Addendum 1

to the Monograph

of 15 May 2001

Bacillus subtilis

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Contents

B.1 Identity ... 2

B.1.1 Identity of the micro-organism ... 2
B.1.1.3 Name and species description (Annex IIB 1.3) ... 2
B.1.3 References relied on ... 2

B.2 Biological properties of the organism - Technical properties of the preparation ... 3

B.2.1 Biological properties of the micro-organism (Annex IIB 2) ... 3
B.2.1.6 Relationships to known plant or animal or human pathogens (Annex IIB 2.6) ... 3
B.2.3 References relied on ... 4

B.7 Residues ... 5

B.7.2 Further information required (Annex IIB 6.2, Annex IIIB 8) ... 5
B.7.2.2 Viable residues(Annex IIB 6.2.2, Annex IIIB 8) ... 5
B.7.4 References relied on ... 5

B.8 Environmental fate and behaviour ... 6

B.8.1 Persistence and multiplication in soil (Annex IIB 7.1.1; Annex IIIB 9) ... 6
B.8.5 References relied on ... 7

B.9 Ecotoxicology ... 9

B.9.2 Effects on aquatic organisms (Annex IIB 8.2, IIIB, 10.2) ... 9
B.9.2.1 Acute toxicity and/or pathogenicity and infectivity to freshwater fish ... 9
B.9.2.2 Toxicity to freshwater invertebrates ... 10
B.9.2.2.1 Acute toxicity test with *Palaemonetes pugio* ... 10
B.9.2.2.2 Chronic (21 d) toxicity test with *Daphnia magna* ... 10
B.9.2.3 Risk assessment for aquatic organisms ... 11
B.9.3 Effects on bees (Annex IIB 8.3; Annex IIIB 10.3) ... 12
B.9.3.5 Field test ... 12
B.9.4 Effects on other arthropod species (Annex IIB 8.4, IIIB, 10.4) ... 13
B.9.4.1 Acute toxicity, pathogenicity and infectivity (Annex IIB 8.4, Annex IIIB 10.4) ... 13
B.9.4.2 Field tests (Annex IIIB 10.4) ... 14
B.9.4.3 Risk assessment for non-target terrestrial arthropods ... 16
B.9.5 Effects on earthworms (Annex IIB 8.5; Annex IIIB 10.6) ... 16
B.9.7 References relied on ... 18

B.1 Identity

B.1.1 Identity of the micro-organism

B.1.1.3 Name and species description (Annex IIB 1.3)

The American Type Culture Collection identified the strain QST 713 as *Bacillus subtilis* using classical biochemical tests. With these methods a strain differentiation is not possible. To distinguish the *Bacillus subtilis* strain QST 713 from any other *B. subtilis* strain a molecular biological method for strain discrimination has been designed.

A strain differentiation protocol was developed using the Riboprinter system (DuPont Qualicon) for automated rRNA operon ribotyping. Ribotyping involves Southern blotting of digested chromosomal DNA of the organism of interest, probing with the *E. coli* rRNA operon, and conducting a computer analysis of the resulting patterns. These patterns may be compared to a database for identification or to other strains for strain differentiation.
AgraQuest developed the Riboprinter protocol in association with the scientists from Accugenix (Newark, DE 19702, USA), a commercial strain identification laboratory. Seven *Bacillus* strains were studied including two replicates of QST 713 and a commercial strain of *B. subtilis*, sold as Kodiak, a fungicide for seed treatment. Several enzyme digests were tested to find a pattern or ”fingerprint” that would allow to differentiate QST 713 from other strains. The enzyme PvuII has been chosen as an appropriate enzyme, that separated QST 713 from other strains in the test group which included *B. subtilis* and other closely related strains from the *B. subtilis* grouping.

B.1.3 References relied on

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BBA registration number	Data protection claimed Y/N	Owner
AIIB-1	Lehmann, L.	2002	Strain Discrimination of Bacillus subtilis QST 713 by Ribotyping statement BWS2002-2	Y	QST

B.2 Biological properties of the organism - Technical properties of the preparation

B.2.1 Biological properties of the micro-organism (Annex IIB 2)

B.2.1.6 Relationships to known plant or animal or human pathogens (Annex IIB 2.6)

Morphological and physiological differentiation of *Bacillus subtilis* from pathogenic *Bacillus* species (HEINS 2001, BMF 2001-55):

Bacillus subtilis strain QST 713 (also referred to as QST 713 here) can be distinguished from *B. anthracis* based on size, motility, spore location and maximum growth temperature. *B. anthracis* is a large (1-1.5 µm x 4-10 µm) non-motile rod with centrally located spores and a maximum growth temperature of 40 °C for the type strain. In contrast, QST 713 is smaller (~1µm x 3-5µm), motile, has subterminal spores (occasionally central) and has a maximum growth temperature of 55 °C. *B. anthracis* can often be characterised by palisade growth when observed microscopically, in contrast, *B. subtilis* strain QST 713 does not demonstrate this growth behaviour.

QST 713 can be distinguished from *B. cereus* and *B. thuringiensis* by size, spore location, and maximum growth temperature. Like *B. anthracis*, *B. cereus* and *B. thuringiensis* are larger than QST 713, have centrally located cylindrical spores and have maximum growth temperatures of less than 40 – 50 °C. Additionally they do not have the mucoid colony morphology that is characteristic of QST 713. *B. thuringiensis* also produces parasporal crystal proteins that are not produced by QST 713 or the other species.

The biochemical test api 50 CHB (API, Analytical Profile Index) is used to identify bacilli. Therefore it can be used to distinguish QST 713 from the pathogenic species *B. anthracis*, *B. cereus* and *B. thuringiensis*.

