cyclic Lipopeptides

Surfactin

Surfactin is a macrolide containing the heptapeptide sequence Glu-Leu-Leu-Val-Asp-Leu-Leu and a lipid portion which is a mixture of several β-hydroxy-fatty acids with chain length of 13 to 15 carbon atoms (Kalinovskaya *et al*. 2002). Surfactin is primarily renowned for its exceptional surfactant power (Peypoux *et al*. 1999, Carrillo *et al*. 2003). Investigations of its other physiological or biochemical actions have shown that it has a range of pharmacological applications, including antibacterial, antiviral, anti-adhesive and anti-inflammatory uses (Vollenbroich *et al*., 1997; Cooper *et al*., 1981, Shaligram and Singhal, 2010), is a valuable inhibitor of fibrin clot formation (Arima *et al*. 1968) and an antibacterial, antitumoral and hypocholesterolemic agent (Tsukagoshi *et al*. 1970; Kameda *et al*. 1972). Surfactin is able to form mixed micelles with iturin, which substantially augments the latter's antifungal activity (Maget-Dana *et al*. 1992).

Surfactin is of moderate toxicity (intraperitoneal LD_{50} = 200 mg/kg[2], intravenous LD_{50} = 105 mg/kg).

[2] *CRC Handbook of antibiotic compounds. Berdy, J.; Boca Raton, F.L. CRC Press.*

Reference: Kalinovskaya, N.I.; Kuznetsova, T.A.; Ivanova, E.P.; Romanenko, L.A.; Voinov, V.G.; Huth, F.; Laatsch, H.: Characterization of Surfactin-like Cyclic Depsipeptides Synthesized by *Bacillus pumilus* from *Ascidian Halocynthia aurantium*. Mar. Biotechnol. 2002 4, 179–188.

Abstract: A marine bacterium (KMM 1364), identified as *Bacillus pumilus*, was isolated from the surface of ascidian *Halocynthia aurantium.* Structural analysis revealed that the strain KMM 1364 produced a mixture of lipopeptide surfactin analogs with major components with molecular masses of 1035, 1049, 1063, and 1077. The variation in molecular weight represents changes in the number of methylene groups in the lipid and/or peptide portions of the compounds. Structurally, these lipopeptides differ from surfactin in the substitution of the valine residue in position 4 by leucine, and have been isolated as two carboxy-terminal variants, with valine or isoleucine in position 7. As constituents of the lipophilic part of the peptides, only _-hydroxy-C15-, _-hydroxy- C16-, and a high amount of _-hydroxy-C17 fatty acid were determined.

Reference: Peypoux, F.; Bonmatin, J.M.; Wallach, J.: Recent trends in the biochemistry of surfactin. Appl Microbiol Biotechnol 1999 51: 553-563.

Abstract: The name surfactin refers to a bacterial cyclic lipopeptide, primarily renowned for its exceptional surfactant power since it lowers the surface tension of water from 72 mN m^{-1} to 27 mN m^{-1} at a concentration as low as 20 μM. Although surfactin was discovered about 30 years ago, there has been a revival of interest in this compound over the past decade, triggered by an increasing demand for effective biosurfactants for difficult contemporary ecological problems. This simple molecule also looks very promising as an antitumoral, antiviral and anti-*Mycoplasma* agent. Structural characteristics show the presence of a heptapeptide with an LLDLLDL chiral sequence linked, via a lactone bond, to a *β*-hydroxy fatty acid with 13–15 C atoms. In solution, the molecule exhibits a characteristic "horse saddle" conformation that accounts for its large spectrum of biological activity, making it very attractive for both industrial applications and academic studies. Surfactin biosynthesis is catalysed non-ribosomally by the action of a large multienzyme complex consisting of four modular building blocks, called the surfactin synthetase. The biosynthetic activity involves the multicarrier thiotemplate mechanism and the enzyme is organized in structural domains that place it in the family of peptide synthetases, a class of enzymes involved in peptidic secondary-metabolite synthesis. The *srfA* operon, the *sfp* gene encoding a 4′-phosphopantetheinyltransferase and the c*omA* regulatory gene work together for surfactin biosynthesis, while the gene encoding the acyltransferase remains to be isolated. Concerning surfactin production, there is no indication whether the genetic regulation, involving a quorum-sensing mechanism, overrides other regulation factors promoted by the fermentation conditions. Knowledge of the modular arrangement of the peptide synthetases is of the utmost relevance to combinatorial biosynthetic approaches and has been successfully used at the gene level to modify the surfactin template. Biosynthetic and genetic rationales have been described for building variants. A fine study of the structure/function relationships associated with the three-dimensional structure has led to the recognition of the specific residues required for activity. These studies will assist researchers in the selection of molecules with improved and/or refined properties useful in oil and biomedical industries.

Reference: Carrillo, C.; Teruel, J.A.; Aranda, F.J.; Ortiz, A.: Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. Biochim Biophys Acta 2003 1611:91–97

Abstract: Surfactin, an acidic lipopeptide produced by various strains of *Bacillus subtilis*, behaves as a very powerful biosurfactant and possesses several other interesting biological activities. This work deals with the molecular mechanism of membrane permeabilization by incorporation of surfactin. The surfactin-induced vesicle contents leakage was monitored by following release of carboxyfluorescein entrapped into unilamellar vesicles made of palmitoyloleoylphosphatidylcholine (POPC). The effect of the addition of cholesterol, dipalmitoylphosphatidylcholine (DPPC) and palmitoyloleoylphosphatidylethanolamine (POPE) was also checked. It was observed that surfactin was able to induce content leakage at concentrations far below the onset surfactin/lipid ratio for membrane solubilization to occur, which in our system was around 0.92. Electron microscopy showed that vesicles were present after addition of surfactin at a ratio below this value, whereas no vesicles could be observed at ratios above it. Cholesterol and POPE attenuated the membrane-perturbing effect of surfactin, whereas the effect of DPPC was to promote surfactin-induced leakage, indicating that bilayer sensitivity to surfactin increases with the lipid tendency to form lamellar phases, which is in agreement with our previous observation that surfactin destabilizes the inverted-hexagonal structure. Fourier-transform infrared spectroscopy (FTIR) was used to specifically follow the effect of surfactin on different parts of the phospholipid

bilayer. The effect on the C□O stretching mode of vibration of POPC indicated a strong dehydration induced by surfactin. On the other hand, the C□H stretching bands showed that the lipopeptide interacts with the phospholipid acyl chains, resulting in considerable membrane fluidization. The reported effects could be useful to explain surfactin-induced 'pore' formation underlying the antibiotic and other important biological actions of this bacterial lipopeptide.

Reference: Vollenbroich, D.; Ozel, M.; Vater, J.; Kamp, R.M.; Pauli, G.: Mechanism of inactivation of enveloped viruses by the biosurfactant surfactin from *Bacillus subtilis*. Biologicals 1997 25:289–297

Abstract: The antiviral activity of surfactin, a cyclic lipopeptide antibiotic and biosurfactant produced by*Bacillus subtilis*, was determined for a broad spectrum of different viruses, Semliki Forest virus (SFV), herpes simplex virus (HSV-1, HSV-2), suid herpes virus (SHV-1), vesicular stomatitis virus (VSV), simian immunodeficiency virus (SIV), feline calicivirus (FCV), murine encephalomyocarditis virus (EMCV).*In vitro*experiments showed biphasic virus inactivation kinetics for enveloped viruses during treatment. Inactivation of enveloped viruses, especially herpes- and retroviruses, was much more efficient than that of non-enveloped viruses. For those viruses susceptible to its action, surfactin was active at 25 μMin medium containing 5% fetal calf serum (FCS). Concentrations up to 80 μMof surfactin led to a titre reduction of >4·4 $\log_{10}$CCID$_{50}$/ml for HSV-1 in 15 min and for SIV and VSV in 60 min. The inactivation rate increased linearly with the incubation temperature by a factor 2·4/10°C and logarithmically with the concentration. Serum components, probably proteins and/or lipids, influence the effective surfactin concentration. A disruption of the viral lipid membrane and partially of the capsid was observed by electron microscopy. These findings suggest that the antiviral action, postulated also in other investigations, seems to be due to a physicochemical interaction of the membrane-active surfactant with the virus lipid membrane. Surfactin may be useful for application in virus safety enhancement of biotechnological and pharmaceutical products.

Reference: Cooper, D.G.; MacDonald, C.R.; Duff, S.J.B.; Kosaric, N.: Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. Appl Environ Microbiol 1981 42:408–412

Abstract: The lipopeptide, surfactin, is produced by *Bacillus subtilis.* A study has been made on large-scale production of this surfactant. A good yield was obtained from a glucose substrate fermentation by continuously removing the product by foam fractionation. The surfactin could be easily recovered from the collapsed foam by acid precipitation. The yield was also improved by the addition of either iron or manganese salts. Hydrocarbon addition to the medium, which normally increases biosurfactant production, completely inhibited surfactin production by *B. subtilis.*

Reference: Arima, K.; Kakinuma, A.; Tamura, G.: Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis:* isolation, characterization and its inhibition of fibrin clot formation. Biochem Biophys Res Commun 1968 31:488–494

Abstract: No available abstract.

Reference: Tsukagoshi, N.; Tamura, G.; Arima, K.: A novel protoplast-bursting factor (surfactin) obtained from *Bacillus subtilis* IAM 1213. I. The effects of surfactin on Bacilllus megaterium KM. Biochim Biophys Acta 1970 196: 204-210

Abstract: 1. The protoplast-bursting activity of surfactin was neutralized by membrane fractions which had been solubilized in dilute alkali solution.
2. After digestion of the membranes by trypsin, the residual fraction, containing high contents of lipids, still retained neutralizing activity against surfactin.
3. Phospholipids such as phosphatidylethanolamine and phosphatidylcholine neutralized the activity of surfactin

Reference: Kameda, Y.; Matsui, K.; Hisato, K.; Yamada, T.; Sagai, H.: Antitumor activity of Bacillus natto. III. Isolation and characterization of a cytolytic substance on Ehrlich ascites carcinoma cells in the culture medium of *Bacillus natto KMD 1126.* Chem Pharm Bull (Tokyo) 1972 20: 1551-1553

Abstract: For the purpose of finding out a strain which has stronger cytolytic activity on Ehrlich ascites carcinoma cells, the authors isolated 113 strains of Bacillus natto from straws, which were collected at various areas in Japan, and measured the cytolytic activities by cylinder plate method. As the results, the authors found out a strain of Bacillus natto (tentatively called KMD 2311) which has the strongest cytolytic activity in the 113 strains. There were at least two kind of cytolytic substances on Ehrlich ascites carcinoma cells in the culture medium of

Bacillus natto KMD 2311. One was extracted with AcOEt from the culture medium, which constitute approximately 20% of the cytolytic activity in the culture medium and it was stable. This cytolytic substance was purified and colorless crystalline compound (mp 247-249°) was obtained by recrystallization from acetone-petr. ether. Chemical structure of this compound was examined by elementary analysis, infrared, nuclear magnetic resonance, and mass spectroscopy and study of decomposed products. As the results, this compound was proved to be identical with surfactin (mp 140°), which was obtained from culture medium of Bacillus subtilis by Kakinuma, et al. and from Bacillus natto KMD 1126 by the authors. Melting point of this compound (mp 249°) was higher about 100° than that of surfactin (mp 140°), but high mp compound was obtained from surfactin by recrystallization from acetone-petr. ether. From these results, it was indicated that the cytolytic substance was identified with surfactin and dimorphic.

Reference: Maget-Dana, R.; Thimon, L.; Peypoux, F.; Ptak, M.: Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A. Biochimie 1992 74: 1047-1051

Abstract: Iturin A and surfactin are two lipopeptides extracted from a same strain of *Bacillus subtilis*. Iturin A possesses antibiotic and antifungal activities and surfactin is a strong surfactant. The presence of surfactin, at a concentration at which, alone, it is inactive, increases to a very large extent the haemolysis percent induced by iturin A. This synergistic effect seems to be in relation with interactions between iturin A and surfactin. Iturin A adsorbs to and penetrates into surfactin monolayers. Iturin A and surfactin are miscible and interact specifically in mixed monolayers.

Reference: *CRC Handbook of antibiotic compounds. Berdy, J.; Boca Raton, F.L. CRC Press*

Abstract: No available abstract.

Iturin Group

The iturin family encompasses the closely related cyclic lipoheptapeptides mycosubtilin, the iturins and bacillomycin, which contain one β-amino fatty acid and seven α-amino acids, and exhibit strong antifungal and hemolytic activities and a limited antibacterial activity (Stein, 2005). Biological effects of the iturin family peptides are due to their capability of forming ion-conducting pores (Maget-Dana and Peypoux, 1994). Iturin A and bacillomycin L provoked hemolysis and released potassium from erythrocytes (Aranda *et al*. 2005). Iturin A contains the heptapeptide Asn1-Tyr2-Asn3-Gln4-Pro5-Asn6-Ser7, whereas in the other members the amino acid residues in the heptapeptides vary slightly; e.g. mycosubtilin, that was isolated from *Bacillus subtilis*, has Asn1-Tyr2-Asn3-Gln4-Pro5-Ser6-Asn7.

Clinical trials show that iturin is a valuable anti-fungal drug against dematomycoses because of its low toxicity and low allergenic effects. Iturin A has an intraperitoneal LD_{50} of 75 mg/kg[3], indicating moderate toxicity, by this non-standard exposure route.

Bacillomycin, a member of the iturin family of peptides, contains one β-amino fatty acid and seven α-amino acids in its molecular structure. Bacillomycin homologues are referred to as bacillomycins D, F, and L, and they differ in the composition of their amino acids and length of their fatty acid chain, which varies from C_{14} to C_{17}. Similar to other iturin homologues, these compounds exhibit strong antifungal activities and limited antibacterial activities (Yuan *et al*. 2012).

Reference: Maget-Dana, R.; Peypoux, F.: Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties. Toxicology 1994 87:151–174

Abstract: Iturins are a family of lipopeptides extracted from the culture media of various strains of *Bacillus subtilis*. These amphiphilic compounds are characterized by a peptide ring of seven amino acid residues including an invariable D-Tyr^2, with the constant chiral sequence LDDLLDL closed by a C_{14}–C_{17} aliphatic β-amino acid. They exhibit strong antifungal activities against a wide variety of pathogenic yeasts and fungi but their antibacterial activities are restricted to some bacteria such as *Micrococcus luteus*. The biological activity of the iturin lipopeptides is modulated by the primary structure of the peptide cycle as illustrated by the methylation of the D-Tyr^2 residue which dramatically decreases the activity or by the inversion of the two adjacent Ser^6-Asn^7 residues which makes mycosubtilin more active than iturin A. The antifungal activity is related to the interaction of the iturin lipopeptides with the cytoplasmic membrane of target cells, the K^+ permeability of which is

[3] *CRC Handbook of antibiotic compounds. Berdy, J.; Boca Raton, F.L. CRC Press.*

greatly increased. The ability of iturin compounds to increase the membrane cell permeability is due to the formation of ion-conducting pores, the characteristics of which depend both on the lipid composition of the membrane and on the structure of the peptide cycle. From monolayer experiments it has been suggested that these ionic pores are the consequence of the presence of aggregates (lipopeptide aggregates or lipopeptide/phospholipid complex aggregates) in the phospholipid membrane. It has also been shown that, when active, iturins interact strongly with sterols, forming lipopeptide/cholesterol complexes. Therefore, the biologically efficient structure might be a ternary structure: iturin/phospholipid/sterol.

Reference: Aranda, F.J.; Teruel, J.A.; Ortiz, A.: Further aspects on the haemolytic activity of the antibiotic lipopeptide iturin A. Biochimica et Biophysica Acta 1713 (2005) 51 – 56.

Abstract: The bacterial lipopeptide iturin A is able to cause hemolysis of human erythrocytes in a dose-dependent manner. Hemolysis takes place at iturin concentrations below its critical micellar concentration. Relative kinetics determinations clearly show that K+ leakage occurs prior to hemoglobin release. Furthermore, hemolysis can be prevented by addition to the outer solution of osmotic protectants of appropriate size. Altogether these results indicate that iturin A-induced hemolysis follows a colloid-osmotic mechanism, with the formation of a membrane pore of average diameter 32 Å. Iturin A is capable of inducing leakage of an aqueous fluorescent probe trapped in human erythrocyte ghosts, but not in large unilamellar liposomes made of various lipid compositions. The different permeabilizing effects of iturin A on model and biological membranes are discussed on the light of the presented results.

Reference: Yuan, J.; Li, B.; Zhang, N.; Waseem, R.; Shen, Q.; Huang, Q.: Production of Bacillomycin- and Macrolactin-Type Antibiotics *by Bacillus amyloliquefaciens* NJN-6 for Suppressing Soilborne Plant Pathogens. Journal of Agricultural and Food Chemistry. Published by the American Chemical Society, 4 March 2012.

Abstract: *Bacillus amyloliquefaciens* strains have been used as biocontrol agents for the suppression of several soilborne plant pathogens. A clearer understanding of the antagonistic mechanisms of action of these bacteria will facilitate their use in the control of plant diseases. Antagonistic substances were isolated from the fermentation broth of *B. amyloliquefaciens* strain NJN-6 cultures. These compounds were preconcentrated using an XAD-16 column and were purified using reversed-phase high-performance liquid chromatography (RP-HPLC). Fractions were collected from the column and were analyzed, and two homologues of bacillomycin D [molecular weights of 1030 Da (C14) and 1044 Da (C15)] and three homologues of members of the macrolactin family, macrolactin A, 7-*O*-malonyl macrolactin A, and 7-*O*-succinyl macrolactin A (molecular weights of 402, 487, and 502 Da, respectively) were identified using HPLC/electrospray ionization mass spectrometry (ESI–MS) analysis. An antagonistic assay showed that bacillomycin D and macrolactin exhibited significant antagonistic effects against *Fusarium oxysporum* and *Ralstonia solanacearum*, respectively. A reliable method for the isolation and purification of bacillomycin D and macrolactin from bacterial broth cultures was developed. These data will help elucidate the mechanisms that *B. amyloliquefaciens* NJN-6 uses for the biocontrol of soilborne plant pathogens.

Fengycin
Fengycin is a cyclic lipoheptapeptide, with properties very similar to Iturin A (Sansinenea and Ortiz, 2011).

Amphomycin
Amphomycin is of moderate toxicity (intravenous LD_{50} = 120 mg/kg[4], intraperitoneal LD_{50} = 150 mg/kg, intraperitoneal LD_{50} = 300 mg/kg, *per os* LD_{50} = 500 mg/kg).

Valinomycin
Valinomycin is of high toxicity (intraperitoneal LD_{50} = 0.98 mg/kg [5], SC LD_{50} = 4.14 mg/kg).

The Polyketides

Difficidin and Oxidifficidin

[4] *CRC Handbook of antibiotic compounds. Berdy, J.; Boca Raton, F.L. CRC Press.*
[5] *CRC Handbook of antibiotic compounds. Berdy, J.; Boca Raton, F.L. CRC Press.*

Difficidin and oxidifficidin are highly unsaturated 22-memberedmacrocyclic polyene lactone phosphate esters with broad-spectrum antibacterial activity (Zimmerman *et al*., 1987). They are broad spectrum antibiotics, which have activity *in vitro* against both aerobic and anaerobic strains of bacteria, many of which are pathogenic to humans. Difficidin and Oxidifficidin when administered intraperitoneally protected mice against an otherwise lethal bacteremia caused by *Klebsiella pneumonia* (ED_{50} = 1.31 and 15.6 mg/kg respectively).

Reference: Zimmerman, S. B.; Schwartz, C. D.; Monaghan, R. L.; Pelak, B. A.; Weissberger, B.; Gilfillan, E. C.; Mochales, S.; Hernandez, S.; Currie, S. A.; Tejera, E.; Stapley, E. O. J.: Difficidin and oxydifficidin: novel broad spectrum antibacterial antibiotics produced by *Bacillus subtilis.* I. Production, taxonomy and antibacterial activity. Antibiot. 1987, 40, 1677–1681

Abstract: Difficidin and oxydifficidin, two novel macrocyclic polyene lactone phosphate esters were discovered in fermentation broths of each of two strains of Bacillus subtilis: ATCC 39320 and ATCC 39374. Difficidin and oxydifficidin each showed a broad spectrum of activity against aerobic and anaerobic bacteria. Many of the susceptible aerobes and anaerobes were human pathogens resistant to one or more antibiotics. Difficidin and oxydifficidin when administered intraperitoneally protected mice against an otherwise lethal bacteremia caused by Klebsiella pneumoniae (ED50 in mg/kg of 1.31 and 15.6 respectively). Neither difficidin nor oxydifficidin were effective when administered via the subcutaneous route.

Bacillaene and DiHydroBacillaene

Bacillaene inhibits prokaryotic but not eukaryotic protein synthesis by means of an unknown mechanism, and it exhibits high bacteriostatic activity against a wide spectrum of bacteria. Thus, this compound displays antimicrobial activity against human pathogens such as *Serratia marcescens*, *Klebsiella pneumoniae* and *Staphylococcus aureus* (Patel *et al*., 1995). The hybrid polyketide/non-ribosomal peptide synthase is involved in the synthesis of a series of extremely labile, open-chained isomers with bacillaene and dihydrobacillaene as the most abundant representatives. The final steps of bacillaene biosynthesis in *B. amyloliquefaciens* FZB42 were recently elucidated by Moldenhauer *et al*., 2010 providing direct evidence for β,γ dehydration by trans-acyltransferase polyketide synthase.

Reference: Patel, P. S.; Huang, S.; Fisher, S.; Pirnik, D.; Aklonis, C.; Dean, L.; Meyers, E.; Fernandes, P.; Mayerl, F. J.: Bacillaene, a novel inhibitor of prokaryotic protein synthesis produced by *Bacillus subtilis:* production, taxonomy, isolation, physico-chemical characterisation and biological activity. Antibiot. 1995, 48, 997–1003

Abstract: Bacillaene, a novel polyene antibiotic, was discovered and isolated from fermentation broths of a strain of Bacillus subtilis. The novel antibiotic has a nominal molecular weight of 580 and an empirical formula of C35H48O7. Bacillaene is active against a broad spectrum of bacteria in agar-plate diffusion assays. Studies in vitro indicate that the antibiotic inhibits prokaryotic protein synthesis but not eukaryotic protein synthesis. Cell survival studies performed with strains of Escherichia coli indicate that the antibiotic is a bacteriostatic agent.

Reference: Moldenhauer, J.; Goetz, D. C. G.; Albert, Ch. R.; Bischof, S. K.; Schneider, K.; Suessmuth, R. D.; Engeser, M.; Gross, H.; Bringmann, G.; Piel, J.: The final steps of Bacillaene Biosynthesis in *Bacillus amyloliquefaciens* FZB42: direct evidence for β,γ dehydration by a trans-acyltransferase polyketide synthase. Angew. Chem., Int. Ed. 2010, 49, 1465–1467.

Abstract: No available abstract in the full study.

Macrolactin group

Four members of the macrolactin family were detected in *Bacillus amyloliquefaciens* FZB42: macrolactin A, macrolactin D, and 7-O-malonyl- and 7-O-succinylmacrolactin A. Macrolactin A was isolated by Yuan et al. (2012) and exhibits efficient antibacterial activity, in that it inhibits cell division in *Staphylococcus aureus*.

Plant Growth Hormones

Indole-3-acetic acid (IAA)

The *Bacillus amyloliquefaciens* and *Bacillus subtilis* produces substances with auxin indole-3-acetic acid (IAA)-like activity (Idris *et al*., 2004, Idris *et al*., 2007). IAA is a plant growth hormone.

According to the ECHA classification and labelling website IAA will likely be classified as:

GHS07 Warning
Skin Irrit. 2 H315
Eye Irrit. 2 H319
STOT SE 3 H355

3-Hydroxy-2-butanone and Butane-2,3-diol
Bacillus amyloliquefaciens and *Bacillus subtilis* produce a range of volatile compounds, which are also plant growth compounds: 3-hydroxybutan-2-one (acetoin) and butane-2,3-diol. 3-hydroxybutan-2-one was found to be metabolised in rat liver to butane-2,3-diol (Gabriel *et al*. 1971)

According to the ECHA classification and labelling website 3-hydroxybutan-2-one (acetoin) will likely be classified as:

GHS02; GHS07 Warning
Flam. Sol. 2 H228
Skin Irrit. 2 H315
Eye Irrit. 2 H319

According to the ECHA classification and labelling website butane-2,3-diol will likely be classified as:

GHS07 Warning
Eye Irrit. 2 H319

Or not classified.

Reference: Idris, E. E. S.; Bochow, H.; Ross, H.; Borriss, R. J.: Use of *Bacillus subtilis* as biocontrol agent. VI phytohormone like action of culture filtrates FZB24, FZB42, FZB45 *and Bacillus subtilis* FZB37. Plant Dis. Prot. 2004, 111, 583–597

Abstract: Culture filtrates of plant growth-promoting rhizobacteria (PGPR) *Bacillus amyloliquefaciens* (FZB24, FZB42 and FZB45) and *Bacillus subtilis* FZB37 have a strong growth-promoting activity. Bioassays, as seedling segment elongation and coleoptiles bending, performed with diluted *Bacillus* culture filtrates demonstrated that length growth of maize seedlings is significantly enhanced. *Bacillus amyloliquefaciens* FZB42 exhibited the highest enhancement on plant growth comparable with concentrations of 10–6 to 10–7 mol/l indole-3-acetic acid (IAA). However, only low concentrations of 10–8–10–9 mol/l of IAA were detected in ELISA tests using IAA-specific antibodies. FZB42 was found to produce the highest IAA concentration after growing in Landy medium at low temperature (22 °C) and oxygen limitation (75 rpm). The concentration of IAA, detected by IAA-specific antibodies, is not equivalent to the concentration of IAA necessary to induce the observed bio-effects, indicating that these bio-effects might result from more than one plant growth-promoting substance.

Reference: Idris, E. E. S.; Iglesias, D.; Talon, M.; Borriss, R.: Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. Mol. Plant-Microbe Interact. 2007, 20, 619–626

Abstract: Phytohormone-like acting compounds previously have been suggested to be involved in the phytostimulatory action exerted by the plant-beneficial rhizobacterium *Bacillus amyloliquefaciens* FZB42. Analyses by high-performance liquid chromatography and gas chromatography-mass spectrometry performed with culture filtrates of FZB42 demonstrated the presence of indole-3-acetic acid (IAA), corroborating it as one of the pivotal plant-growth-promoting substances produced by this bacterium. In the presence of 5 mM tryptophan, a fivefold increase in IAA secretion was registered. In addition, in the *trp* auxotrophic strains E101 (*ΔtrpBA*) and E102 (*ΔtrpED*), and in two other strains bearing knockout mutations in genes probably involved in IAA metabolism, E103 (*ΔysnE*, putative IAA transacetylase) and E105 (*ΔyhcX*, putative nitrilase), the concentration of IAA in the culture filtrates was diminished. Three of these mutant strains were less efficient in

promoting plant growth, indicating that the Trp-dependent synthesis of auxins and plant growth promotion are functionally related in *B. amyloliquefaciens*.

Reference: Gabriel, M.A.; Jabara, H.; Al-Khalidi, U.A.S.: Metabolism of Acetoin in Mammalian Liver Slices and Extracts. Biochem. J. (1971) 124, 793-800.

Abstract: 1. [^{14}C]Acetoin was enzymically synthesized from [^{14}C]pyruvate with a pyruvate decarboxylase preparation. Its optical activity was $[a]^{20}_{d}$-78°. 2. Large amounts (1000-fold higher than physiological concentrations) of acetoin were incubated with rat liver mince. Acetoin disappeared but very little $^{14}CO_2$ was evolved. A compound accumulated, which was purified and identified as butane-2,3-diol. Chromatography on borate-impregnated paper indicated the presence of both the erythro and threo forms. 3. Liver extracts capable of interconverting biacetyl, acetoin and butane-2,3-diol were obtained. These interconversions were catalysed by two different enzymes: acetoin dehydrogenase (EC 1.1.1.5) and butane-2,3-diol dehydrogenase (EC 1.1.1.4), previously identified in bacteria. Both required NAD^+ or $NADP^+$ as cofactors and were different from alcohol dehydrogenase. The equilibrium in both cases favoured the more reduced compound. 4. The activity of butane-2,3-diol dehydrogenase was decreased by dialysis against EDTA: the addition of Co^{2+}, Cu^{2+}, Zn^{2+} and other bivalent metal ions restored activity. 5. Biacetyl reductase was resolved into multiple forms by CM-Sephadex chromatography and electrophoresis.

Miscellaneous Antibiotics

Bacilysin

Bacilysin is a non-ribosomally synthesized dipeptide, is composed of L-alanine and the amino acid L-anticapsin and represents one of the simplest peptide antibiotics known with antifungal and antibacterial activities. Bacilysin is effective as a biocontrol agent (Arguelles-Arias *et al*. 2009). It is of moderate toxicity (intraperitoneal LD_{50} = 1000 mg/kg[6], intravenous LD_{50} = 450 mg/kg).

Reference: Arguelles-Arias, A.; Ongena, M.; Halimi, B.; Lara, Y.; Brans, A.; Joris, B.; Fickers, P.: *Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. Microb Cell Fact 2009 8:63–74

Abstract: **Background:** Phytopathogenic fungi affecting crop and post-harvested vegetables are a major threat to food production and food storage. To face these drawbacks, producers have become increasingly dependent on agrochemicals. However, intensive use of these compounds has led to the emergence of pathogen resistance and severe negative environmental impacts. There are also a number of plant diseases for which chemical solutions are ineffective or non-existent as well as an increasing demand by consumers for pesticide-free food. Thus, biological control through the use of natural antagonistic microorganisms has emerged as a promising alternative to chemical pesticides for more rational and safe crop management.
Results: The genome of the plant-associated *B. amyloliquefaciens* GA1 was sample sequenced. Several gene clusters involved in the synthesis of biocontrol agents were detected. Four gene clusters were shown to direct the synthesis of the cyclic lipopeptides surfactin, iturin A and fengycin as well as the iron-siderophore bacillibactin. Beside these non-ribosomaly synthetised peptides, three additional gene clusters directing the synthesis of the antibacterial polyketides macrolactin, bacillaene and difficidin were identified. Mass spectrometry analysis of culture supernatants led to the identification of these secondary metabolites, hence demonstrating that the corresponding biosynthetic gene clusters are functional in strain GA1. In addition, genes encoding enzymes involved in synthesis and export of the dipeptide antibiotic bacilysin were highlighted. However, only its chlorinated derivative, chlorotetaine, could be detected in culture supernatants. On the contrary, genes involved in ribosome-dependent synthesis of bacteriocin and other antibiotic peptides were not detected as compared to the reference strain *B. amyloliquefaciens* FZB42.
Conclusion: The production of all of these antibiotic compounds highlights *B. amyloliquefaciens* GA1 as a good candidate for the development of biocontrol agents.

RMS's comment:

[6] *CRC Handbook of antibiotic compounds. Berdy, J.; Boca Raton, F.L. CRC Press.*

Applicant provided a literature research on metabolites produced by *Bacillus amyloliquefaciens* in agreement with information presented in different report[78] submitted to EFSA and in other Draft assessment reports of *Bacillus subtilis*.

The mode of action of *Bacillus amyloliquefaciens strain* MBI600 against plant pathogenic fungi is partly based on the production and secretion of metabolites that disrupts hyphal growth and prevents spore germination (see part B2.1.2.2). It makes sense to find surfactin and iturin in the broth fermentation of *Bacillus amyloliquefaciens strain* MBI600.
These substances belong to the lipopeptide family, strong surfactants showing membrane damaging properties *in vitro*. Effects observed in laboratory tests, e.g. cytotoxicity to particular animal cell lines (Mikkola *et al*. (2004)), are due to those surface-active compounds targeting biological membranes but not the genome. Moreover, lytic activity activity of these substances appears to be inhibited under *in vivo* conditions.

B.6.1.2.4. Cell culture study

Cell culture studies are not required, since *Bacillus* does not enter the cytoplasm to replicate intra-cellularly.

B.6.1.2.5. Information on short-term toxicity and pathogenicity

The acute oral and acute intravenous studies demonstrate that *Bacillus amyloliquefaciens* strain MBI600 is of low toxicity to mammals (acute oral LD_{50} >5000 mg/kg bw). There is no evidence of toxicity, pathogenicity or infectivity following administration. *Bacillus amyloliquefaciens* strain MBI600 is rapidly eliminated from mammals, predominantly via the faeces. During the 22 day observation period, no adverse effects were noted. Therefore short term studies are not required.

The acute inhalation study demonstrates that *Bacillus amyloliquefaciens* strain MBI600 is of low toxicity to mammals (acute inhalation LC_{50} >5.31 mg/L).

B.6.1.2.5.1. Health effects after repeated inhalatory exposure

B.6.1.2.6. Proposed treatment: first aid measures, medical treatment

Skin contact:	In case of contact with skin wash off immediately with soap and water.
Eye contact:	In the case of contact with eyes, rinse immediately with plenty of water for at least 15 minutes.
Ingestion:	Drink plenty of water. Do not induce vomiting. Seek medical advice.
Inhalation:	Move to fresh air and ventilate suspected area.
Notes to the physician	
Symptoms:	No specific symptoms are known.
Treatment:	No specific antidote. Initial treatment should be symptomatic and supportive.

B.6.1.2.7. Other/special studies

Bacillus amyloliquefaciens strain MBI600 has been demonstrated to be of low toxicity via the oral, inhalation and intravenous routes. The mortality observed by intratracheal route has been attributed to the mode of administration. There is no evidence of pathogenicity or infectivity following all routes of administration. Therefore further testing is not required.

The following studies, although not required, are available and therefore are submitted for completeness.

[7] *C. Martel et al. Bibliographic review on the potential microorganisms, microbial products and enzymes to induce respiratory sensitization- Scinetif/Technical report submitted to EFSA*
[8] *S. Mudgal et al. Scientific support, literature review and data collection and analysis for risk assessment on microbial organisms used as active substance in plant protection products –Lot 1 Environmental Risk characterisation- EFSA supporting publication 2013:EN-518*

Reference:	[redacted] (1989) Acute dermal toxicity to rabbits of MBI600. [redacted] Unpublished report No. 89270D/AGC 1/1/AC
Guideline / GLP:	EPA FIFRA 152A-11/ Yes
Deviation:	None
Acceptability:	**Yes**
Test substance / purity:	*Bacillus subtilis* strain MBI600 [later confirmed to be *Bacillus amyloliquefaciens* strain MBI600] (NCIB 12376) spore/metabolite suspension
Dose	2.0 ml/kg bw

Materials and methods:
Ten New Zealand White Rabbits (5 males, 5 females) were assigned to the treatment group. Approximately 24 hours before treatment, the dorso-lumbar region of each rabbit was clipped free of fur, exposing approximately 10% of the total body surface area.

Test material (2.0 ml/kg bw equivalent to 2.0 x 10^{10} spore/kg bw), as supplied, was applied to the exposed skin. The application site was covered with a gauze patch and held in place with Elastoplast.

After approximately 24 hours exposure, the dressing was removed and the skin was gently washed. Animals were observed soon after doing and at frequent intervals on Day 1. On subsequent days, they were observed twice daily and dermal reactions were scored according to Draize.

Following the observation period, rabbits were sacrificed by intravenous overdose of pentobarbitone sodium and subjected to gross pathological examination, consisting of external examination and opening of the abdominal and thoracic cavities.

Results:
Mortality
One male died within 24 hours of dosing. The death was not considered to be treatment related.

Clinical signs
No clinical signs were noted in any of the animals.

Dermal irritation
Slight erythema with or without slight oedema was observed in all surviving animals after removal of the dressings. The reactions had completely resolved by Day 3.

Details are presented in following table below:

Table B6.1.2.7-1 Irritation indices following a topical treatment of *Bacillus amyloliquefaciens* strain MBI600 to the skin of rabbits

Rabbit No. and sex	E = Erythema O = Oedema	Observation time point (after patch removal)					
		0.5h	24h	48h	72h	7d	14d

Rabbit No. and sex	E = Erythema O = Oedema	Observation time point (after patch removal)					
		0.5h	**24h**	**48h**	**72h**	**7d**	**14d**
1 (male)	E O	1 1	0 0	0 0	0 0	0 0	0 0
2 (male)	E O	1 1	0 0	0 0	0 0	0 0	0 0
3 (male)	E O	Deceased					
4 (male)	E O	1 1	0 0	0 0	0 0	0 0	0 0
5 (male)	E O	1 0	0 0	0 0	0 0	0 0	0 0
6 (female)	E O	1 1	0 0	0 0	0 0	0 0	0 0
7 (female)	E O	1 0	0 0	0 0	0 0	0 0	0 0
8 (female)	E O	1 0	0 0	0 0	0 0	0 0	0 0
9 (female)	E O	1 0	0 0	0 0	0 0	0 0	0 0
10 (female)	E O	1 1	0 0	0 0	0 0	0 0	0 0
Mean score	E O	1 0.6	0 0	0 0	0 0	0 0	0 0

Bodyweight
Slightly low body weight gains were recorded for 2 males and three females in the second week of the study. All other animals achieved expected bodyweight gains throughout the study.