Bacillus licheniformis can be distinguished from *B. subtilis* based on morphological parameters as type of growth. In contrast to *B. subtilis B. licheniformis* occurs in long chains. The colonies of B. licheniformis are strongly attached to agar and hair like outgrowths are common (Bergey`s manual of Systematic Bacteriology, Vol. 2, 1986). Additionally *B. licheniformis* can be distinguished from *B. subtilis* by pyrolysis gas-liquid chromatography, deoxyribonucleic acid-deoxyribonucleic acid hybridisation and API identification systems (O`Donnell *et al*., 1980).

B.2.3 References relied on

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BBA registration number	Data protection claimed Y/N	Owner
AIIB-2.6	O`Donnell A.G., Norris J.R., Berkely R.C.W., Kaneko N., Nozaki R.	1980	Characterization of *Bacillus subtilis*, *Bacillus pumilus, Bacillus licheniformis* and *Bacillus amyloliquefaciens* by Pyrolysis Gas-Liquid Chromatography, Deoxyribonucleic Acid-Deoxyribonucleic Acid Hybridization, Biochemical and API Systems not GLP, published International Journal of Systematic Bacteriology BWS2002-1	N	-
AIIB-2.6	Heins, S.	2001	Report: Morphological and Physiological Differentiation of Bacillus subtilis From Pathogenic Bacillus species not GLP, published Stellungnahme BMF2001-55	Y	QST
AIIB-2.6	Sneath, P.H.A.	1986	Bergey`s manual of Systematic Bacteriology, Vol. 2, page 1133 not GLP, published BWS 2002-8	N	-

B.7 Residues

B.7.2 Further information required (Annex IIB 6.2, Annex IIIB 8)

B.7.2.2 Viable residues(Annex IIB 6.2.2, Annex IIIB 8)

A new study (RIP 2002-985, Ryder Fox, 2001) was conducted to show the amount of *Bacillus subtilis* strains on grapes after application of Serenade WP.

Material and Methods
Serenade WP (QRD132) was applied to to vine-plots in the field at application rates of 5 and 10 kg/ha. Samples of grapes were taken 4 hr, 1, 2, 4, 7, 10, 14,21,28 days after treatment.

Findings
Populations of *Bacillus subtilis* strain QST 713 decline from 7.2 x 10^4 CFU/g berries to 3.9 x 10^3 CFU/g berries (application rate 5 kg/ha) and from 9.4 x 10^4 CFU/g berries to 7.1 x 10^3 CFU/g berries (application rate 10 kg/ha). There were no CFU of *Bacillus subtilis* strain QST 713 on grapes of untreated control plots. The background counts of non QST 713 *Bacillus* spp. were low.

B.7.4 References relied on

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BBA registration number	Data protection claimed Y/N	Owner[1]
AIIB-6.2.2	Ryder Fox, J.	2001	Serenade WP Residues on Wine Grapes. not GLP, unpublished RIP2002-985	Y	QST

[1] Only notifier listed

B.8 Environmental fate and behaviour

B.8.1 Persistence and multiplication in soil (Annex IIB 7.1.1; Annex IIIB 9)

Milus & Rothrock (1992) investigated the ability of different soil bacteria to colonise the rhizosphere of wheat, which is essential for bacteria to function as biological control agents of soil-borne plant pathogens. Two investigated *Bacillus subtilis* strains (D-39 Sr and D-60 R) were able to colonise roots of soft red winter wheat from seed-borne inoculum in two different soils in Arkansas (silty clay soil and silt loam soil). At planting time, the population size of both strains varied between $10^{5.5}$ – $10^{7.1}$ cfu/g of seeds. In fall, 4-6 weeks after planting, the rhizosphere population sizes on seminal roots were between $10^{4.5}$ and $10^{7.1}$ cfu/g dry weight of roots. In spring of the following year the values had further decreased to $10^{3.7}$ - $10^{5.7}$ cfu/g dry weight of roots.

Pandey et al. (2001) investigated natural populations of dominant fungi in the rhizosphere of established tea bushes in the Himalaya region (subtropical to temperate climate) and the interaction with the two most dominant rhizosphere bacteria, *Bacillus subtilis* and *B. mycoides*. The populations of *Penicillium* and *Trichoderma* species were inversely correlated with the populations of both bacteria. The study by Pandey et al. (2001) demonstrates the existence of a kind of antagonism under natural conditions in the rhizosphere of established tea bushes. The data also showed the saisonal variation in the composition of the rhizosphere microflora.

In soil microcosm studies Pantastico-Caldas (1992) investigated the population dynamics of bacteriophages and *Bacillus subtilis* (host). The sterilised acidic peat soil were treated with bacteria or phage alone (controls) or with bacteria (wildtype or auxotrophic strains) and bacteriophages (temperate or virulent). The acidic soil used in these experiments caused rapid and permanent inactivation of free phages, making the continuos interaction of phage and host essential for persistence of phage. The phage and bacterial spore densities were checked over the whole period of the study.
Within 2 days the control populations of *B. subtilis* declined from initial densities near 10^7 cfu per gram of soil to a constant density of approx. 8 x 10^5 cfu/g soil (study duration 60 d). In the mixed microcosm with the temperate phages the density of the bacteria dropped sharply in the first 24 h to nearly 10^5 cfu/g soil. The bacteria population then quickly rose back to nearly its initial density and over the next 10 to 20 d dropped again to the density of the control population.
An initial drop of the bacterial population due to phage infections occurred, followed by stable equilibria lasting weeks to months. A host density threshold for phage epidemics occurred at approx. 5 x 10^6 cfu/g soil.
The temperate phages did not depress the equilibrium host density, while the virulent phages lowered it by a factor of ten.

The survival pattern of two B. *subtilis* strains (NB22-1 and YB8-1, spontaneous streptomycin-resistance mutants) in four non-sterile and one sterile soil was investigated by Tokuda et al. (1993). The different soil types were: Fujimi - loamy coarse sand, low humus Andasol, pH 6.6; Hiratsuka – coarse sandy loam, pH 5.7; Kagoshima – loam, abundant humus, pH 5.4; Engei – artificial soil, pH 6.6.
In non-sterile soil (Hiratsuka) the number of cfu`s of the strain YB8-1 (initial concentration: $10^8$ cfu/g dry soil) decreased quickly, and after about two days the strain was not detectable anymore. In the case of the strain NB22-1 the number of cfu`s in four non sterile soils (initial

concentration: approx. 10^7 cfu/g dry soil) decreased during the first 10 d and thereafter the number of cfu`s 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 to the end of the experiment (50 days). In sterile soils the decrease in the cell numbers was slower than in non-sterile ones. However, there was no difference in the final level of the population between the two systems.

Conclusions:
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.

B.8.5 References relied on

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BBA registration number	Data protection claimed Y/N	Owner
AIIB 7.1.1	Bochow, H. and Gantcheva, K.	1995	Soil introductions of Bacillus subtilis as biocontrol agent and its population and acivity dynamic not GLP, published Acta Horticulturae 382 BOD2002-551	N	-
AIIB 7.1.1	Casida, L. E., Jr.	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 not GLP, published Appl. Environ. Microbiol. 54 BOD2002-554	N	-
AIIB 7.1.1	Milus, E. A. and Rothrock, C. S	1993	Rhizosphere colonization of wheat by selected soil bacteria over diverse environments Canadian Journal of Microbiology 39 not GLP, published BOD2002-555	N	-
AIIB 7.1.1	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 Microbiol. Research 156 not GLP, published BOD2002-556	N	-
AIIB 7.1.1	Pantastica-Caldas, M. and Duncan, K. E.	1992	Population dynamics of bacteriophage and *Bacillus subtilis* in soil not GLP, published Ecology 73 (5) BOD2002-557	N	-

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BBA registration number	Data protection claimed Y/N	Owner
AIIB 7.1.1	Tokuda, Y., Ano, T. and Shoda, M.	1993	Survival of *Bacillus subtilis* NB22, an Antifungal-Antibiotic Iturin Producer, and its transformant in Soil-Systems J. of Fermentation and Bioengineering 75 (2) BOD2002-558	N	-

B.9 Ecotoxicology

B.9.2 Effects on aquatic organisms (Annex IIB 8.2, IIIB, 10.2)

B.9.2.1 Acute toxicity and/or pathogenicity and infectivity to freshwater fish

Title: **QST 713 Technical: A Five-Concentration Toxicity and Pathogenicity Test with the Rainbow Trout (*Oncorhynchus mykiss*)**

Author: Drottar, K.R., Flaggs, R.S., Krueger, H.O. (2001)
BBA-Ref.-No.: WAT2002-442

Test substance: Technical *Bacillus subtilis*
Purity: 1.61×10^{11} CFU/g

Guideline: US-EPA Microbial Pesticide Test Guidelines OPPTS No 885.4200 (1)
Test species: *Oncorhynchus mykiss*
Exposure mode: Semi-static
Conc. levels (nom.): negative control/sterile filtrate control/attenuated control/broth concentrate control/2.3×10^6/4.0×10^6/9.2×10^6/ 1.84×10^7/ 3.68×10^7 CFU/mL
Conc. levels (meas.): negative control/sterile filtrate control/attenuated control/broth concentrate control/1.7×10^6/4.0×10^6/7.7×10^6/ 1.7×10^7/ 3.5×10^7 CFU/mL (74 - 95 %)

Results (CFU/ml) related to measured concentrations

Time	End point	NOEC	LOEC	Effect (%) at LOEC	EC50
720 h	Mortality	1.7×10^6			1.4×10^7

Remarks: Mortality in the highest QST 713 Technical treatment group was 90 % after 30 days. Mortality in rainbow trouts exposed to the attenuated control at a concentration equal to the highest QST 713 Technical treatment group was 100 %. It cannot be decided, if the mortality observed was due to the physical nature of the test solutions (test solutions were turbid) or, for example an effect of toxin-production.
However, within the tissue-preparations (3 fish per conc-entration) of gills, stomache and muscles no signs of infectivity were observed.

valid: yes
GLP compliance: yes

B.9.2.2 Toxicity to freshwater invertebrates

B.9.2.2.1 Acute toxicity test with *Palaemonetes pugio*

Title: **QST 713 Technical Powder – Infectivity and Pathogenicity to Grass Shrimp (*Palaemonetes pugio)* during a 30-Day Static Renewal Test**

Author: Machado, M.W. (2001)
BBA-Ref.-No.: WAT2002-446

Test substance: Technical *Bacillus subtilis*
Purity: 14.6 % (6.3 x 10^6 CFU/g)

Guideline: US-EPA Microbial Pesticide Test Guidelines OPPTS No 885.4280
Test species: *Palaemonetes pugio*
Conc. levels (nom.): negative control/sterile filtrate control/attenuated control/broth concentrate control /3.7 x 10^6 CFU/g (dietary dosage)
Conc. levels (meas.): negative control/sterile filtrate control/attenuated control/broth concentrate control /2.8 x 10^6 CFU/g (dietary dosage, 76-110 %)