Necropsy
Gross necropsy revealed no observable abnormalities in all surviving animals.

In the rabbit that died, congestion of blood vessels was observed in the heart, lungs, liver, kidneys and stomach. The stomach and small intestine were gaseous and distended. The small intestine was liquid filled and the large intestine contained loose faeces. The colon was liquid filled and had red coloured mucosa.

Dose quantification
The stock solution of *Bacillus amyloliquefaciens* strain MBI600 was estimated to contain 10^{10} cfu per ml.

Conclusions:
Based on the above results, the median lethal dose (LD_{50}) to male and female rabbits of *Bacillus amyloliquefaciens* strain MBI600 after a single dermal dose is >2 ml/kg bw.

Bacillus amyloliquefaciens strain MBI600 is considered to be non-irritant to rabbit skin.

Reference: [redacted] (2011b) Acute dermal toxicity study in rats. [redacted] Unpublished report No. 15627-11

Guideline / GLP: OCSPP 870.1200, OCDE 402/ Yes

Deviation: Temperature and relative humidity was at times outside of the protocol range. Female weight was over the protocol range. This was not considered to affect the results of the study.

Acceptability: **Yes**

Test substance / purity: *Bacillus subtilis* strain MBI600 Technical [later confirmed to be *Bacillus amyloliquefaciens* strain MBI600]
Lot No.: BS7211
Purity: 8.07 x 10^{11} spores/g

Dose 5050 mg/kg bw equivalent to 4.08 x 10^{11}spore/kg bw

Materials and methods:
Ten Albino Sprague-Dawley rats (5 males, 5 females) were assigned to the treatment group. Approximately 24 hours before treatment, the dorsal region of each rat was clipped free of fur, exposing approximately 10% of the total body surface area.

Test material (5050 mg/kg bw equivalent to 4.08 x 10^{11}spore/kg bw), moistened with 1.0 ml/g de-ionised water, was applied to the exposed skin. The application site was covered with a gauze patch and held in place with non-irritating tape. The trunk of each animal was wrapped in a veterinary bandage.

After approximately 24 hours exposure, the dressing was removed and the skin was gently washed with water. Animals were observed three times on Day 1. On subsequent days, they were observed once daily. Dermal reactions were scored according to Draize 60 minutes after removing the bandages and on days 4, 7, 11 and 14.

Following the observation period, rats were sacrificed by carbon dioxide asphyxiation and subjected to gross pathological examination.

Results:
Mortality
No mortality was observed in any of the rats.

Clinical signs
No clinical signs were noted in any of the animals.

Dermal irritation
Very slight erythema was observed on Day 1.

Details are presented in Table B6.1.2.7-2 below:

Table B6.1.2.7-2 Irritation indices following a topical treatment of *Bacillus amyloliquefaciens* strain MBI600 to the skin of rats

Rabbit No. and sex	E = Erythema O = Oedema	Observation time point (after patch removal)				
		1d	4d	7d	11d	14d
1	E	1	0	0	0	0
(male)	O	0	0	0	0	0
2	E	1	0	0	0	0
(male)	O	0	0	0	0	0
3	E	1	0	0	0	0
(male)	O	0	0	0	0	0
4	E	1	0	0	0	0
(male)	O	0	0	0	0	0
5	E	1	0	0	0	0
(male)	O	0	0	0	0	0
6	E	1	0	0	0	0
(female)	O	0	0	0	0	0
7	E	1	0	0	0	0
(female)	O	0	0	0	0	0
8	E	1	0	0	0	0
(female)	O	0	0	0	0	0
9	E	1	0	0	0	0
(female)	O	0	0	0	0	0
10	E	1	0	0	0	0
(female)	O	0	0	0	0	0
Mean	E	1	0	0	0	0
score	O	0	0	0	0	0

Bodyweight
Slightly low body weight gains were recorded for 2 males and three females in the second week of the study. All other animals achieved expected bodyweight gains throughout the study.

Necropsy
Gross necropsy revealed no observable abnormalities.

Conclusions:
Based on the above results, the median lethal dose (LD_{50}) to male and female rats of *Bacillus amyloliquefaciens* strain MBI600 after a single dermal dose is >5050 mg/kg bw equivalent to 4.08 x 10^{11}spore/kg bw.

Bacillus amyloliquefaciens strain MBI600 is considered to be non-irritant to rat skin.

Reference: [redacted] (2011c) Acute dermal irritation study in rabbits. [redacted] Unpublished report No. 15630-11

Guideline / GLP: OCSPP 870.2500, OCDE 404/ Yes

Deviation: Relative humidity was at times outside of the protocol range. This was not considered to affect the results of the study.

Acceptability: **Yes**

Test substance / purity: *Bacillus subtilis* strain MBI600 Technical [later confirmed to be *Bacillus amyloliquefaciens* strain MBI600]
Lot No.: BS7211
Purity: 8.07 x 10^{11} spores/g

Materials and methods:
Three Albino New Zealand White rabbits (2 males, 1 female) were assigned to the treatment group. Approximately 24 hours before treatment, the dorsal region of each rabbit was clipped free of fur, exposing an area approximately 8 x 8 cm.

Test material (500 mg), moistened with 1.5 ml de-ionised water, was applied to the exposed skin. The application site was covered with a gauze patch and held in place with non-irritating tape. The trunk of each animal was wrapped in a veterinary bandage.

After approximately 4 hours exposure, the dressing was removed and the skin was gently washed with water. Dermal reactions were scored according to Draize at 1, 24, 48 and 72 hours after removal of the bandages.

Results:
Very slight erythema was observed in one animal at the one hour observation time point only. Oedema was not observed during the study. No other signs of irritation were noted during the study.

Details are presented in Table B6.1.2.7-3 below:

Table B6.1.2.7-3 Irritation indices following a topical treatment of *Bacillus amyloliquefaciens* strain MBI600 to the skin of rabbits

Rabbit No. and sex	E = Erythema O = Oedema	Observation time point (after patch removal)					
		1h	24h	48h	72h	7d	14d
1 (male)	E O	1 0	0 0	0 0	0 0	-	-
2 (male)	E O	0 0	0 0	0 0	0 0	-	-
3 (female)	E O	0 0	0 0	0 0	0 0	-	-
Mean score	E O	0.33 0	0 0	0 0	0 0	-	-

Conclusions:
Bacillus amyloliquefaciens strain MBI600 is considered to be non-irritant to rabbit skin.

Report:	[redacted] (1989) Primary eye irritation and infectivity of MBI600. [redacted] Unpublished report No. 89399D/AGC 1/4/SE
Guideline/GLP:	EPA FIFRA 152A-14/ Yes
Deviation:	-
Acceptability:	Yes
Test substance / purity:	Bacillus subtilis strain MBI600 Technical [later confirmed to be *Bacillus amyloliquefaciens* strain MBI600] Lot No.: BS7211 Purity: 8.07×10^{11} spores/g

Materials and methods:
Six Albino New Zealand White rabbits (6 females) were assigned to the treatment group. Test material (0.1 ml – providing 10^9 viable spores/animal) was placed into the lower everted eyelid of one eye of each animal. The eyelids were gently held together for 1 second before releasing. The eye was not washed. The untreated eye served as a control.

Reactions were scored according to Draize at 1 hour and 1, 2, 3, 4, 7, 14 and 21 days after instillation.

The eyes and eyelids of the animals were examined for the presence of bacterial spores by swabbing. Swabs were taken prior to dosing, then at 1, 2, 3, 4, 7, 14 and 21 days after instillation.

Results:
Ocular reactions
No effects on the cornea or iris were noted during the study.

At 1, 24 and 48 hours post instillation, conjunctival redness was observed in the treated eyes of all the rabbits. At 72 hours post instillation, the treated eye of 2 rabbits revealed conjunctival redness and reactions had fully resolved in the other 4 rabbits. On day 4, post instillation, the treated eyes of all the rabbits had recovered completely and appeared normal.

No abnormalities were detected in the control eyes of the rabbits.

Details are presented in Table B6.1.2.7-4 below:

Table B6.1.2.7-4 Irritation indices following instillation of *Bacillus amyloliquefaciens* strain MBI600 into the conjunctival sac of rabbits

Rabbit No. and sex	Reaction	Observation time point (after instillation)					Mean of 24, 48, 72 h scores
		1h	24h	48h	72h	4d	

Rabbit No. and sex	Reaction	Observation time point (after instillation)					Mean of 24, 48, 72 h scores
		1h	24h	48h	72h	4d	
1 (female)	Cornea opacity	0	0	0	0	0	0
	Iris lesion	0	0	0	0	0	0
	Conjunctival redness	1	1	1	0	0	0.67
	Conjunctival oedema	0	1	1	0	0	0.67
2 (female)	Cornea opacity	0	0	0	0	0	0
	Iris lesion	0	0	0	0	0	0
	Conjunctival redness	1	1	1	1	0	1
	Conjunctival oedema	0	1	1	1	0	1
3 (female)	Cornea opacity	0	0	0	0	0	0
	Iris lesion	0	0	0	0	0	0
	Conjunctival redness	1	1	1	1	0	1
	Conjunctival oedema	0	1	1	0	0	0.67
4 (female)	Cornea opacity	0	0	0	0	0	0
	Iris lesion	0	0	0	0	0	0
	Conjunctival redness	1	1	1	0	0	0.67
	Conjunctival oedema	0	1	1	0	0	0.67
5 (female)	Cornea opacity	0	0	0	0	0	0
	Iris lesion	0	0	0	0	0	0
	Conjunctival redness	1	1	1	0	0	0.67
	Conjunctival oedema	0	1	1	0	0	0.67
6 (female)	Cornea opacity	0	0	0	0	0	0
	Iris lesion	0	0	0	0	0	0
	Conjunctival redness	1	1	1	0	0	0.67
	Conjunctival oedema	0	1	1	0	0	0.67

Dose quantification
The stock solution of *Bacillus amyloliquefaciens* strain MBI600 was estimated to contain 10^{10} cfu per ml.

Presence of *Bacillus amyloliquefaciens* strain MBI600 in the eye
No *Bacillus amyloliquefaciens* spores were found prior to dosing. Day 1 and 2 swabs are only available for 1 rabbit due to heavy contamination with a gram-negative organism, which made counting *Bacillus amyloliquefaciens* impossible.

3, 4 and 7 days after instillation, small numbers of *Bacillus amyloliquefaciens* spores were found in the control eye, which was attributed to grooming. Large numbers were found in the treated eye, particularly from the lids, with relatively small numbers being found in the conjunctival fluid.

After 14 and 21 days, virtually no spores were recovered, with the exception of relatively small numbers from the treated eye lids.

Conclusions:
Bacillus amyloliquefaciens strain MBI600 is considered to be slightly irritant to rabbit eyes.

Report: [redacted] (2011d) Acute eye irritation study in rabbits. [redacted] Unpublished report No. 15629-11
Guideline/GLP: OCSPP 870.2400, OECD 405/ Yes
Deviation: -
Acceptability: Yes
Test substance / purity: *Bacillus subtilis* strain MBI600 Technical [later confirmed to be *Bacillus amyloliquefaciens* strain MBI600]
Lot No.: BS7211
Purity: 8.07 x 10^{11} spores/g

Materials and methods:
Three Albino New Zealand White rabbits (1 male, 2 females) were assigned to the treatment group. Test material (100 mg), as supplied was placed into the conjunctival sac of the right eye of each animal. The eyelids were gently held together for 1 second before releasing. The eye was not washed. The untreated eye served as a control.

Reactions were scored according to Draize at 1, 24, 48 and 72 hours and 4 days after instillation.

Results:
A slight dulling of the cornea was observed in all animals at 24 and 48 hours after instillation. All reactions were resolved at 72 hours.

No effects on the iris were noted during the study.

At 1 hour post instillation, conjunctival redness was observed in the treated eyes of all the rabbits. 48 and 72 hours post instillation, the treated eye of all rabbits revealed conjunctival redness, with a diffuse crimson colour. On day 4, post instillation, the treated eyes of all the rabbits had recovered completely and appeared normal.

No abnormalities were detected in the control eyes of the rabbits.

Details are presented in Table B6.1.2.7-5 below:

Table B6.1.2.7-5 Irritation indices following instillation of *Bacillus amyloliquefaciens* strain MBI600 into the conjunctival sac of rabbits

Rabbit No. and sex	Reaction	Observation time point (after instillation)					Mean of 24, 48, 72 h scores
		1h	24h	48h	72h	4d	
1 (male)	Cornea opacity	0	0	0	0	0	0
	Iris lesion	0	0	0	0	0	0
	Conjunctival redness	1	2	2	1	0	1.67
	Conjunctival oedema	1	2	2	0	0	1.33
2 (female)	Cornea opacity	0	0	0	0	0	0
	Iris lesion	0	0	0	0	0	0
	Conjunctival redness	1	2	2	1	0	1.67
	Conjunctival oedema	1	2	2	1	0	1.67
3 (female)	Cornea opacity	0	0	0	0	0	0
	Iris lesion	0	0	0	0	0	0
	Conjunctival redness	1	2	2	1	0	1.67
	Conjunctival oedema	1	2	2	1	0	1.67

Conclusions:
Conjunctival redness was observed in the treated eye of all rabbits, with the mean of 24, 48 and 72h scores for each animal being 1.67, 1.67 and 1.67. Conjunctival chemosis was observed in all rabbits, with the mean of 24, 48 and 72h scores for each animal being 1.33, 1.67 and 1.67. All reactions had fully resolved in 4 days.

Bacillus amyloliquefaciens strain MBI600 is considered to be slightly irritant to rabbit eyes.

B.6.1.3. Summary and conclusions of Tier I studies

EFSA has considered that *Bacillus amyloliquefaciens* is suitable for Qualified Presumption of Safety (QPS) assessment with the qualification of absence of toxigenic activity.

Toxicological studies performed with the active substance *Bacillus amyloliquefaciens* strain MBI600 revealed a low toxicity via the oral, dermal and intravenous routes and does not show any evidence of pathogenicity or infectivity. After intratracheal instillation of *Bacillus amyloliquefaciens* strain MBI600, deaths were seen shortly after administration, which is not consistent with an infection. Moreover, no trends of increase of body temperature at 24 hours were observed and no observable abnormalities were lesions were revealed at necropsy. This study did not show evidence of pathogenicity or infectivity, a pattern of clearance was established. Mortality in inhalation toxicity studies on microbial pesticides is not unusual, and these deaths seem to be the result of physical action rather than inherent toxicity of the substance. Furthermore, no mortality was observed in an intravenous study and in the inhalation toxicity study performed with the technical microorganism as currently marketed with a more realistic exposure pathway, no mortality was observed.
Bacillus amyloliquefaciens strain MBI600 is not irritating to skin or eyes. As many micro-organisms, this strain may have the potential to provoke sensitising reactions after skin and/or inhalation contact.

The fermentation broth of *Bacillus subtilis* strain MBI600 was not mutagenic in this bacterial test system either in the presence or absence of metabolic activation in the strains tested.

Bacillus amyloliquefaciens species is known to be a producer of a large range of secondary metabolites, especially non ribosomal cyclic lipopeptides. According to a screening test performed on *Bacillus amyloliquefaciens* MBI600, surfactin and iturin were the principal secondary metabolites produced in broth fermentation condition. These substances belong to the lipopeptides family, surface-active compounds targeting biological membranes but not the genome. Moreover, activity of these substances appears to be inhibited under *in vivo* conditions.

B.6.2. Tier II – the active micro-organism

B.6.2.1. Specific toxicity, pathogenicity and infectiveness studies (Annex IIB 5.5.1)

No adverse effects were observed in acute toxicity studies with *Bacillus amyloliquefaciens* MBI600. Therefore no further studies on specific toxicity, pathogenicity and infectiveness are required.

B.6.2.2. *In vivo* studies in somatic cells (Annex IIM 5.5.2)

No *in vivo* study in somatic cells was conducted and is not considered necessary based on the information and data provided.

B.6.2.3. Genotoxicity – *In vivo* studies in germ cells (Annex IIM 5.5.3)

There is no evidence for a probable mutagenic activity of *Bacillus amyloliquefaciens* strain MBI600.
No *in vivo* study in germ cells was conducted and is not considered necessary based on the information and data provided.

B.6.2.4. Summary and conclusions of Tier II studies

No further studies are required. Tier II were not considered necessary.

B.6.3. Summary of mammalian toxicity, pathogenicity and effectiveness and overall evaluation of the active micro-organism (Annex IIM 5.6)

EFSA has considered that *Bacillus amyloliquefaciens* is suitable for Qualified Presumption of Safety (QPS) assessment with the qualification of absence of toxigenic activity. Case reports on infection or other incidents in humans related to *Bacillus amyloliquefaciens* or *Bacillus subtilis* were scarce and occurred mainly in immune suppressed people.
Bacillus amyloliquefaciens is not listed in the directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work.
No evidence of adverse health effects have been observed in any of the employees involved in the production, handling and application of *Bacillus amyloliquefaciens* strain MBI600.

Two sets of studies were provided by the applicant:

- Studies performed in 1989: these studies were run with fermentation broth. The broth was designed to provide optimal conditions for *Bacillus amyloliquefaciens* MBI600 to produce metabolites. The studies therefore tested the toxicity of spores, other life-stages and a representative metabolite profile which the organism would produce under optimal conditions.
- Studies performed in 2011 and 2012 on *Bacillus amyloliquefaciens* strain MBI600 Technical. Tested batches were representative of current commercial lots.

Toxicological studies performed with the active substance *Bacillus amyloliquefaciens* strain MBI600 revealed a low toxicity via the oral, dermal and intravenous routes and does not show any evidence of pathogenicity or infectivity. After intratracheal instillation of *Bacillus amyloliquefaciens* strain MBI600, deaths were seen shortly

after administration, which is not consistent with an infection. Moreover, no trends of increase of body temperature at 24 hours were observed and no observable abnormalities were lesions were revealed at necropsy. This study did not show evidence of pathogenicity or infectivity, a pattern of clearance was established. Mortality in inhalation toxicity studies on microbial pesticides is not unusual, and these deaths seem to be the result of physical action rather than inherent toxicity of the substance. Furthermore, no mortality was observed in an intravenous study and in the inhalation toxicity study performed with the technical microorganism as currently marketed with a more realistic exposure pathway, no mortality was observed.
Bacillus amyloliquefaciens strain MBI600 is not irritating to skin or eyes. As many micro-organisms, this strain may have the potential to provoke sensitising reactions after skin and/or inhalation contact.
Bacillus amyloliquefaciens is not related to species known to produce genotoxins. The fermentation broth of *Bacillus subtilis* strain MBI600 was not mutagenic in this bacterial test system either in the presence or absence of metabolic activation in the strains tested.

Bacillus amyloliquefaciens species is known to be a producer of a large range of secondary metabolites, especially non ribosomal cyclic lipopeptides. According to a screening test performed on *Bacillus amyloliquefaciens* MBI600, surfactin and iturin were the principal secondary metabolites produced in broth fermentation condition. These substances belong to the lipopeptides family, surface-active compounds targeting biological membranes but not the genome. Moreover, activity of these substances appears to be inhibited under *in vivo* conditions.
In the light of the acute studies results and the literature data, no further studies with *Bacillus amyloliquefaciens* strain MBI600 were not considered necessary.

Table: B.6.3 -1. Summary of the toxicity studies performed with *Bacillus amyloliquefaciens* strain MBI600

Study	Item/Vehicle	Dose levels	Results
Skin sensitisation GLP/OECD 406 Guinea pig	fermentation broth of *Bacillus amyloliquefaciens* StrainMBI600/ Water or mixture of FCA with water	Epidermal induction: 0.1ml/site at 5% test item solution in vehicle challenge: 0.2ml site at 50% test item solution in vehicle	100% sensitisation response. Responses generally persisted up to the end of the observation period, with necrosis and severe oedema observed in some test animals
Acute oral Toxicity/Pathogenicity Study in Rats GLP/ EPA FIFRA 152A-10	fermentation broth of *Bacillus amyloliquefaciens* StrainMBI600/ Distilled water	10^9 viable spores/animal	LD 50 >10^9 viable spores Large numbers of spores survived passage through the intestinal tract, but were rapidly eliminated within 7 days. Clearance of live microorganism from all tissues analysed was complete 21 days after the dosing. No evidence of pathogenicity or infectivity.
Acute oral Toxicity/Pathogenicity Study in Rats GLP/OCSPP 870.1100 OECD 425	*Bacillus amyloliquefaciens* strain MBI600 technical (Batch BS7211)/ Water	5000 mg/kg bw 4.04 x 10^{11} spores/kg bw	LD_{50} > 4.04 10^{11} spores/kg bw
Acute pulmonary Toxicity/Pathogenicity Study in Rats GLP/ EPA FIFRA 152A-12	fermentation broth of *Bacillus amyloliquefaciens* StrainMBI600/ -	3.5 x 10^8 viable spore/animal	Mortality (shortly after administration) No evidence of pathogenicity or infectivity Clear pattern of clearance
Acute inhalation Toxicity GLP/OCSPP 870.1300 OECD 403	*Bacillus amyloliquefaciens* strain MBI600 technical (Batch BS7211)/-	5.31 mg/L 4.29 x 10^9 spores/mL	No mortality CL50> 5.31 mg/L

Acute intravenous toxicity and infectivity/pathogenicity in Rats GLP/EPA FIFRA 152A-13	fermentation broth of *Bacillus amyloliquefaciens* StrainMBI600/ Physiological saline	4.5 x 10^7 viable spores/animal	No evidence of pathogenicity or infectivity Clear pattern of clearance
Bacterial reverse mutation test of the supernatant of the fermentation broth of *Bacillus amiloliquefaciens* StrainMBI600	Supernatant of the fermentation broth of *Bacillus subtilis* strain MBI600 / -	4.88, 19.5, 78.1, 313, 1250 and 5000 µg/plate	Not mutagenic in absence and presence of S9 mix
Acute dermal toxicity study in rabbit GLP/EPA FIFRA 152A-11	fermentation broth of *Bacillus amyloliquefaciens* StrainMBI600/ -	2,0ml/ kg bw	No mortality Not irritating
Acute dermal toxicity study in rats GLP/OCSPP 870.1200, OCDE 402	*Bacillus amyloliquefaciens* strain MBI600 technical (Batch BS7211)/ moistened with 1.0 ml/g de-ionised water	5050 mg/kg bw equivalent to 4.08 x 10^{11} CFU/kg bw	No mortality Not irritating LD_{50} >5050 mg/kg bw equivalent to 4.08 x 10^{11} CFU/kg bw
Acute dermal irritation study in rabbit GLP/OCSPP 870.2500, OCDE 404	*Bacillus amyloliquefaciens* strain MBI600 technical (Batch BS7211)/ moistened with 1.5 ml/g de-ionised water	500 mg/ animal	Slightly irritating
Primary eye irritation and infectivity in rabbit GLP/EPA FIFRA 152A-14	fermentation broth of *Bacillus amyloliquefaciens* StrainMBI600/-	0.1 ml/animal equivalent to 10^9 viable spores/animal	Slightly irritating
Acute eye irritation study in rabbit GLP/ OCSPP 870.2400, OECD 405	*Bacillus amyloliquefaciens* strain MBI600 technical (Batch BS7211)/-	100 mg/animal	Slightly irritating

Taking together the results of experimental studies, published literature, the QPS statute of *Bacillus amyloliquefaciens*, the experience of safe production and application of *Bacillus amyloliquefaciens* strain MBI600 as plant protection product and the natural occurrence of *Bacillus amyloliquefaciens* it is appropriate to state that no concern has been raised with regard to human health.

The derivation of reference values for the microorganism is not considered necessary based on the lack of toxicity, infectivity or pathogenicity in the available studies

B.6.4. Effects on human health – the preparation

Subtilex® is formulated as a wettable powder (WP) and contains as active substance spores of *Bacillus amyloliquefaciens* MBI600 (11% w/w). Subtilex® is to be used on grape vines to control Botrytis. Subtilex® may be applied via a tractor mounted air assisted sprayer or a knapsack sprayer at a rate of 0.5 kg/ha of formulated product, which is equivalent to a minimum of 2.75 x 10^{13} cfu/ha. It is applied in 400 to 1000 L/ha of water, thus the concentration is between 2.75 x 10^{10} and 6.88 x 10^{10} cfu/L.

B.6.4.1. Basic acute toxicity studies – the preparation (Annex IIIM 7)

B.6.4.1.1. Acute oral toxicity

Report:	[redacted] (2011a) Acute oral toxicity (UDP) in rats. [redacted] Unpublished report No. 15387-11
Guideline/GLP:	OCSPP 870.1100, OECD 425/ yes
Deviation:	Relative humidity was at times outside of the protocol range. This was not considered to affect the results of the study.
Acceptability:	Yes
Test substance / purity:	Subtilex® Lot No.: 53357 Purity: 7.38 x 10^{10} spores/g

Materials and methods:
An acute oral toxicity study was conducted on rats using the up and down procedure.

Animals were fasted overnight for 16 hours prior to dosing. Dose solutions were freshly prepared prior to dosing using de-ionised water as a vehicle. All rats received a dose volume of 12.5 ml/kg bw of 40% Subtilex® (*Bacillus amyloliquefaciens* strain MBI600 11% w/w) by gavage equivalent to 5000 mg/kg bw of Subtilex®.

Following dosing, animals were observed for signs of toxicity and mortality three times on the day of dosing, then once daily during the 14-day observation period. Body weights were recorded on the day of dosing (Day 0) and on Days 7 and 14.

Following the 14-day observation period, rats were sacrificed by carbon dioxide asphyxiation and subjected to gross pathological examination.

Results:
Mortality
No mortality was observed in any of the rats.

Clinical signs
All animals appeared normal for the duration of the study.

Bodyweight
All animals achieved expected bodyweight gains throughout the study.

Necropsy
Gross necropsy conducted at termination of the study revealed no observable abnormalities.

Conclusions:
Based on the above results, the median lethal dose (LD_{50}) to female rats of Subtilex® (*Bacillus amyloliquefaciens* strain MBI600 11% w/w) after a single oral dose is >5000 mg/kg bw.

Based on the above results, Subtilex® (*Bacillus amyloliquefaciens* strain MBI600 11% w/w) does not warrant classification according to Directive 67/548/EEC or Regulation 1272/2008.

B.6.4.1.2. Acute inhalation toxicity

Report:	[redacted] (2011) Acute inhalation toxicity study in rats. [redacted] Unpublished report No. 15389-11
Guideline/GLP:	OCSPP 870.1300, OECD 403/ yes
Deviation:	Relative humidity was at times outside of the protocol range. This was not considered to affect the results of the study.
Acceptability:	Yes

Test substance / purity: Subtilex®
Lot No.: 53357
Purity: 7.38 x 10^{10} spores/g

Materials and methods:
Five male and 5 female Sprague-Dawley albino rats were exposed via the inhalation route to Subtilex® (*Bacillus amyloliquefaciens* strain MBI600 11% w/w) in air for 4 hours. Rats were exposed to a nose only concentration of 5.23 mg/L. The concentration of test substance in the breathing zone of the animals was determined gravimetrically twice per hour and nominally at the end of exposure. The particle size was monitored twice during the exposure using a cascade particle impactor.

Animals were observed for signs of toxicity and mortality frequently on the day of exposure. Subsequently, the rats were observed at least once daily during the 14-day observation period for mortality and clinical signs. Individual body weights were recorded on days 0, 7 and 14.

Following the 14-day observation period, rats were sacrificed by intraperitoneal injection of Fatal Plus® and subjected to gross pathological examination.

Results:
Mortality
No mortality was observed in any of the rats.

Clinical signs
Clinical signs included decreased activity and piloerection. All animals appeared normal by Day 4.

Bodyweight
All animals achieved expected bodyweight gains throughout the study.

Necropsy
Gross necropsy conducted at termination of the study revealed no observable abnormalities.

Conclusions:
Based on the above results, the median lethal concentration (LC_{50}) to rats of Subtilex® (*Bacillus amyloliquefaciens* strain MBI600 11% w/w) after a 4h inhalation dose is >5.23 mg/L.

Based on the above results, Subtilex® (*Bacillus amyloliquefaciens* strain MBI600 11% w/w) does not warrant classification according to Directive 67/548/EEC or Regulation 1272/2008.

B.6.4.1.3. Acute percutaneous toxicity

Report: [redacted] (2011b) Acute dermal toxicity study in rats. [redacted]
Unpublished report No. 15388-11
Guideline/GLP: OCSPP 870.1200, OECD 402/ yes
Deviation: Relative humidity was at times outside of the protocol range. This was not considered to affect the results of the study.
Acceptability: Yes
Test substance / purity: Subtilex®
Lot No.: 53357
Purity: 7.38 x 10^{10} spores/g

Materials and methods:
Ten Albino Sprague-Dawley rats (5 males, 5 females) were assigned to the treatment group. Approximately 24 hours before treatment, the dorsal region of each rat was clipped free of fur, exposing approximately 10% of the total body surface area.

Test material (5050 mg/kg bw), moistened with 1.0 ml/g de-ionised water, was applied to the exposed skin. The application site was covered with a gauze patch and held in place with non-irritating tape. The trunk of each animal was wrapped in a veterinary bandage.

After approximately 24 hours exposure, the dressing was removed and the skin was gently washed with water. Animals were observed three times on Day 0. On subsequent days, they were observed once daily. Dermal reactions were scored according to Draize 60 minutes after removing the bandages (Day 1) and on days 4, 7, 11 and 14. Individual body weights were recorded on days 0, 7 and 14.

Following the observation period, rats were sacrificed by carbon dioxide asphyxiation and subjected to gross pathological examination.

Results:
Mortality
No mortality was observed in any of the rats.

Clinical signs
No clinical signs were noted in any of the animals.

Dermal irritation
There were no signs of dermal irritation at any observation during the study.

Bodyweight
Slightly low body weight gains were recorded for 1 female in the second week of the study. All other animals achieved expected bodyweight gains throughout the study.

Necropsy
Gross necropsy revealed no observable abnormalities.

Conclusions:
Based on the above results, the median lethal dose (LD_{50}) to male and female rats of Subtilex® (*Bacillus amyloliquefaciens* strain MBI60 11% w/w) after a single dermal dose is >5050 mg/kg bw.

B.6.4.2. Additional acute toxicity studies – the preparation

B.6.4.2.1. Skin irritation

Report:	[redacted] (2011c) Acute dermal irritation study in rabbits. [redacted] Unpublished report No. 15391-11
Guideline/GLP:	OCSPP 870.2500, OECD 404/ yes
Deviation:	Relative humidity was at times outside of the protocol range. This was not considered to affect the results of the study.
Acceptability:	Yes
Test substance / purity:	Subtilex® Lot No.: 53357 Purity: 7.38 x 10^{10} spores/g

Materials and methods:
Three Albino New Zealand White rabbits (2 males, 1 female) were assigned to the treatment group. Approximately 24 hours before treatment, the dorsal region of each rabbit was clipped free of fur, exposing an area approximately 8 x 8 cm.

Test material (500 mg), moistened with 1.0 ml de-ionised water, was applied to the exposed skin. The application site was covered with a gauze patch and held in place with non-irritating tape. The trunk of each animal was wrapped in a semi-permeable dressing (orthopaedic stockinette).

After approximately 4 hours exposure, the dressing was removed and the skin was gently washed with water. Dermal reactions were scored according to Draize at 1, 24, 48 and 72 hours after removal of the bandages.

Results:
Mortality

One animal was found dead on Day 1 of the study. The death was not considered to be related to the administration of the test substance.

Dermal irritation
There were no signs of dermal irritation at any observation during the study.

Details are presented in Table B6.4.2.1-1 below:

Table B6.4.2.1-1 Irritation indices following a topical treatment of *Subtilex® (Bacillus amyloliquefaciens* strain MBI600 11% w/w) to the skin of rabbits

Rabbit No. and sex	E = Erythema O = Oedema	Observation time point (after patch removal)					
		1h	24h	48h	72h	7d	14d
0710 (male)	E	0	0	0	0	-	-
	O	0	0	0	0	-	-
0712 (male)	E	0	0	0	0	-	-
	O	0	0	0	0	-	-
0701 (female)	E	0	Deceased			-	-
	O	0				-	-
Mean score	E	0	0	0	0	-	-
	O	0	0	0	0	-	-

Conclusions:
Subtilex® (*Bacillus amyloliquefaciens* strain MBI600 11% w/w) is considered to be non-irritant to rabbit skin.

Based on the above results, Subtilex® (*Bacillus amyloliquefaciens* strain MBI600 11% w/w) does not warrant classification according to Directive 67/548/EEC or Regulation 1272/2008.

B.6.4.2.2. Eye irritation

Report: [redacted] (2011d) Acute eye irritation study in rabbits. [redacted] Unpublished report No. 15390-11
Guideline/GLP: OCSPP 870.2400, OECD 405/ yes
Deviation: Relative humidity was at times outside of the protocol range. This was not considered to affect the results of the study.
Acceptability: Yes
Test substance / purity: Subtilex®
Lot No.: 53357
Purity: 7.38 x 10^{10} spores/g

Materials and methods:
Three Albino New Zealand White rabbits (2 males, 1 female) were assigned to the treatment group. Test material (100 mg), as supplied was placed into the conjunctival sac of the right eye of each animal. The eyelids were gently held together for 1 second before releasing. The eye was not washed. The untreated eye served as a control.

Reactions were scored according to Draize at 1, 24, 48 and 72 hours and 4 days after instillation.

Results:
No effects on the iris or the cornea were noted during the study.

At 1 hour post instillation, conjunctival redness was observed in the treated eyes of all the rabbits, persisting to 48, 72 and 72 hours respectively. On day 4, post instillation, the treated eyes of all the rabbits had recovered completely and appeared normal.

Conjunctival chemosis was observed in all rabbits one hour after instillation, persisting to 72 hours in one rabbit only. Reactions had completely resolved in 2 animals 24 hours post dosing and in all animals by Day 4.

No abnormalities were detected in the control eyes of the rabbits.

Details are presented in Table B6.4.2.2-1 below:

Table B6.4.2.2-1 Irritation indices following a instillation of *Subtilex® (Bacillus amyloliquefaciens* strain MBI600 11% w/w) into the conjunctival sac of rabbits

Rabbit No. and sex	Reaction	Observation time point (after instillation)					Mean of 24, 48, 72 h scores
		1h	24h	48h	72h	4d	
0704 (male)	Cornea opacity	0	0	0	0	0	0
	Iris lesion	0	0	0	0	0	0
	Conjunctival redness	1	2	2	1	0	1.67
	Conjunctival oedema	1	1	1	1	0	1
0706 (male)	Cornea opacity	0	0	0	0	0	0
	Iris lesion	0	0	0	0	0	0
	Conjunctival redness	1	0	1	1	0	0.67
	Conjunctival oedema	1	0	0	0	0	0
0667 (female)	Cornea opacity	0	0	0	0	0	0
	Iris lesion	0	0	0	0	0	0
	Conjunctival redness	1	1	1	0	0	0.67
	Conjunctival oedema	1	0	0	0	0	0

Conclusions:
Conjunctival redness was observed in the treated eye of all rabbits, with the mean of 24, 48 and 72h scores for each animal being 1.67, 0.67 and 0.67. Conjunctival chemosis was observed in all rabbits, with the mean of 24, 48 and 72h scores for each animal being 1, 0 and 0. All reactions had fully resolved in 4 days.

Based on the above results, Subtilex® (*Bacillus amyloliquefaciens* strain MBI600 11% w/w) does not warrant classification according to Directive 67/548/EEC or Regulation 1272/2008.

B.6.4.2.3. Skin sensitisation

Report: [redacted] (2003) Skin sensitization study in guinea pigs. [redacted] Unpublished report No. 7831-03
Guideline/GLP: OPPTS 870.2600/ yes
Deviation: -
Acceptability: Not suitable for micro-organisms based product.
Test substance / purity: Subtilex®
Certificate of analysis not provided by applicant

Materials and methods:
A skin sensitisation study was conducted on Subtilex® (*Bacillus amyloliquefaciens* strain MBI600 11% w/w) according to the Buehler method. Two male and 2 female guinea pigs were selected for irritation screening to determine the maximum dose producing no more than moderate irritation and the maximum non-irritating dose. 25, 50 and 75% dilutions in de-ionised water were tested.

Twenty short-haired albino guinea pigs (10 males, 10 females) were assigned to the test group and ten (5 males, 5 females) were assigned to the control group. On the day prior to each treatment, the treatment site was clipped free of hair to expose an area at least 8 x 10 cm.