Results (CFU/g) related to nominal concentrations

Time	End point	NOEC	LOEC	Effect (%) at LOEC	EC50
720 h	Mortality	3.7 x 10^6 (2.8 x 10^6 real)			

Remarks: No mortality or other effects on normal behaviour and development were observed in the tests animals. There were no signs of pathogenicity.

valid: yes
GLP compliance: yes

B.9.2.2.2 Chronic (21 d) toxicity test with *Daphnia magna*

Title: **QST 713 Technical: A 21-Day Life-Cycle Toxicity and Pathogenicity Test with the Cladoceran (*Daphnia magna***

Author: Drottar, K.R., Flaggs, R.S., Krueger, H.O. (2001)
BBA-Ref.-No.: WAT2002-449

Test substance: Technical *Bacillus subtilis*
Purity: 1.61 x 10^{11} CFU/g

Guideline: US-EPA Microbial Pesticide Test Guidelines OPPTS No. 885.4240

Test species: *Daphnia magna*
Exposure mode: Semi-static
Conc. levels (nom.): negative control/sterile filtrate control/attenuated control/broth concentrate control/2.3 x 10^6/4.6 x 10^6/9.2 x 10^6/ 1.84 x 10^7/ 3.68 x 10^7 CFU/ml
Conc. levels (meas.): negative control/sterile filtrate control/attenuated control/ 2.4 x 10^6/7.9 x 10^4/1.8 x 10^6/3.4 x 10^6/7.3 x 10^6/2.0 x 10^7 mg/l (34 – 54 %)

Results (CFU/ml) related to measured concentrations

Time	End point	NOEC	LOEC	Effect (%) at LOEC	EC50
21 d	Mortality	7.9 x 10^5	1.8 x 10^6		1.6 x 10^6
21 d	Growth	7.9 x 10^5			
21 d	Reproduction	7.9 x 10^5			

Remarks: No signs of pathogenicity were observed. Mortality in the highest QST 713 Technical treatment group was 100 % after 21 days. Mortality in daphnids exposed to the attenuated control at a concentration equal to the highest QST 713 Technical treatment group was also 100 %. It cannot be decided, if the mortality observed was due to the physical nature of the test solutions (test solutions were turbid) or for example an effect of toxin-production.

valid: no. Although they were aerated, within some of the vessels the amount of dissolved oxygen decreased under 60 %. Taking that into account the reason for the observed effects is not clear.

GLP compliance: yes

Conclusion: The submitted test is not valid. However, independent of the reason of the observed effects the observed NOEC is considered to be a worst-case and therefore can be used for the risk assessment.

B.9.2.3 Risk assessment for aquatic organisms

The results of the additional studies described above indicate that QST 713 TP is of low toxicity to aquatic organisms. In addition to that no signs of infectivity were observed in the histological investigations.
The predicted initial environmental concentration in surface water were calculated taking into account the current Ganzelmeier drift rates. When applied during early growth stages in fruit crops (worst case), spray drift amounts to 29.2 % in a distance of 3 m from application. On the basis of the maximum application rate of 15 kg/ha (i.e. 1.5 kg as/ha), and giving an average of 5 x 10^9 CFU/g of Serenade, the resultant CFU-load per treated hectare is 7.5 x 10^{13} CFU/ha. This corresponds to an initial PEC_{sw} of 7,3 x 10^6 CFU/l (or 7,3 x 10^3 CFU/ml) in the top 30 cm water layer of lentic water bodies.

Table B.9.3-1 summarises TER-values calculated for a single application with the maximum application rate of 15 kg/ha (i.e. 1.5 kg as/ha).

Table B.9.3-1: QST 713 TP – Toxicity exposure ratios (TER) for aquatic organisms (single application of 1.5 kg as/ha; 29.2 % spray drift in a distance of 3 m; early growth stage in fruit crops)

Test species	Toxicity [CFU/ml]	Initial PEC_{sw} [CFU/ml]	TER	Annex VI Trigger
O. mykiss	$LC_{50;\,4d}$: 1.4 x 10^7	7,3 x 10^3	1918	100
D. magna	$NOEC_{21\,d}$: 7.9 x 10^5	7,3 x 10^3	108	10

The TER-values are above the relevant Annex VI trigger. In the grass shrimp *Palaemonetes pugio* only dietary exposure was tested. No mortality, no signs of pathogenicity or other effects on normal behaviour and development were observed at a dose rate of 3.7 x 10^6 CFU/g.

In conclusion, the overall risk to aquatic organisms is considered to be acceptable.

B.9.3 Effects on bees (Annex IIB 8.3; Annex IIIB 10.3)

B.9.3.5 Field test

Title: **Honey bee field study of Serenade biofungicide wettable powder in Alfalfa**

Author: Mayer, D.F. (2000)
BBA-Ref.-No.: BIE 2002-14
Test substance: Serenade WP (100 g/kg *Bacillus subtilis* strain QST 713)
Guideline: EPA Ecological Effects Test Guidelines
Test species: *Apis mellifera* L.
Testdesign: The test product was applied 6 times to flowering alfalfa field in intervals of 3-6 days using an amount of 11,21 kg Serenade WP/ha.
Evaluation criteria: Mortality flight intensity in the field, influence to the brood

Conclusions:
The test design is not harmonising with the principles of the EPPO-guideline 170, as there is no worst case situation for the test animals: The plots for test product, water control and toxic standard were situated close to each other, so that the bees were able to avoid the treated plots because of an eventual repellency of the test product and the toxic standard. Moreover the application was carried out in the early morning and not during the intensive bee flight. The test was performed according to GLP-prescriptions and is confirming the results of the laboratory test: Honeybees will not be set at risk by a practical use of *Bacillus subtilis*-containing products.

B.9.4 Effects on other arthropod species (Annex IIB 8.4, IIIB, 10.4)

The following additional studies have currently been made available by the notifier and were taken into account for the final risk assessment:

B.9.4.1 Acute toxicity, pathogenicity and infectivity (Annex IIB 8.4, Annex IIIB 10.4)

Laboratory tests

Parasitoids

Title:	***Bacillus subtilis* strain QST 713: A Dietary Pathogenicity and Toxicity Study With the Parasitic Hymenopterian (*Nasonia vitripennis*)**
Author:	Bryan et al. (2000)
BBA-Ref.-No.:	ANA2002-288
Test substance:	QST 713 Technical Powder, Lot # 54-63-4TP (5.25 x 10^{10} CFU/g)
Guideline:	Series 885 US EPA´s Microbial Pesticide Test Guidelines, OPPTS No. 885.4340 (1996)
Test species:	*Nasonia vitripennis*
Developmental stage:	Adults
Substrate:	Paper container (9 cm diameter, 9 cm high)
Exposure route:	oral uptake (cotton swab coated with diet at concentrations of 295, 1730, 10200, 60000 ppm, ad lib)
Exposure duration:	10 d

Results:

Application rate	Mortality[1)]	Sublethal effects[2)]
attenuated control	24.1 %	1.3 % immobile
sterile filtrate	13.0 %	1.3 % lethargic
295 ppm	1.9 %	1.3 % lethargic
1730 ppm	0 %	2.6 % lethargic, 1.3 % immobile
10200 ppm	25.9 %	4 % lethargic
60000 ppm	74.1 %	2.6 % lethargic, 1.3 % immobile

[1)] corrected using Abbott´s formula (total number of wasps per treatment 75, control mortality 28 %)
[2)] unadjusted incidental clinical signs

Remarks: The dietary LC50 value was determined to be 24739 ppm, the NOEC was 1730. The observed effects appeared to be a result of toxicity rather than pathogenicity, since mortality in the attenuated control group was observed to be app. equal to that in the 10200 ppm test group and since the 295 and 1730 ppm treatment groups showed no apparent treatment related mortality. Additionally, there were no apparent clinical signs typical of a disease process.

Validity:	positive
GLP compliance:	positive

B.9.4.2 Field tests (Annex IIIB 10.4)

Predatory mites

Title:	**Effects of Serenade WP on predatory mites (*Typhlodromus pyri*) under typical vine culture conditions on grape vines, Germany 2000**
Author:	Ipach, R. (2000a)
BBA-Ref.-No.:	ANA2002-286
Test substance:	Formulation Serenade WP *Bacillus subtilis* strain QST 713 ($\geq 10^6$ CFU/g; nominal 10^9 CFU/g)
Guideline:	Typhlodromus pyri (BBA-guideline 23-2.3.4, 1991)
Species:	*Typhlodromus pyri*
Developmental stage:	Field populations
Substrate:	Vineyard (starting on June at BBCH 68)
Exposure route:	direct spray and residue uptake
Exposure duration:	9th June (1st application) to 30th August (final check 28 d after 4th application)
Calculation of effects:	Reduction of abundance according to Henderson & Tilton

Results:

Field rates g/ha	Number of application	BBCH[1)]	Effects[2)] %
7575	1	68	n.a.
10395	2	73	20 (7 d after 2nd)
12255	3	77-79	n.a.
12120	4	81	16 (8 d after 4th)/17 (28 d after 4th)

[1)] acc. to Lorenz et al. 1994
[2)] corrected using Henderson & Tilton´s formula

Remarks: It needs to be mentioned that other fungicides were applied during the test period. However, effects less than 40 % indicate that four applications of Serenade WP at the recommended field rates are harmless to field populations of *Typhlodromus pyri*. The pairwise comparison of arithmetic means according to Scheffée showed no significant differences (α 0.05).

Validity:	positive
GLP compliance:	positive

Title:	**Effects of Serenade WP on predatory mites (*Typhlodromus pyri*) under typical vine culture conditions on grape vines, Germany 2000**
Author:	Ipach, R. (2000b)
BBA-Ref.-No.:	ANA2002-287
Test substance:	Formulation Serenade WP *Bacillus subtilis* strain QST 713 ($\geq 10^6$ CFU/g; nominal 10^9 CFU/g)
Guideline:	Typhlodromus pyri (BBA-guideline 23-2.3.4, 1991)

Species: *Typhlodromus pyri*
Developmental stage: Field populations
Substrate: Vineyard (starting on June at BBCH 65-68)
Exposure route: direct spray and residue uptake
Exposure duration: 9th June (1st application) to 4th September (final check 28 d after 4th application)
Calculation of effects: Reduction of abundance according to Henderson & Tilton

Results:

Field rates g/ha	Number of application	BBCH[1)]	Effects[2)] %
7556	1	65-68	n.a.
10579	2	73	27 (3 d after 2nd)
12225	3	77-79	n.a.
12645	4	81	33 (7 d after 4th)/25 (28 d after 4th)

1) acc. to Lorenz et al. 1994
2) corrected using Henderson & Tilton´s formula

Remarks: It needs to be mentioned that other fungicides were applied during the test period. However, effects less than 40 % indicate that four applications of Serenade WP at the recommended field rates are harmless to field populations of *Typhlodromus pyri*. The pairwise comparison of arithmetic means according to Scheffée showed no significant differences (α 0.05).