Induction
Induction treatments were performed on Days 1, 8 and 15. The same treatment site (back of the trunk) was used for all 3 induction treatments. Test material (400 mg), moistened with 0.3 ml de-ionised water, was applied to the exposed skin. The application site was covered with a gauze patch and held in place with non-irritating tape. A strip of clear polythene film was placed over the patch and securely taped. After 6 hours the dressing was removed.

The control group were not treated during the induction phase.

Challenge
On Day 29, test and control animals were treated on a virgin test site (right rear quadrant). Test material (400 mg), moistened with 0.3 mL de-ionised water, was applied to the exposed skin. The application site was covered with a gauze patch and held in place with non-irritating tape. A strip of clear polythene film was placed over the patch and securely taped. After 6 hours the dressing was removed.

The dermal reactions were scored according to Draize and were noted at approximately 24 hours after each treatment and additionally at 48 hours after the first induction and challenge treatments.

Findings:
There were no signs of dermal irritation in either the test or control groups.

Conclusions:
Subtilex® (*Bacillus amyloliquefaciens* strain MBI600 11% w/w) elicited a 0 % sensitisation response under the conditions of this study and is therefore considered not to be a skin sensitiser.

Based on the above results, Subtilex® (*Bacillus amyloliquefaciens* strain MBI600 11% w/w) does not warrant classification according to Directive 67/548/EEC or Regulation 1272/2008.

B.6.4.3. Data on exposure – the preparation (Annex IIIM 7.2)

The product Subtilex® is formulated as a wettable powder (WP) and contains as active substance spores of *Bacillus amyloliquefaciens* strain MBI600 (11% w/w). Subtilex® is to be used on grape vines to control Botrytis. Subtilex® may be applied via a tractor mounted air assisted sprayer or a knapsack sprayer at a rate of 0.5 kg/ha of formulated product, which is equivalent to a minimum of 2.75×10^{13} cfu/ha. It is applied in 400 to 1000 L/ha of water, thus the concentration is between 2.75×10^{10} and 6.88×10^{10} cfu/L. (table 12).

Table B6.4.2.3-1 Intended uses

Crop	Scenario	Number of applications	Max. dose (kg product/ha)	Application rate (kg MPCA/ha)	Water (l/ha)
Vines	Field	1-10	0.5 kg/ha	0.055 (2.75×10^{13}cfu/ha)	400-100

Operator exposure

No toxicological reference values were derived from the studies performed.
Meanwhile, the acute and short term toxicity/pathogenicity studies submitted in support of the registration demonstrate that *Bacillus amyloliquefaciens* strain MBI600 is not toxic or infective and is non-pathogenic to mammals.

As no especially designed models for microorganisms are available, the exposure is qualitatively estimated.
The primary routes of exposure for mixer/loaders and applicators would be dermal and/or inhalation exposure. The product Subtilex® is a wettable powder (WP). Exposure to *Bacillus amyloliquefaciens* strain MBI600 spores is therefore expected during mixing and loading of the product.
Furthermore, protective gloves, coverall, goggles and a respiratory mask (a filter mask with a type P3 filter) have to be used by operators handling Subtilex® due to the potentially sensitising properties of the active substance.
In these conditions, the operator exposure could be considered as negligible.
Taking into account the results of toxicity/pathogenicity studies, the rapid clearance, literature data and the very low exposure, no unacceptable risk is anticipated for the operator.

Bystander exposure

Following the above given reasons for abstaining from an estimation of operator risk assessment, this also applies with regard to bystanders. *Bacillus amiloliquefaciens* preparations including the formulation Subtilex® are considered safe for bystanders as well.

Worker exposure

Following the above given reasons for abstaining from an estimation of operator risk assessment, this also applies with regard to workers. *Bacillus amiloliquefaciens* preparations including the formulation Subtilex® are considered safe for workers as well.

In case of any post-application activities where contact to treated crop can occur, workers should also wear appropriate personal protective equipment (protective gloves).

B.6.4.4. Available toxicological data relating to non-active substances – the preparation (Annex IIIM 7.4)

The product Subtilex® contains contains as active substance spores of *Bacillus amyloliquefaciens* strain MBI600 and non-active ingredients, which do not affect the overall toxicity of the preparation considering the results of toxicity studies, as, indicated in the MSDS of the formulants. The properties of non-active ingredients and their toxicological data are provided in volume 4.

B.6.4.5. Supplementary studies for combinations of plant protection products (Annex IIIM 7.5)

The formulation is not recommended to be combined with another adjuvant or pest control product.

B.6.5. Summary and evaluation of health effects – the preparation (Annex IIIM 7.6)

All toxicological studies performed with the MPCP Subtilex® (*Bacillus amyloliquefaciens* strain MBI600 11% w/w) revealed no effects to human or animal health. It is of low toxicity via the oral, dermal and inhalation routes. It is not irritating to skin or eyes and is not a skin sensitizer (however sensitizing tests are not considered suitable for micro-organisms based products). The MPCA, *Bacillus amyloliquefaciens* strain MBI600 does not show any evidence of pathogenicity or infectivity.

Proposed labelling phrase for the formulated product Subtilex® is:
"Contains *Bacillus amyloliquefaciens* strain MBI600. Micro-organisms may have the potential to provoke sensitising reactions."
Therefore appropriate personal protective equipment should be worn when handling Subtilex®.

Subtilex® is intended on grape vines to control Botrytis. The derivation of an AOEL is not necessary based on the lack of adverse effect related to the microorganism in the available studies.

Operator exposure is considered acceptable when PPE and RPE are used. Worker exposure is considered acceptable when PPE is used. Bystander exposure is considered acceptable under the proposed conditions of use.

Table B6.4.2.3-1 Summary of toxicity studies performed on the formulated product Subtilex®.

Study	Test item	Dose levels	Finding

Acute oral toxicity GLP/ OCSPP 870.1100 OECD 425 rat	Subtilex® Batch n°53357	5000 mg/kg bw	LD_{50} > 5000 mg/kg bw
Acute inhalation GLP/ OCSPP 870.1300 OECD 403 rat	Subtilex® Batch n°53357	5.23 mg/L	LC_{50}> 5.23 mg/L
Acute percutaneous toxicity GLP/ OCSPP 870.1200 OECD 402 rat	Subtilex® Batch n°53357	5050 mg/kg bw	LD_{50} > 5050 mg/kg bw
Skin irritation GLP/ OCSPP 870.2500 OECD 404 rabbit	Subtilex® Batch n°53357	500 mg/animal	Not irritating
Eye irritation GLP/ OCSPP 870.2400 OECD 405 rabbit	Subtilex® Batch n°53357	100 mg/animal	Slightly irritating
Skin sensitisation GLP/OPPTS 870.2600 Buehler 3 applications Guinea pig	Subtilex® Batch n°53357	400 mg/animal	Not sensitising

B.6.6. References relied on

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
Annex II Data and Information					
IIM, 5/02	Kloepper, J.W.	2012	Identification of MBI600 by 16S rRNA gene sequencing using 8F and 1492R primers Auburn University, USA Becker Underwood No report No. Not GLP Unpublished **Supporting information, study not fully summarised**	Y	BASF Agricultural Specialities Ltd
IIM, 5/03	Yang, G.P.	2005	Identification of MBI600 by 16S rRNA gene sequencing Becker Underwood No report No. Not GLP Unpublished **Supporting information, study not fully summarised**	Y	BASF Agricultural Specialities Ltd

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIM, 5/04	Joshi, R.; McSpadden Gardener, B.B.	2006	Identification and characterisation of novel genetic markers associated with biological control activities in *Bacillus subtilis* Phytopathology 96 (2): 145-154. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5/05	Logan, N.A.	1988	*Bacillus* species of medical and veterinary importance J. Med. Microbiol. 25:157-165. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5/06	Claus, D.; Berkeley, R.C.W.	1986	Genus *Bacillus* Cohn 1872, pp. 1105-1139. In: P.H.A. Sneath, *et al*. (eds.) Bergey's Manual of Systematic Bacteriology, Vol. 2. Williams and Wilkins Co., Baltimore, MD. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5/07	Priest, F.G.; Goodfellow, M.; Shute, L.A.; Berkeley, R.C.W.	1987	*Bacillus amyloliquefaciens* sp. nov., nom. rev. Int. J. Syst. Bacteriol., 1987, 37, 69-71 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5/08	Welker, N.E.; Campbell, L.L.	1967	Unrelatedness of *Bacillus amyloliquefaciens* and *Bacillus subtilis* Journal of Bacteriology 1967, 94(4): 1124-1130. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5/09	EFSA	2011	Scientific Opinion Technical guidance on the assessment of the toxigenic potential of *Bacillus* species use in human nutrition EFSA Journal 2011; 9(11):2445 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.1/01	EFSA	2007	Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA Opinion of the Scientific Committee The EFSA Journal (2007) 587, 1-16 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIM, 5.2.1/01	Izu, S.	2012	Exposure to, and adverse health effects from *Bacillus subtilis*, strain MBI 600 Becker Underwood No report No. Not GLP Unpublished **Supporting information, paper not fully summarised**	Y	BASF Agricultural Specialities Ltd
IIM, 5.2.2/01	Caballero M.L.; Gomez M.; Gonzalez-Munoz, M.; Reinoso, L.; Rodrigues-Perez, R.; Moneo, I.	2007	Occupational sensitisation to fungal enzymes used in animal feed industry Int Arch Allergy Immunol 2007; 144:231-239 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.2/02	Johnson, C.L.; Bernstein, I.L.; Gallagher, J.S.; Bonventre, P.F.; Brooks, S.M.	1980	Familial hypersensitivity pneumonitis induced by *Bacillus subtilis* American Review of Respiratory Disease 1980; 122:339-348 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.2/03	Green, B.J.; Beezhold, D.H.	2011	Industrial fungal enzymes: an occupational allergen perspective Journal of allergy Vol. 2011, article ID 628574 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.3/01	Mikkola, R.; Andersson, M.A.; Teplova, V.; Grigoriev, P.; Kuehn, T.; Loss, S.; Tsitko, I.; Apetroaie, C.; Saris, N.E.L.; Veijalainen, P.; Salkinoja-Salonen, M.S.	2007	Amylosin from *Bacillus amyloliquefaciens*, a K+ and Na+ channel-forming toxic peptide containing a polyene structure Toxicon, 2007 49, 1158-1171 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.3/02	Apetroaie-Constantin, C.; Mikkola, R.; Andersson, M.A.; Teplova, V.; Suominen, I.; Johanssonand, T.; Salkinoja-Salonen, M.	2009	*Bacillus subtilis* and *B. mojavensis* strains connected to food poisoning produce the heat stable toxin amylopsin Journal of Applied Microbiology 2009 ISSN 1364-5072 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIM, 5.2.3/03	From, C.; Pukall, R.; Schumann, P.; Hormazábal, V.; Granum, P.E.	2005	Toxin producing ability among *Bacillus* spp. outside the *Bacillus cereus* group Applied and Environmental Microbiology, 2005 71, 1178-1183 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.3/04	Claus, D.; Berkeley, R.C.W.	1986	Genus *Bacillus* Cohn 1872, pp. 1105-1139. In: P.H.A. Sneath, *et al*. (eds.) Bergey's Manual of Systematic Bacteriology, Vol. 2. Williams and Wilkins Co., Baltimore, MD. Not GLP Published ⇨ 5/06 **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.3/05	EFSA	2011	Scientific Opinion Technical guidance on the assessment of the toxigenic potential of *Bacillus* species use in human nutrition EFSA Journal 2011; 9(11):2445 Not GLP Published ⇨ 5/09 **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.3/06	de Boer, A.S. and Diderichsen, B.	1991	On the safety of *Bacillus subtilis and B. amyloliquefaciens*: a review Appl. Microbiol. Biotechnol., 36, 1991, 1-4 TOX2000-1212 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.3/07	Ihde, D.C.; Armstrong D.	1973	Clinical spectrum of infection due to *Bacillus* species Amer. J. Med., Vol. 55, 1973, 839-845 BMF2000-152 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.3/08	Abo-State, M.A.M.; Husseiny, H.M.; Helimish, F.A.; Zickry A.R.A.	2012	Contamination of Eye Drops with *Bacillus* Species and Evaluation of Their Virulence Factors World Applied Sciences Journal 19 (6): 847-855, 2012 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.4/01	Gupta, D.K.; Vyas, K.M.	1989	Efficacy of *Bacillus subtilis* against mosquito Larvae (*Anophelis culicifacies*) Zeitschrift fuer Angewandte Zoologie 1989, 76(1) 85-91 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

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IIM, 5.2.4/02	Fossum, K.; Herikstad, H.; Binde, M.; Pettersen K.E.	1986	Isolations of *Bacillus subtilis* in connection with Bovine Mastitis Nordisk Veterinärmedicine, Vol. 38, 1986, 233-236 BMF2000-151 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.4/03	Mikkola, R.; Andersson, M.A.; Grigoriev, P.; Teplova, V.V; Saris, N.E.L.; Rainey, F.A.; Salkinoja-Salonen, M.S.	2004	*Bacillus amyloliquefaciens* strains isolated from moisture-damaged buildings produced surfactin and a substance toxic to mammalian cells. Arch Microbiol (2004) 181 : 314–323 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.3.1/01	PRAPeR	2009	Report of PRAPeR expert meeting M2. Peer Review Programme under Directive 91/414/EEC. Meeting to discuss the approach for assessment of plant protection products containing micro-organisms as the active substance Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.3.1/02	[redacted]	1989	Delayed contact hypersensitivity in the guinea pig with MBI600 [redacted] Report No. 89429D/AGC 2/SS GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM, 5.3.2/01	[redacted]	1989a	Acute oral toxicity and infectivity/pathogenicity to rats of MBI600 [redacted] Report No. 89396D/AGC 1/0/AC GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM, 5.3.2/02	[redacted]	2011a	Acute oral toxicity study (UDP) in rats [redacted] Report No. 15626-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM, 5.3.3/01	[redacted]	2011	Acute inhalation toxicity study in rats [redacted] Report No. 15628-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM, 5.3.3/02	[redacted]	1989b	Acute pulmonary toxicity and infectivity/pathogenicity to rats of MBI600 [redacted] Report No. 89397D/AGC 1/2/EC GLP Unpublished	Y	BASF Agricultural Specialities Ltd

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIM, 5.3.4/01	[redacted]	1989c	Acute intravenous toxicity and infectivity/pathogenicity to rats of MBI600 [redacted] Report No. 89398D/AGC 1/3/AC GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM, 5.3.5/01	EFSA	2007	Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA Opinion of the Scientific Committee The EFSA Journal (2007) 587, 1-16 Not GLP Published ⇨ 5.1/01 **Supporting information, paper not fully summarised**	N	Public
IIM, 5.3.5/02	Hashimoto, J.	2012	Bacterial reverse mutation test of the supernatant of the fermentation broth of *Bacillus subtilis* MBI600 UBE Scientific Analysis Laboratory Inc Becker Underwood Report No. USA-R-12224 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM, 5.4/01	Du Toit Schabort	2011	Secondary compound analysis of *Bacillus subtilis* strain MBI600 Becker Underwood No report No. Not GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM, 5.4/02	Sansinenea, E.; Ortiz, A.	2011	Secondary metabolites of soil *Bacillus* spp. Nordisk Biotechnol Lett (2011), 33, 1523-1538 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/03	Hamdache, A.; Lamarti, A.; Aleu, J.; Collado, I.G.	2011	Non-peptide metabolites from the genus *Bacillus* Journal of Natural Products Published by the American Chemical Society and American Society of Pharmacognosy 14 March 2011 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/04	Wulff, E.G.; Mguni, C.M.; Mansfeld-Giese, K.; Fels, J.; Lubeck, M.; Hockenhull, J.	2002	Biochemical and molecular characterization of *Bacillus amyloliquefaciens, B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris pv. Campestris* Plant Pathology (2002) 51 , 574–584 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIM, 5.4/05	Stein, T.	2005	*Bacillus subtilis* antibiotics: structures, syntheses and specific functions Molecular Microbiology (2005) 56 (4), 845–857 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/06	Kalinovskaya, N.I.; Kuznetsova, T.A.; Ivanova, E.P.; Romanenko, L.A.; Voinov, V.G.; Huth, F.; Laatsch, H.	2002	Characterization of Surfactin-like Cyclic Depsipeptides Synthesized by *Bacillus pumilus* from Ascidian *Halocynthia aurantium* Mar. Biotechnol. 2002 4, 179–188 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/07	Peypoux, F.; Bonmatin, J.M.; Wallach, J.	1999	Recent trends in the biochemistry of surfactin Appl Microbiol Biotechnol 1999 51: 553–563 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/08	Carrillo, C.; Teruel, J.A.; Aranda, F.J.; Ortiz, A.	2003	Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin Biochim Biophys Acta 2003 1611:91–97 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/09	Vollenbroich, D.; Ozel, M.; Vater, J.; Kamp, R.M.; Pauli, G.	1997	Mechanism of inactivation of enveloped viruses by the biosurfactant surfactin from *Bacillus subtilis* Biologicals 1997 25:289–297 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/10	Cooper, D.G.; MacDonald, C.R.; Duff, S.J.B.; Kosaric, N.	1981	Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions Appl Environ Microbiol 1981 42:408–412 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/11	Shaligram, N.S.; Singhal, R.S.	2010	Surfactin–a review on biosynthesis, fermentation, purification and applications Food Technol Biotechnol 2010 48:119–134 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIM, 5.4/12	Arima, K.; Kakinuma, A.; Tamura, G.	1968	Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation Biochem Biophys Res Commun 1968 31:488–494 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/13	Tsukagoshi, N.; Tamura, G.; Arima, K.	1970	A novel protoplast-bursting factor (surfactin) obtained from *Bacillus subtilis* IAM 1213. I. The effects of surfactin on *Bacilllus megaterium* KM Biochim Biophys Acta 1970 196: 204-210 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/14	Kameda, Y.; Matsui, K.; Hisato, K.; Yamada, T.; Sagai, H.	1972	Antitumor activity of *Bacillus natto*. III. Isolation and characterization of a cytolytic substance on Ehrlich ascites carcinoma cells in the culture medium of *Bacillus natto* KMD 1126 Chem Pharm Bull (Tokyo) 1972 20: 1551-1553 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/15	Maget-Dana, R.; Thimon, L.; Peypoux, F.; Ptak, M.	1992	Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A Biochimie 1992 74: 1047-1051 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/16	Bérdy, J.; Boca Raton, F.L.	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/17	Maget-Dana, R.; Peypoux, F.	1994	Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties Toxicology 1994 87:151–174 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/18	Aranda, F.J.; Teruel, J.A.; Ortiz, A.	2005	Further aspects on the haemolytic activity of the antibiotic lipopeptide iturin A Biochimica et Biophysica Acta 1713 (2005) 51 – 56Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIM, 5.4/19	Bérdy, J.; Boca Raton, F.L.	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/20	Yuan, J.; Li, B.; Zhang, N.; Waseem, R.; Shen, Q.; Huang, Q.	2012	Production of Bacillomycin- and Macrolactin-Type Antibiotics by *Bacillus amyloliquefaciens* NJN-6 for Suppressing Soilborne Plant Pathogens Journal of Agricultural and Food Chemistry. Published by the American Chemical Society, 4 March 2012 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/21	Bérdy, J.; Boca Raton, F.L.	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/22	Bérdy, J.; Boca Raton, F.L.	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/23	Bérdy, J.; Boca Raton, F.L.	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/24	Zimmerman, S. B.; Schwartz, C. D.; Monaghan, R. L.; Pelak, B. A.; Weissberger, B.; Gilfillan, E. C.; Mochales, S.; Hernandez, S.; Currie, S. A.; Tejera, E.; Stapley, E. O. J.	1987	Difficidin and oxydifficidin: novel broad spectrum antibacterial antibiotics produced by *Bacillus subtilis*. I. Production, taxonomy and antibacterial activity Antibiot. 1987, 40, 1677–1681 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/25	Patel, P. S.; Huang, S.; Fisher, S.; Pirnik, D.; Aklonis, C.; Dean, L.; Meyers, E.; Fernandes, P.; Mayerl, F. J.	1995	Bacillaene, a novel inhibitor of prokaryotic protein synthesis produced by *Bacillus subtilis*: production, taxonomy, isolation, physico-chemical characterisation and biological activity Antibiot. 1995, 48, 997–1003 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIM, 5.4/26	Moldenhauer, J.; Goetz, D. C. G.; Albert, Ch. R.; Bischof, S. K.; Schneider, K.; Suessmuth, R. D.; Engeser, M.; Gross, H.; Bringmann, G.; Piel, J.	2010	The final steps of Bacillaene Biosynthesis in *Bacillus amyloliquefaciens* FZB42: direct evidence for β,γ dehydration by a trans-acyltransferase polyketide synthase Angew. Chem., Int. Ed. 2010, 49, 1465–1467 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/27	Idris, E. E. S.; Bochow, H.; Ross, H.; Borriss, R. J.	2004	Use of *Bacillus subtilis* as biocontrol agent. VI phytohormone like action of culture filtrates FZB24, FZB42, FZB45 and *Bacillus subtilis* FZB37 Plant Dis. Prot. 2004, 111, 583–597 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/28	Idris, E. E. S.; Iglesias, D.; Talon, M.; Borriss, R.	2007	Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42 Mol. Plant-Microbe Interact. 2007, 20, 619–626 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/29	Gabriel, M.A.; Jabara, H.; Al-Khalidi, U.A.S.	1971	Metabolism of Acetoin in Mammalian Liver Slices and Extracts Biochem. J. (1971) 124, 793-800 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/30	Arguelles-Arias, A.; Ongena, M.; Halimi, B.; Lara, Y.; Brans, A.; Joris, B.; Fickers, P.	2009	*Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens Microb Cell Fact 2009 8:63–74 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/31	Bérdy, J.; Boca Raton, F.L.	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/32	Bérdy, J.; Boca Raton, F.L.	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIM, 5.4/33	Bérdy, J.; Boca Raton, F.L.	N/A	CRC Handbook of antibiotic compounds CRC Press Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/35	Anon	N/A	http://www.lookchem.com/Bacilysin/ Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.5.1/01	[redacted]	1989	Acute dermal toxicity to rabbits of MBI600 [redacted] Report No. 89270D/AGC 1/1/AC GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM, 5.5.1/02	[redacted]	2011b	Acute dermal toxicity study in rats [redacted] Report No. 15627-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM, 5.5.1/03	[redacted]	2011c	Acute dermal irritation study in rabbits [redacted] Report No. 15630-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM, 5.5.1/04	[redacted]	1989	Primary eye irritation and infectivity of MBI600 [redacted] Report No. 89399D/AGC 1/4/SE GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM, 5.5.1/05	[redacted]	2011d	Acute eye irritation study in rabbits [redacted] Report No. 15629-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIM, 5.6/01	EFSA	2007	Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA Opinion of the Scientific Committee The EFSA Journal (2007) 587, 1-16 Not GLP Published ⇨ 5.1/01 **Supporting information, paper not fully summarised**	N	Public
Annex III Data and Information					
IIIM1 7.1.1/01	[redacted]	2011a	Acute oral toxicity (UDP) in rats [redacted] Report No. 15387-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIIM1 7.1.2/01	[redacted]	2011b	Acute dermal toxicity study in rats [redacted] Report No. 15388-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIIM1 7.1.3/01	[redacted]	2011	Acute inhalation toxicity study in rats [redacted] Report No. 15389-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIIM1 7.1.4/01	[redacted]	2011c	Acute dermal irritation study in rabbits [redacted] Report No. 15391-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIIM1 7.1.5/01	[redacted]	2011d	Acute eye irritation study in rabbits [redacted] Report No. 15390-11 GLP Unpublished	Y	BASF Agricultural Specialities Ltd
IIIM1 7.1.6/01	[redacted]	2003	Skin sensitization study in guinea pigs [redacted] Report No. 7831-03 GLP Unpublished	Y	BASF Agricultural Specialities Ltd

Draft Assessment Report

Bacillus amyloliquefaciens strain MBI600

Volume 3
Annex B.7
Residue data

Rapporteur Member State : France

Version History of Volume 3 B7

Date	**Reason for revision**
December 2014	Initial DAR

Table of contents

B.7. Residues in or on treated products, food and feed (Annex IIM 6 and IIIB 8)

The applicant produces a biocontrol agent against fungal plant pathogens (exemplified in this submission by *Botrytis spp*) made of *Bacillus amyloliquefaciens MBI600* to be used on vineyards. The product is intended to be applied only by foliar spray.

Strain MBI600 was previously identified as *Bacillus subtilis*; however recent studies with more modern techniques have identified *Bacillus amyloliquefaciens* as a new specie (Priest et *al*., 1987[1]).
In some cases *Bacillus subtilis* literature data are referred to in this report. For the reasons stated above, it is considered that these data are equally applicable to *Bacillus amyloliquefaciens*, which is part of the *Bacillus subtilis* group (EFSA, 2011[2])

The bacterial strain is a naturally occurring, indigenous wild type and was isolated from leaf washings from leaves of broad bean plants (*Vicia faba*) in UK.

The applicant produced the following **rationale for Waiver of residue data.**
All toxicological studies prepared with the active substance *Bacillus amyloliquefaciens* strain MBI600 revealed no effects on human or animal health. It is of low toxicity via the oral, dermal, inhalation and intravenous routes and does not show any evidence of pathogenicity or infectivity. It is not irritating to skin or eyes, but is a skin sensitiser (see B.6).

Furthermore EFSA has considered Bacillus amyloliquefaciens along with its assessment of Bacillus species and has concluded an absence of emetic food poisoning toxins with surfactant activity and an absence of enterotoxic activity. Based on this assessment Bacillus amyloliquefaciens is included in their list of microorganisms which warrant a Qualified Presumption of Safety (QPS)[3].

It is also reported that *Bacillus subtilis* is consumed in large quantities in the Japanese food “Natto” (de Boer and Diderichsen, 1991[4]).

B.7.1. Persistence and likelihood of multiplication in or on crops, feedingstuffs or foodstuffs (Annex IIB 6.3 and Annex IIIB 8)

Use patterns: *Bacillus amyloliquefaciens* strain MBI600 should be used as foliar treatment on grapevine.
The intended application method of the product is performed via foliar treatment. Critical Good Agricultural Practices (cGAPs) are presented on table B.7.1-1 below. The critical GAP include 10 applications at a maximum application rate of 0.5 kg/ha which correspond to up to 3.03×10^{12} CFU/ha.

[1] *Priest, F.G.; Goodfellow, M.; Shute, L.A. and Berkeley, R.C.W. Bacillus amyloliquefaciens sp. nov., nom. rev. Int. J. Syst. Bacteriol., 1987, 37, 69-71*
[2] *EFSA Journal 2011; 9(11):2445 Scientific Opinion Technical guidance on the assessment of the toxigenic potential of Bacillus species use in human nutrition.*
[3] *The EFSA Journal (2007) 587, 1-16 Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA Opinion of the Scientific Committee.*
[4] *De Boer, A.S.; Diderichsen, B.: On the safety of Bacillus subtilis and B. amyloliquefaciens: a review. Appl. Microbiol. Biotechnol., 36, 1991, 1-4 TOX2000-1212*

Table B.7.1-1: List of intended uses and cGAPs of *Bacillus amyloliquefaciens* MBI600

Crop and/ or situation (a)	Zone	Product code	F G or I (b)	Pests or Group of pests controlled (c)	Formulation MPCP**		Application				Application rate per treatment			PHI (days) (l)	Remarks: (m)
					Type (d-f)	Minimum conc. of MPCA* (i)	method kind (f-h)	growth stage & season (j)	number min max (k)	interval between applications (min)	kg MPCA /hL min max	water L/ha min max	kg MPCA /ha min max		
Grapes	SEU	BUEXP1780 S	F	*Botrytis* spp., powdery mildew	WP	5.5 x 10^{10} cfu/g 50 g/kg	Spray tractor-mounted air assisted or knapsack sprayers	All	1 - 10	7 days	0.014 - 0.0055	400 - 1000	0.055 kg MPCA/ ha*	-	Equivalent to 0.5 kg MPCP /ha

B.7.1.1. Data from published literature

The applicant made reference to several data from publish literature to demonstrate the natural occurrence and the persistence of *B. amyloliquefaciens* in the environement. As most of these data have been already discussed in the DAR for *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747, these references were considered by applicant to have been reviewed by the RMS (Germany) and assessed as being reliable according to Uniform Principles. Therefore corresponding individual studies/publications were therefore not submitted to France (details of submitted and not submitted publications are reported in foot note references).

Both, *B. amyloliquefaciens* and *B. subtilis* occur ubiquitously and are prevalent in soils and on different plant surfaces. Colonisation of different foodstuffs is common, but largely ignored because the species are generally accepted to be non-pathogenic (Priest, 1993[5]).

The abundance of indigenous *B. subtilis* in soil reported in the literature varies from 3×10^4 CFU/g in desert soils (Pantastico-Caldas et al., 1992[6]) to 7 × 104 CFU/g soil in the rhizosphere of tea bushes (Pandey et al., 2001[7]).
An examination of the distribution of vegetative cells and spores of *B. subtilis* within the upper 20 cm in a pine forest soil was performed (Siala et al., 1974[8]). It was found that cells and spores were predominately associated with decaying organic matter particles, although 85% of the total soil particles were of mineral origin. Vegetative cells were mainly found in the near-surface organic layer (67.5% of total *B. subtilis* counts), containing large amounts of particles of decaying pine leaves and stems, while spores mostly persisted in the mineral horizon below root clusters (81% of total *B. subtilis* counts were spores). Cell counts of *B. subtilis*, however, were very similar in both horizons, ranging between 7.1×10^4 and 7.6×10^4 CFU/g dw soil.

Several studies support the assumption that conditions for growth of *B. subtilis* and *B. amyloliquefaciens* are expected to be most favourable in the rhizosphere where root exudates provide sufficient nutrients whereas in the surrounding soil numbers of vegetative cells strongly decline within a few days after inoculation. Among the members of the *B. subtilis* group, *B. amyloliquefaciens* may exhibit superior root colonisation abilities (Reva et al., 2004[9]). More information and references about the persistence of *B. amyloliquefaciens* in soil and water are reported in volume B.8.

Product BUEXP1780S containing *B. amyloliquefaciens* MBI600 is intended to be applied to the plant foliage. It works by preventing further growth of fungi already present, but in addition forms a protective layer on the surface of the plant to prevent further fungal growth. Environmental conditions on leaf surface are usually unfavourable (lack of water and nutrients) and restrict microbial growth (Campbell, 1989[10]). Then it is expected that growth and spread of *B. amyloliquefaciens* will be limited following its application in vine plant foliage and grapes.

B.7.1.2. Data specific to strain MBI 600 provided by the applicant

A study has been provided by the applicant to observe the persistence of *Bacillus amyloliquefaciens* MBI600 following a single application of BUEPXP1780S (*Bacillus subtilis* strain MBI600, 5.5 $x10^{10}$ cfu/g) on the foliage of vines.

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[5] *Priest, F.G. (1993). Systematics and Ecology of Bacillus. Bacillus subtilis and other gram-positive bacteria. American Society of Microbiology, Washington D.C. (ed.), 1993, 3-16 BMF2000-57. (Publication not provided by the applicant)*
[6] *Pantastico-Caldas M, Duncan K, Istock C and Bell J (1992) Population dynamics of bacteriophage and Bacillus subtilis in soil. Ecology 73: 1888–1902. (Publication not provided by the applicant).*
[7] *Pandey A, Palni LM and Bisht D (2001) Dominant fungi in the rhizosphere of established tea bushes and their interaction with the dominant bacteria under in situ conditions. Microbiological research 156: 377–382. (Publication not provided by the applicant).*
[8] *Siala A, Hill IR and Gray TRG (1974) Populations of Spore-forming Bacteria in an Acid Forest Soil, with Special Reference to Bacillus subtilis. Journal of General Microbiology 81(1): 183–190.. (Publication not provided by the applicant).*
[9] *Reva ON, Dixelius C, Meijer J and Priest FG (2004) Taxonomic characterization and plant colonizing abilities of some bacteria related to Bacillus amyloliquefaciens and Bacillus subtilis. FEMS Microbiology Ecology 48: 249–259. (Publication not provided by the applicant).*
[10] *Campbell, R.: Biocontrol on leaf surfaces. Biological control of microbial plant pathogens, Cambridge University Press, Cambridge, Department of Botany, University of Bristol, Chapter 3, 1989, 66-94 BMF2000-100*

Report:	Martin, G. (2011) Foliar persistence of BUEXP1780S on vine leaves. Becker Underwood. Unpublished report No. R&D071101
Guideline:	None
GLP:	No

Materials and methods

BUEPXP1780S was applied at a rate of 1.43 g/L (corresponds approximately to an application of *ca* 1kg of BUEPXP1780S/ha or 6.05 x 10^{12} CFU/ha considering a water volume of 700 l/ha) to the leaf surface of 5 vines using a calibrated sprayer. Five controls received water only. Plants were then kept in a greenhouse to maintain exposure to daily fluctuations in heat and UV radiation but not wind and rainfall.

The number of colony forming unit (cfu) was monitored over an 11 week period. The concentration on the controls was taken as background and then any increase in spore numbers over this value in the treated plants were considered to be a result of the BUEXP1780S applications. At each time point, a total ten leaves were sampled, five from treated plants and five from the controls.
Samples were extracted with Reverse Osmosis water and diluted. At each time point, one sample was heat treated in a water bath at 70°C for 10 minutes before quickly being cooled in an ice bath for a further 10 minutes. The number of cfu from each sample was assessed using the drop method. Three 0.01 ml drops from each dilution 10^{-1} to 10^{-5} are plated out onto sterilised 20 ml nutrient agar plates (pH = 7, concentration 2.825 g per 100 ml of RO water). The plates were then incubated at 28°C for 24-48 hours. The number of cfu in each drop was recorded and an average from the three per dilution calculated. From this data the original concentration of cfu per ml of dilution 0 (the flask containing the vine leaf) was obtained.

Results

The results are summarised in the figures below. Application of BUEXP1780S to vine leaves resulted in a significant increase in the presence of bacteria on leaves (Figure 1). By comparing the number of CFU between the BUEXP1780S treated leaves and the control leaves after 11 weeks there was found to be significantly more spores on the treated leaves compared to the control. This demonstrates that *B. amyloliquefaciens* MBI600 is capable of persisting in the foliar environment after application. Over the 11 week period the number of cfu on treated leaves declined by 12%.

Because prior to BUEXP1780S application all ten plants were taken from the same location, the difference in spores counts between control plant and treated plants was considered to be due to *B. amyloliquefaciens* applied to the crop in BUEXP1780S.

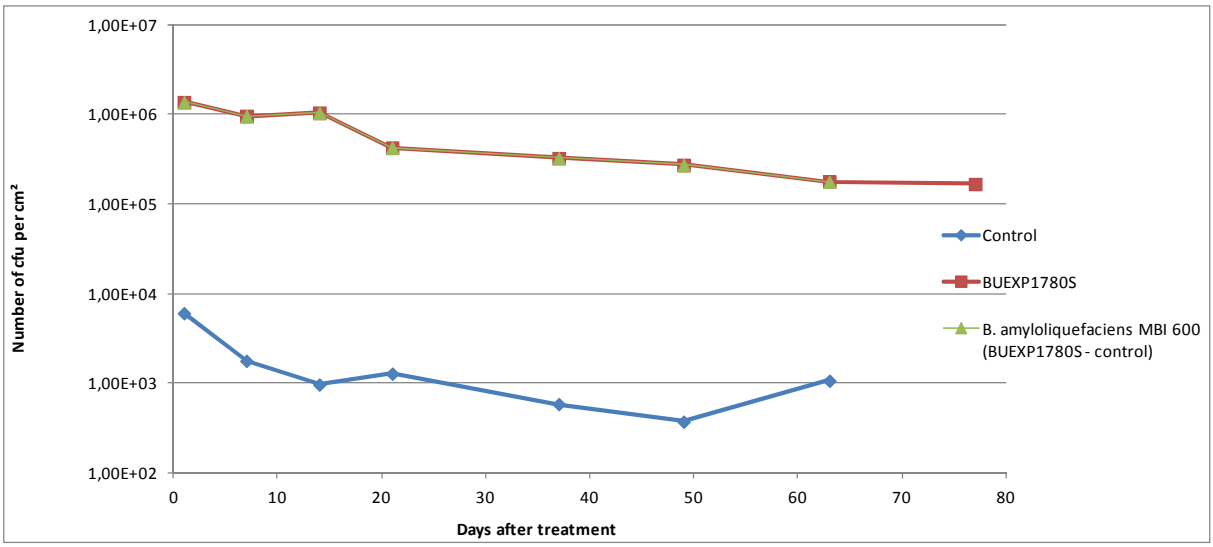

Figure 1: Number of CFU present on vine leaves treated with water (control) or BUEXP1780S and determination of persistence of CFU of *B. amyloliquefaciens* following application of BUEXP1780S.

The effect of heat treatment was found not to affect significantly the number of cfu recorded on the leaf surface (Figure 2 and 3). This suggests that the majority of bacteria recorded as being present on treated leaves or control leaves were in resting spore stage.