Validity: positive
GLP compliance: positive

Summary of arthropod toxicity data

Table B.9.4-1 Summary of arthropod toxicity data

Test material	Species	Stage	Rate[1)] ppm	Effects[2)] lethal %	sublethal %
	Parasitoids				
QST 713	*Nasonia vitripennis*	Adults	295	1.9	1.3
Technical Powder			1730	0	3.9
			10200	25.9	4
			60000	74.1	3.9

1) oral uptake (cotton swab coated with diet at concentrations of 295, 1730, 10200, 60000 ppm, ad lib)
2) Mortality corrected using Abbott´s formula (total number of wasps per treatment 75, control mortality 28 %), sublethal effects unadjusted incidental clinical signs

Summary of arthropod field and semi-field testing

Table B.9.4-2 Summary of arthropod field and semi-field testing

Test material	Species	Test	No. of applications	Field rate per application g/ha	Effect [1)] Final check %
	Predatory mites				
Serenade WP	*T. pyri*	Field	4	7575 -12120	16 / 17
Serenade WP	*T. pyri*	Field	4	7556 -12645	33 / 25

[1)] corrected using Henderson & Tilton´s formula

B.9.4.3 Risk assessment for non-target terrestrial arthropods

Risk assessment
Non-target arthropods are likely to be exposed to formulated *Bacillus subtilis* by direct spray, contact on fresh or dry residues. However, due to the nature of the microbial agent oral uptake which might occur via contaminated pollen, nectar and honey dew, prey or via host organisms was considered using a specific study design.

The field rates tested correspond to the intended uses given above. According to the data submitted a low oral toxicity was demonstrated in basic laboratory tests on the hymenopteran species *Nasonia vitripennis.* In field tests with *T. pyri* Serenade WP was harmless at recommended field rates to field populations.

It is therefore concluded, that the use of *Bacillus subtilis* as outlined in this monograph has no unacceptable influence on non-target arthropods, represented by species of two ecological groups.

B.9.5 Effects on earthworms (Annex IIB 8.5; Annex IIIB 10.6)

The objective of the study was the assessment of the acute toxicity of QST 713 WP (Serenade WP) to *Eisenia fetida*. For the assessment of the infectivity and pathogenicity of *Bacillus subtilis* strain QST 713 the acute study was combined with histopathological investigations.

Title:	**Acute Toxicity of QST 713 WP (Serenade WP) on Earthworms, *Eisenia Fetida* Using an Artificial Soil Test**
Author:	Stäbler, D. (2002)
BBA-Ref.-No.:	ARW2002-117
Test substance:	Formulation QST-00713-F-O-WP *Bacillus subtilis* 100 g/kg

Guideline: OECD 207
Test species: *Eisenia fetida*
Exposure duration: 14 d
Worms per treatment: 4 x 10
Conc. levels (nom): 0/100/178/316/562/1000 mg/kg

Findings:
LC50: > 1000 mg/kg
Lowest lethal conc.: > 1000 mg/kg

valid: yes
GLP compliance: yes

Documentation and Results of Histopathological Investigations

After the final assessment of the acute study, 14 days after application, three organisms per treatment group (0/100/178/316/562/1000 mg Serenade/kg soil) were selected for the histopathological investigations. Additionally one earthworm from the 562 mg/kg treatment group, which had died after study termination during the excretion, was investigated separately.

After excretion the earthworms were prepared for light microscopic examination of tissues belonging to the cerebral ganglion, the genital region and the clitellum.

Findings:
The investigated earthworms treated with 562 mg/kg or 1000 mg Serenade/ kg soil showed the following sublethal symptoms:

- Enormous increase of bacterial colonisation on the peritoneum and between the body wall musculature without, or not worth mentioning, immune reactions. With the technique used it was not possible to determine the species of the colonising bacteria.
- Degeneration of the nucleoli in the nucleus of the nerve cells in the cerebral ganglion and in the ventral nerve cord.
- Enlargement of the blood vessels, especially in the blood sinusoids.

The investigated earthworms treated with 316, 178, 100 and 0 mg Serenade/kg soil showed the following symptoms:

- Moderate colonisation by bacteria on the peritoneum and between the longitudinal muscle fibres with immune reactions
- No degeneration, especially nucleoli divisions or fragmentations, of the nucleus structure in the nerve cells
- No, or in some cases moderate enlargement of the blood vessels in the intestine.

The reason for the death of the one earthworm treated with 562 mg/kg soil could not be determined.

Conclusions
The investigator hypothesises that the enormous increase of bacterial colonisation in the test groups treated with 562 and 1000 mg Serenade/kg soil was due to a breakdown of the immune reactions. However, because the identity of the invading bacteria was not known it

was unclear whether the overflow with bacteria in these test groups was due to active invasion of *Bacillus subtilis* strain QST 713. The investigator further supposes that the observed increase in bacterial colonisation may have been due to malnourishment of the earthworms during the testing period (the earthworms were not fed for a period of 18 days except with the test substance). Even if these interpretations can not be verified from the results of the study described above it can be concluded that Serenade WP is of low acute toxicity to earthworms. Concerning the sublethal effects the NOEC was 316 mg Serenade/kg soil.

Considering that a rate of 50 % of the applied amount of product will reach the soil surface (maximum application rate: 16 x 15 kg/ha), the maximal predicted initial environmental concentration in soil is 160 mg/kg corresponding to 8 x 10^5 cfu/g soil. This value is most probably still overestimated, because it can be expected that part of the spores in the product will not start vegetative growth when reaching the ground (e.g. due to lack of fresh organic matter). Instead, these bacteria will remain in the metabolically inactive spore state. Furthermore it can be assumed that both vegetative cells and dormant spores of *Bacillus subtilis* strain QST 713 will be subject to natural competition within the saprophytic soil microflora and that cell numbers will decline.
Therefore the risk to earthworms is considered to be acceptable.