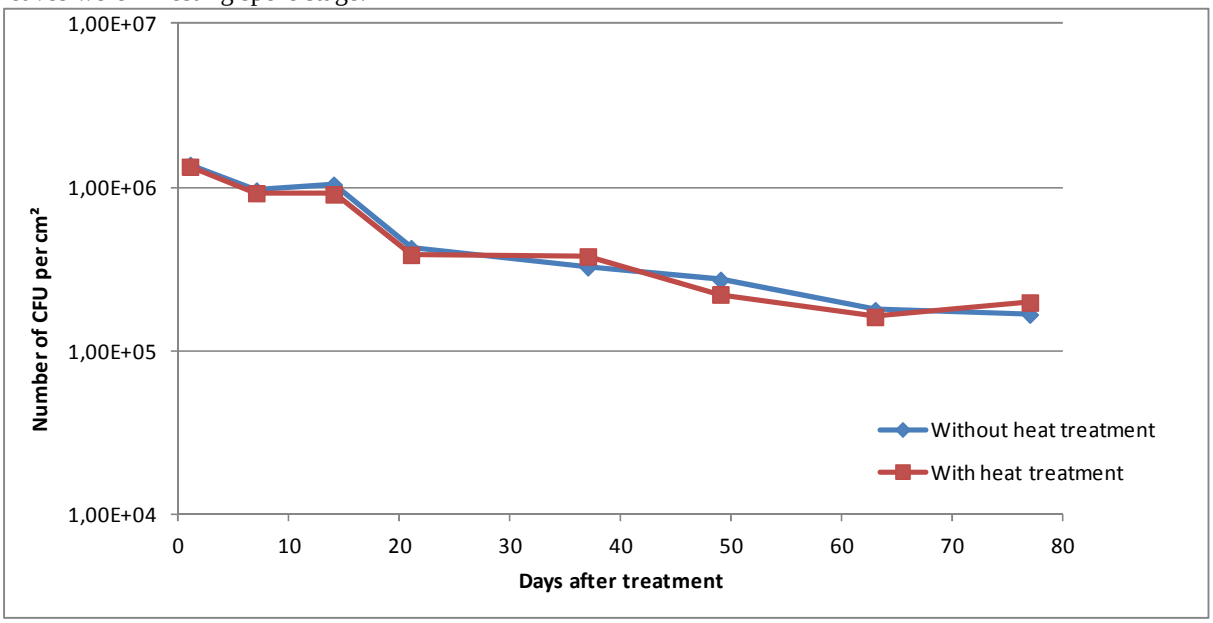

Figure 2: Effect of heat treatment on the number of cfu on vine leaves treated with BUEXP1780S

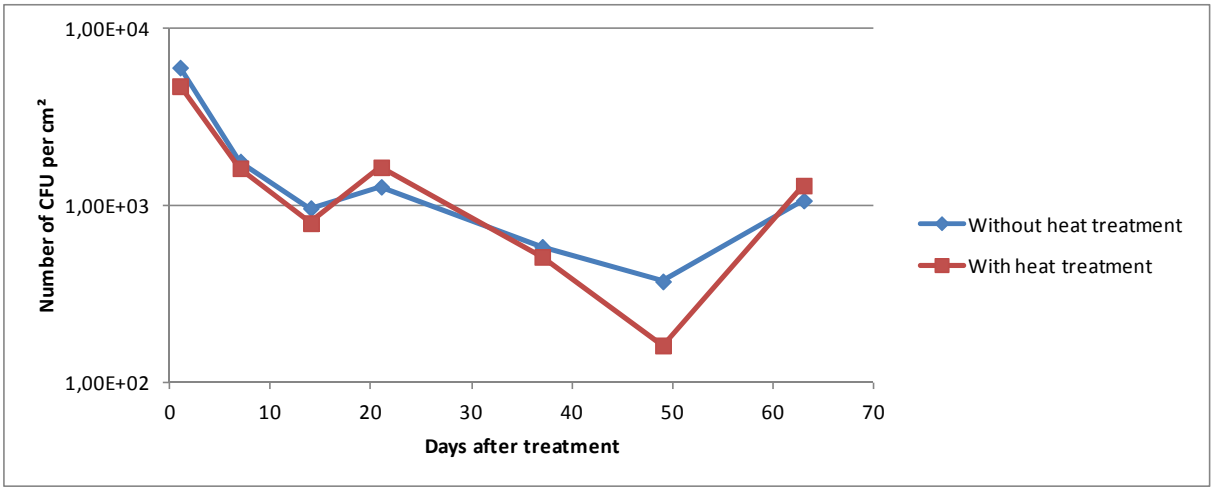

Figure 3: Effect of heat treatment on the number of cfu on vine leaves treated with water

Conclusion

This trial demonstrated that spores of *Bacillus amyloliquefaciens* strain MBI600 applied to a foliar environment will persist at the applied level and the number of cfu will not increase. This persistence occurs in the resting spore life-stage allowing the bacteria to wait until suitable environment to germinate and grow colonies.

The above work was carried out on an artificial environment where the vines were not exposed to rainfall. It is likely that rainfall would result in a reduction in resting spores by physical loss through runoff and spore germination when exposed to damp leaf conditions. Loss of persistence as a result of ultra violet radiation is also expected to be greater on vines grown outside and in warmer climates such as the Mediterranean ecological zone, the main wine growing region in Europe.

B.7.2. Exposure to consumers (Annex IIB 6.2 and Annex IIIB 8)

B.7.2.1. Non-viable residues

B. amyloliquefaciens strain MBI600 does not produce any relevant metabolites or toxins.

B. amyloliquefaciens strain MBI600 is considered to produce antimicrobial compounds at the treatment site (leaves and fruits) to successful compete against other microorganisms including plant pathogens. However, it should be noted that the mode of action of *B. amyloliquefaciens* is not only related to the production of antifungal substances, but also to competition for nutrients and space and the release of lytic enzymes.

In the literature, *Bacillus amyloliquefaciens* has been shown to produce many antibiotics, antifungals and siderophores including surfactin, iturin (bacillomycin D, F, L and FC, and iturin A and C, mycosubtilin), macrolactin, azalomycin F, the auxin indole-3-acetic acid (IAA), 3-hydroxybutan-2-one (acetoin), butane-2,3-diol, bacilysin, fengycin, amphomycin, acivicin, arthrobactin, difficidin, oxidifficidin, bacillaene, diHydroBacillaene, valinomycin, enterobactin and nocardamin (Sansinenea and Ortiz 2011, Hamdache *et al*. 2011, Wulff *et al*. 2002, Stein 2005, Toure *et al*., 2004). Welker and Campbell (1967) also report the production of α-amylase. None of these compounds are a concern for human health or the environment (see B.2).

For different antagonistic compounds (iturin, surfactin, fengycin, bacillomycin) produced by *B. subtilis* upon treatment of melon leaves it was demonstrated that they do not accumulate on the treatment site and disappear within ~12 days post application (Romero et al., 2007[11]).

In a recent study (Crane et al., 2013[12]) monitored the persistence of iturin and *B. amyloliquefaciens* populations upon treatment of wheat either in the greenhouse or in the field for 14 days. It was found that under greenhouse conditions initial levels of iturin were three times higher than after field application. However, in both settings the iturin levels rapidly decreased within the first 3 days post treatment, although significant populations of the bacterium were still present. It can be therefore concluded that antifungal compounds or other substances are probably involved in antagonistic activity of *B. amyloliquefaciens* strain MBI600 and will only be produced at the treatment site during direct interaction with the target pathogen and only for a period of a few days. The substances will not accumulate and their persistence is considerably shorter than that of the microorganism itself. In addition, none of the metabolites eventually produced by the strain are known to exhibit toxic properties.

B.7.2.2. Viable residues

EFSA has considered *Bacillus amyloliquefaciens* in its assessment of *Bacillus* species and has concluded an absence of emetic food poisoning toxins with surfactant activity and an absence of enterotoxic activity. Based on this assessment *Bacillus amyloliquefaciens* is included in their list of microorganisms which warrant a Qualified Presumption of Safety (QPS). In addition, *Bacillus amyloliquefaciens* and close relatives are regarded as non-pathogenic micro-organisms accepted as "GRAS" (generally regarded as safe) by the U.S. Food and Drug Administration.
Bacillus amyloliquefaciens strain MBI600 is not hazardous to humans and/or animals. It is of low toxicity and does not show any evidence of pathogenicity or infectivity.
Furthermore studies performed on grapes did not show an increasing number of CFU within 11 weeks after application.

B.7.3. Summary and evaluation of residue behaviour (Annex IIM6.5 and Annex IIIB 8)

B. amyloliquefaciens MBI 600 is a naturally occurring, indigenous wild type and was isolated from the surface of broad bean leaves (*Vicia faba*) in UK.

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[11] *Romero D, de Vicente A, Rakotoaly RH, Dufour SE, Veening J-W, Arrebola E, Cazorla FM, Kuipers OP, Paquot M and Pérez-García A (2007) The iturin and fengycin families of lipopeptides are key factors in antagonism of Bacillus subtilis toward Podosphaera fusca. Molecular plant-microbe interactions : MPMI 20(4): 430–40. (Publication not provided by the applicant).*
[12] *Crane JM, Gibson DM, Vaughan RH and Bergstrom GC (2013) Iturin levels on wheat spikes linked to biological control of Fusarium head blight by Bacillus amyloliquefaciens. Phytopathology 103(2): 146–55. (Publication not provided by the applicant).*

B. amyloliquefaciens is ubiquitous in the environment. Colonisation of different foodstuffs is common, but largely ignored because the species are generally accepted to be non-pathogenic.
Bacillus amyloliquefaciens strain MBI600 is intended to be used as foliar treatment on grapevine. It works by preventing further growth of fungi already present, but in addition forms a protective layer on the surface of the plant to prevent further fungal growth. It has been demonstrated that environmental conditions on leaf surfaces are usually unfavourable for bacterial growth and that it is expected that the number of cfu will persist at the initial level of application but will not increase.
Antifungal compounds or other substances are involved in antagonistic activity of *B. amyloliquefaciens* strain MBI600 and will probably only be produced at the treatment site during direct interaction with the target pathogen and only for a period of a few days. The substances will not accumulate and their persistence is considerably shorter than that of the microorganism itself.
EFSA has considered *Bacillus amyloliquefaciens* along with its assessment of *Bacillus* species and has concluded an absence of emetic food poisoning toxins with surfactant activity and an absence of enterotoxic activity. Based on this assessment Bacillus amyloliquefaciens is included in the list of microorganisms which warrant a Qualified Presumption of Safety. According to the provide data *B. amyloliquefaciens* strain MBI 600 is eligible to QPS status and therefore a risk for consumer is not expected following its use as plant protection product. Consequently no residue definition was deemed necessary and it is proposed to include *Bacillus amyloliquefaciens* MBI 600 to the Annex IV of Reg. 396/2005.

B.7.4. References

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
Annex II Data and Information					
IIM, 5/07	Priest, F.G.; Goodfellow, M.; Shute, L.A.; Berkeley, R.C.W.	1987	*Bacillus amyloliquefaciens* sp. nov., nom. rev. Int. J. Syst. Bacteriol., 1987, 37, 69-71 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5/09	EFSA	2011	Scientific Opinion Technical guidance on the assessment of the toxigenic potential of *Bacillus* species use in human nutrition EFSA Journal 2011; 9(11):2445 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.2.3/06	De Boer, A.S. and Diderichsen, B.	1991	On the safety of *Bacillus subtilis and B. amyloliquefaciens*: a review Appl. Microbiol. Biotechnol., 36, 1991, 1-4 TOX2000-1212 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/02	Sansinenea, E.; Ortiz, A.	2011	Secondary metabolites of soil *Bacillus* spp. Nordisk Biotechnol Lett (2011), 33, 1523-1538 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIM, 5.4/03	Hamdache, A.; Lamarti, A.; Aleu, J.; Collado, I.G.	2011	Non-peptide metabolites from the genus *Bacillus* Journal of Natural Products Published by the American Chemical Society and American Society of Pharmacognosy 14 March 2011 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/04	Wulff, E.G.; Mguni, C.M.; Mansfeld-Giese, K.; Fels, J.; Lubeck, M.; Hockenhull, J.	2002	Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris pv. Campestris* Plant Pathology (2002) 51 , 574–584 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5.4/05	Stein, T.	2005	*Bacillus subtilis* antibiotics: structures, syntheses and specific functions Molecular Microbiology (2005) 56 (4), 845–857 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 5/08	Welker, N.E.; Campbell, L.L.	1967	Unrelatedness of *Bacillus amyloliquefaciens* and *Bacillus subtilis* Journal of Bacteriology 1967, 94(4): 1124-1130. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 2.1/03	Toure Y.; Ongena M.; Jacques P.; Guiro A.; Thonart P.	2004	Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple Journal of applied microbiology 2004, 96:1151-1160. Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 6.1/01	EFSA	2007	Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA Opinion of the Scientific Committee The EFSA Journal (2007) 587, 1-16 Not GLP Published **Supporting information, paper not fully summarised**	N	Public

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
IIM, 6.2/01	Campbell, R.	1989	Biocontrol on leaf surfaces Biological control of microbial plant pathogens, Cambridge University Press, Cambridge, Department of Botany, University of Bristol, Chapter 3, 1989, 66-94 BMF2000-100 Not GLP Published **Supporting information, paper not fully summarised**	N	Public
IIM, 6.3/01	Martin, G.	2011	Foliar persistence of BUEXP1780S on vine leaves Becker Underwood R&D071101 Not GLP Unpublished	Y	BASF Agricultural Specialities Ltd
Reference to Published literature not provide to RMS (see B.8)					
	Priest, F.G.	1993	Systematics and ecology of *Bacillus* *Bacillus subtilis* and other gram-positive bacteria; American Society of Microbiology, Washington D.C. (ed.) 3-16 GLP: N, published: Y 2040017 /	N	Public
	Pantastico-Caldas, M., Duncan, K.E. and Istock, C.A.	1992	Population dynamics of bacteriophage and *Bacillus subtilis* in soil Ecology 73, 1888-1902 GLP: N, published: Y	N	Public
	Pandey, A., Palni, L.M.S. and Bisht, D.	2001	Dominant fungi in the rhizosphere of established tea bushes and their interaction with the dominant bacteria under in situ conditions Microbiological Research 156, 377-382 GLP: N, published: Y	N	Public
	Siala, A., Hill, I.R. and Gray, T.R.G.	1974	Populations of spore-forming bacteria in an acid forest soil, with special reference to *Bacillus subtilis* J Gen Microbiol 81, 183 - 190 GLP: N, published: Y	N	Public
	Reva, O.N., Dixelius, C., Meijer, J. and Priest, F.G.	2004	Taxonomic characterisation and plant colonising abilities of some bacteria related to *Bacillus amyloliquefaciens* and *Bacillus subtilis* FEMS Microbiol Ecol 48, 249-259 GLP: N, published: Y 2040019 / 2040052	N	Public
	Romero, D., de Vicente, A., Rakotoaly, R.H., Dufour, S.E., Veening, J-W., Arrebola, E., Cazorla, F.M. and Kuipers, O.P., Paquot, M. and Perez-Garcia, A.,	2007	The Iturin and Fengycin Families of Lipopeptides Are Key Factors in Antagonism of Bacillus subtilis Toward Podosphaera fusca Molecular Plant-Microbe Interactions (MPMI) 20, 430-440 GLP: N, published: Y	N	Public

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****
	Crane JM, Gibson DM, Vaughan RH and Bergstrom GC	2013	turin levels on wheat spikes linked to biological control of Fusarium head blight by Bacillus amyloliquefaciens. *Phytopathology* 103(2): 146–55 GLP: N, published: Y	N	Public
Annex III Data and Information					
Not required					

Draft Assessment Report

Bacillus amyloliquefaciens strain MBI600

Volume 3

Annex B.8

Fate and behaviour in the environment

Rapporteur Member State: France

Version History of Volume 3 B8

Date	**Reason for revision**
December 2014	Initial DAR

Table of contents

B.8. Fate and behaviour in the environment (Annex IIM 7 and IIIB 9)

General information:

Species:	*Bacillus amyloliquefaciens*
First description:	1987
Strain:	MBI600
Genus:	*Bacillus*
Family:	*Bacillaceae*
Division:	Bacteria

B. amyloliquefaciens strain MBI600 is deposited in the National Collection of Industrial (Scotland) and in the American Type Culture Collection (USA). *B. amyloliquefaciens* strain MBI600 was originally isolated from a bean leaf in the UK.

GAP:

The Subtilex® formulation is intended for field application to grape vines (all growth stages) to control botrytis and mildew. Subtilex® is a WP formulation containing at least 5.5×10^{10} cfu/g[1] , equivalent to 110 g *Bacillus amyloliquefaciens* MBI600/kg[2]. Subtilex® is applied at a rate of 0.5 kg/ha of formulated product (equal to 0.055 kg MPCA/ha), which is equivalent to a minimum of 2.75×10^{13} cfu/ha.

The formulated product Subtilex® may be applied up to 10 times per season with a minimum application interval of 7 days between applications, at all growth stages. Application should be made before infestation for preventative control. Duration of protection is at least 7 days, with the maximum number of applications giving control up to harvest.

Supported uses:

Crop and/or situation	Product code	Pests or Group of pests controlled	Formulation MPCP**		Application				Application rate per treatment
			Type	Conc. of MPCA*	method kind	growth stage & season	number min max	interval between applications (min)	kg MPCA /ha
Grapes	Subtilex®	*Botrytis* spp., powdery mildew	WP	5.5×10^{10} spores/g 110 g/kg Equivalent to 0.5 kg MPCP /ha	Spray tractor-mounted air assisted or knapsack sprayers	All	1 - 10	7 days	0.055 kg MPCA/ ha*

* MPCA: Microbial Pest Control Agent
** MPCP: Microbial Pest Control Product

Mode of action:

The mode of action of *B. amyloliquefaciens* strain MBI600 is fungicidal and fungistatic, working by preventing further growth rather than killing the hyphae already present. *B. amyloliquefaciens* disrupts the growth of the hyphae and prevents spore germination, following contact with the fungal pathogen at the leaf surface. In addition, *B. amyloliquefaciens* exhibits strong fungicidal properties via production of iturin A and surfactin, which are antagonistic to the fungal pathogen, resulting in an exclusion zone around the application site. *B. amyloliquefaciens* strain MBI600 colonises developing shoot systems of plants, suppressing by competition, disease organisms such as *Botrytis*, *Fusarium*,

[1] based on the minimum specification of the active substance and nominal concentration of active substance in the product
[2] nominal concentration

Rhizoctonia, and *Alternaria* as well as those organisms causing powdery mildew and anthracnose. Maximum efficacy is achieved if *B. amyloliquefaciens* is able to colonise the leaf surface before fungal attack has occurred. Besides antagonism nutrient competition is involved in the mode of action and more importantly *B. amyloliquefaciens* induces systemic resistance response of the plant, indicated by enhanced peroxidase production.

Literature search:
A search of the scientific peer-reviewed open literature on the active substance and its relevant metabolites/toxines, dealing with side-effects on health, the environment and non-target species and published within the last 10 years before the date of submission of dossier, was not conducted by the notifier in accordance with the Guidance of EFSA on the submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009 (EFSA, 2011).

Note 1:
A full copy of all cited articles was not provided by the notifier while the request was done earlier in the process. The articles, available in the notifier dossier, are clearly identified in the list of references at the end of this document. Furthermore, some articles were provided too late in the process. These articles were thus not assessed by RMS, and listed in the list of references. Therefore, data gap has been identified. Notifier to made available to RMS a full copy of all cited articles.

Note 2:
Some of the following articles were previously assessed by Germany as RMS for the active substance *B. amyloliquefaciens* subsp. *plantarum* strain D747. Therefore, the notifier proposes the same summaries as presented in the volume 3 B.8 of *B. amyloliquefaciens* subsp. *plantarum* strain D747 for *B. amyloliquefaciens* strain MBI 600. To be consistent with the previous assessment on these articles, RMS (FR) followed the conclusions of the Rapporteur Member State German. The articles, assessed by DE are clearly identified in the list of references at the end of this document.
Concerning the articles not already assessed by DE and for which a full copy is not available or provided too late in the process, a brief summary are presented by RMS as informative data, but cannot be used to finalise the assessment.

B.8.1. Persistence and multiplication (Annex IIM 7.1)

- **Sufficient information on the origin, properties, survival and residual metabolites of the microorganism to assess its fate and behaviour in the environment. Information provided in parts 2 - 6 may suffice. Viability/population dynamics, persistence, multiplication and mobility**

B. amyloliquefaciens is a non-pathogenic, naturally occurring, non-modified bacterium, phenotypically closely related to *B. subtilis.* The close similarity between the two species has historically resulted in *B. amyloliquefaciens* being incorrectly classified as a subspecies of *B. subtilis,* rather than a separate species. Due to the close relationship and physiological similarity of the two species information on the fate of *B. amyloliquefaciens,* which in many studies would have been included in the total cells counts for *B. subtilis* in different environmental compartments can be deduced from data on *B. subtilis*.

Natural Background Concentrations

Both, *B. amyloliquefaciens* and *B. subtilis* are known saprophytes occurring predominantly and without any geographic restriction in soils particularly in the rhizosphere (Priest, 1993) from which they are spread to associated environments.

Bacillus species, including *B. amyloliquefaciens*, are commonly found in soils, including agricultural settings, and are naturally present on fresh produce. Bacilli contribute to nutrient cycling when biologically active due to the production of extracellular enzymes. Although the actual numbers in soil for *B. amyloliquefaciens* has not been determined, bacilli occur at population levels of *ca.* 10^6 to *ca.* 10^7 CFU per gram of soil (Alexander, 1977). However, unless a soil has been recently amended with organic matter providing readily utilisable substrate, the bacilli exist in the endospore stage. It is thought that 60 to 100% of soil bacilli populations exist in the inactive endospore state (Alexander, 1977).
An examination of the distribution of vegetative cells and spores of *B. subtilis* within the upper 20 cm in a pine forest soil was performed by Siala *et al*. (1974a). It was found that cells and spores were predominately associated with decaying organic matter particles, although 85% of the total soil particles were of mineral origin. Vegetative cells were mainly found in the near-surface organic layer (67.5% of total *B. subtilis* counts), containing large amounts of particles of decaying pine leaves and stems, while spores mostly persisted in the mineral horizon below root clusters (81% of total *B. subtilis* counts were spores). Cell counts of *B. subtilis*, however, were very similar in both horizons, ranging between 7.1×10^4 and 7.6×10^4 CFU/g dw soil.

The abundance of indigenous *B. subtilis* in soil reported in the literature varies from 3×10^4 CFU/g in desert soils (Pantastico-Caldas *et al*., 1992), and 7×10^4 CFU/g soil in the rhizosphere of tea bushes (Pandey *et al*., 2001) to 7.6×10^4 CFU/g soil in a pine forest soil (Siala *et al*., 1974a).

Activity of *B. subtilis* and its close relatives under natural conditions in soils primarily depends on nutrient availability, which is determined by pH, moisture of the soil, content of biomass, and competition with other native soil microbes. Several studies support the assumption that conditions for growth of *B. subtilis* and *B. amyloliquefaciens* are expected to be most favourable in the rhizosphere where root exudates provide sufficient nutrients whereas in the surrounding soil numbers of vegetative cells strongly decline within a few days after inoculation. Among the members of the *B. subtilis* group, *B. amyloliquefaciens* may exhibit superior root colonisation abilities (Reva *et al*., 2004).

B.8.1.1. Soil (Annex IIB 7.1.1)
The notifier addressed the persistence and multiplication of *B. amyloliquefaciens* strain MBI 600 in soil by supplying information from open scientific literature. The information is presented by RMS below:

Population dynamics of introduced *B. subtilis/amyloliquefaciens* strains

The fate of introduced *B. subtilis / amyloliquefaciens* strains was analysed in numerous studies.

As mentioned in the previous response, several studies found that the rhizosphere is a favoured environment in soil for vegetative *B. subtilis / amyloliquefaciens* cells. The authors Milus and Rothrock (1993) analysed eight different bacterial species and strains, respectively for their ability to colonise roots. Rapid and lasting root colonisation by two different *B. subtilis* strains, D-39Sr and D-60R B applied as wheat seed treatment at a dose of 10^6-10^7 CFU/g seed in fumigated soil was observed. For strain D-39Sr, population sizes 36 h post summer planting, were ~10^5 CFU/g dw root and remained stable over winter until spring of the following year. Population densities of strain D-60R showed higher fluctuations (10^3 to 10^7 CFU/g dw root) depending on the year and the root environment (seminal roots/crown roots developing from late fall to early spring). However,

population averaged over all sampling points, soils and root sections were nearly the same for both strains (86% and 88%, respectively).

In maize, seed coating with a spore suspension of 10^7 CFU/mL was shown to result in high population densities on root surfaces after 57 days of plant growth (Krebs *et al*., 1998), with CFU counts reaching values of about 10^8/g dw root after 28 days declining only slightly to 2-7 × 10^7 CFU/g dw root over following 4 weeks. These results are generally confirmed by a study (Bochow and Gantcheva, 1995), investigating population and activity dynamics of a phytosanitary *B. subtilis* isolate after seed treatment or direct soil application. When applied on maize seeds at dosages of 10^5, 10^7 and 10^9 CFU/mL dipping suspension, *B. subtilis* was shown to quickly colonise the rhizosphere of growing plants and was found at high numbers (10^3 - 10^6 CFU/g soil) around the roots in soil depths up to 15 cm for more than 2 months after planting. The largest lateral distribution of *B. subtilis* along maize roots was found at more than 50 cm from the treated seeds. Afterwards the number of vegetative *B. subtilis* cells declined and were not detectable in two of three test series after 4 month. However, endospores (possibly formed during the decline of vegetative cells) were probably not well represented, as no activation of spores was conducted. According to Moir and Smith (1990; cited in Moir, 1992) activation is inessential for *B. subtilis* but increases the rate of germination. Direct application to soil prior to planting of pea seedlings resulted in similar root colonisation. In dry, greenhouse stored soils drenched with 10^8 spores/L, *B. subtilis* populations were found to be stable for two years after soil drenching and also maintained antagonistic activity against *Rhizoctonia solani*. In a study by Kim *et al*. (2012) it is clearly demonstrated that populations of *B. subtilis* in cropped greenhouse soils strongly decline with time. Less than 10% of the initial number of DNA copies where detectable in the soil after 22 weeks, whereby the presence of DNA copies provides no information on the viability of the corresponding microorganism.

Van Elsas *et al.* (1986) did not observe significant differences between the development of *B. subtilis* population in cropped and non-cropped soils. Shortly after introduction of 3.7 × 10^7 vegetative cells/g soil in non-cropped soils ~10^6 CFU/g soil were retrieved. The number of CFU followed a rapid decline in non-cropped soil stabilising at ~3 × 10^3 and ~5 × 10^3 CFU/g dry soil in loamy sand and silty loam, respectively. Except for sampling on Day 7 (silt loam) and Day 30 (loamy sand) the bulk of population was found to consist mainly of endospores (60 - 100 %) suggesting unfavourable conditions for survival of vegetative cells. Endospores were formed and persisted until the end of the study period. Comparable survival patterns of the introduced *B. subtilis* populations were observed in the root free phase and in the rhizosphere of cropped soils. Numbers of colony forming units stabilised at ~2 × 10^2 CFU/g dry soil (loamy sand) and 2.5 × 10^3(CFU/g dry soil (silty loam).

Report: Bennett, A.J., Leifert, C. and Whipps, J.M. (2003) Survival of the biocontrol agents *Coniothyrium minitans* and *Bacillus subtilis* MBI600 introduced into pasteurised, sterilised and non-sterile soils. *Soil Biology & Biochemistry* 35: 1565 – 1573. Published.
Guideline: N/A
GLP: No

Executive Summary

B. amyloliquefaciens strain MBI 600 was added to three soil types that had been either sterilised, pasteurised or left non-sterile. MBI600 survived well following introduction as a cell suspension into sterilised soil at a rate of 1 × 10^6 cfu/g soil. Endospores were formed rapidly and after 14 days, the introduced microorganism survived in this form rather than as vegetative cells. However, in non-sterile soil, the introduced microorganism did not compete well and decreased in number, with endospores being formed in low numbers. Survival of MBI600 in pasteurised soil was variable, but resembled the survival seen in non-sterile soil more than that seen in sterilised soil. More MBI600 endospores were formed in pasteurised soil than in non-sterile soil, however, and may have been important for survival in pasteurised soil. In conclusion, this work has shown that although soil

pasteurisation does improve establishment of MBI600 compared to non-sterile soil, survival is relatively poor when applied as cells. The best survival of MBI600 occurred as endospores in sterilised soil.

Materials and Methods

Test item: *Bacillus amyloliquefaciens* (incorrectly referred to as *B. subtilis*) MBI600 (NCIMB 12376) stored at -80°C in a bacterial preservation system.

Test system: Three soil types were used (Table 8.1.1-01).

Table 8.1.1-01: Test Soil Characteristics

Soils	Textures	pH ($CaCl_2$)	C (%)	N (%)
Wellesbourne	Sandy clay loam	5.6 ± 0.03	0.76 ± 0.06	0.29 ± 0.02
Dalcross Old	Very fine sandy loam	5.3 ± 0.00	4.29 ± 0.27	0.53 ± 0.05
Rayne	Sandy loam	5.9 ± 0.06	4.81 ± 0.23	0.97 ± 0.03

Soil was allowed to dry at room temperature for 2-3 days and then sieved (<2 mm). Sieved soil (100 g) was placed in wide-necked glass jars and samples were left untreated (non-sterile) at room temperature, or either sterilised or pasteurised. Soil was sterilised by autoclaving the samples twice (0.1 MPa), once each on consecutive days, at 121°C for 30 min. Soil was pasteurised by autoclaving the soil at 80°C for 3 min. The pasteurisation process was standardised by placing the autoclave temperature probe in cold water at the start of the cycle, standardising the water level in the autoclave before each run, and unlocking the autoclave immediately the cycle was complete. Autoclaved samples (sterilised or pasteurised) were left overnight before the experiments were set up the following day.

The moisture contents used were 25% field capacity for Wellesbourne soil, 40% field capacity for Dalcross soil and 35% field capacity for Old Rayne soil to obtain similar handling characteristics in all three soils.

Study Design

An inoculum dose 1×10^6 cfu/g soil was used. All processes were carried out under aseptic conditions in a laminar flow bench. The required suspension of bacterial cells, plus sterile water as necessary to adjust the moisture content of the soil, was pipetted onto the soil in the glass jars. The sample was mixed thoroughly using a sterile spatula and then transferred to a sterile Duran bottle (100 mL). Control samples had sterile water added only using the same procedure. The Duran bottles were incubated in the dark at 18°C for 30 d.

Three replicates were set up for each soil type and soil state. On each sampling occasion, a sub-sample of soil (1 g) was removed aseptically from each sample bottle and placed in sterile water (10 mL), shaken by hand for 1 min and left to stand for 4 min. A dilution series in sterile water was set up (1 mL into 9 mL), and serial dilutions of 0.1 mL were plated onto various media to isolate bacteria. Heat treatment of soil dilutions (70°C for 15 min) allowed endospore formation to be assessed, and this also showed for how long the introduced organism survived as endospores or cells. Sampling was carried out on setting up the experiment and at intervals of 1, 3, 7, 14 and 30 d. Assessments for endospore formation were made from 1 d onwards. In the experiment investigating the survival in non-sterile and sterilised Wellesbourne soil, soil samples were assessed at 21 d instead of 14 d. For each individual 1 g soil sample derived from a different bottle, colony forming units (cfu) were counted on three repeat plates of the appropriate dilution (cfu within the range 30-300 colonies per plate). Final colony counts were made after 7 d at 20°C.

Counts from the dilution plates were first converted to the number of cfu/g fresh weight soil. To satisfy the assumption of homogeneity of variance, all counts were $\log_{10}$ transformed prior to analysis, including the addition of a constant value of 0.375 to allow the inclusion of counts of zero. A single value was obtained for each replicate sample as the mean of the $\log_{10}$ transformed counts from the three repeat dilution plates.

For all analyses, a completely randomised design was assumed.

All numerical data were subjected to analysis of variance (ANOVA). Significant differences between treatment means at the 5% significance level were assessed against the appropriate least significant difference (LSD), where LSD = $t_v \times$ SED. SED is the standard error of the difference between two means derived from the residual mean square obtained from each analysis, and t_v is the critical value (P = 0.05) of the Student's t distribution on v degrees of freedom.

Results and discussion

In sterilised Dalcross soil, the total counts of MBI600 increased initially before declining slowly over the 30 d. The same pattern occurred with Wellesbourne soil, and although the initial increase was not seen in Old Rayne soil, the same slight decline was found as with the other two soil types. However, the final numbers of MBI600 in sterilised soil were high, at between 5 and 7 $\log_{10}$ cfu/g soil. Endospores were formed quickly in all three soils and similar numbers were recorded as the total count for the introduced bacteria by 14 d in all three soils, suggesting that by then MBI600 was surviving as endospores.

In non-sterile Dalcross soil, the introduced bacteria decreased in number to be virtually unrecoverable after 30 d. In the non-sterile Old Rayne soil the bacteria decreased in number initially before increasing again and levelling off at about 4 $\log_{10}$ cfu/g soil. This rate of about 4 $\log_{10}$ cfu/g soil was also maintained in the non-sterile Wellesbourne soil. Few endospores were formed (under 2 $\log_{10}$ cfu/g soil) in all three non-sterile soils and there was a significant difference ($P < 0.05$) in the number of endospores formed in sterilised and non-sterile soil.

The response of the introduced bacteria in pasteurised soil was varied. In all instances, the number of introduced bacteria decreased from their initial number. In Dalcross soil there was an increase again to over 4 $\log_{10}$ cfu/g soil by 14 d. An increase was also seen in Old Rayne soil, but only to just over 2 $\log_{10}$ cfu/g soil by 30 d. No increase was seen in pasteurised Wellesbourne soil. In all pasteurised soils, the final endospore numbers were low, with a maximum of about 3 $\log_{10}$ cfu/g soil in Wellesbourne soil.

Discussion

It was found that MBI600 added as cells to sterilised soil soon converted to endospores and survived well in this form, either remaining at the level of introduction or increasing in number. In non-sterile soil this was not the case, and survival of the introduced bacteria was poor, with low endospore formation. In pasteurised soil, there was a similar decline in numbers to that seen in non-sterile soil, but endospore formation was higher in pasteurised soil than non- sterile soil.

Conclusions

Soil survival of MBI600 when applied as cells is poor. Survival is facilitated by sporulation, which is low in natural soils.

RMS comment:
The study is considered acceptable and gives some information on the persistence of *B. amyloliquefasciens* in soil.

Effluence of several environmental factors on the fate of vegetative cells (germination of endospores)

A variety of factors are important for the survival of microorganisms in soil, and include biotic factors such as the influence of root exudates, predation, competition, the influence of other microorganisms in the soil, e.g. production of antibiotics or lytic enzymes, as well as abiotic factors such as water tension, organic carbon, inorganic nutrients, pH and temperature. It should be considered that the presence of even large numbers of endospores does not equate to large numbers of vegetative cells (the active stage in relation to fungal control) and that numbers of cells in soil will be regulated and stabilised by local environmental conditions such as those mentioned above (Liang *et al.*,1982). Though endospores can be resistant to predation, newly germinated vegetative cells are not. Furthermore, unless the soil conditions have changed significantly (*e.g.* introduction of organic matter) newly germinated cells will be subject to the same competitive disadvantage which results in sporulation and elimination of vegetative cells from soil. For these reasons, persistence of endospores *per se* is not considered to be a significant factor controlling the number of active cells in soil.

a) Autochthonous soil microorganisms

Vegetative cells of *B. subtilis* are subject to competition with autochthonous soil microorganisms.

Several studies are cited for the following:

1. Comparing the fate of introduced *B. subtilis* in sterilised and non-sterilised soil.
2. Investigating the interaction with soil fungi.
3. Showing the significance of the ability to produce antimicrobial metabolites for successful competition.

These points are discussed in further detail below:

1. The survival patterns of two *B. subtilis* strains (NB22-1 and YB8-1, spontaneous streptomycin-resistant mutants) inoculated into different non-sterile and sterilised agricultural soils were investigated by Tokuda *et al.* (1993).

   Vegetative cells of strain YB8-1 introduced in one non-sterile soil (initial concentration: 10^8 CFU/g dry soil) decreased quickly and were not detectable after ~2 days. For NB22-1, the number of CFUs in four non-sterile soils (initial concentration ~10^7 CFU/g dry soil) decreased during the first days and stabilised at a level of 10^4 to 10^5 CFU/g dry soil. After stabilisation, the *B. subtilis* population persisted at these levels in all four non-sterile soils for 50 days. In sterile soils, cell numbers decreased slower and more gradually in the early period compared to the non-sterile soils. However, there was no difference in the final level of the population between the two systems. In this experiment only vegetative cells were detected; no information about the formation and numbers of endospores is given. A faster decline in population levels of vegetative *B. subtilis* cells introduced into non-sterile silty loam soil (pH 6.7, 4.7 % organic matter) was observed by Liang *et al.* (1982). Here, initial cell numbers of about 10^5 CFU/g soil decreased to 24 CFU/g within 3 days accompanied by weak sporulation.

   In sterilised soil samples, vegetative *B. subtilis* cells initially grew, with peak population of ~10^7 CFU/g after 2 days. Thereafter, the number of vegetative cells fell slowly accompanied by high sporulation. The Authors concluded that *B. subtilis* is able to persist in environments in which the supply of organic nutrients permits the formation of a resistant structure, but not in environments where the nutrient level is reduced by competing microbes so that spores are not produced.

2. Competition with rhizosphere fungi was shown to affect *Bacillus* populations under environmental conditions. Pandey *et al.* (2001) investigated natural populations of *Penicillium* and *Trichoderma* associated with the roots of tea bushes in the Himalaya region (subtropical to temperate climate) and their interaction with the two most abundant rhizosphere bacteria, *B. subtilis* and *B. mycoides.* The population of *Penicillium* was found to be highest when the bacterial populations were lowest and *vice versa*. Lowering of *Trichoderma* densities due to the increased populations of *B. subtilis* and *B. mycoides* were observed several times. The negative correlation between the fungal and the bacterial rhizosphere populations suggests a kind of antagonism under environmental conditions. This is confirmed by the results of Pandey *et al.* (1979) (cited in Pandey *et al.*, 2001) with *in vitro* assays with bacterial and fungal rhizosphere isolates; concurrent application of fungicides with *B. subtilis* seed treatment significantly increased (5-10 fold) rhizosphere populations in field soil. This increase could be due to inhibition of competitive fungi and bacteria in the rhizospere (Mahaffee and Backmann 1993; Siala and Gray, 1974b) observed that spore germination of *B. subtilis* spores and proliferation of vegetative cells in acid forest soils were stimulated by the growth and development of fungal hyphae. Neither spore germination nor proliferation of vegetative cells occurred in sterilised soil. This effect was later ascribed to antifungal activity causing death of the fungus and the *B. subtilis* spores living off the products of hyphae lysis (Nicholson, 2002).

3. Results of a study conducted by Asaka *et al.* (1996) suggest the significance of the ability to produce the antimicrobial metabolites, iturin A and surfactin on the survival/death rate of introduced vegetative cells, as well as on the rate of sporulation in non-sterile soils.

After introduction of three *B. subtilis* strains into sterile and non-sterile soil total cell numbers as well as spore numbers were determined at several points during 30 days incubation at 30 °C and at 15 °C. The used strains differed in their ability to produce iturin A and surfactin. The parent strain (R14C) carries the gene lpa-14 in its genome. It is responsible for synthesis of both metabolites. The second strain (RΔ1) was derived from the parent strain by deleting lpa-14 and thus did not produce iturin A and surfactin. The possibility to synthesise both metabolites was intended to be restored in the third strain [RΔ1(C115)] by reintroduction of lpa-14 *via* plasmid transfer.
No significant differences of population dynamics between the three strains were found at 30°C. Generally the conditions for vegetative cells seemed to be less favourable at 30°C. The total cell number, and thus the number of vegetative cells declined within two days accompanied by an intensive sporulation. The number of spores corresponded to the total cell count from day 2 up to day 30.

Trials conducted at 15°C support the assumption that the production of antimicrobial metabolites is essential for vegetative cells of *B. subtilis* to compete with other autochthonous soil microorganisms:

- In non-sterile soils total cell numbers, and therefore numbers of vegetative cells of the lpa-14 deleted strain declined significantly faster than cell numbers of the parent strain.
- In sterile soil the total (vegetative) cell count of the lpa-14 deleted strain remained stable at the introduced number for a significantly longer time than in non-sterile soil.

In non-sterile soil the total cell count (survival/death rate of vegetative cells) of the third strain carrying the reintroduced 1pa-14 gene were comparable with total cell counts of the lpa-14 deleted strain. This appears to be contradictory, as the restored ability to produce iturin A and the partly restored ability to produce surfactin should result in an improved survivability of vegetative cells in non-sterile soil.

The assumption that the production of the two considered metabolites affects the rate of sporulation is supported by significantly reduced sporulation rate of the lpa-14 deleted strain compared to the parent strain in non-sterile soil at 15 °C. However, it was not clear why the rate of sporulalron is similar between both strains at 30 °C.

b) Temperature

Temperature was shown to significantly influence activity of *B. subtilis* (Zimmer *et al.*, 1998). *B. subtilis* applied either on seed alone or on substrate under different temperatures between 10 °C and 30 °C showed root and substrate population density increasing with rising temperatures.

Results obtained by Asaka *et al.* (1996) indicated that the conditions for vegetative cells seemed to be less favourable at 30 °C, compared to trails conducted at 15°C. Survival time of vegetative cells was lower, sporulation occurred faster. However, *B. subtilis* was applied on bare soils without planting, and therefore without rhizosphere.

A new study (Morgan and Brown, 2011) is included as IIM 2.8.2 demonstrating the effect of temperature on growth of *B. amyloliquefaciens* strain MBI600 compared to *B. subtilis* strain QST713. The results shows that at temperature range investigated (0-50°C) there is no difference in the effect of temperature on growth of both microorganisms (measured as colony diameter).

Report: IIM 2.8.2; Morgan R. and Brown A. (2011), Effect of temperature on the growth rate of BLUEXP1780, Serenade and *Botrytis cinerea* strains, report R&D0411001
Guideline: N/A
GLP: No

Executive Summary

- Both *B. subtilis* products, BUEXP1780 and Serenade (AgraQuest) were seen to be active at the same temperature range. Both grew at 15-50°C and there was no growth at temperatures lower than this. The upper temperature limit of growth was not determined for either strain.
- At temperatures 20-50°C both took approximately 48 hours to complete the vegetative growth phase of colonies. Both strain also grew slowly at 15°C and took up to 14 days to reach full colony size.
- *Botrytis cinerea* strains 32, B05-10 and T4 were able to grow within the range 4-28°C. No strain grew at 32.5°C. The optimum temperature for growth of the strain 32 and B05-10 was 25°C and for strain T4, 28°C.
- There is likely to be a narrow window of activity for both *B. subtilis* strains of approximately 20-28°C in which effective control is possible against *B. cinerea,* though this range needs to be further defined.
- Although BUEXP1780 is shown to grow at mammalian body temperatures, reviews published by the Envrionmental Protection Agency (EPA) in the USA have concluded that *B. subtilis* is safe for mammals.

Materials and Methods:

This trial comprised of temperature growth assessments of both the Becker Underwood strain of *B. subtilis*, MBI600, assigned the experimental code BUEXP1780 and the market leading competitor Serenade (AgraQuest, Davis, CA, USA), *B. subtilis* strain QST 713. Similar growth assessments were carried out using three different strains of grey mould, *Botrytis cinerea.* Samples of three cultures of

Botrytis cinerea (strains 32, T4 and BO510) were originally supplied to Becker Underwood by INRA (Thiverval-Grignon, France). Disease cultures were maintained at Becker Underwood UK laboratories on Potato Dextrose Agar (PDA) plates in an incubator at 4°C.

All assessments took place on 90 mm diameter Petri dishes using PDA at 39g / Litre as the growing media. Bacteria were assessed through spread plating onto agar at the recognised dilution to give an uncongested agar plate in which adjacent bacterial colonies did not inhibit each other. For both bacteria tested this was the 10^{-9} dilution. Fungi were tested by placing a pinched sample of mycelia from a stock culture into the centre of a Petri dish using forceps. Plates were assessed at a range of temperatures (from 0 to 50°C).

Colony growth was assessed in each case by measuring the diameter every 24 hours until growth ceased, reached the side of the Petri dish or was deemed not to occur at the temperature. In the case of bacteria this could be multiple colonies on each plate while with fungi it was just the central seeded colony. Five repetitions were made for each bacteria or disease strain at each temperature tested.

Results and discussion:

After 24 hours, growth of both *B. subtilis* products has occurred at the same temperatures from 20 to 50°C (Table 8.1.1-02). The growth of BUEXP1780 is far more consistent across the temperatures than Serenade. BUEXP1780 shows colony sizes of no more than 4 mm up to 37.5°C and then 5.5mm at 50°C. Serenade shows consistently faster growth than BUEXP1780 including a peak of 8.2mm at 32.5°C though drops to 2.6mm at 50°C.

Table 8.1.1-02: Mean colony growth (mm) of *B. subtilis* strain MBI600 and QST713 and three strains of *Botrytis cinerea* supplied by INRA (France) on potato dextrose agar plates at a range of temperatures after 24 hours.

	Temperature (ºC)								
	0	10	15	20	25	28	32.5	37.5	50
Bacillus subtilis QST713	0	0	0	1.9	4	3.2	8.2	4.1	2.6
Bacillus subtilis MBI600	0	0	0	2.6	2	2	2.5	4.1	5.6
Botrytis cinerea strain 32	0	9.3	3.67	5.8	3.6	4.75	0	0	0
Botrytis cinerea strain T4	0	2.0	3.67	3.6	4.6	2.67	0	0	0
Botrytis cinerea strain B05 10	0	3.3	2	3.2	3.4	3	0	0	0

After 48 hours, growth of both *B. subtilis* products again occurs at the same temperatures from 20 to 50°C (Table 8.1.1-03). The growth of BUEXP1780 is much more similar to that of Serenade after this time. This is likely to be due to both strains having reached the limit of vegetative growth with the much slower motile growth stages to follow. At 20°C both strains show growth of close to 5.5mm and remain near to this size up to 28°C. At 32.5°C, BUEXP1780 remains at a similar size while Serenade increases sharply again to 10.4mm. Both strains show similar growth at the higher temperatures of 37.5°C and 50°C at 8mm and approximately 5.5mm respectively.

Table 8.1.1-03: Mean colony growth (mm) of *B. subtilis* strain MBI600 and QST713 and three strains of *Botrytis cinerea* supplied by INRA (France) on potato dextrose agar plates at a range of temperatures after 48 hours.

	Temperature (ºC)								
	0	10	15	20	25	28	32.5	37.5	50
Bacillus subtilis QST713	0	0	0	4.4	6	4.5	10.4	7.9	5.1
Bacillus subtilis MBI600	0	0	0	5.3	5.2	5	5.3	8.1	5.5
Botrytis cinerea strain 32	0	24.67	25	33.4	30.6	11.75	0	0	0
Botrytis cinerea strain T4	0	13	11.67	23.6	34.8	15	0	0	0
Botrytis cinerea strain B05 10	0	10.67	11	26.4	23.8	12	0	0	0

While the upper limit for neither *B. subtilis* strain was seen, the lower limit for growth was in fact found to be at most 15°C. Colonies at 15°C were not visible until the 5th day of incubation but would continue growing to reach the motile phase for a further 9 days reaching maximum size after 14 days. No growth occurred at 10°C and below.

Discussion
The activity of both *B. subtilis* strains appears very similar. While *B. subtilis* strain QST713 my exhibit stronger initial growth than *B. subtilis* strain MBI600, it could well be limitations of the assessment technique used that limit the definition possible. Measuring single colony growth once every 24 hours is a very imprecise technique given that the colonies are so small, as are changes in hourly growth, making measurement very difficult. This then makes differences between strains and different temperature difficult to ascertain. A more reliable technique could be to assess growth in a liquid media in Trytone Soya Broth (TSB) and shaker flasks. Measuring the light absorbance every 2 hours would be fast and a more accurate measure of bacterial growth at different temperatures. It would also allow the important upper limit of *B.subtilis* growth to be determined if a water bath was used in place of an incubator.

Accurate determination of the lower limit of growth of bacteria will be necessary as will determination of the lower limit for vigorous growth. At the moment the lower limit of growth is defined as between 10 and 15°C while the lower vigorous growth limit is between 15 and 20°C.

Comment RMS:
This study is considered as acceptable.

c) pH

The pH of the plant cultivar and the spermosphere environment were shown to affect growth of *B. subtilis* after seed treatment (Mahaffee and Backmann 1993). The Authors proposed lower populations in the spermosphere of seeds was due to a surface pH < 3.5 causing a delay in spore germination.

Siala *et al.* (1974b) observed growth of vegetative cells of *B. subtilis* as well as germination of *B. subtilis* spores in non-sterile acid forest soils (A_1 horizon). Whereas introduced vegetative cells did not grow and declined rapidly in alkaline forest soils (C-horizon). Number of spores introduced into alkaline soils remained stable, almost no spore germination took place.

Degradation of *B. amyloliquefaciens / subtilis* vegetative cells and endospores by phage infection and protozoan grazing

Effects of phage infection

Pantastico-Caldas *et al*. (1992) investigated population dynamics of *B. subtilis* in the presence and absence of a wild temperate phage and a virulent mutant of the well-known *B. subtilis* phage (SP10) over a period of 60 days in soil microcosms containing sterilised acidic peat soil. Without phage infection, populations of *B. subtilis* declined from about 10^7 CFU/g soil to a constant density of ~8×10^5 CFU/g soil within 2 days. With the temperate phage treatment, the density of the bacteria dropped sharply to ~10^5 CFU/g within 24 h while the phage density concurrently increased by a factor of ten. The bacterial population then rose back to initial densities and fell to the density of the control populations within the following 20 days. In contrast to the temperate phages, virulent phages depressed *B. subtilis* equilibrium densities by a factor of ten.

In all cases an initial epidemic of phage occurred, followed by stable equilibrium lasting weeks to months. A threshold host density for phage outbreak occurs at 5×10^6 CFU/g soil. At equilibrium the phage, both temperate and virulent, were much less abundant than bacteria.

Reduction by protozoa and bacterial predators

Predatory activity of soil protozoa is considered as one of the major factors decreasing the numbers of beneficial bacteria introduced into soils for plant protection or promotion. In contrast to autochthonous microbes, these strains often suffer from the missing development of feeding resistance structures. Also amoebae, which represent typical soil protists, were shown to grow moderately with *B. subtilis* as food source. However, it is also stated that *B. subtilis* as a typical soil microorganism may prevent more effective grazing by means of defence mechanisms, like toxic pigments or special outer membrane structure (Weekers *et al*., 1993).

Bacterial predators like *Cupriavidus necator* are known to attack *B. subtilis* cells and spores in soils (Zeph *et al*, 1986; cited in Casida, 1988).

Influences of clay amendments to soils on survival or degradation in soils

A review regarding survival in soil by England *et al.* (1993) suggests that different compositions of soils may influence the degradation of soil bacteria by protozoa as well as bacteriophages. Robert and Marshall (1974; cited in England *et al.*, 1993) presented evidence that *Eschericha coli* was protected from phage lysis by sediments, montmorillonite or organic matter. At low electrolyte concentrations, this protection was provided by an envelope of colloidal materials sorbed to cells. At high electrolyte concentrations, protection resulted from both the colloid envelope around cells and sorption of cells and phages to solid particles.

The survival of inoculated *Rhizobium leguminosarum* in non-sterile loamy sand with added bentonite, was higher (cell numbers remained at the initial level of ~10^7 CFU/g dry soil) than in non-sterile loamy sand without bentonite (initial level of 10^7 CFU/g dry soil dropped to 3×10^5 CFU/g dry soil). In sterile loamy sand with or without added bentonite, an increase of cell counts (from 10^7 to 3×10^8 CFU/g dry soil) was observed. Bacteria introduced at log 7.4 to 7.7 CFU/g dry soil into three unamended loamy sand with 3 % clay content, loamy sand amended with kaolinite and loamy sand amended with bentonite resulted in cell counts of log 5.5, 6.4 and 8.05 CFU/g dry soil, respectively (van Elsas *et al*., 1986).

The effect of additional clay in soils on the survival of bacterial cell was explained by shielding microorganisms from predation by protozoals by forming protective microhabitats (Heijnen *et al*., 1988; Heijnen and van Veen, 1991; cited in England *et al.,* 1993). It is suggested that the number of protective

pore spaces increased with addition of bentonite (Heijnen and van Veen, 1991; cited in England *et al.*, 1993).

Discussion

B. amyloliquefaciens spores introduced into soils are likely to germinate, grow and exhibit plant protective activity in the rhizosphere, as it provides sufficient concentration of nutrients for induction of germination. Due to their ability to produce antimicrobial metabolites including iturin A and surfactin, vegetative cells can compete with autochthon microorganisms to some extent, although studies have shown a significant difference between fate of introduced cells in sterile and non-sterile soil. Survival of vegetative cells also depends on abiotic factors like pH, temperature and UV-light. Temperatures between 27 - 37 °C and pH-values between 6 - 7 are considered as optimal. Endospores introduced into soils with poor nutrient content and without planting (without rhizolphere) will not germinate. Sporulation of vegetative cells occurs if environmental conditions become unfavourable. As endospores are very resistant to abiotic environmental factors and do not compete with autochthonous microorganisms for nutrients, they can survive in soil for a long period of time. Grazing by protozoa and by bacterial predators is one of the main reduction factors of vegetative cells and endospores in soil. Infection by bacteriophages is further factors responsible for decline of vegetative cells. High clay contents of soil are considered to be protective concerning grazing and infection. However, influence of environmental factors cause a steady decline of vegetative cells and spores until an equilibrium is reached. The concentration of equilibrium is variable, dependent on local environmental conditions.

The potential production for metabolites such as indol acetic acid, surfactant, iturins, polyketides and amylosin (that may be formed following endospore germination).

B. amyloliquefaciens strain MBI600 is considered to produce antimicrobial compounds at the treatment site (leaves and fruits) to successful compete against other microorganisms including plant pathogens. However, it should be noted that the mode of action of *B. amyloliquefaciens* is not only related to the production of antifungal substances, but also to competition for nutrients and space and the release of lytic enzymes. Activity is related to the presence of the microorganism as actively growing cells, and can therefore not be considered or assessed independently from the active ingredient *B. amyloliquefaciens* strain MBI600.

For different antagonistic compounds produced by *B. subtilis* upon treatment of melon leaves (iturin, surfactin, fengycin, bacillomycin) it was demonstrated that they do not accumulate on the treatment site but disappear within ~12 days post application (Romero *et al*., 2007).

In a recent study of Crane *et al.* (2013) monitored the persistence of iturin and *B. amyloliquefaciens* populations upon treatment of wheat either in the greenhouse or in the field for 14 days. It was found that under greenhouse conditions initial levels of iturin were three times higher than after field application. However, in both settings the iturin levels rapidly decreased within the first 3 days post treatment, although significant populations of the bacterium were still present. It can be therefore concluded that antifungal compounds or other substances are probably involved in antagonistic activity of *B. amyloliquefaciens* strain MBI600 and will only be produced at the treatment site during direct interaction with the target pathogen and only for a period of a few days. The substances will not accumulate and their persistence is considerably shorter than that of the microorganism itself. It can therefore be concluded that exposure to non-target organisms to any substance eventually produced by *B. amyloliquefaciens* strain MBI600 after field application is negligible. In addition, none of the metabolites eventually produced by the strain are known to exhibit toxic properties against non-target organisms including birds and mammals.

Additionally, production of antagonistic compounds is a general trait of natural microbial communities in soil environments. This property leads to the phenomenon of suppressive soils, in these soils antagonistic activity of the indigenous microflora is able to suppress soil diseases representing a kind of natural control of bacterial and fungal pathogens. It can therefore be concluded that non-target organisms and in particular those dwelling in soils are naturally exposed to antimicrobial compounds. It can also be assumed that in particular in soils, organic substances such as secondary metabolites are readily degraded due to the presence of a highly diverse and abundant microflora.

As indicated in the above, these anti-fungal agents are produced by vegetative cells only. As such their concentration is not fixed and cannot be reliably predicted for all soil types and conditions. However, due to their transient appearance and general impersistence, the notifier states that the increase in antimicrobial compound levels reaching soil or groundwater environments, due to *B. amyloliquefaciens* strain MBI600 field application, will be negligible.

Concerning the potential transfer of genetic material from the micro-organism to other organisms, some information were provided by the notifier.
Little information on the genetic stability of natural *Bacillus* strains in general and *B. amyloliquefaciens* strains in particular is available. The main focus of studies regarding genetic stability is on genetically engineered strains and the fate of the inserted DNA or other genetic modifications.

Genes responsible for the mode of action are genome located and not located on plasmids (Chen *et al*., 2007). Exchange of genomic (chromosomal) DNA was shown to occur between strains of *B. subtilis* grown together in soil culture at high densities. Linked markers for antibiotic resistance were presumably acquired by transformation. Apparently, selection pressure was present that led to the shift in genotypes (Graham and Istock, 1979). Sporulation capacity was chosen as a marker for genome stability by Maughan *et al*. (2009). *B. subtilis* strains were cultured for 6000 generations in the absence and presence of selective pressure for sporulation. Populations cultured in the absence of selective pressure lost the ability to sporulate. Point mutations or small indels, in regulatory elements of the genome, were responsible for a loss in sporulation initiation whereas no large deletions were detected.

Gene exchange can also occur through plasmid uptake or recombination of chromosomal DNA from other *Bacillus strains*. Bacterial conjugation is a mechanism of genetic exchange that requires cell-to-cell contact in the medium. However, cells need to be not only in a vegetative state, but also in a "competent" stage to take up external plasmids. This event may theoretically take place under application conditions, but the probability is very low due to the restricted populations of possible DNA donor strains and the low proportion of competent cells. Interspecies gene transfer has been demonstrated by van Elsas *et al*. (1987), who suggested conjugation between *B. cereus* and *B. subtilis* as the transfer mechanism of a plasmid carrying tetracycline resistance. The plasmid transfer from *B. cereus* to *B. subtilis* was detectable in sterile, nutrient amended soil at 27 °C at a frequency of 1.6×10^6, but transconjugants were not detectable in non-sterile soil, unless bentonite clay was added which may have modified the physicochemical soil environment. At 15 °C and a lower moisture content, plasmids were also transferred but at a lower rate. High population densities of both the donor and the receptor strains ($> 5 \times 10^6$) were essential for conjugation to occur (van Elsas *et al*., 1987). Even if gene exchange is theoretically possible and was observed *in vitro*, Vilas-Boas *et al*. (2002) could not find evidence that horizontal gene transfer occurs randomly in sympatric natural isolates of *B. cereus* and *B. thuringiensis* under natural conditions in soil. Similarly, Duncan *et al*. (1994) found in a hierarchical population genetics analysis, that populations of *B. subtilis* were genetically differentiated from sympatric populations of *B. licheniformis*. The Authors speculate that there is probably modest genetic exchange, if any, between the species in nature. The level of recombination between different *Bacillus* species was found to be much lower than within the same species (Vilas-Boas *et al*., 2002).

All available literature information on exchange of genetic material between *Bacillus* species or members of the genus and other bacteria species indicate that gene transfer within the species *B. amyloliquefaciens* or between *B. amyloliquefaciens* and related species may occur in nature but is a rare event. All Authors observed that under natural conditions in soil, the degree in exchange of genetic material was reduced compared to that observed in manipulated soil (sterilized, nutrient amended, single populations, addition of growing cells) or was even no longer detectable. Vilas-Boas *et al.* (2002) for example, states:
"*..although the horizontal transfer of plasmids between B. thuringiensis and B. cereus has been demonstrated under laboratory conditions, our results, although limited to two populations, provide no evidence that such transfer occurs randomly in sympatric natural isolates of B. cereus and B. thuringiensis*".

The authors also concluded that only empirical studies under realistic natural conditions will bring us closer to assessing the safety of pest control based on bacilli.

In a very recent study, such experiments have been carried out by Kim *et al.* (2012). To investigate the possibility of horizontal gene transfer between agricultural microorganisms and soil microorganisms in the environment, *B. subtilis* strain KB producing iturin and a recombinant strain of the plant growth promoting rhizobacterium *Pseudomonas fluorescens* (strain MX1 producing mannityl opine cyclase from *Agrobacterium tumefaciens*) were used as model microorganisms. The microbes were added to soil samples of cucumber or tomato plants cultivated in pots or in the greenhouse and monitored for a six month period by PCR, real-time PCR, Southern hybridization, and T-RFLP fingerprinting. The analyses demonstrated that the genomic DNA of the two microbes strongly decreased within the observation period. After 10 weeks only 20% of the initial genomic DNA copies were still present in the soils, decreasing to < 10% at the end of the observation period (22 weeks). Following the engineered genes (iturin and mop cyclase) of the two model microbes by Southern blot revealed a quick degradation of the genes in the soils. The authors therefore concluded that gene transfer from model strains to soil bacteria does not occur under natural conditions in soil.

Even if genetic exchange occurs, it will only affect single cells in a population of soil bacteria. Proliferation will only happen if the new recombinant strain has a selective advantage compared to indigenous strains. This scenario is very unlikely as it would necessitate a single gene that confers a competitive advantage to all other microorganisms competing for the same ecological niche.

❑ Predicted environmental concentrations in soil (PEC_S)

Worst-case initial PECs values for spray drift have been calculated by applicant for use in the environmental risk assessment.
The calculation is based on 40% interception of the application by the foliage (lowest FOCUS crop interception value for grape vines at any growth stage) and uniform distribution in 5 cm soil (bulk density 1.5 g/cm). PEC_S values have been calculated for a single application and up to 10 applications, following the proposed use. The calculation is based on each application being at the maximum recommended rate of 0.5 kg product/ha.

The PEC_S for the product and active substance were calculated according to FOCUS guidance[3], using the following equation:

$$PEC_S \text{ initial} = \frac{\text{Rate} \times (1 - \text{interception})}{\text{Soil Depth} \times \text{Soil Density} \times 100}$$

Where:
Rate = 500 g MPCP/ha / 55 g MBI600/ha / 2.75×10^{13} cfu/ha

[3] *FOCUS (1997) Soil persistence models and EU Registration - The Final Report of the Soil Modelling Workgroup of FOCUS (Forum for the Co-ordination of Pesticide Fate Models and their Use) – 29 February 1997.*

Interception = 0.4 (40% FOCUS minimum interception vines)
Soil Depth = 5 cm
Soil Density = 1.5 $g.cm^{-3}$
Multiple applications are additive, no degradation or dissipation is considered. The PEC_S based on cfu is calculated from the product PEC_S (converted to g) multiplied by the concentration in the product as cfu (*i.e.* 5.5×10^{10} cfu/g of MPCP).

Initial PECs values are presented in the following table:

Table 8.1.1-04: Initial PEC_S for formulated product and *B. amyloliquefaciens* strain MBI600

Product mg MPCP/kg		***B. amyloliquefaciens* strain MBI600**			
		mg MPCA/kg		**cfu/kg**	
1 Application	10 Applications	1 Application	10 Applications	1 Application	10 Applications
0.400	4.00	0.044	0.440	2.20×10^7	2.20×10^8

MPCP: Microbial pest control product
MPCA: Microbial pest control agent

The above multiple application PECs values asre considered to be overly conservative as the active life stage is the vegetative cell of *B. amyloliquefaciens*, and not the dormant endospore as applied. Due to the rapid decline of vegetative cells derived from MBI600 following product application, it can be assumed that there will be no significant carry-over of vegetative cells soil between applications. Endopores will likely remain in the soil both directly from the application and from naturally occurring cells and cells derived from MBI600. However germination of viable endospores will be limited by the environmental conditions and availability of organic carbon, i.e. numbers of vegetative cells will be controlled by the environment, not by the number of endospores. The study of Bennett *et al.* (2003) showed that populations of *B. amyloliquefaciens* are influenced by biotic environmental factors. Introduced *B. amyloliquefaciens* populations are subject to competition by the indigenous microflora (bacteria and fungi) and may also be affected by infectious agents like phages. As a result, initial population numbers resulting from application of *B. amyloliquefaciens* endospores will rapidly decline and reach a natural equilibrium, with the population stabilising as endospores.

Initial PECsoil were calculated by RMS according to the PRaPer M2 (2009) recommendations without taken into account crop interception is calculation.

Table 8.1.1-05: Initial PEC_S for Subtilex® and *B. amyloliquefaciens* strain MBI600

Subtilex® mg MCPP/kg		***B. amyloliquefaciens* strain MBI600**			
		mg MPCA/kg		**cfu/kg**	
1 Application	10 Applications	1 Application	10 Applications	1 Application	10 Applications
0.67	6.67	0.07	0.73	3.67×10^7	3.67×10^8

B.8.1.2. Water (Annex IIB 7.1.2)

The notifier addressed the fate and the behaviour of *B. amyloliquefaciens* in surface water by supplying information from open scientific literature. This information supplying by applicant is summarised below by RMS.

Bacilli in the *subtilis* group, such as *B. amyloliquefaciens*, occur frequently in fresh, estuarine and coastal aquatic environments, and endospores have been reported in sediments and in the ocean (Priest, 1993). However, members of this group of bacilli are not regarded as autochthonous inhabitants of aquatic environments and do not find optimal conditions for growth, as waters are often poor in organic carbon. Therefore, proliferation is not likely to occur. Bacterial cells and especially endospores may survive, but will be subject to natural competition in the diverse micro-flora of natural waters.

Population dynamics and fate

Liang *et al.* (1982) studied the survival of *B. subtilis* introduced into sewage and lake water in microcosm experiments under non-sterile as well as under sterile conditions. When added to raw sewage vegetative cells of *B. subtilis* strongly decreased from 6.5×10^5 CFU/mL to 15 CFU/mL, sporulation was almost absent. In sterilised sewage vegetative cells of *B. subtilis* first increased and then declined concurrent to endospore density increase. At the end of the incubation period (15 d) all cells remaining in the microcosms were endospores (~10^5 endospores/mL). Vegetative cells introduced into lake water rapidly declined with no differences found between sterilised or non-sterile samples. Sporulation was not observed. *B. subtilis* persisted in environments in which the supply of organic nutrients permitted the formation of a resistant structure, but not in environments where the nutrient level was reduced (i.e. by other inhabitants) so that the resistant structure was not produced. Therefore this bacterium formed spores in large numbers in nutrient-rich sterile sewage and soil but not in nutrient-poor sterile lake water or in non-sterile samples of all three environments.

Report: Liang, L.N., Sinclair, J.L., Mallory, L.M. and Alexander, M. (1982) Fate in model ecosystems of microbial species of potential use in genetic engineering. *Appl. Environ. Microbiol*. 44, 708-14. Published.
Guideline: N/A
GLP: No

Executive Summary

The changes in populations of *B. subtilis* were measured after introduction into samples of sewage, lake water, and soil. By the end of the study, fewer than 2 cells/mL sewage and lake water, and 25 cells/g soil could be detected. Vegetative cells of *B. subtilis* failed to survive in samples of sewage and lake water. In sterile sewage, *B. subtilis* grew, whereas in sterile lake water the number of vegetative cells of *B. subtilis* declined rapidly. The population of *B. subtilis* declined in non-sterile soil, but grew in sterile soil.

Materials and Methods:

Test item: Streptomycin resistant *B. subtilis* (strain CU155) grown in trypticase soy broth

Test system: Three media were used (Table 8.1.2-01).

Table 8.1.2-01: Test Media Characteristics

Soil	Sewage	Lake water
Silty Loam pH 6.7 4.7% Organic matter 90% Field Moisture Capacity	Raw sewage Ithaca, N.Y.	Lake Beebe Ithaca, N.Y.

Soil samples were dried in air and passed through a 2 mm sieve and 10 g aliquots were placed in sterile bottles. In instances in which sterile soil was used, the soil was sterilized by irradiation with 2.5 Mrad from a ^{60}Co source. The soil samples were moistened to 90% of field capacity by adding 3.0 mL of cell suspension, and they then were mixed with the inoculum. The bottles were incubated at 30°C in the dark. Samples of raw sewage from the Ithaca, N.Y. sewage treatment plant and water samples from Beebe Lake, Ithaca, N.Y., were taken immediately before the experiments were conducted. If sterile sewage or lake water was desired, portions were sterilized either by filtration of fresh samples through a 0.22 µm membrane filter or by irradiation of frozen samples with 2.5 Mrad from a ^{60}Co source. Frozen samples were used to prevent microbial destruction of the organic matter before sterility was achieved. After irradiation, samples of soil, sewage, and lake water, subsamples inoculated into a glucose-asparagine broth (1.0% glucose, 0.05% asparagine, 0.05% K_2HPO_4) failed to show growth in 7 days. Portions of sewage and lake water (100 mL) were placed in either 250 or 500 mL Erlenmeyer flasks, and the liquids were inoculated with the test organism and incubated at 30°C in the dark on a rotary shaker operating at 120 rpm. Each treatment was replicated three times.

Counts were made by the spread-plate technique with 250 µg of cycloheximide per mL of medium to inhibit fungi. *B. subtilis* was counted after incubation at 37°C for 48 h on half-strength trypticase soy agar with 50 µg of rifampin and 1.0 mg of streptomycin per mL. Agar containing rifampin was prepared the day it was used, and the plates were dried for 2 h at 50°C. Endospore counts of *B. subtilis* were made by heating appropriate dilutions at 80°C for 10 min before plating.

Results and discussion:

After addition of the microorganism to separate samples of raw sewage at densities of 3.3 x 10^3 to 6.3 x 10^5 cells per mL, the population of *B. subtilis* declined rapidly. The population of *B. subtilis* had fallen to 15 cells/mL at day 1. In sterile sewage, *B. subtilis* increased in abundance, and then the number of vegetative cells declined; however, the endospore density rose so that all cells that remained at the end of the incubation period were endospores.

In non-sterile lake water the population of *B. subtilis* fell from an initial value of 4.1 × 10^5 cells/mL to 8 cells/mL at day 2, whereas the endospore density increased from 5 to only 8 endospores/mL in the same period. In sterile lake water the density of vegetative cells of *B. subtilis* fell to 12 cells/mL at day 2 and endospores were not produced under these conditions. Thus, *B. subtilis* was essentially eliminated from the non-sterile and sterile lake water.

The size of the population of *B. subtilis* introduced into non-sterile soil fell with time, reaching 24 cells/g at day 10. *B. subtilis* grew in sterile soil, reaching populations in excess of 10^7 cells/g after which the number of viable cells fell slowly with time, with large numbers still present at the end of the test period. Under these conditions *B. subtilis* initially sporulated profusely, followed by a rapid decline of endospore numbers.

Discussion

B. subtilis declined rapidly in both non-sterile and sterile sewage and lake water. This short persistence is considered due either to sensitivity to abiotic factors (e.g., pH or the presence of inorganic or

organic inhibitors), inability to obtain nutrients from the environment coupled with an intrinsic susceptibility to elimination by starvation, or both.

B. subtilis declined rapidly in non-sterile soil samples but persisted in sterile samples. This species is therefore not destroyed by abiotic stresses in soil, evidently being able to obtain organic nutrients from the habitat in the absence of competition, but they may fail to do so in the presence of other species. Their elimination in non-sterile samples indicates that they either are susceptible to predation, parasitism, or lytic enzymes or toxins produced by the indigenous community or are eliminated because they are prone to rapid loss of viability during starvation and are not getting the nutrients they require in the presence of an established community.

Conclusions

B. subtilis persisted in environments in which the supply of organic nutrients permitted the formation of a resistant structure, but not in environments where the nutrient level was reduced by other inhabitants so that the resistance structure was not produced. Thus this bacterium formed endospores in large numbers in nutrient rich sterile sewage and soil but not in nutrient poor sterile lake water or in non-sterile samples in all three environments. The number of endospores reduced rapidly after an initial peak.

Based on data obtained by Liang *et al.* (1982) and Gurijala and Alexander (1990), mortality rates of 42% and 50% were calculated for vegetative cells of *B. subtilis* in lake water by Leff *et al*., 1998. Gurijala and Alexander (1990) described that in contrast to other model strains persisting at high levels at least in sterile phosphate buffer, *B. subtilis* readily lost viability and was not detectable in lake water, sterilised lake water and buffer after 2-3 days. However, it was not clearly documented if possible sporulation was fully detectable by methods used in this study, since heat activation was not described.

These results confirmed previous observations of (Sinclair and Alexander, 1984) demonstrating the inability of *B. subtilis* cells to persist in phosphate buffer as well as in non-sterile and sterile samples of both nutrient-poor lake water and nutrient-rich sewage. The rapid diminution was ascribed to its susceptibility to starvation. Sporulation was not detected.

Population decline of other bacterial species was mainly attributed to protozoan grazing as heterotrophic flagellates increased in numbers while bacterial numbers dropped and were sometimes completely eliminated. This observation was delayed or even absent in the presence of eukaryotic inhibitors.