In addition Kristufek *et al.* (1993) showed that a high proportion of streptomycete strains isolated from earthworm guts (*Lumbricus rubellus* and *Octolasion montanum*, examined in autumn) produced antibiotics active against the Gram-positive *Bacillus subtilis*. No gut strains were, however, active against the Gram-negative *Escherichia coli*. This could circumstantiate that earthworms are adapted to Gram-positive bacteria like *B. subtilis* in their guts.

B.9.7 References relied on

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BBA registration number	Data protection claimed Y/N	Owner
IIB, 8.2	Drottar, K.R., Flaggs, R.S., Krueger, H.O.	2001	QST 713 Technical: A Five-Concentration Toxicity and Pathogenicity Test with the Rainbow Trout (*Oncorhynchus mykiss*) 489A-108 GLP unpubl. WAT2002-442	Y	QST
IIB, 8.2	Machado, M.W.	2001	QST 713 Technical Powder – Infectivity and Pathogenicity to Grass Shrimp (*Palaemonetes pugio)* during a 30-Day Static Renewal Test 13759.6101 GLP unpubl. WAT2002-446	Y	QST

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BBA registration number	Data protection claimed Y/N	Owner
IIB, 8.2	Drottar, K.R., Flaggs, R.S., Krueger, H.O.	2001	QST 713 Technical: A 21-Day Life-Cycle Toxicity and Pathogenicity Test with the Cladoceran (*Daphnia magna*) 489A-107A GLP unpubl. WAT2002-449	Y	QST
IIB 8.3	Mayer, D.F.	2000	Honey Bee Field Study of Serenade Biofungicide Wettable Power in Alfalfa GLP unpubl. BIE2002-14	Y	QST
IIB 8.4, IIIB, 10.4	Bryan et al.	2000	Bacillus subtilis strain QST 713: A Dietary Pathogenicity and Toxicity Study With the Parasitic Hymenopterian (*Nasonia vitripennis*) GLP unpubl. ANA2002-288	Y	QST
IIB 8.4, IIIB, 10.4	Ipach, R.	2000 a	Effects of Serenade WP on predatory mites (*Typhlodromus pyri*) under typical vine culture conditions on grape vines, Germany GLP unpubl. ANA2002-286	Y	QST
IIB 8.4, IIIB, 10.4	Ipach, R.	2000 b	Effects of Serenade WP on predatory mites (*Typhlodromus pyri*) under typical vine culture conditions on grape vines, Germany GLP unpubl. ANA2002-287	Y	*Agra Quest*
AIIB-8.5	Kristufek, V., Ravasz, K. & Pizl, V.	1993	Actinomycete communities in earthworm guts and surrounding soil Pedobiologia 37, 379-384 ARW 2002-167	N	-
AIIB-8.5 AIIIB-10.5	Stäbler, D.	2002	Acute Toxicity of QST 713 WP (Serenade WP) on Earthworms, Eisenia Fetida Using an Artificial Soil Test 20011062/01 ARW2002-117	Y	QST

Addendum 2

to the Draft Assessment Report

of 15 May 2001

(relating to Volume 1 + 3)

Bacillus subtilis

30 September 2005

<u>Rapporteur Member State: Germany</u>

Contents

To Volume 1: **1**

Level 2 Reasoned statement of the overall conclusions **1**

2.1 Identity 1
2.1.1 Name and species description 1
2.1.2 Biological properties of the micro-organism 1
2.3 Impact on human and animal health 2
2.3.1 Effects having relevance to human and animal health arising from exposure to the micro-organism or to impurities contained in the organism, its residual traces and metabolites 2
2.3.1.2 Genotoxicity 2
2.3.1.4 Short-term toxicity, pathogenicity and infectivity 2
2.6 Effects on non-target species 2
2.6.3 Effects on bees 2
2.6.5 Effects on earthworms 3

Level 3 Proposed decision with respect to the application for inclusion of the active substance in Annex I **4**

3.1 Background to the proposed decision 4
3.2 Proposed decision 4

To Volume 3: **5**

B.1 Identity **5**

B.1.1 Identity of the micro-organism 5
B.1.1.3 Name and species description (Annex IIB 1.3) 5
B.1.3 References relied on 7

B.2 Biological properties of the organism – Technical properties of the preparation **9**

B.2.1 Biological properties of the micro-organism (Annex IIB 2) 9
B.2.1.2 Information on target organism(s) (Annex IIB 2.2) 9
B.2.1.2.2 Mode of action (Annex IIB 2.2.2) 9
B.2.1.8 Information on the production of metabolites (especially toxins) Annex IIB 2.8) 11
B.2.1.9 Antibiotics and other anti-microbial agents (Annex IIB 2.9) 11
B.2.2 Physical, chemical and technical properties of the plant protection product (Annex IIIB 2) 12
B.2.3 References relied on 12

B.6 Toxicity, pathogenicity and infectivity **15**

B.6.1 Step I – Basic studies (micro-organism) (Annex IIB 5.1) 15
B.6.1.2 Genotoxicity testing (Annex IIB 5.2.3) 15
B.6.1.4 Short-term toxicity, pathogenicity and infectivity (Annex IIB 5.2.5.1) 15
B.6.12 References relied on 22

B.9 Ecotoxicology 24
B.9.3 Effects on bees (Annex IIB 8.3; Annex IIIB 10.3) 24
B.9.2 References relied on 27

To Volume 1:

Level 2 Reasoned statement of the overall conclusions

2.1 Identity

2.1.1 Name and species description

To distinguish the Bacillus subtilis strain QST 713 from other *B. subtilis* strains a molecular biological method for strain discrimination has been designed. The PvuII restriction enzyme in the ribotyping method demonstrates that it is a suitable tool for distinguishing *B. subtilis* strain QST 713 from strains in different subgroups as well as in the same subgroup, isolated from similar environments and with similar bioactivity characteristics.