Gurijala and Alexander (1990) revealed that vegetative cells of *B. subtilis* grown in a 0.05% TSB medium were significantly reduced (~ 10^6 CFU/mL to ~10^2 CFU/mL) by the fresh-water protozoan *Tetrahymena thermophila* after 24 h. Thereafter the population of *B. subtilis* remained constant at the level of 10^2 CFU/mL. Furthermore, the Authors found that the level of survival depends on the growth rate. Adding chloramphenicol to the 0.05 % TSB medium the bacterial growth stopped. Under these circumstances vegetative *B. subtilis* cells were completely eliminated.

Based on microcosm stirring experiments with a model strain (not *B. subtilis / amyloliquefaciens*), Leff *et al.* (1998) stated that current water speed strongly influences bacterial survival in streams with sediments as the major environment for bacterial accumulation. Nevertheless, within one month the abundance of the model strain declined to about 2%. As the strain flourished in autoclaved environments, the Authors concluded that competition, grazing and phage infection contributed to the loss of the introduced population.

Discussion

Contamination of surface waters by applications of plant protection products containing endospores of *B. amyloliquefaciens* (e.g. MBI 600) may occur *via* spray drift. Endospores are known to be metabolically inactive. Thus, they do not consume nutrients and do not grow/multiply.

Germination of endospores is dependent on availability of sufficient nutrients. Hence, the formation of metabolically active vegetative cells might be theoretically possible in eutrophic surface waters or when nutrient-rich sewage reaches surface waters continuously. However, all studies cited above reveal that vegetative cells of *B. subtilis* introduced into different non-sterile aqueous environments, including nutrient-rich sewage, declined rapidly. Therefore, due to competition with indigenous microorganisms germination of endospores appears to be highly unlikely, although the concentration of nutrients might be sufficient in some cases. The total number of endospores decreases over a certain period of time due to grazing protozoans or sediment-dwelling organisms.

- **Predicted environmental concentration in surface water (PEC_{SW})**

Surface water may be exposed via spray drift, whereas other entry routes such as deposition following volatilisation are not expected to occur. Entry via run-off and/or drainage is also not considered as no adequated method for calculation is available.

Worst-case initial drift PEC_{SW} values have been calculated for use in the environmental risk assessment. The calculation is based on application to vines, assuming that spraying takes place within 3, 5 and 10 m of a static water body with a depth of 0.3 m. PEC_{SW} values have been calculated for a single application and up to 10 applications, following the proposed use. The calculation is based on each application being at the maximum recommended rate of 0.5 kg product/ha.

The PEC_{SW} for each application of the product and active substance were calculated according to the following equation SANCO, 2001[4]; multiple applications are additive, no degradation or dissipation is considered:

$$PEC_{SW}(\mu g.L^{-1}) = \frac{R\ (kg.ha^{-1}) \times 10^{9} (\mu g.kg^{-1}) \times \frac{F}{100}}{10^{4} (m^{2}.ha^{-1}) \times D\ (m) \times 10^{3} (L.m^{-3})}$$

Where:

R = Application rate 0.5 kg product/ha / 55 g MBI600/ha / 2.75×10^{13} cfu/ha
F = Rautmann Drift Factor (worst-case late applications to vines)
Single application: 8.02, 3.62 and 1.23% for 3, 5 and 10 m distance from crop margin
Multiple applications (≥3): 6.90, 3.07 and 1.02% for 3, 5 and 10 m distance from crop margin
D = Depth of water body 0.3 m

The PEC_{SW} based on cfu is calculated from the product PEC_{SW} (converted to g) multiplied by the concentration in the product as cfu (*i.e.* 5.5×10^{10} cfu/g).

Initial PECs values are presented in Table 8.1.2-02 below:

[4] *SANCO/3268/2001 (version 1, 1st October 2001) Working Document; Guidance Document on Aquatic Ecotoxicity in the Frame of Directive 91/414/EEC*

Table 8.1.2-02: Initial PEC_{SW} for Subtilex® and MBI600

Distance	Subtilex® µg MPCP/L		MBI600 *			
			µg MPCA/L		cfu/L	
	1 Application	10 Applications	1 Application	10 Applications	1 Application	10 Applications
3 m	13.367	133.37	1.470	14.70	7.35×10^5	7.35×10^6
5 m	6.033	60.33	0.664	6.64	3.32×10^5	3.32×10^6
10 m	1.700	17.000	0.226	2.26	9.35×10^4	9.35×10^5

MPCP: Microbial pest control product
MPCA: Microbial pest control agent
* based on the minimum specification of the formulation (5.5×10^{10} cfu/g)

The above multiple application PEC_{SW} values are considered to be overly conservative as bacilli in the same group as *B. subtilis* are not regarded as an autochthonous inhabitant of aquatic environments and do not find optimal conditions for growth, e.g. waters are poor in organic carbon. Liang *et al.* (1982) measured the numbers of cells *B. subtilis* after application to natural lake water. Vegetative cells failed to survive lake water. Therefore, proliferation is not likely to occur. Bacterial cells and especially endospores may survive, but will be subject to natural competition in the diverse micro-flora of natural waters.

RMS notes when considering the maximum test item specification, the calculated TER for a single application could exceed the TER trigger value (see volume 3 section B9). However, the maximum test item specification is not available (see data gap reported in Volume 4).

The following applicant's statement demonstrates that the specific strain would not cause interference with the analytical systems for the control of drinking water Directive 98/83/EC, methods ISO 12780, ISO 93808-1 and ISO 7899-2. Despite the restricted persistence of *B. amyloliquefaciens* strain MBI600 in surface waters it may be possible that single cells or spores can reach drinking water treatment plants. There is no evidence that the presence of *B. amyloliquefaciens* strain MBI600 might affect drinking water monitoring systems. Drinking water quality is monitored by screening for microbial indicator species using highly selective media on which the strain does not grow. This was clearly demonstrated by the outcome of 5-batch analyses of microbial contaminants in the technical material, which included these indicator bacteria. However, *B. amyloliquefaciens* strain MBI600 is not expected to be relevant for human health as the microorganism is not pathogenic and does not produce metabolites of toxicological concern, e.g. B. cereus toxins or amylosin.
Potential interference with the analytical systems for the control of the quality of drinking water according to Council Directive 98/83/EC for drinking water coliforms or *E. coli*, enterococci, and *Pseudomonas aeruginosa* are monitored in drinking water. The methods used are ISO 9308-1, ISO 12780 and ISO 7899-2. In principle, drinking water monitoring requires a concentration step (usually membrane filtration) as drinking water does not contain high densities of micro-organisms. Afterwards, the concentrated samples are subjected to cultivation procedures on media which are highly selective for the above mentioned indicator species. In most cases the media contain specific substrates which are metabolized by the indicator species resulting in a particular colour reaction which is then used as a discrimination criterion. Quality assurance for the specific media always includes testing of false positive signals for other indicator species whereby in most case *E. coli*, Enterococcus and *P. aeruginosa* are used which never interfere with the methods used. This indicates

that the media are highly specific and it is very unlikely that another bacterial species grow on the media and/or exhibits the required enzymatic activities for metabolism of the applied substrates used for identification of the indicator species.

The technical material of *B. amyloliquefaciens* stain MBI600 was screened for the presence of the above mentioned indicator bacteria using the similar microbiological methods or identification principles. Despite a high density of *B. amyloliquefaciens* strain MBI600 in the test material, no false positives or any other kind of interference with the applied methods has been observed. It can be therefore concluded, that *B. amyloliquefaciens* strain MBI600 will not interfere with the microbiological methods used for drinking water monitoring.

B.8.1.3. Air (Annex IIB 7.1.3 and IIIB 9)

Specific data, which indicate the survivability of *B. amyloliquefaciens* in the atmosphere after release, are currently unavailable. Survival of vegetative cells during aerosolization is typically limited due to stresses such as shear forces, desiccation, temperature, and UV light exposure. However, its ability to survive in a broad habitat range and produce endospores suggests that this organism may survive after release. As with naturally-occurring strains, human exposure may occur via inhalation as the organisms are dispersed in the atmosphere attached to dust particles, or lofted through mechanical or air disturbance.

Endospores are suitable for aerial distribution as they are easily blown by wind (Priest, 1993). Therefore, under conditions of use drift spacious transport may occur. However, germination and multiplication of MBI600 in the air, aerosols or clouds can be excluded due to lack of organic matter substrate and lack of mineral matrix to adhere to. In addition, during aerolization vegetative cells of MBI600 would be exposed to severe environmental stress factors (desiccation, UV-radiation, temperature), therefore survival of vegetative cells is unlikely.

B.8.2. Mobility (Annex IIB 7.1.1 and IIIB 9)

No specific data on the soil mobility of *B. amyloliquefaciens* cells was provided by the notifier.
Mobility of vegetative cells is limited by short persistence times in soil. Clearly the ability of endospores to disperse through multiple pathways means that endospores are likely to be found in many environments. However, as germination is limited by suitable environmental conditions, proliferation outside a soil environment is highly unlikely.

In principle, the usually employed model calculations on the persistence and mobility of chemical substances are not applicable to an active ingredient being a viable organism. Unlike a chemical substance a microorganism does not follow first order kinetics in degradation. Therefore, no ground water concentrations can be calculated for the different environmental media.

Bacilli in the same group as *B. subtilis*, such as *B. amyloliquefaciens*; are frequently occurring in different aquatic environments, as fresh water, estuarine and coastal waters, and endospores have been detected in sediments and in the open ocean. However, Bacilli in the same group as *B. subtilis* are not regarded as an autochthonous inhabitant of aquatic environments and do not find optimal conditions for growth, e.g. waters are poor in organic carbon. Liang *et al.* (1982) measured the numbers of cells *B. subtilis* after application to natural lake water. Vegetative cells failed to survive lake water. Therefore, proliferation is not likely to occur. Bacterial cells and especially endospores may survive, but will be subject to natural competition in the diverse micro-flora of natural waters. It is therefore considered that the risk to groundwater is acceptable.

Based on data provided by DE as RMS for the active substance *B. amyloliquefaciens* subsp. *plantarum strain* D747, leaching of *B. amyloliquefaciens* endospores through soil can occur (Jiang

***et al*., 2005[5]). RMS underlines that the notifier statement on the absence of risk to groundwater contamination for *B. amyloliquefaciens* is not informed enough. Therefore a data gap is proposed. Notifier to provide more information to confirm the low risk of groundwater contamination for *B. amyloliquefaciens strain* MBI600.**

- **Other/special studies**

No data are available to address this data point. Additional Fate and Behaviour studies are not needed because sufficient information is available from the studies summarised under point 7.1 above to support a risk assessment for the proposed uses of MBI600.

[5] *Jiang, G., Noonan, M.J., Buchan, G.D., Smith, N. (2005): Transport and deposition of* Bacillus subtilis *through an intact soil column. Australian Journal of Soil Research, 43, 695-703*

B.8.3. References relied on

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N	Owner **	Full copy provided by the notifier Y/N	Study already assessed by DE as RMS Y/N
Annex II Data and Information							
IIM 7/01	Alexander, M.	1977	Introduction to Soil Microbiology. John Wiley and Sons, Inc., New York. Not GLP Published	N	Public	Y	N
IIM 7.1.1/02	Asaka, O., Ano, T. and Shoda, M.,	1996	Persistence of *Bacillus subtilis* RB14 and its derivative strains in soil with respect to the lpa-14 gene. Journal of Fermentation and Bioengineering 81, 1–6. Not GLP Published	N	Public	Y	Y
IIM 7.1.1/03	Bennett, A.J., Leifert, C. and Whipps, J.M.	2003	Survival of the biocontrol agents *Coniothyrium minitans* and *Bacillus subtilis* MBI600 introduced into pasteurised, sterilised and non-sterile soils. Soil Biology & Biochemistry 35: 1565 – 1573. Not GLP Published	N	Public	Y	N
	Bochow H and Gantcheva K	1995	Soil introductions of *Bacillus subtilis* as biocontrol agent and its population and activity dynamic. *Acta - Horti.* 382: 164–172 Not GLP Published	N	Public	Y	Y
	Casida LE	1988	Response in Soil *of Cupriavidus necator* and Other Copper-Resistant Bacterial Predators of Bacteria to Addition of Water, Soluble Nutrients, Various Bacterial Species, or Bacillus thuringiensis Spores and Crystals. *Applied and environmental microbiology* 54: 2161–2166 Not GLP Published	N	Public	Y	Y

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****	**Full copy provided by the notifier** **Y/N**	**Study already assessed by DE as RMS** **Y/N**
	Crane JM, Gibson DM, Vaughan RH and Bergstrom GC	2013	Iturin levels on wheat spikes linked to biological control of Fusarium head blight by *Bacillus amyloliquefaciens*. *Phytopathology* 103(2): 146–55 Not GLP Published	N	Public	Y; not assessed submitted too late in the process	N
	Chen XH, Koumoutsi A, Scholz R, Eisenreich A, Schneider K, Heinemeyer I, Morgenstern B, Voss B, Hess WR, Reva O, Junge H, Voigt B, Jungblut PR, Vater J, Süssmuth R, Liesegang H, Strittmatter A, Gottschalk G and Borriss R	2007	Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. *Nature biotechnology* 25(9): 1007–14 Not GLP Published	N	Public	Y; not assessed submitted too late in the process	N
	Duncan KE, Ferguson N, Kimura K, Zhou X and IStock CA	1994	Fine-Scale Genetic and Phenotypic Structure in Natural Populations of *Bacillus subtilis* and *Bacillus licheniformis*: Implications for Bacterial Evolution and Speciation. *Evolution* 48: 2002–2025 Not GLP Published	N	Public	Y; not assessed submitted too late in the process	N
	England LS, Lee H and Trevors JT	1993	Bacterial survival in soil: Effect of clays and protozoa. *Soil Biology and Biochemistry*, 525–531 Not GLP Published	N	Public	Y	Y

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****	**Full copy provided by the notifier** **Y/N**	**Study already assessed by DE as RMS** **Y/N**
	Graham JP and Istock CA	1979	Gene exchange and natural selection cause *Bacillus subtilis* to evolve in soil culture. *Science (New York, N.Y.)* 204: 637–639 Not GLP Published	N	Public	Y; not assessed submitted too late in the process	N
	Gurijala KR and Alexander M	1990	Explanation for the decline of bacteria introduced into lake water. *Microbial Ecology*, 231–244 Not GLP Published	N	Public	Y	Y
	Heijnen	1988	Reference unknown. Cited in England *et al.* (1993)	N	Public	N	N
	Heijnen and van Veen	1991	Reference unknown. Cited in England *et al.* (1993)	N	Public	N	N
	Kim SE, Moon JS, Choi WS, Lee SH and Kim SU	2012	Monitoring of horizontal gene transfer from agricultural microorganisms to soil bacteria and analysis of microbial community in soils. *Journal of microbiology and biotechnology* 22(4): 563–6 Not GLP Published	N	Public	Y; not assessed submitted too late in the process	N
	Krebs B, Höding B and Kübart S	1998	Use of *Bacillus subtilis* as biocontrol agent. I. Activities and characterization of *Bacillus subtilis* strains. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* 105: 181–197 Not GLP Published	N	Public	Y	Y
	Leff LG, McArthur J V. and Shimkets LJ	1998	Persistence and dissemination of introduced bacteria in freshwater microcosms. *Microbial Ecology* 36: 202–211 Not GLP Published	N	Public	Y	Y

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****	**Full copy provided by the notifier** **Y/N**	**Study already assessed by DE as RMS** **Y/N**
IIM 7.1.2/02	Liang, L.N., Sinclair, J.L., Mallory, L.M. and Alexander, M.	1982	Fate in model ecosystems of microbial species of potential use in genetic engineering. *Appl. Environ. Microbiol*. 44, 708-14. Not GLP Published.	N	Public	Y	Y
	Mahaffee WF and Backman PA	1993	Effects of seeds factors on spermosphere and rhizosphere colonisation of cotton by *Bacillus subtilis* GB03. *Phytopathology* 83: 1120–1125 Not GLP Published	N	Public	Y	Y
	Maughan H, Birky CW and Nicholson WL	2009	Transcriptome divergence and the loss of plasticity in *Bacillus subtilis* after 6,000 generations of evolution under relaxed selection for sporulation. *Journal of bacteriology* 191(1): 428–33 Not GLP Published	N	Public	Y; not assessed submitted too late in the process	N
	Milus EA and Rothrock CS	1993	Rhizosphere colonization of wheat by selected soil bacteria over diverse environments. *Canadian Journal of Microbiology*. NRC Research Press 39(3): 335–341 Not GLP Published	N	Public	Y	Y
	Moir and Smith (1990)		Reference unknown. Article cited in Moir (1992)	N	Public	Y; not assessed submitted too late in the process	N
	Moir A	1992	Spore germination Doi, R.H. & Mc Gloughlin, M (eds) Biology of Bacilli. Application to Industry, Chapter 2, 22-28 Not GLP Published	N	Public	Y	Y

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****	**Full copy provided by the notifier** **Y/N**	**Study already assessed by DE as RMS** **Y/N**
IIM 2.8.2	Morgan R. and Brown A.	2011	Effect of temperature on the growth rate of BLUEXP1780, Serenade and *Botrytis cinerea* strains, report R&D0411001 Not GLP Not published	?	BASF Agricultural Specialities Ltd	Y	N
	Nicholson WL	2002	Roles of Bacillus endospores in the environment. *Cellular and Molecular Life Sciences*, 410–416 Not GLP Published	N	Public	Y	Y
	Pandey NK and Aronson AI	1979	Properties of the *Bacillus subtilis* Spore Coat. *J. Bactriol.* 137, 3, 1208-1218 Not GLP Published Article cited in Pandey *et al.* (2001)	N	Public	Y; not assessed submitted too late in the process	N
	Pandey A., Palni S. and Coulomb N.	1997	Antifungal activity of bacteria isolated from the rhizosphere of established tea bushes. Microbiol. Res. 152, 105-112 Not GLP Published	N	Public	Y; not assessed submitted too late in the process	N
	Pandey A., Man L, and Palni S.	1997	Bacillus species/ the dominant bacteria of the rhizosphere of established tea bushes. Microbiol. Res. 152, 359-365 Not GLP Published	N	Public	N	N
	Pandey A, Palni LM and Bisht D	2001	Dominant fungi in the rhizosphere of established tea bushes and their interaction with the dominant bacteria under in situ conditions. *Microbiological research* 156: 377–382 Not GLP Published	N	Public	Y	Y

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****	**Full copy provided by the notifier** **Y/N**	**Study already assessed by DE as RMS** **Y/N**
	Pantastico-Caldas M, Duncan K, Istock C and Bell J	1992	Population dynamics of bacteriophage and *Bacillus subtilis* in soil. *Ecology* 73: 1888–1902 Not GLP Published	N	Public	Y	Y
	Priest	1993	Priest, F.G. (1993). Systematics and Ecology of *Bacillus*. *Bacillus subtilis* and other gram-positive bacteria, American Society of Microbiology, Washington D.C. (ed.), 1993, 3-16 BMF2000-57 Not GLP Not published	N	Public	N	Y
	Reva ON, Dixelius C, Meijer J and Priest FG	2004	Taxonomic characterization and plant colonizing abilities of some bacteria related to *Bacillus amyloliquefaciens* and *Bacillus subtilis*. *FEMS Microbiology Ecology* 48: 249–259 Not GLP Published	N	Public	Y	Y
	Roper MM and Marshall KC	1974	Modification of the interaction between *Escherichia coli* and bacteriophage in Saline Sediment. *Microbial Ecology*. 1, 1-13 Not GLP Published Cited in England *et al.* (1993)	N	Public	Y; not assessed submitted too late in the process	N
	Romero D, de Vicente A, Rakotoaly RH, Dufour SE, Veening J-W, Arrebola E, Cazorla FM, Kuipers OP, Paquot M and Pérez-García A [2]	2007	The iturin and fengycin families of lipopeptides are key factors in antagonism of Bacillus subtilis toward Podosphaera fusca. *Molecular plant-microbe interactions : MPMI* 20(4): 430–40 Not GLP Published	N	Public	Y; not assessed submitted too late in the process	N

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****	**Full copy provided by the notifier** **Y/N**	**Study already assessed by DE as RMS** **Y/N**
	Siala A, Hill IR and Gray TRG	1974a	Populations of Spore-forming Bacteria in an Acid Forest Soil, with Special Reference to *Bacillus subtilis*. *Journal of General Microbiology* 81(1): 183–190 Not GLP Published	N	Public	Y	Y
	Siala A and Gray TRG	1974b	Growth of *Bacillus subtilis* and Spore Germination in Soil Observed by a Fluorescent-antibody Technique. *Journal of General Microbiology* 81: 191–198 Not GLP Published	N	Public	Y	Y
	Sinclair JL and Alexander M	1984	Role of resistance to starvation in bacterial survival in sewage and lake water. *Applied and environmental microbiology* 48: 410–415.*Not GLP Not GLP Published	N	Public	Y	Y
	Tokuda Y, Ano T and Shoda M	1993	Survival of *Bacillus subtilis* NB22, an antifungal-antibiotic iturin producer, and its transformant in soil-systems. *Journal of Fermentation and Bioengineering* 75(2): 107–111 Not GLP Published	N	Public	Y	Y
IIM 7.1.1/01	Van Elsas J, Dijkstra A, Govaert J and Vanveen J	1986	Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. *FEMS Microbiology Ecology* 38(3): 151–160 Not GLP Published	N	Public	Y	Y
	Van Elas	1987	Reference unknown	N	Public	N	N

Annex point / reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data Protection Claimed** **Y/N**	**Owner ****	**Full copy provided by the notifier** **Y/N**	**Study already assessed by DE as RMS** **Y/N**
	Vilas-Boas G, Sanchis V, Lereclus D, Lemos MVF and Bourguet D	2002	Genetic Differentiation between Sympatric Populations of *Bacillus cereus* and *Bacillus thuringiensis*. *Applied and Environmental Microbiology*, 1414–1424 Not GLP Published	N	Public	Y; not assessed submitted too late in the process	N
	Weekers PH, Bodelier PL, Wijen JP and Vogels GD	1993	Effects of Grazing by the Free-Living Soil Amoebae Acanthamoeba castellanii, Acanthamoeba polyphaga, and Hartmannella vermiformis on Various Bacteria. *Applied and environmental microbiology* 59: 2317–2319 Not GLP Published	N	Public	Y	Y
	Zeph	1986	Reference unknown. Cited in Casida (1988)	N	Public	Y; not assessed submitted too late in the process	N
	Zimmer J, Issoufou I, Schmieddek necht G and Bocow H	1998	Poupulations dynamik, phytoeffektivität und antagonistische Wirksamkeit von *Bacillus subtilis* als nutzbakterium, Mitt a.d. Biol. Bundesanst. H. 357	N	Public	Y	Y
Annex III Data and Information							
-	-	-	-	-	-	-	-

Draft Assessment Report

Bacillus amyloliquefaciens strain MBI600

Volume 3

Annex B.9

Effects on non-target organisms

Rapporteur Member State: France

Version History of Volume 3 B9

Date	**Reason for revision**
December 2014	Initial DAR

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B.9. Effects on non-target organisms

Subtilex® is a WP formulation containing at least 5.5×10^{10} cfu/g[1] , equivalent to 110 g *Bacillus amyloliquefaciens* MBI600/kg[2]. A single application of 0.5 kg product/ha is equivalent to 55 g *Bacillus amyloliquefaciens* MBI600/ha, which in turn is equivalent to 2.75×10^{13} cfu/ha.

Ecotoxicological studies on the microbial pesticide control agent, *Bacillus amyloliquefaciens* MBI600 are reported in the Annex II dossier and are used in the risk assessment for Subtilex®. Due to the inert nature of the main co-formulant used in the manufacturing process, additional studies with the formulation were not performed since it is possible to extrapolate from data obtained with the formulation.

The Subtilex® formulation is intended for field application to grape vines (all growth stages) to control *Botrytis*. The worst-case GAP is a maximum of 10 applications of 0.5 kg product/ha, with a minimum application interval of 7 days, at all growth stages.

This document reviews the potential risk to a range of non-target terrestrial and aquatic organisms from the proposed use pattern of Subtilex® as shown in table 9-1. This risk assessment is conducted in accordance with the appropriate EU guidance.

Table 9-1: Intended Proposed use patterns for Subtilex® in Southern Europe

Crop	Scenario	Number of applications	Minimum application interval (days)	Application rate (kg MPCA/ha)	Application timing
Vines	Field	1-10	7	0.055	All season

Strain MBI600 was previously identified as *Bacillus subtilis*[3]; however recent studies with more modern techniques have identified the organism as *Bacillus amyloliquefaciens*[4].

Previous genetic identification work on the amplification of 16S rRNA using fD1 and rD1 primers[5] and Ba1F and Ba2R primers[6] identified the organism as *Bacillus subtilis*. Recent taxonomic work with *Bacillus subtilis* organisms has resulted in their re-classification and division into smaller groups. As a result, some species that had previously been identified as *Bacillus subtilis* are now classified as *Bacillus amyloliquefaciens*. Work carried out at Auburn University, using more accurate methods, has resulted in the re-classification of *Bacillus subtilis* strain MBI600 as *Bacillus amyloliquefaciens* strain MBI600.

This is simply a re-classification; the organism has not changed and therefore all proprietary studies performed and reported with the strain previously identified as *Bacillus subtilis* strain MBI600 have

[1] based on the minimum specification of the active substance and nominal concentration of active substance in the product
[2] nominal concentration
[3] *Bruch, M.K. MicrobbioTest Inc. Identification of microorganisms. Report No. 186-102. 18 December, 1990.*
[4] *Kloepper, J.W. 2012. Identification of MBI600 by 16S rRNA gene sequencing using 8F and 1492R primers.*
[5] *Yang, G.P. 2005. Identification of MBI600 by 16S rRNA gene sequencing.*
[6] *Joshi, R.; McSpadden Gardener, B.B. 2006. Identification and characterisation of novel genetic markers associated with biological control activities in Bacillus subtilis. Phytopathology 96 (2): 145-154.*

actually been carried out with the organism that is now identified as *Bacillus amyloliquefaciens* strain MBI600.

Historically, *Bacillus subtilis* was a term given to all aerobic endospore-forming bacilli (Logan, 1988[7]). Numerous species that appeared in the early literature are no longer recognized as official species. Although in the past it has been designated as a separate species, the latest edition of Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986[8]) listed *Bacillus amyloliquefaciens* as a member of the species *Bacillus subtilis*. However, it has again achieved the status of a separate species (Priest *et al*., 1987[9]); differences are noted between the two species (Welker and Campbell, 1967[10]), notably increased production of α-amylase by *Bacillus amyloliquefaciens*, amongst other differences.

In some cases *Bacillus subtilis* literature data are referred to in this dossier. For the reasons stated above, it is considered that these data are equally applicable to *Bacillus amyloliquefaciens*, which is part of the *Bacillus subtilis* group (EFSA, 2011[11]).

B.9.1. Effects on birds (Annex IIM 8.1; Annex IIIB 10.1)

B.9.1.1. Toxicity

ACTIVE INGREDIENT

To address the effects of *Bacillus amyloliquefaciens* strain MBI 600 on birds, a toxicity, infectiveness and pathogenicity study was provided.

Report	**IIM 8.1/01** Campbell, S.M., Grimes, J. and Jaber M.J. (1993); *Bacillus subtilis* Strain MBI600: An avian oral pathogenicity and toxicity study in the bobwhite. Project Number: 301-102.
Guidelines	FIFRA Guideline 154A-16
GLP	Yes

Materials and Methods
The study was conducted during the period 18 May 1992 to 7 January 1993 by Wildlife International Ltd, Easton, Maryland, USA. The test items were *Bacillus amyloliquefaciens* MBI600 technical concentrate (batch #0347-51), washed spores of MBI600 (batch #CLW6LP001 pg.69 Tmt3), and water-soluble metabolites (batch # CLW6LP001 pg.69 Tmt2). The technical material was reported to contain 1.5 x 10^{11} spores/g and the washed spores to contain 1.63 x 10^{11} spores per gram.

The test species was 21 day-old northern bobwhite quail (*Colinus virginianus*).
The birds were immature and could not be differentiated by sex. Each bird was identified with a wing band. Seventy-two birds were distributed into twelve pens, with six birds in each pen. Pens were galvanised sheet and wire mesh cages approximately 72 x 90 x 23 cm and test conditions were as follows: temperature 23.2 $\pm$ 1.6˚C, humidity 58 $\pm$ 14% and a 16:8 photoperiod with light levels of approximately 594 lux.

[7] *Logan, N.A. 1988. Bacillus species of medical and veterinary importance. J. Med. Microbiol. 25:157-165.*
[8] *Claus, D. and Berkeley, R.C.W. 1986. Genus Bacillus Cohn 1872, pp. 1105-1139. In: P.H.A. Sneath, et al. (eds.), Bergey's Manual of Systematic Bacteriology, Vol. 2. Williams and Wilkins Co., Baltimore, MD.*
[9] *Priest, F.G. et al., (1987) Bacillus amyloliquefaciens sp. nov., nom. rev. Int. J. Syst. Bacteriol., 1987, 37, 69-71*
[10] *Welker, N.E.; Campbell, L.L.: Unrelatedness of Bacillus amyloliquefaciens and Bacillus subtilis. Journal of Bacteriology 1967, 94(4): 1124-1130.*
[11] *EFSA Journal 2011; 9(11):2445 Scientific Opinion Technical guidance on the assessment of the toxigenic potential of Bacillus species use in human nutrition.*

Body weights were recorded on four pre-treatment occasions, at the start of the study and after 11, 18, 25 and 30 days. Birds were fed Wildlife International Ltd. specified game bird ration and average estimated feed consumption was measured for days 0-4, 5-11, 12-18, 19-25 and 26-30 by measurement of the change of weight in feed presented to the birds over a given period. Although consumption was measured accurately the data are considered to be estimates due to unavoidable wastage by the birds.

There were three test item treatments, each administered to the birds by oral gavage for five days.

- *Bacillus amyloliquefaciens* MBI600 was administered to a total of 30 birds in six pens at a rate of approximately 4000 mg/kg body weight per day for five days (6×10^{11} spores/kg body weight per day). The resulting total dosage was approximately 3×10^{12} spores/kg body weight over the five day dosing period.
- Washed spores of *Bacillus amyloliquefaciens* MBI600 were administered to ten birds in two pens at a rate of approximately 3680 mg/kg per day for five days (6×10^{11} spores/kg bw/d). The resulting total dosage was approximately 3×10^{12} spores/kg body weight over the five day dosing period.
- Water soluble metabolites of *Bacillus amyloliquefaciens* MBI600 were administered to ten birds in two pens at a rate of approximately 240 mg/kg body weight per day for five days. The resulting total dosage was 1,200 mg/kg body weight over the five day dosing period.

There was a negative control of deionised water (administered with deionised water at a rate of 1% (v/w) of body weight daily for five days) comprising five birds in two replicates and an untreated infectivity control bird was included in each pen.

The infectivity control group served as sentinels to assess the potential for infectivity and were not dosed with any solutions during the test period.

Observations of mortality, signs of toxicity and abnormal behaviour were made at least twice daily until day 30 after the start of the study. Samples of the test substance stock solutions were taken on day 0 to verify the test concentrations administered to the birds. Samples were sent to the sponsor for analysis.

Findings

There were no mortalities in the negative control or infectivity control groups and all birds were normal in appearance and behaviour throughout the study period.

There were no mortalities or overt signs of toxicity among birds administered *Bacillus amyloliquefaciens* MBI600 (GUS 378 Concentrate), washed spores of *Bacillus amyloliquefaciens* MBI600 or water soluble metabolites of *Bacillus amyloliquefaciens* MBI600.

There were no signs of pathogenicity in the group treated with *Bacillus amyloliquefaciens* MBI600 (GUS 378 Concentrate). One bird in this group was noted with a head lesion on Days 25-28 which was attributed to pen wear. All other birds in the treatment groups were normal in appearance and behaviour throughout the study period.

When compared to the negativity and infectivity controls, there were no apparent treatment related effects upon body weight or feed consumption in any of the test item treated groups.

None of the findings in the treatment or infectivity control groups indicated that there was any evidence of pathogenicity or other treatment related effects. One female administered the water soluble metabolites of *Bacillus amyloliquefaciens* MBI600 was noted with a pale spleen and one male administered with *Bacillus amyloliquefaciens* MBI600 was noted with a slightly enlarged and pale spleen. Necropsy of all other birds was unremarkable.

Mean measured values of the dosing stocks for *Bacillus amyloliquefaciens* MBI600 and washed spores were 103 and 100% of nominal respectively.

Conclusion:
Bacillus amyloliquefaciens strain MBI600 showed no apparent pathogenicity, toxicity or effect upon the survival of young bobwhite when administered by oral gavage over a five day period at approximately 4000 mg/kg of body weight per day for five days (6×10^{11} spores/kg of body weight per day for five days equivalent to a total dosage of 3×10^{12} spores/kg body weight over the five day dosing period).

RMS comments on study 8.1/01:
The study is considered for information because it provides results to address pathogenicity and infectivity. The certificate of analysis is not included but the spore concentrations are reported in the study report (footnotes 2 and 3 in page 14 of the study report). Furthermore, an analytical check of the dosing solutions was conducted (results page 21). It is not clear whether the spores are viable spores or not, however since the method refers to "potency" (page 21), it is assumed that the counts are based on viable spores.

TOXIN/METABOLITE FROM ACTIVE INGREDIENT

The administration by oral gavage for 5 days of water-soluble metabolites at 240 mg/kg bw/d (total rate of 1200 mg/kg bw) did not result in signs of toxicity but a pale spleen in one female (study IIM 8.1/01).

The notifier declares that no relevant toxin/metabolite was produced by *Bacillus amyloliquefaciens* MBI600 (see volume 1).

PLANT PROTECTION PRODUCT
No specific study on birds, which is carried out using the plant protection product, is available. Due to the inert nature of the main co-formulant used in the manufacturing process, additional studies with the formulation were not performed since it is possible to extrapolate from data obtained with the formulation.

B.9.1.2. Infectiveness

No sign of infectiveness was observed in study IIM 8.1/01.

B.9.1.3. Pathogenicity

No sign of pathogenicity was observed in study IIM 8.1/01.

B.9.1.4. Risk assessment for terrestrial vertebrates

Effects on birds

Table 9.1.4-1: Effects on bird

Species	Test duration	Dose range	Results/Endpoint	Observations	Reference
TOXICITY, INFECTIVENESS, PATHOGENICITY					
Colinus virginianus	5 dosing days by gavage, 30 observation days , GLP	4000 mg technical concentrate/kg bw/dor 3680 mg washed spores/kg bw/d (6.0×10^{11} spores/kg bw/d)	- No mortality, no sign of toxicity except on male with a pale spleen	survival, signs of toxicity and abnormal behaviour, gross necropsy	IIM 8.1/01
		240 mg water soluble metabolites/kg bw/d	No mortality, one female with a pale spleen		
INFECTIVENESS					
Colinus virginianus	30 observation days, GLP	-	No evidence of infectiveness	-	IIM 8.1/01
PATHOGENICITY					
Colinus virginianus	5 dosing days by gavage, 30 observation days, GLP	-	No evidence of pathogenicity	-	IIM 8.1/01

Effects on mammals

The assessment of the following studies is available in the section B.5.

IIM 5.3.2/01

Report: [redacted] (1989a) Acute oral toxicity and infectivity/pathogenicity to rats of MBI600. [redacted] Unpublished report No. 89396D/AGC 1/0/AC

Large numbers of spores survived passage through the intestinal tract, but were rapidly eliminated within 7 days. *Bacillus amyloliquefaciens* strain MBI600 showed no evidence of pathogenicity or infectivity when administered via the oral route. There was no mortality or evidence of toxicity following oral administration of *Bacillus amyloliquefaciens* strain MBI600 and therefore the acute oral LD_{50} is $>10^9$ viable spores/animal.

IIM 5.3.2/02

Report: [redacted] Acute oral toxicity study (UDP) in rats. [redacted] Unpublished report No. 15626-11

Based on the above results, the median lethal dose (LD_{50}) to female rats of *Bacillus amyloliquefaciens* strain MBI600 after a single oral dose is >5000 mg/kg bw.

IIIM1 7.1.1/01

Report: [redacted] (2011a) Acute oral toxicity (UDP) in rats. [redacted] Unpublished report No. 15387-11

Based on the above results, the median lethal dose (LD_{50}) to female rats of Subtilex® (*Bacillus amyloliquefaciens* strain MBI600 11% w/w) after a single oral dose is >5000 mg/kg bw.

Exposure of birds and mammals

Bacillus amyloliquefaciens MBI600 is a ubiquitous micro-organism in the environment, found in soil, plants and animal foodstuffs, with which birds and mammals will naturally be in contact.

Any potential risk to birds and mammals from Subtilex® will occur due to ingestion of food (plants and insects) containing residues of the microbial pest control agent (MPCA) *Bacillus amyloliquefaciens* MBI600.

A standard risk assessment has been conducted to determine a worst-case dietary exposure level in accordance with the EFSA Guidance for the Risk Assessment for Birds and Mammals (2009)[12].

The screening assessment according to the current EFSA Guidance Document on birds and mammals has been developed for sprayed organic chemicals and is thus not directly applicable to a microbial pesticide. However, the risk has been assessed based on the test results of acute studies and the assessment scheme applicable to standard pesticides, to represent a worst case situation that makes no allowance for rapid equilibration of the MPCA in the environment. An initial screening step to assess the risk to an 'indicator species' was undertaken according to Module 1: Acute dietary risk assessment for birds and mammals (Sections 4.1 and 4.2 of the EFSA Guidance Document (2009)). In this assessment the daily dietary dose (DDD) is defined by the food intake of the species of concern, the body weight of the species of concern, the concentration of a substance in/on fresh diet and the fraction of the diet obtained in the treated area. The estimated food intake rates are based on the daily expenditure of the species of concern, the energy in the food, the 'energy' assimilation efficiency of the species of concern, and the moisture content of the food. The above information is combined into a single value for a specific species-crop-combination and termed a short-cut value (SV).

Table 9.1.4-2: Acute DDD for birds and mammals – screening step

Crop	Indicator species	Rate (kg as/ha)	Max. No. Applns.	MAF_{90} 2)	Shortcut value (SV) for acute assessment 1)	DDD (mg/kg/d)
Vines	Small omnivorous bird	0.055	1	not relevant	95.3	5.24
Vines	Small omnivorous bird	0.055	10	not relevant	95.3	52.4
Vines	Small herbivorous mammal	0.055	1	not relevant	136.4	7.5
Vines	Small herbivorous mammal	0.055	10	not relevant	136.4	75

1) Short cut value based on the 90th percentile of residues provided in EFSA Guidance document 2009.
2) Multiple application factor used for chemical substance is not relevant for microorganisms.

[12] *Guidance of EFSA Risk assessment for Birds and Mammals, EFSA Journal 2009; 7(12):1438*

Table 9.1.4-3: Acute Toxicity/exposure ratios for birds and mammals– screening step

Test substance	Crop, use pattern	Crop scenario, indicator species	Toxicity endpoint (mg a.s./kg bw)	DDD (mg a.s./kg bw)	TER	TER risk assessment trigger
Bacillus amyloliquefaciens MBI600	Vines	Small omnivorous bird	>4000	52.4	>76	10
Bacillus amyloliquefaciens MBI600	Vines	Small herbivorous mammal	>5000	75	>67	10

The screening step gives TER values above the trigger of 10 for acute risk for 10 cumulative applications and the active substance was demonstrated in two studies with the bobwhite quail, and a rat study to have no pathogenicity to the test species. Thus the risk to birds and mammals is considered as acceptable from the proposed use of Subtilex®.

B.9.2. Effects on aquatic organisms (Annex IIB 8.2; Annex IIIB 10.2)

The primary habitat of *Bacillus amyloliquefaciens* MBI600 is soil (Sneath, 1986)[13]. From soil, aerobic spore formers of the *Bacillus* genus can contaminate everything by dust or other means. Habitats such as fresh water may acquire *Bacillus amyloliquefaciens* naturally from soil by run-off, dust and infected plant materials, etc. In the *Bacillus subtilis* strain QST 713 Monograph (2001)[14], the RMS commented that: '*B. subtilis* is not regarded as an autochthonous inhabitant of aquatic environments and does not find optimal conditions for growth, e.g. waters are poor in organic carbon. Therefore proliferation is not likely to occur. Bacterial cells and especially endospores may survive, but will be subject to natural competition in the diverse micro-flora of natural waters' and concludes 'Survival of introduced QST 713 strain of *B. subtilis* will not cause any environmental or health impact.'

B.9.2.1. Effects on fish (Annex IIM 8.2; Annex IIIB 10.2)

B.9.2.1.1. **Toxicity**

ACTIVE INGREDIENT

An acute toxicity study with rainbow trout, *Oncorhynchus mykiss* has been conducted.

Report	**IIM 8.2/01**, [redacted] (2013a); *Bacillus amyloliquefaciens* strain MBI600 Rainbow Trout (*Oncorhynchus mykiss*) 96-Hour Acute Toxicity Test. Laboratory study No. 17142-13.
Guidelines	OECD Guideline for Testing of Chemicals: Fish, Acute Toxicity Test No. 203, 17 July 1992
GLP	Yes

[13] *Sneath, P.H.A. (1986) Endospore-forming gram-positive rods and cocci, Genus Bacillus. In Sneath et al (eds) Bergerys Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore, Vol2, Section 12 pp 1104-1139.*
[14] *Monograph, Bacillus subtilis strain QST 713, Volume 1 Report and Proposed Decision. 15 May 2001. Rapporteur Member State: Germany.*

Materials and methods

The study was conducted during the period 7-12 February 2013 by [redacted] The test item was *Bacillus amyloliquefaciens* MBI600, Batch/Lot Number BS11001 (seed stock 1.06 x 10^{12} cfu/g according to the certificate of analysis).

A limit test was conducted in a 96-hour static, non-renewal test, using the test substance at a nominal concentration of 100 mg/L (equivalent to an enumerated concentration of 9.97 x 10^{10} cfu/L) administered to the test system, *Oncorhynchus mykiss*, in moderately hard laboratory freshwater. Three replicates of ten fish were treated at this concentration. A control group containing thirty organisms was not exposed to the test substance.

Dissolved oxygen, conductivity and pH measurements were recorded at dosing and daily throughout the study period. Observations of mortality were made at 6, 24, 48, 72 and 96 hours after treatment. The test was terminated after 96 $\pm$ 1 hours of exposure. Samples of the treatment solutions were collected at 0 and 96 hours for dose cfu/mL count verification.

Findings

Temperature, pH and dissolved oxygen were recorded during the experimentations and were within acceptable ranges.
The validity criterion for control mortality was fulfilled and the study is therefore considered to be valid.

Dosing concentrations were enumerated to establish a colony forming unit (cfu) count of the test solutions. The 100 mg/L equivalent count for freshly poured solutions and 96-hour aged solutions were 9.9 x 10^7 cfu/mL and 1.7 x 10^7 cfu/mL respectively.

Enumerations of test solutions from the treated containers for the limit test are reported in the following table:

Solution	Replicate 1	Replicate 2	Replicate 3	Mean CFU/ml
0hr-100mg/L	$7.5x10^7$	$1.23x10^8$	TMTC	$9.9x10^7$
96hr-100mg/L	$1.8x10^7$	$1.59x10^7$	$1.63x10^7$	$1.67x10^7$

TMTC-Too many to count

The variation in values is likely to be due to the test item settling out or due to natural inaccuracies in enumerating high counts of *Bacillus* spp. The initial concentration is closed to the targeted nominal concentration (10^8 cfu/mL). The results of the study are presented based on nominal concentration.

There was no mortality in either the control or test item concentration (100 mg/L) and the EC_{50} is therefore considered to be > 100 mg *Bacillus amyloliquefacien*s MBI600/L, equivalent to 9.9 x 10^{10} cfu/L based on initial measured concentration. No sub-lethal effects were recorded.

Conc. (mg/L)	Rep.	Number of Surviving Organisms						
		0 Hrs	6 Hrs	24 Hrs	48 Hrs	72 Hrs	96 Hrs	Survival Rate
Control	A	10	10	10	10	10	10	100%
	B	10	10	10	10	10	10	
	C	10	10	10	10	10	10	
100	A	10^a	$10^{a, b}$	$10^{a, b}$	$10^{a, b}$	$10^{a, b}$	$10^{a, b}$	100%
	B	10^a	$10^{a, b}$	$10^{a, b}$	$10^{a, b}$	$10^{a, b}$	$10^{a, b}$	
	C	10^a	$10^{a, b}$	$10^{a, b}$	$10^{a, b}$	$10^{a, b}$	$10^{a, b}$	

Conc. - Nominal Concentration; Rep. - Replicate; Hrs - Hours; a – heavily murky cloudy water, visibility low; b – brown residue at bottom of tank.

Conclusions
In a limit test conducted at 100 mg *Bacillus amyloliquefaciens* MBI600/L, there was no mortality and the EC_{50} for *O. mykiss* is therefore determined to be > 100 mg/L, equivalent to 9.9×10^{10} cfu/L based on initial measured concentration. The NOEC (no-observed effect concentration) is determined to be 100 mg/L nominal concentration, equivalent to 9.9×10^{10} cfu/L based on initial measured concentration. Results presented are based on nominal concentrations.

RMS comment on study 8.2/01:
The study is valid. In a 96-h static test conducted with *Bacillus amyloliquefaciens* MBI600, Batch/Lot Number BS11001 (seed stock 1.06×10^{12} cfu/g), no toxicity was observed on *Oncorhynchus mykiss* at 100 mg/L equivalent to 9.9×10^{7} cfu/mL (measured initial concentration).

Any increase in population numbers of *Bacillus amyloliquefaciens* in surface waters that result from application of the MPCA is likely to be transient and therefore long-term exposure is not considered appropriate. However, the following non-GLP study was conducted to determine the long term effects on *Cyprinus carpio*.

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B.9.2.2. Effects on freshwater invertebrates (Annex IIM 8.3; Annex IIIB 10.2)

B.9.2.2.1. **Toxicity**

An acute toxicity study has been conducted with *Daphnia magna.*

Report	**IIM 8.3/01**, Mikulas, J. (2013b); *Bacillus amyloliquefaciens* strain MBI600 *Daphnia magna* 48-Hour Acute Toxicity Test. Laboratory study No. 16672-12.

Guidelines	OECD Guideline for Testing of Chemicals: Daphnia sp., Acute Immobilisation Test No. 202, 13 April 2004
GLP	Yes

Materials and methods

The study was conducted during the period 19-22 September 2012 by STILLMEADOW Inc. Sugar Land, Texas, USA. The test item was *Bacillus amyloliquefaciens* MBI600, Batch/Lot Number: BS11001 (seed stock 1.06 x 10^{12} cfu/g according to the certificate of analysis).

A limit test was conducted in a 48-hour static, non-renewal test, using the test substance at a concentration of 100 mg/L (equivalent to an enumerated concentration of 1.9 x 10^{10} cfu/L) administered to the test system, *Daphnia magna* in moderately hard synthetic freshwater (as the OECD guideline). Four replicates of five daphnids were treated at this concentration. A control group containing four replicates, each of five daphnids was not exposed to the test substance. Daphnids were not fed during the test.

Dissolved oxygen, conductivity and pH measurements were recorded at dosing and daily throughout the study period. Observations of immobility were made at daily. The test was terminated after 48 $\pm$ 1 hours of exposure. Samples of the treatment solutions were collected at 0 and 96 hours for dose cfu/mL count verification.

Temperature was maintained at 20-21°C.

Findings

Temperature, pH and dissolved oxygen were recorded during the experimentations and were within acceptable ranges.
The validity criterion for control immobility was fulfilled (< 10%) and the study is therefore considered to be valid.

Dosing concentrations were enumerated to establish a colony forming unit (cfu) count of the test solutions. Enumerations of test solutions from the treated containers as well as the seed stock are reported in the following table:

Solution	Replicate 1	Replicate 2	Replicate 3	Mean CFU/ml
Stock	$2.95x10^{11}$	$2.98x10^{11}$	$2.87x10^{11}$	$2.9x10^{11}$
0hr-100mg/L	$1.35x10^{7}$	Too Many Too Count	$2.5x10^{7}$	$1.9x10^{7}$
48hr-100mg/L	$1.38x10^{7}$	$1.40x10^{7}$	$1.64x10^{7}$	$1.5x10^{7}$

The counts for the seed stock were 2.9 x 10^{11} cfu/mL. Based on nominal values, a concentration of 100 mg/L should yield a count of 2.9 x 10^{7} cfu/mL. The counts for freshly poured solutions and 48 hour old solutions were 1.9 x 10^{7} cfu/mL and 1.5 x 10^{7} cfu/mL, respectively. The dose verification results suggest that the spores neither replicated nor dissipated from the system since the cfu count remained stable during the study period and it was concluded that the cfu count remained stable over the study period. The results of the study are therefore presented based on nominal concentration.

The biological results are reported in the Table 9.2.1-01 and Table 9.2.1-02.

Table 9.2.1-01: Results of immobility observations

Test item concentration (mg/L)	Replicate	Number of mobile daphnids			Mobility %
		0 hours	24 hours	48 hours	
0 (control)	A	5	5	5	100
	B	5	5	5	
	C	5	5	5	
	D	5	5	5	
100	A	5	5	5	90
	B	5	4	4	
	C	5	4	4	
	D	5	5	5	

Table 9.2.1-02: Results of immobility observations

Test item concentration (cfu/L)*	Replicate	Number of mobile daphnids			Mobility %
		0 hours	24 hours	48 hours	
0 (control)	A	5	5	5	100
	B	5	5	5	
	C	5	5	5	
	D	5	5	5	
1.9×10^{10} cfu/L	A	5	5	5	90
	B	5	4	4	
	C	5	4	4	
	D	5	5	5	

*Based on initial measured concentration

There was no immobilisation in the control and in the test item concentration (100 mg/L) there was 10% immobilisation. The EC_{50} is therefore considered to be >100 mg *Bacillus amyloliquefaciens* MBI600/L based on nominal concentration, equivalent to 1.9×10^{10} cfu/L based on initial measured concentration. No sub-lethal effects were recorded.

Conclusions:
In a limit test conducted at 100 mg *Bacillus amyloliquefaciens* MBI600/L, there was 10% immobilisation. The EC_{50} for *Daphnia magna* was determined to be >100 mg/L (equivalent to 1.9×10^{10} cfu/L initial measured concentration).

RMS comment on study IIM 8.3/01:
The study is valid. In a 48-h static test conducted with *Bacillus amyloliquefaciens* MBI600, Batch/Lot Number BS11001 (seed stock 1.06×10^{12} cfu/g), 10% immobilisation of *Daphnia magna* was observed at 100 mg/L equivalent to 1.9×10^{10} cfu/L (measured initial concentration).

Any increase in population numbers of *Bacillus amyloliquefaciens* in surface waters that result from application of the MPCA, is likely to be transient and therefore long term risk assessment is not considered appropriate for the MPCA. However, the following, non-GLP study was conducted to determine the long term effects on *Daphnia magna*.

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B.9.2.3. Effects on algae growth (Annex IIM 8.4; Annex IIIB 10.2)

ACTIVE INGREDIENT

A 72-hour toxicity study has been conducted with the green alga, *Pseudokirchneriella subcapitata*.

Report	**IIM 8.4/01**, Mikulas, J. (2013b); *Bacillus amyloliquefaciens* strain MBI600 *Pseudokirchneriella subcapitata* 72-hour Algal Inhibition Test. Laboratory study No. 17141-13.
Guidelines	OECD Guideline for Testing of Chemicals: Freshwater Alga and Cyanobacteria, Growth Inhibition Test No. 201, 23 March 2006
GLP	Yes

Materials and methods
The study was conducted during the period 28 January to 01 February 2013 by STILLMEADOW Inc. Sugar Land, Texas, USA. The test item was *Bacillus amyloliquefaciens* MBI600, Batch/Lot Number: BS11001; seed stock 1.06 x 10^{12} cfu/g according to the certificate of analysis).

A limit test was conducted in a 72-hour test, using the test substance at a concentration of 100 mg/L, equivalent to an enumerated concentration of 5.8 x 10^{11} cfu/L, administered to the test system, *Pseudokirchneriella subcapitata* in sterile algal medium. For the test concentration, six flasks containing the freshwater algae were treated with the appropriate concentration of the test substance. A control group comprised six test flasks contain sterile medium and the algal test culture only. A reference item, zinc chloride was tested in parallel with three replicates. The cell density in each test, reference and control container was measured daily using a haemocytometer.
Temperature was maintained at 24 ± 2°C. The pH of each test and control container was determined at test termination. The test temperature and daily maximum and minimum temperatures were recorded daily. Light intensity was measured across five locations of the testing area daily. The test was

terminated after 72 ± 2 hours of exposure. Samples of the treatment solutions were collected at 0 and 72 hours for dose cfu/mL count verification.

Findings
The validity criteria for control growth were fulfilled and the study is therefore considered to be valid.

Enumeration of test solutions from the treated containers as well as the seed stock are presented in the following table:

Table 3: Enumeration Values

Solution	Replicate 1	Replicate 2	Replicate 3	Mean CFU/ml
0hr	$6.3x10^8$	$7.2x10^8$	$3.9x10^8$	$5.8x10^8$
72hr	$3.7 x10^7$	$6.2 x10^7$	$7.5 x10^7$	$5.8 x10^7$

The dosing concentrations were enumerated to establish a colony forming unit (cfu) count of the test solutions. The counts for the seed stock were 2.9 x 10^{11} cfu/mL. Based on nominal values, a concentration of 100 mg/L should yield a count of 2.9 x 10^{10} cfu/L. The counts for freshly poured solutions were 5.8 x 10^{11} cfu/L. The 72 hour concentration solution was 5.8 x 10^{10} cfu/L. The dose verification results suggest that the spores did not replicate within the system, but there appears to have been some loss in spores over the 72 hour period, evident from the cfu count dropping between a full log over the length of this study. However, due to the algae growth inhibition test being a more dynamic test system, it is possible that the test substance was absorbed by the increasing algal biomass, making the exposure concentration difficult to define, therefore, it cannot be concluded that the test substance concentration was held constant during the length of this study.

Initial cell density was 50000 cells/mL. Daily cell counts are presented in Table 9.2.3-01 (biomass) and growth rate and growth curve area are shown in Table 9.2.3-02. The reference item, zinc chloride grew significantly less than the negative control and thus demonstrated that the test population responded to a toxic response. There was no significant difference in growth rates or growth curve area in the test item treatment compared to the control.

Table 9.2.3-01: Biomass determination

Test item concentration (mg/L)	Replicate	Cell density x 10^4 cells/mL		
		24 hours	48 hours	72 hours
0 (control)	A	22	30	109
	B	18	37	128
	C	17	40	138
	D	18	28	123
	E	15	39	139
	F	23	45	146
100 (equivalent to 5.8 x 10^{11} cfu/L measured concentration)	A	11	35	118
	B	14	48	143
	C	8	34	120
	D	10	43	139
	E	12	49	145
	F	10	45	137
Reference item (Zinc chloride)	A	6	7	1
	B	7	8	1
	C	6	6	2

Table IIM 8.4/01-02: Growth Rate/Curve Area

Test item concentration (mg/L)	Replicate	Growth Rate			Average Growth Rate	Growth Curve Area
		24 hours	48 hours	72 hours		
0 (control)	A	0.062	0.013	0.054	0.043	2256
	B	0.053	0.030	0.052	0.045	2556
	C	0.051	0.036	0.052	0.046	2724
	D	0.053	0.018	0.062	0.044	2280
	E	0.046	0.040	0.053	0.046	2664
	Mean	0.055	0.027	0.053	0.045	2594.000
	S.D.	0.007	0.010	0.004	0.001	308.605
	% CV	12.248	37.124	8.100	3.245	11.897
100 (equivalent to 5.8 x 10^{11} cfu/L measured concentration)	A	0.033	0.048	0.051	0.044	2220
	B	0.043	0.051	0.045	0.047	2904
	C	0.020	0.060	0.053	0.044	2148
	D	0.029	0.061	0.049	0.046	2640
	E	0.036	0.059	0.045	0.047	2904
	F	0.029	0.063	0.046	0.046	2664
	Mean	0.032	0.057	0.048	0.046	2580.000
	S.D.	0.008	0.006	0.003	0.001	327.668
	% CV	25.011	10.197	6.221	2.739	12.7000

The EC_{50} is therefore considered to be > 100 mg *Bacillus amyloliquefaciens* MBI600/L, equivalent to 5.8 x 10^{11} cfu/L based on initial measured concentration.

Conclusions:
In a limit test conducted at 100 mg *Bacillus amyloliquefaciens* MBI600/L EC_{50} for *Pseudokirchneriella subcapitata* was therefore determined to be > 100 mg/L based on initial concentration. The NOEC (no-observed effect concentration) was determined to be 100 mg/L (nominal) equivalent to 5.8 x 10^{11} cfu/L based on initial measured concentration.

RMS comments on study 8.4/01:
The study is valid.
In a 72-h static test conducted with *Bacillus amyloliquefaciens* MBI600, Batch/Lot Number BS11001 (seed stock 1.06 x 10^{12} cfu/g), no toxicity on *Pseudokirchneriella subcapitata* was observed at 100 mg/L equivalent to 5.8 x 10^{11} cfu/L (measured initial concentration).

TOXIN/METABOLITE FROM ACTIVE INGREDIENT
The notifier declares that no relevant toxin/metabolite was produced by *Bacillus amyloliquefaciens* MBI600 (see volume 1).

PLANT PROTECTION PRODUCT
Due to the inert nature of the main co-formulant used in the manufacturing process, additional studies with the formulation were not performed since it is possible to extrapolate from data obtained with the formulation.

B.9.2.3.1. **Infectiveness**

No sign of infectiveness was observed in study IIM 8.4/01.

B.9.2.3.2. **Pathogenicity**

No sign of pathognencity was observed in study IIM 8.4/01.

B.9.2.4. Effects on plants other than algae (Annex IIM 8.5; Annex IIIB 10.2)

Bacillus amyloliquefaciens strain MBI600 is not known to be a plant pathogen or phytotoxic agent and therefore no study is required.

ACTIVE INGREDIENT
No specific study on plants, which is carried out using the active substance, is available.

TOXIN/METABOLITE FROM ACTIVE INGREDIENT
The notifier declares that no relevant toxin/metabolite was produced by *Bacillus amyloliquefaciens* MBI600 (see volume 1).

PLANT PROTECTION PRODUCT
No specific study on plants, which is carried out using the plant protection product, is available.

B.9.2.4.1. **Infectiveness**

Bacillus amyloliquefaciens strain MBI600 is not known to be a plant pathogen.

B.9.2.4.2. **Pathogenicity**

Bacillus amyloliquefaciens strain MBI600 is not known to be a plant pathogen.

B.9.2.5. Summary of the studies on aquatic organisms toxicity, infectiveness and pathogenicity

Table 9.2.5-1: Summary of the effects on aquatic organisms treated with the active substance.

Species	Test duration	Dose range	Results/Endpoint	Observations	Reference
TOXICITY					
Oncorhynchus mykiss	96 h static, GLP	100 mg/L (seed stock)	No effect at 9.9 x 10^7 cfu/mL (maximum mean measured initial concentration)	Survival, signs of toxicity	IIM 8.2/01
Cyprinus caprio	30 d semi-static, not GLP	2.5 to 250 mg/L (technical concentrate)	No effect up to 1.3 to 1.6 x 10^8 cfu/mL (maximum measured concentrations)	Survival, signs of toxicity, growth, gross pathology	IIM 8.2/02
Daphnia magna	48 h static, GLP	100 mg/L (seed stock)	No effect at 1.9 x 10^7 cfu/mL (maximum mean measured initial concentration)	Mobility, signs of toxicity,	IIM 8.3/01
Daphnia magna	21 d semi-static, not GLP	2 x 10^5 cfu/mL to 2 x 10^8 cfu/mL (technical)	No effect at 2.7 x 10^4 cfu/mL (measured initial concentration at the lowest test concentration)	Mobility, signs of toxicity, reproduction parameters	IIM 8.3/02
Pseudokirchneriella subcapitata	72 h static, GLP	100 mg/L (seed stock)	No effect at 5.8 x 10^8 cfu/mL (maximum mean measured initial concentration)	Biomass, growth rate	IIM 8.4/01
INFECTIVENESS					
Cyprinus carpio	30 days semi-static, not GLP	-	No evidence of infectiveness	-	IIM 8.2/02
Daphnia magna	21 d semi-static, not GLP	-	No evidence of infectiveness	-	IIM 8.3/02
PATHOGENICITY					
Cyprinus carpio	30 days semi-static, not GLP	-	No evidence of pathogenicity	-	IIM 8.2/02
Daphnia magna	21 d semi-static, not GLP	-	No evidence of pathogenicity	-	IIM 8.3/02

B.9.2.6. Risk assessment for aquatic organisms

The information provided from studies conducted on the microbial pest control agent *Bacillus amyloliquefaciens* strain MBI600 and summarised above is directly applicable to the microbial pest control product. Formulation components are considered to be inert and no further tests for toxicity of Subtilex® to aquatic organisms have been conducted.

The following worst-case endpoints (Table B.9.2.6-01) determined from recent, GLP-compliant studies conducted in accordance with the current OECD Guidelines are used in the risk assessment:

Table B.9.2.6-01: Aquatic organisms - ecotoxicological endpoints

Risk type	Species	Endpoint	Value	Study reference
Acute	*Oncorhyncus mykiss*	96-hour LC_{50}	>100 mg MPCA/L > 9.9 x 10^{10} cfu/L	Mikulas, J. (2013a) Report No:17142-13 IIM 8.2/01
Acute	*Daphnia magna*	48-hour EC_{50}	>100 mg MPCA/L > 1.9 x 10^{10} cfu/L	Mikulas, J. (2013b) Report No:16672-12 IIM 8.3/01
Long-term	*Pseudokirchneriella subcapitata*	72-hour EC_{50}	>100 mg MPCA/L > 5.8 x 10^{11} cfu/L	Mikulas, J. (2013c) Report No:17141-13 IIM 8.4/01

Additional long-term aquatic studies on fish and an aquatic invertebrate were not conducted in accordance with current OECD guidelines and are not GLP compliant. However, they are included here to provide supporting information.

Table B.9.2.6-02: Additional aquatic ecotoxicological studies for supporting information

Risk type	Species	Endpoint	Value	Study reference
Long-term	*Cyprinus carpio*	30-day NOEC (Semi-static)	250 mg MPCA/L 1.3 to 1.6 x 10^{11} cfu/L No pathological effects observed	Hagino. S. (1997) Report No. STS(2)-97004 IIM 8.2/02
Long-term (reproduction)	*Daphnia magna*	21-day NOEC (Semi-static)	2.7 x 10^{7} cfu/L	Fujii, Y.(1997) Report No:597040201-001 IIM 8.3/02

Aquatic exposure levels are taken from the PEC_{SW} values reported in fate and behaviour in environment. The maximum peak PEC_{SW} value of 14.7 µg MPCA/L (equivalent to 7.35 x 10^{6} cfu/L was estimated following 10 applications to vines at 0.055 kg MPCA/ha and a minimum buffer distance of 3 m. As a worst-case estimate, this value was used for TER calculations.

TER_A for fish:

The acute TER for fish is shown below in table B.9.2.6-03.

Table B.9.2.6-03: TER_A for fish

Species	Endpoint	Toxicity (mg as/L)	PEC_{SW}	TER	TER Trigger value
Oncorhyncus mykiss	96-hour LC_{50}	>100 mg MPCA/L > 9.9 x 10^{10} cfu/L	14.7 µg MPCA/L 7.35 x 10^{6} cfu/L (10 applications)	> 6803 > 13469	100

The TER exceeds the trigger value of 100 specified in COMMISSION REGULATION (EU) No 546/2011. It is concluded that Subtilex® does not present a significant acute risk to fish when used according to the proposed GAP.

An assessment of potential pathogenicity was carried out with *C. carpio* (IIM 8.2/01 Hagino, S. (1997); Toxicity study of MBI-600 on carp (*Cyprinus carpio*) Project Number: STS (2)-97004). Fish were exposed for 30 days under semi-static condition. The highest measured concentrations were within 1.3 to 1.6 x 10^{11} cfu/L.

There were no toxicity and no gross pathological findings at the end of the study period and therefore none would be anticipated following acute exposure to *B. amyloliquefaciens* strain MBI600 following application of Subtilex® according to the proposed GAP.

It can therefore be concluded that *Bacillus amyloliquefaciens* MBI600 is not pathogenic to fish and is unlikely to cause adverse long-term effects.

TER_A for *Daphnia magna:*

The acute TER for *Daphnia* is shown below in table B.9.2.6-04.

Table B.9.2.6-04: TER_A for *Daphnia*

Species	Endpoint	Toxicity (mg as/L)	PEC_{SW}	TER	TER Trigger value
Daphnia magna	48-hour EC_{50}	>100 mg MPCA/L > 1.9 x 10^{10} cfu/L	14.7 µg MPCA/L 7.35 x 10^6 cfu/L (10 applications)	> 6803 > 2585	100

The TER exceeds the trigger value of 100 specified in COMMISSION REGULATION (EU) No 546/2011. It is concluded that Subtilex® does not present a significant acute risk to aquatic invertebrates when used according to GAP.

A non-GLP compliant, 21-day study was conducted to determine the long term effects of *Bacillus subtilis* NCIB12376 on *Daphnia magna* (IIM 8.3/02, Fujii, Y. (1997) Reproductive Toxicity on *Daphnia*. Project Number: 597040201-001). Daphnids were exposed for 21 days under semi-static condition at four concentrations of *B. amyloliquefaciens* MBI600 (2 x 10^8 to 2 x 10^{11} cfu/L, nominal concentrations).

The results of the 21-d toxicity study are not unequivocal since there were no significant differences between the test item and the inactivated test item at equivalent concentrations. There were, however, significant differences in mortality in the parental generation and thus in reproduction, between the control and the highest test item treatment (2 x 10^{11} cfu/L, nominal concentration) in both the test item and the inactivated test item. The author concluded that the effect may have been a result of physical factors, not as a result of the biological effects of the test item. There were no effects on survival of adult daphnids at any of the lower concentrations of the test item and thus suggests that there was no pathogenic activity in *Daphnia magna*. Based on effects on reproduction observed at the two intermediate concentrations (2 x 10^9 and 2 x 10^{10} cfu/L), a no effect concentration was determined to be 2 x 10^8 cfu/L.

TER_{LT} for *Daphnia magna:*

The long-term TER for *Daphnia* is shown below in table B.9.2.6-05.

Table B.9.2.6-05: TER_{LT} for *Daphnia*

Species	Endpoint	Toxicity (mg as/L)	PEC_{SW}	TER	TER Trigger value
Daphnia magna	21-days EC_{50}	2.7 x 10^7 cfu/L	7.35 x 10^6 cfu/L (10 applications)	**3.7**	10
Daphnia magna	21-days EC_{50}	2.7 x 10^7 cfu/L	7.35 x 10^5 cfu/L (1 application)	37	10

When 10 cumulative applications are considered, the TER is 3.7 below the trigger value of 10 specified in COMMISSION REGULATION (EU) No 546/2011. However, the cumulative rate is a very unrealistic scenario giving an unrealistic PECsw which remains below the no effect concentration. In addition, for 1 application, the TER is 37, indicating that long-term risk is acceptable.

RMS notes when considering the maximum test item specification, the calculated TER for a single application could exceed the TER trigger value. However, the maximum test item specification is not available (see data gap reported in Volume 4).

The risk assessment for the sensitive aquatic crustacean *Daphnia magna* demonstrates that the use of Subtilex® in accordance with the GAP will not represent a risk to aquatic invertebrates.

TER_{LT} for algae:

The long-term TER for algae is shown below in table B.9.2.6-05.

Table B.9.2.6-05: TER_{LT} for algae

Species	Endpoint	Toxicity (mg as/L)	PEC_{SW}	TER	TER trigger value
Pseudokirchneriella subcapitata	72-hour EC_{50}	>100 mg MPCA/L (>1.1 x 10^{11} cfu/L) > 5.8 x 10^{11} cfu/L	14.7 µg MPCA/L 7.35 x 10^{6} cfu/L (10 applications)	> 6803 > 79000	10

The TER exceeds the trigger value of 10 specified in COMMISSION REGULATION (EU) No 546/2011. It is concluded that Subtilex® does not present a significant long-term risk to algae when used according to GAP.

Overall conclusion
Bacillus amyloliquefaciens MBI600 is not toxic to a fish *Oncorhynchus mykiss* at 100 mg/L for 96-h (9.9 x 10^{10} cfu/L), an invertebrate *Daphnia magna* at 100 mg/L for 48-h (1.9 x 10^{10} cfu/L) and a green algae *Pseudokirchneriella subcapitata* at 100 mg for 72 h (5.8 x 10^{11} cfu/L). Based on a 30-d study with *Cyprinus carpio*, it can be conclude that *Bacillus amyloliquefaciens* MBI600 is not pathogenic to fish and is unlikely to cause adverse long-term effects. However, *Bacillus amyloliquefaciens* MBI600 may affect reproduction of *Daphnia magna* in a 21-d study with a no effect level at 2.7 x 10^{7} cfu/L.
The TER exceeds the trigger values specified in COMMISSION REGULATION (EU) No 546/2011 for a single application and 10 cumulative applications, with the exception of the long-term TER for invertebrate calculated for 10 cumulative applications. However, the unrealistic exposure estimate for 10 cumulative applications is below the no effect level on the reproduction to *Daphnia magna*. It is therefore concluded that Subtilex® does not present an unacceptable risk to aquatic organisms when used according to the proposed GAP.

B.9.3. Effects on bees (Annex IIM 8.7; Annex IIIB 10.3)

B.9.3.1. Toxicity

ACTIVE INGREDIENT

An acute contact toxicity test was conducted with *Bacillus amyloliquefaciens* strain MBI600.

Report **IIM 8.7/02**, Younger, C. (2013); *Bacillus amyloliquefaciens* strain MBI60. Honey Bee Acute Contact Toxicity Limit Test. Laboratory Study No.

	16674-12
Guidelines	OECD Guideline for Testing of Chemicals: Honeybees, Acute Contact Toxicity Test No. 214, 21 September 1998
GLP	Yes

Materials and methods
The study was conducted during the period 5 to 7 October 2012 by STILLMEADOW Inc. Sugar Land, Texas, USA. The test item was *Bacillus amyloliquefaciens* MBI600, Batch/Lot Number: BS11001 (seed stock 1.06 x 10^{12} cfu/g).

The study was designed to assess the acute contact toxicity of the test substance, *Bacillus amyloliquefaciens* strain MBI600 when administered to the honey bee, *Apis mellifera*, at a rate of 100 µg/bee, equivalent to 4.4 x 10^{8} cfu/bee based on measured concentration in the dosing solution (2.2 x 10^{11} cfu/mL). The test substance was administered as a single topical dose to the dorsal side of the thorax to each bee following immobilisation using CO_2.

There was one rate of the test item, 100 µg/bee (in 2 µL deionized water) and a negative control dosed with deionized water, each comprising six replicates of 10 bees per replicate. A reference item, dimethoate, was tested in parallel with three application rates of 0.01, 0.1 and 1.0 µg a.s./bee. After application of treatments, bees were returned to containers and were observed after 4, 24 and 48 hours. Observations were made for mortality and clinical signs of toxicity, particularly ataxia, lethargy, hypersensitivity, etc.

Findings
The validity criteria for control mortality was fulfilled and the study is therefore considered to be valid.

Mortality in the control and test item treatments is shown in Table B.9.3.1-01 below:

Table B.9.3.1-01: Percentage mortality

Test item concentration	Percentage mortality at 4 hours	Percentage mortality at 24 hours	Percentage mortality at 48 hours
Control	0	0	0
100 µg/bee (equivalent to 4.4 x 10^{8} cfu/bee*)	0	0	3.3
Inactive test item	0	1.7	5.0

*based on enumerated concentration in the dosing solution

After 48 hours, mortality in the test item treatment at 100 µg/bee (4.4 x 10^{8} cfu/bee) was 3.3 % and was not significantly different from the water control. In the inactivated test item treatment mortality was 5.0%. The LD_{50} is therefore considered to be > 100 µg *Bacillus amyloliquefaciens* MBI600/bee equivalent to 4.4 x 10^{8} cfu/bee. The estimated LD50 for the reference item, dimethoate, was 0.32 µg a.s./bee with 95% confidence intervals of 0.22 to 0.52 µg a.s./bee, which is within the expected range of 0.1 to 0.3 µg a.s./bee.

Conclusions
At 48 hours after dosing, the mortality for bees in the active test substance group was 3.3% and 5.0% for bees in the inactive test substance group. The LD_{50} for the toxic standard was 0.32 µg a.i./bee and the LD_{50} for the test substance, *Bacillus amyloliquefaciens* strain MBI600, at 48 hours was greater than 100 µg/bee, equivalent to 4.4 x 10^{8} cfu/bee based on measured concentration.

RMS comment on study 8.7/02:
The study is valid.

The nominal concentration of the test item is 50 mg/mL equivalent to 5.3 x 10^{10} cfu/mL and a dose rate of 1.06 x 10^{8} cfu/bee. The enumeration analysis of the test substance solution resulted in a count of 2.2 x 10^{11} cfu/mL (mean of replicates). **Therefore, 100 µg/bee is equivalent to 4.4 x 10^{8} cfu/bee.**
The enumeration analysis of the inactivated test substance resulted in a count of 4.8 x 10^{2} cfu/mL (mean of replicates).
In a 48-h contact toxicity study conducted with *Bacillus amyloliquefaciens* MBI600, Batch/Lot Number BS11001 (seed stock 1.06 x 10^{12} cfu/g), no toxicity on *Apis mellifera* was observed at 100 µg/bee equivalent to 4.4 x 10^{8} cfu/bee (based on measured concentration).

The following non-GLP study was conducted to determine the long term effects of oral exposure on honeybees.

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

B.9.4. Effects on arthropods other than bees (Annex IIM 8.8; Annex IIIB 10.4)

Exposure of non-target terrestrial arthropods following use of *Bacillus amyloliquefaciens* MBI600 according to the proposed GAP is therefore likely to be similar to constant ambient exposure and for this reason no new regulatory studies are considered necessary. However, non-GLP studies have been provided as supportive information.

B.9.4.1. Toxicity

ACTIVE INGREDIENT
Two non-GLP studies have been conducted to determine effects on non-target arthropods and summaries are included here to provide supporting data. The first examined effects on three non-target arthropods from three different arthropod orders and the second examined effects on silkworm larvae. The studies demonstrated little or no effect of *Bacillus amyloliquefaciens* MBI600 on four non-target arthropod species tested.

Report	**IIM 8.8/01**, Mori, K. (1997b) Studies on the Effect of Microbial Pesticides on Environmental Organisms: Effect of IK-1080 WP on Off-target Insects.
Guidelines	Not stated
GLP	No

1. *Harmonia axyridis* (Predatory Coleopteran)

Materials and methods

The study was conducted during the period 6 to 27 February 1997 by Research Institute, Japan Plant Protection Association, Ibaragi, Japan. The test item was IK-1080 WP Technical (*Bacillus subtilis* NCIB 12376; Batch/Lot # S1-4; 8 x 10^{11} cfu/g). Strain NCIB 12376 is declared to be the same strain as MBI600 and IK-1080WP is declared to be the development code number of biofungicide containing MBI600 in Japan.

The test organism was the predatory coccinellid beetle, *Harmonia axyridis*. The test used adult and larval stages that were exposed to a test item rate that represented an 800 fold dilution of IK-1080 WP Technical, *Bacillus amyloliquefaciens* NCIB12376, equivalent to 1 x 10^9 cfu/mL (or 1 x 10^{12} cfu/L) as stated in the study report, prepared in distilled water and mixed with drone powder, the food of *Harmonia*. There was an untreated control prepared with dilution water without the test item. The treated and control food mixtures were pasted onto glass plates and the plates, together with a sponge soaked in water, were provided to *H. axyridis* in Petri dishes.

Larvae were fed with the food for 24 hours and adults for 48 hours after which the test organisms were provided with untreated food for the duration of the study. For the adults, one beetle was confined to each Petri dish with a total of 30 replicates (Petri dishes) and for juveniles six were confined to each Petri dish, with a total of 5 replicates. Observations were made for 7 to 10 days after treatment. In order to confirm application of the test item, residual drone powder from the treated Petri dishes was diluted and pasted onto Poamedia agar culture medium. The plates were incubated at 37˚C for 16 hours and the number of colonies formed was counted.

Findings

The concentration of IK-1080 *Bacillus amyloliquefaciens* (NCIB12376) in the food source was found to be 1.5 x 10^5 cfu/mg powder. At the end of the observation period, there were 2 dead larvae and 2 dead adults in the control treatment. In the test item treatment there were 3 dead larvae and 3 dead adults. Mean food consumption was 33.6 mg per larva in the test item treatment (5 x 10^6 cfu/larvae) compared with 38.2 mg in the control and in adults was 21.6 mg/ beetle in the test item treatment (3.24 x 10^6 cfu/adult) compared with 22.7 in the control.

Table 1 The effect of IK-1080 WP Technical on larval *Harmonia*

	Consumption of food (per larvae)		Day 3	Day 7	Day 10
IK-1080 WP Technical	33.6 mg	Larvae	29	0	0
		Nymphs	1	27	0
		Adults	0	0	27
		Dead	0	3	3
Untreated control	38.2 mg	Larvae	29	0	0
		Nymphs	1	28	0
		Adults	0	0	28
		Dead	0	2	2

* The amount consumed in 24 hours

Table 2 The effect of IK-1080 WP Technical on adult *Harmonia*

	Consumption of food (per larvae)		Day 3	Day 7	Day 10
IK-1080 WP Technical	21.6 mg	Existence	28	28	27
		Dead	2	2	3
Untreated control	22.7				
		Existence	27	24	23
		Dead	3	6	7

* The amount consumed in 48 hours

Conclusion
The test item was not considered to have had any effect on *Harmonia axyridis*.

2. ***Chrysoperla carnea*** (Predatory neuropteran)

Materials and methods
The study was conducted during the period 7 February to 5 March 1997 by Research Institute, Japan Plant Protection Association, Ibaragi, Japan. The test item was IK-1080 WP Technical (*Bacillus subtilis* NCIB 12376; Batch/Lot # S1-4; 8 x 10^{11} cfu/g). Strain NCIB 12376 is declared to be the same strain as MBI600 and IK-1080WP is declared to be the development code number of biofungicide containing MBI600 in Japan.

The test organism was the predatory neuropteran, *Chrysoperla carnea*. The test used eggs and second and third larval instar stages that were exposed to a test item rate equivalent to 10-fold the standard application rate that represented an 800 fold dilution of IK-1080 WP Technical, *Bacillus amyloliquefaciens* NCIB12376, equivalent to 1 x 10^{9} cfu/mL (or 1 x 10^{12}cfu/L) prepared in distilled water. Eggs were dipped into the treatment solution for 20 seconds then were air dried and placed in Petri dishes with an ample supply of red flour beetles for food. The number of larvae hatched was counted five days after treatment. For the larvae, the test item was diluted with distilled water and mixed with drone powder as food for the larvae. The treated and control food mixtures were pasted onto glass plates and the plates, together with a sponge soaked in water, were provided to *C. carnea* in Petri dishes. After 72 hours the plates were replaced with the eggs of red flour beetles as the food source. For the control treatment, distilled water was used in place of the test item solution. Feed was weighed before and after the feeding phase. For the larvae, one individual was confined to each Petri dish with a total of 30 replicates (Petri dishes) and for eggs, 10 were placed on each Petri dish with three replicates of the test item and four for the control. Observations were made for 5 days (eggs) and 21 days (larvae) after treatment.

Findings
At the end of the observation period for the eggs, there was 97.5% hatching success in the control compared with 93.3% in the test item treatment (3.3% observed dead at the larval stage). The following table reports the results after treatment of eggs (10^{9} cfu/mL).

Table 1 The effect of IK-1080 WP Technical on larval *Chrysoperla carnea*

		Day 3
IK-1080 WP Technical	Larvae	93.3 %
	Nymphs	3.3
	Dead*	3.3
	Unknown	0
Untreated control	Larvae	97.5 %
	Nymphs	0
	Dead	0
	Unknown	2.5

*Dead at larval stage

For larvae, all 30 (100%) in the test item treatment group successfully emerged as adults compared with 28 (93.3%) in the control.

Table 2 The effect of IK-1080 WP Technical on larval *Chrysoperla carnea*

	Consumption of food (per larvae)		Day 3	Day 7	Day 10	Day 14	Day 21
IK-1080 WP Technical	33.6 mg	Larvae	29	3	0	13	0
		Nymphs	1	27	30	17	0
		Adults	0	0	0	0	30
		Dead	0	0	0	0	0
Untreated control	38.2 mg	Larvae	30	2	0	13	0
		Nymphs	0	28	30	17	0
		Adults	0	0	0	0	28
		Dead	0	0	0	0	2

* The amount consumed in 72 hours

The concentration of IK-1080 *Bacillus amyloliquefaciens* (NCIB12376) in the food source was found to be 1.5 x 10^5 cfu/mg powder. Food consumption was 33.6 mg per larva in the test item treatment (5 x 10^6 cfu/larvae) compared with 38.2 mg in the control.

Conclusion
The test item IK-1080 *Bacillus amyloliquefaciens* (NCIB12376) was not considered to have had any effect on *Chrysoperla carnea.*

3. ***Phytoseiulus persimilis*** (Predatory Acarina)

Materials and methods
The study was conducted during the period 14 to 17 March 1997 by Research Institute, Japan Plant Protection Association, Ibaragi, Japan. The test item was IK-1080 WP Technical (*Bacillus subtilis* NCIB 12376; Batch/Lot # S1-4; 8 x 10^{11} cfu/g). Strain NCIB 12376 is declared to be the same strain as MBI600 and IK-1080WP is declared to be the development code number of biofungicide containing MBI600 in Japan.

The test organism was the predatory mite, *Phytoseiulus persimilis.* The test used adult mites that were exposed to a test item rate that represented an 800 fold dilution of IK-1080 WP Technical, *Bacillus amyloliquefaciens* NCIB12376, equivalent to 1 x 10^9 cfu/mL as stated in the study report (equivalent to 1 x 10^{12} cfu/L) prepared in distilled water. The test item solution and control treatment (distilled water) was sprayed onto Petri dishes containing kidney bean leaf discs on agar (4 mg/cm^2 equivalent to 400 L/ha and 4 x 10^{14} cfu/ha). The leaves were infested with adults of *Phytoseiulus persimilis.* There were four replicates (Petri dishes) for the test item and the control and each replicate comprised 10 mites giving a total of 40 mites per treatment. Observations were made at 24, 48 and 72 hours after treatment. The test was conducted at 25 ± 1˚C and with a 16:8 photoperiod.

Findings
At the end of the study period there was 1 dead and 1 missing ('unidentified') mite in the control, representing 2.5% mortality, and in the test item treatment there were 5 dead and 2 missing mites, representing 12.5% mortality.

Table 1 The effect of IK-1080 WP Technical on adults of *Phytoseiulus persimilis*

		After 24 hrs	After 48 hrs	After 72 hrs
IK-1080 WP Technical	Living	36	34	33
	Dead	2	4	5
	Unidentified	2	2	2
Untreated control	Living	38	38	38
	Dead	1	1	1
	Unidentified	1	1	1

Conclusion
The test item IK-1080 *Bacillus amyloliquefaciens* (NCIB12376) was considered to have had little effect on the predatory mites when compared to the control at a dose rate equivalent to 4 x 10^{14} cfu/ha on leaf disc.

Overall study conclusion
The test item IK-1080 *Bacillus amyloliquefaciens* (NCIB12376) applied in a test item treatment solution of 1 x 10^9 cfu/mL (equivalent to 1 x 10^{12} cfu/L) was considered to have had little or no effect on any of the three non-target arthropod species tested when compared to the control.

Report	**IIM 8.8/02**, Wada, Y. (1996) Studies on the Effect of Microbial Pesticides on Environmental Organisms: Effect of IK-1080 WP on Silkworm Larvae.
Guidelines	Not stated
GLP	No

Materials and methods
The study was conducted during the period 12 October to 1 November 1996 by Research Institute, Japan Plant Protection Association, Ibaragi, Japan. The test item was IK-1080 WP Technical (*Bacillus subtilis* NCIB 12376; Batch/Lot # S1-4; 8 x 10^{11} cfu/g). Strain NCIB 12376 is declared to be the same strain as MBI600 and IK-1080WP is declared to be the development code number of biofungicide containing MBI600 in Japan.

The test organism was the silkworm. The test used the fourth instar larval stage (waking up from dormancy) that were exposed to a test item rate equivalent to 83-fold the standard application rate of IK-1080 WP Technical, *Bacillus amyloliquefaciens* NCIB12376, equivalent to to a 500 fold dilution

of IK-1080 WP Technical, *Bacillus amyloliquefaciens* NCIB12376, equivalent to 1.66 x 10^9 cfu/mL (or 1.66 x 10^{12} cfu/L), prepared in distilled water. There was an untreated control comprising dilution water without the test item and a reference item, Thuricide WP (active substance *Bacillus thuringiensis* 10%) applied at 2000 times dilution. Suspensions of the test and reference items were prepared using distilled water and 0.02% of Tween 40 as an emulsifier.

Ample amounts of the suspensions were brushed on to mulberry leaves (approximately 45 leaves for 2 days) and the treated leaves were air-dried for one hour. At the start of the test the leaves were provided to the silkworms and remaining leaves were stored. The untreated control comprised mulberry leaves treated with distilled water containing the emulsifier alone. For each treatment there were 2 replicates each of 50 silkworms. The silkworms were provided with treated leaves for 48 hours and thereafter were provided with untreated leaves every day until they began to emit silk. The larvae were raised in an insectron regulated at 25˚C with a 16:8 photoperiod. Daily observations were made of dead silkworms and quality of cocoons (mean weight of female and male cocoons, weight of cocoon layers and percentage of cocoon layers) and any toxic symptoms. A pathological examination was conducted on insects that died during the study.

Findings
Although the test was carried out at a high test item concentration of 83 times the maximum application concentration (to be compared to the EU data), the number of dead insects, days at fourth and fifth instar and quality of female/male cocoons were similar in the test item treatment and the control plots. Three insects were found dead in the test item treatment (compared with a mean value of 3.5 in the control) but from the pathological examination the test item was considered to be unrelated to the deaths. In the reference item treatment there was 100% mortality by day 2 after treatment.

Name of test substances Concentration	No. of insects tested (4th instar silkworms exiting up form dormancy)	Daily death of silkworms (number)													No. of silkworms dead				
		4th Instar					5th Instar										Spinning		
		1	2	3	4	5	1	2	3	4	5	6	7	8	9	4th Instar	5th Instar	cycle	Cocooning	Total
IK-1080 WP Technical, 1.66 X 10^{10}CFU/ml	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0.5	0	0.5	0.5	2	3
Thuricide WP, Diluted into 2000 times	50	46.5	3.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Untreated control	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1.5	3.5

Name of test substances Concentration	Percent pupated (%)	Days at (Day and hr)		Weight of cocoons (g)		Weight of cocoon layers (cg)		Percent cocoon layers (%)		Toxic symptoms
		4th Instar	5th Instar	♂	♀	♂	♀	♂	♀	
IK-1080 WP Technical, 1.66 X 10^{10}CFU/ml	94	4.16	8.23	1.48	1.98	39.21	43.81	26.5	22.1	-
Thuricide WP, Diluted into 2000 times	0	-	-	-	-	-	-	-	-	-
Untreated control	93	4.16	8.23	1.48	1.93	38.76	42.44	26.1	22	-

The numbers are the average of 2 plots.

Conclusion:
The test item IK-1080 *Bacillus amyloliquefaciens* (NCIB12376) was considered to have had little effect on silkworm larvae fed with treated mulberry leaves when compared to the control.

Comments:
The test item IK-1080 *Bacillus amyloliquefaciens* (NCIB12376) when applied to mulberry leaves in a solution containing 1.66 x 10^9 cfu/mL (equivalent to 1.66 x 10^{12} cfu/L) was considered to have had little or no effect on any of the three non-target arthropod species tested when compared to the control.

A summary of endpoints is given in the table below.

Table 9.4.1-01: Toxicity effects of IK-1080 *Bacillus amyloliquefaciens* (NCIB12376) to non-target arthropods

Test species	
Toxicity	
Harmonia axyridis	Larvae, oral exposure: NOEC = 5 x 10^6 cfu/larvae Adults, oral exposure: NOEC = 3.24 x 10^6 cfu/adult
Chrysoperla carnea	Eggs, dipping exposure: NOEC = 10^9 cfu/mL Larvae, oral exposure: NOEC = 5 x 10^6 cfu/larvae
Phytoseiulus persimilis	Adults, spray exposure at 4 x 10^{14} cfu/ha on leaf disc: little to no effect
silkworm larvae	Larvae, exposure on brushed mulberry leaves in a solution containing 1.66 x 10^9 cfu/mL (equivalent to 1.66 x 10^{12} cfu/L): little to no effect

TOXIN/METABOLITE FROM ACTIVE INGREDIENT
The notifier declares that no relevant toxin/metabolite was produced by *Bacillus amyloliquefaciens* MBI600 (see volume 1).

PLANT PROTECTION PRODUCT
No specific study on arthropds, which is carried out using the plant protection product, is available. Due to the inert nature of the main co-formulant used in the manufacturing process, additional studies with the formulation were not performed since it is possible to extrapolate from data obtained with the formulation.

B.9.4.2. Infectiveness
The supportive data do not indicate infectiveness however the study duration may be too short to address this endpoint. However, non-target arthropods are naturally exposed to *Bacillus amyloliquefaciens*.

B.9.4.3. Pathogenicity
The supportive data do not indicate pathogenicity however the study duration may be too short to address this endpoint. However, non-target arthropods are naturally exposed to *Bacillus amyloliquefaciens*.

B.9.4.4. Summary and risk assessment for non-target arthropod species other than bees
The microbial pest control agent *Bacillus amyloliquefaciens* MBI600 is a naturally occurring, predominantly soil borne bacterium. It is a ubiquitous soil organism and occurs without geographical restriction. It is spread to associated environments including plants and plant materials (straw and composts) by dust or other means. In the target crop, grapevines, terrestrial arthropods other than bees will be constantly exposed to *Bacillus amyloliquefaciens*, present at ambient background levels.

The standard risk assessment scheme for chemicals is not considered applicable to micro-organisms. The ratio of a maximum application rate of the MPCA to an LD_{50} value would not represent the

potential risk of a micro-organism since an infective agent would be able to reproduce and colonise the host regardless of the exposure. In the following risk assessments for Subtilex®, the proposed field application rate for MBI600 (2.75×10^{13} cfu/ha in a volume of 400 L water) has been compared with the test item treatment solutions for the non-target arthropod studies in terms of cfu/L and assuming an application volume of 400 L/ha to represent an equivalent field application rate according to the proposed GAP.

Formulation components are considered to be inert and tests for toxicity of Subtilex® to terrestrial arthropods other than bees have therefore not been conducted.

Two non-GLP studies were conducted with *Bacillus amyloliquefaciens* MBI600 to determine effects on four non-target arthropods (IIM 8.8/01 by Mori (1997b) and 8.8/02 by Wada (1996)).

The first of these studies examined effects on three non-target arthropods from three different arthropod orders (the predatory coccinellid beetle, *Harmonia axyridis*; the predatory neuropteran, *Chrysoperla carnea* and the predatory mite, *Phytoseiulus persimilis*) and the second examined effects on silkworm larvae. The studies demonstrated little or no effect of *Bacillus amyloliquefaciens* MBI600 on the four non-target arthropod species tested at test item treatment application rates, equivalent to 1×10^{12} cfu/L (IIM 8.8/01) or 1.66×10^{12} cfu/L (IIM 8.8/02).

The test item treatment concentration is equivalent to a field rate of 4×10^{14} cfu/ha in the first study and 6.64×10^{14} cfu/ha in the second study, assuming application in 400 L volume as the proposed GAP. Both are higher than the proposed single field application rate for *Bacillus amyloliquefaciens* MBI600 of 2.75×10^{13} cfu/ha. It is therefore considered that exposure to *Bacillus amyloliquefaciens* MBI600 following application of Subtilex® according to the proposed GAP will not represent a risk to non-target arthropods.

No additional acute/short-term or higher tier studies have been performed or considered necessary.

B.9.5. Effects on earthworms (Annex IIM 8.9.1; Annex IIIB 10.5)

Bacillus amyloliquefaciens MBI600 is a naturally occurring, predominantly soil borne bacterium. It is a ubiquitous soil organism and occurs without geographical restriction. It is spread to associated environments including plants and plant materials (straw and composts) foods, animals and faeces. The Review Report for the active substance *Bacillus subtilis* QST 713 (2006) states that 'Different studies have shown that populations of *B. subtilis* are influenced by biotic environmental factors. Introduced *B. subtilis* populations are subject to competition by the indigenous microflora (bacteria and fungi) and may also be affected by infectious agents like phages. As a result, high initial population numbers resulting from application *Bacillus subtilis* will decline and reach a natural equilibrium.' Any local effects that result from the use of *Bacillus amyloliquefaciens* MBI600 according to the proposed GAP are likely to be transient as populations rapidly equilibrate in the soil.

Earthworms are therefore likely to be exposed to *Bacillus amyloliquefaciens* MBI600 in the soil environment and no adverse effects are anticipated from exposure following applications of Subtilex®. In addition, earthworms are extremely resistant to pathogens and have a very active immune system (there are no known pathogens of earthworms)[15]

B.9.5.1. Toxicity

[15] Report of the second OECD Biopesticides Steering Group Seminar on the fate in the environment of microbial control agents and their effect on non-target organisms. Series on Pesticides No. 64.ENV/JM/MONO(2011)42 29 September 2011

ACTIVE INGREDIENT

A 28-day reproduction and growth study was conducted with *Bacillus amyloliquefaciens* strain MBI600.

Report	**IIM 8.9.1/01**, Whittaker, M. (2013); Effects of *Bacillus amyloliquefaciens* MBI600 on reproduction and growth of the earthworm *Eisenia fetida* in an artificial soil substrate. Laboratory study No. BUBA001.
Guidelines	OECD Guideline for Testing of Chemicals: Earthworm Reproduction Test (*Eisenia fetida* / *Eisenia Andrei*). No. 222, 13 April 2004
GLP	Yes

Materials and methods
The laboratory phase of the study was conducted during the period 05 March to 06 May 2013 by APIS, Knaresborough, UK. The test item was *Bacillus amyloliquefaciens* MBI600, Label identification: BS7211; purity 5 x 10^{11} cfu/g nominal.

The objective of the study was to determine the effects of the microbial pest control agent (MPCA) *Bacillus amyloliquefaciens* strain MBI600 on the survival, reproduction and growth of the earthworm *Eisenia fetida* in an artificial soil substrate.

The study was conducted as a limit test with a single test item treatment of MBI600 at a nominal concentration of >1.1 x 10^{9} cfu/g dry soil. A non-infective MPCA control (NI MPCA) was included in the test series at the same rate using test item that had been deactivated by autoclaving. A reference item (carbendazim at a concentration of 5 mg active substance/kg dry soil) and water control were tested concurrently. There were eight replicates of each test and reference item treatment and control. The concentration of the test item in soil from the control and all three treatments was determined at the start and end of the study.

The artificial soil substrate was prepared according to the OECD guideline (No. 222). Treatments were prepared by mixing 1kg batches of soil with deionised water, sufficient to achieve 50% of water holding capacity, and appropriate quantities of test and reference items. 500 g of the substrate were added to each test vessel. Test vessels were 2000 mL glass beakers covered in fine gauze, secured by elastic bands to prevent escape whilst still permitting air exchange. Test vessels were incubated in a temperature controlled chamber set to 20 +/- 2°C with a 16:8 hour light:dark cycle.

Earthworms used in the study were from a cohort aged between 2 and 12 months. At day 0, all worms were clitellate, appeared healthy and were within the acceptable weight range of 300 to 600 mg. At the start of the study worms were rinsed in deionised water and blotted dry with paper towel. Individual weights were recorded and ten worms were placed in each of eight test vessels per treatment group. Individual vessel weights were recorded and the vessels transferred to a growth chamber. During the study the worms were fed with approximately 5g porridge oats, moistened with approximately 5 mL of deionised water. Food was replaced every 2 to 3 days to avoid the growth of mould. Each time food was replaced, soil water content was maintained by placing test vessels on a balance and spraying with deionised water until the vessel returned to its starting weight.

On day 28, the adult worms were removed from each test vessel and the soil substrate was replaced into the test vessels, vessels were re-weighed and returned to the test chamber for a further period of 28 days.

The adult worms were re-weighed and mean weight per vessel was determined. The clearance of the MPCA from adult worms in the test item treatment was assessed 0, 2 and 4 days after removal from the soil by comparing recovery rates of the microorganism to those from the control group.

On day 56, juvenile worms were removed from each vessel using a heat extraction method.

Adult mortality, body weight and reproductive capacity in the test and reference item treatment groups was compared to the controls. Data sets were tested for normality and homogeneity of variance using Kolmogorov-Smirnov's test and Levene's tests respectively and then analysed using ANOVA. Adult mortality data in the MPCA treatment were non-normally distributed and were compared to the control with the non-parametric Kruskal-Wallis test. Data were analysed using Minitab 16.2.4.

Findings

Validity criteria
Adult control mortality was 7.5 at day 28. This is below the guideline validity threshold of 10% control mortality. Reproduction in the control was between 31 and 51 juveniles per vessel and is thus greater than the validity threshold of 30 per vessel. The coefficient of variance in the control was 6.33% below the validity of 30%. As all three validity criteria were met, the study is considered to be valid.

Microbial monitoring
Results of the microbial monitoring are shown in Table 9.5.1-01:

Table 9.5.1-01: Results of microbial monitoring – microbial content in control, test and reference item treatments (cfu/g dry soil)

Time point	Water control	NI MPCA	MPCA	Toxic reference
Study start	1.7×10^8 cfu/g	1.0×10^8 cfu/g	2.9×10^9 cfu/g	1.6×10^8 cfu/g
Study end	2.4×10^8 cfu/g	2.0×10^8 cfu/g	3.5×10^9 cfu/g	1.7×10^8 cfu/g

Adult mortality
Adult mortality results at 0 and 28 days after treatment (DAT) are shown in Table 9.5.1-02. No sub-lethal effects were observed in any treatment group at 28 DAT with the exception of a single moribund worm in the NI MPCA group which showed no signs of lesions, discolouration or other abnormality.

Table 9.5.1-02: Adult mortality at 0 and 28 days after treatment

	Survivorship at		Number dead	% Mortality
Treatment	**0 DAT**	**28 DAT**		
Water control	80	74	6	7.50
Toxic reference	80	3	77	96.25
MPCA	80	75	5	6.25
NI MPCA	80	73	7	8.75

There was no significant difference between the water control and the MPCA treatment for adult mortality and the 28-day NOEC for mortality is thus > 1.1×10^9 cfu/g soil.

Adult weight
Adult weight results at 0 and 28 days after treatment (DAT) are shown in Table 9.5.1-03.

On day 0, adult weight was 0.46 ± 0.01 g (mean ± SEM) in the water control and 0.44 ± 0.01 g in the MPCA treatment. At day 28, a slight decrease in mean weight was observed in all groups with mean weight in both the water control and the MPCA treatment being 0.37 ± 0.01 g.

Table 9.5.1-03: Mean adult worm weight at 0 and 28 days after treatment

Treatment	Weight (g) at 0 DAT			Weight (g) at 28 DAT			Difference (g) 0-28 DAT
	Mean weight	SEM	rSEM (%)	Mean weight	SEM	rSEM (%)	
Water control	0.46	0.01	2.23	0.37	0.01	3.21	-0.09
Toxic reference	0.43	0.01	2.38	0.21	0.05	24.29	-0.22
MPCA	0.44	0.01	2.18	0.37	0.01	2.67	-0.07
NI MPCA	0.40	0.01	2.19	0.33	0.01	3.42	-0.07

There was no significant difference in adult weight change between the water control and the MPCA treatments. The 28-day NOEC for adult weight is thus > 1.1 x 10^9 cfu/g dry soil.

Reproduction

Results of the reproduction assessment are shown in Table 9.5.1-06.

Table 9.5.1-06: Mean number of juvenile worms at 56 days after treatment

Treatment	Number of juvenile worms at 56 DAT		
	Mean	SEM	rSEM (%)
Water control	40.43	2.56	6.34
Toxic reference	0	0	0
MPCA	35.88	3.07	8.55
NI MPCA	32.50	3.08	9.48

Although the study validity criterion was met for the number of juvenile worms produced in the control, the number was lower than anticipated and this is considered to be a result of the diet of oats. Although there were signs that the oats had been consumed and partially incorporated into the upper soil layer, the slight decrease in adult weight and lower than expected reproduction across all treatments suggests that the diet was not optimal.
The mean number of juveniles per vessel was 40.43 $\pm$ 3.07 in the water control and 35.88 $\pm$ 3.07 in the MPCA treatment. There was no significant difference in juvenile production between the water control and MPCA treatments and the 56-day NOEC for reproduction is thus >1.1 x 10^9 cfu/g.

Test item clearance

Results for clearance of the test item from the adult worms is shown in Table 9.5.1-07. The recovery of the MPCA from material spiked pre- and post-homogenising demonstrates that the homogenisation process had no detrimental effect on the viability of the MPCA.

Table 9.5.1-07: Microbial loading of adult worms at 0, 2 and 4 days after removal from test soil

Treatment	Microbial load (cfu/g)		
	Day 0	Day 2	Day 4
Water control	1.0 x 10^7	-	
Spiked pre-homogenising	3.2 x 10^{11}	-	
Spiked post-homogenising	3.3 x 10^{11}	-	
MPCA	5.2 x 10^8	1.2 x 10^8	9.4 x 10^6

MPCA levels at day 0 were approximately 50 times higher in the MPCA treatment than the water control, but had dropped slightly below the water control value by day 4, indicating that the MPCA is readily cleared from the test organism.

Conclusion

The test item *Bacillus amyloliquefaciens* MBI600 had no significant effect on mortality, weight change or reproduction in adult *Eisenia fetida* compared to untreated controls. The NOEC for all parameters was determined to be >1.1 x 10^9 cfu/g dry soil, equivalent to >1.1 x 10^{12} cfu/kg dry soil based on nominal test item concentration. The NOEC for all parameters based on initial measured test item concentration was determined to be 2.9 x 10^9 cfu/g dry soil, equivalent to 2.9 x 10^{12} cfu/kg dry soil.

RMS comment on study IIM 8.9.1/01: The study is valid.

TOXIN/METABOLITE FROM ACTIVE INGREDIENT
The notifier declares that no relevant toxin/metabolite was produced by *Bacillus amyloliquefaciens* MBI600 (see volume 1).

PLANT PROTECTION PRODUCT
No specific study on earthworms, which is carried out using the plant protection product, is available. A 28-day reproduction and growth study was conducted with *Bacillus amyloliquefaciens* strain MBI600. The information provided from the study conducted on the microbial pest control agent *Bacillus amyloliquefaciens* strain MBI600 is directly applicable to the microbial pest control product. Formulation components are considered to be inert and no further tests for toxicity of Subtilex® to aquatic organisms have been conducted.

B.9.5.2. Infectiveness

No sign of infectiveness was observed in study IIM 8.9.1/01.

B.9.5.3. Pathogenicity

No sign of pathognencity was observed in study IIM 8.9.1/01.

B.9.5.4. Summary and risk assessment for earthworms

The following endpoints (Table 9.5.4-01) determined from a recent, GLP-compliant study conducted in accordance with the current OECD Guidelines, are used in the risk assessment:

Table 9.5.4-01: Earthworms - ecotoxicological endpoints

Risk type	Species	Endpoint	Value	Study reference
Long-term – growth and reproduction	*Eisenia fetida*	NOEC (mortality, growth and reproduction)	>1.1 x 10^9 cfu/g (nominal) 2.9 x 10^9 cfu/g (measured at study start)	Whitaker, M. (2013) Report No: BUBA001 Section 6 Doc M Annex IIM Section 8.9.1/01

*cfu = colony forming unit

Soil exposure levels are taken from the PEC_s values reported under volume 3 B.8. The maximum peak PEC_S value of 3.67 x 10^8 cfu/kg soil was estimated following 10 applications of Subtilex^{TM} to vines at 2.75 x 10^{13} cfu MBI 600/ha. This value has been used in the TER calculation (Table 9.5.4-02).

Table 9.5.4-02: TER_{LT} for earthworms

Species	Endpoint	Toxicity (cfu/kg soil dry weight)	Max PEC_S (following 10 applications)	TER
Eisenia fetida	56-day NOEC (survival, growth and reproduction	$>1.1 \times 10^{12}$ (nominal)	3.67×10^8	>3000
		$>2.9 \times 10^{12}$ (measured at study start))		>7900

The TER exceeds the trigger value of 5 specified in Annex VI to Directive 91/414/EEC when the toxicity is expressed as either nominal or measured concentration of MBI 600 in soil. It is concluded that Subtilex® does not present a significant risk to earthworm survival, growth or reproduction when used according to the proposed GAP.

B.9.6. Effects on non-target soil micro-organisms (Annex IIB 8.10; Annex IIIB 10.6)

Bacillus amyloliquefaciens MBI600 is a naturally occurring, predominantly soil borne bacterium. It is a ubiquitous soil organism and occurs without geographical restriction. The Review Report for the active substance *Bacillus subtilis* QST 713 (2006) states that 'Different studies have shown that populations of *B. subtilis* are influenced by biotic environmental factors. Introduced *B. subtilis* populations are subject to competition by the indigenous microflora (bacteria and fungi) and may also be affected by infectious agents like phages. As a result, high initial population numbers resulting from application of *Bacillus subtilis* will decline and reach a natural equilibrium.' Any local effects that result from the use of Subtilex® according to the proposed GAP are likely to be transient as populations rapidly equilibrate in the soil. Microbial communities are extremely variable and environmental populations normally represent the most competitive species that have adapted to their niche and it is considered that introduction of *Bacillus amyloliquefaciens* MBI into natural soil will not impede the natural micro-flora.

Tests for toxicity of *Bacillus amyloliquefaciens* MBI600 to soil micro-organisms have therefore not been considered appropriate. Formulation components are considered to be inert and tests for toxicity of Subtilex® to soil micro-organisms have therefore not been considered appropriate and no studies have been conducted.

B.9.7. Effects on terrestrial plants (Annex IIB 8.6; Annex IIIB 10.7)

This is not an EC data requirement and therefore no study is required.

B.9.8. Additional studies (Annex IIM 8.11; Annex IIIB 10.7)

The additional studies might include further acute studies on additional species or processes (such as sewage systems) or higher tier studies such as chronic, sub-lethal or reproductive studies on selected non-target organisms.

B.9.9. References relied on

Section 1, (Annex IIA, Point 1)

Annex point/ reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N	Owner
Annex II Data and information					
IIM 8.1/01	[redacted]	1993	*Bacillus subtilis* Strain MBI 600: An avian oral pathogenicity and toxicity study in the bobwhite. [redacted] 301-102 GLP, unpublished	Y	BASF Agricultural Specialities Ltd
IIM 8.2/01	[redacted]	2013(a)	*Bacillus amyloliquefaciens* strain MBI600 Rainbow Trout (*Oncorhynchus mykiss*) 96-Hour Acute Toxicity Test [redacted] 17142-13 GLP, unpublished	Y	BASF Agricultural Specialities Ltd
IIM 8.2/02	[redacted]	1997	Toxicity study of MBI-600 on carp (*Cyprinus carpio*) [redacted] STS (2)-97004 Non-GLP, unpublished	Y	BASF Agricultural Specialities Ltd
IIM 8.3/01	Mikulas, J.	2013(b)	*Bacillus amyloliquefaciens* strain MBI600 *Daphnia magna* 48-Hour Acute Toxicity Test STILLMEADOW, Inc. 16672-12 GLP, unpublished	Y	BASF Agricultural Specialities Ltd
IIM 8.3/02	Fujii, Y.	1997	Reproductive toxicity on *Daphnia*. Japan Food Research Laboratories 597040201-001 Non-GLP, unpublished	Y	BASF Agricultural Specialities Ltd
IIM 8.4/01	Mikulas, J.	2013(c)	*Bacillus amyloliquefaciens* strain MBI600 *Pseudokirchneriella subcapitata* 72-Hour Algal InhibitionTest STILLMEADOW, Inc. 17141-13 GLP, unpublished	Y	BASF Agricultural Specialities Ltd
IIM 8.7/01	Mori, K.	1997(a)	Studies on the effect of microbial pesticides on environmental organisms: Effect of IK-1080 WP on honeybee Research Institute, Japan Plant Protection Association. Non-GLP, unpublished	Y	BASF Agricultural Specialities Ltd
IIM 8.7/02	Younger, C.	2012	*Bacillus amyloliquefaciens* strain MBI600 Honey Bee Acute Contact Toxicity Limit Test STILLMEADOW, Inc. 16674-12 GLP- Unpublished	Y	BASF Agricultural Specialities Ltd
IIM 8.8/01	Mori, K.	1997 (b)	Studies on the effect of microbial pesticides on environmental organisms: Effect of IK-1080 WP on off-target insects Research Institute, Japan Plant Protection Association. Non-GLP, unpublished	Y	BASF Agricultural Specialities Ltd
IIM 8.8/02	Wada, Y.	1996	Studies on the Effect of Microbial Pesticides	Y	BASF

Annex point/ reference number	**Author(s)**	**Year**	**Title** **Source (where different from company)** **Company, Report No** **GLP or GEP status (where relevant), Published or not**	**Data Protection Claimed** **Y/N**	**Owner**
			on Environmental Organisms: Effect of IK-1080 WP on Silkworm Larvae Research Institute, Japan Plant Protection Association. Non-GLP, unpublished		Agricultural Specialities Ltd
IIM 8.9.1/01	Whittaker, M.	2013	Effects of *Bacillus amyloliquefaciens* MBI600 on reproduction and growth of the earthworm *Eisenia fetida* in an artificial soil substrate. Laboratory study No. BUBA001. GLP, unpublished	y	BASF Agricultural Specialities Ltd
Annex III Data and Information					
-	-	-	-	-	-