2.1.2 Biological properties of the micro-organism

Mode of action
Additional references were submitted addressing the issue of secondary metabolites with antimicrobial activities produced by *B. subtilis* QST 713 and their role for its mode of action. The detected and discussed metabolites belong to the class of lipopeptides and include iturins, plipastatins and surfactins. More detailed information are included in Volume 3.

Presence of secondary metabolites
Secondary metabolites produced by *B. subtilis* strain QST 713 were characterised. The detected metabolites belong to the known class of lipopeptides and include iturins, plipastatins and surfactins.

The species *B. subtilis* has been reported to produce subtilisin, which may cause allergic or hypersensitive reactions. The QST 713 strain was tested for production of this compound. The content of subtilisin was < 1 µg/mL. The analysis was performed with only one production batch.

Information about the analysis of the metabolites is included in Volume 4 (Addendum 3).

Antibiotics and other anti-microbial agents
A susceptibility profile of antibiotics was established for *Bacillus subtilis* strain QST 713 (Lehmann, 2001). Nine antibiotics were used representative of multiple structural classes including ß-lactams, aminoglycosides, macrolids, glycopeptids, peptides, sulfonamides and of multiple modes of action (inhibition of cell wall synthesis, interference of protein synthesis and inhibition of folic acid synthesis). QST 713 proved susceptible to all antibiotics tested except bacitracin. The susceptibility especially to ampicillin, erythromycin and tetracyclin is very important since these antibiotics can be used in case of an human infection with the anthrax bacillus, *B. anthracis*. Therefore, medically relevant antiobitic resistance caused by the intended use of *B. subtilis* is not anticipated.

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 contained in the organism, its residual traces and metabolites

2.3.1.2 Genotoxicity

Bacillus subtilis produces several different secondary metabolites and two entries were found in the fermentation broth of *B. subtilis* strain QST 713: Surfactin and Iturin compounds are cyclic lipoheptapeptides and amphiphilic membrane-active biosurfactants and peptide antibiotics with potent antimicrobial activities. No genotoxicity studies have been performed. In the opinion of the RMS, there is no need to investigate mutagenicity of these secondary metabolites based on the facts, that no public literature reports are available on this well-studied Bacillus species indicating a genotoxic or carcinogenic hazard, whereas several reports demonstrated the harmlessness of *B. subtilis*: Neither toxic nor pathogenic actions against higher organisms were seen. Medical data confirms the low human health risk attributed to *B. subtilis*. Considering the low risk potential of *Bacillus subtilis* and its ubiquitous distribution, even in foods, genotoxicity testing appeared to be dispensable.

Cytotoxicity
The cytotoxic potential of another strain of *B. subtilis* (MB 1252) has been assessed and was tested as nontoxic to CHO-K1 cells. Additionally it was demonstrated that the industrially used strain of *B. subtilis* did not react with antibodies against *B. cereus* enterotoxins.

2.3.1.4 Short-term toxicity, pathogenicity and infectivity

An additional subchronic study in rats with a four-week exposure period by nose-only inhalation of Serenade biofungicide at a dose of 5 x10^8 cfu per animal per day revealed minor clinical signs and organ weight increases. It was shown that viable spores were no longer present in lungs and draining lymph nodes eight weeks after the last exposure. Due to the complete clearance from all organs within the recovery period, it is concluded that repeated exposure to viable spores of *B. subtilis* QST 713 via inhalation will not cause adverse health effects in humans.

2.6 Effects on non-target species

2.6.3 Effects on bees

A test on the dietary effects of QST 713 technical powder on larval honeybee development shows that *Bacillus subtilis* is not acting as pathogen in honeybees. No behavioural or morphological abnormalities in any treatment were observed. Additionally a review on published studies regarding the possible effects of *Bacillus subtilis* and other species of the genus Bacillus to honeybees was submitted. One laboratory study was done to estimate the toxic and pathogenic effects of QST 713 technical powder to honeybees during a 30-day exposure period. The LC_{50} was estimated at 8900 µg. A 30-day field study with Serenade wettable powder was conducted on a blooming alfalfa field. The results of the study

demonstrate that Serenade WP was non-toxic to honeybees. Another study was conducted to assess acute toxicity. The results of the test suggest that Serenade WPO is not more toxic to bees than 50 % sucrose.

The studies submitted confirm that there will be an acceptable risk for honeybees when *Bacillus subtilis* products are applied as intended.

2.6.5 Effects on earthworms

A study on the effects on earthworms has been submitted and is described in Addendum 1. From this study it can be concluded that the risk to earthworms can be considered acceptable.

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

3.1 Background to the proposed decision

Identity
The additional submitted data concerning strain discrimination of B. subtilis QST 713 by ribotyping demonstrates that it is possible to distinguish *B. subtilis* QST 713 from other strains of the same species.

Biological properties of the micro-organism
With morphological and physiological methods it is possible to differentiate *B. subtilis* from the pathogenic *Bacillus* species *B. anthracis, B. cereus* and *B. licheniformis*.

Toxicology
The available data on toxicology, pathogenicity and infectivity allow a risk evaluation of *Bacillus subtilis* (strain QST 713) in humans. It can be concluded that there is no evidence of any adverse health effects in humans under proposed conditions of use.

Ecotoxicology
From the studies submitted it can be concluded that *Bacillus subtilis* QST 713 poses an acceptable risk to honeybees.
A study on the effects on earthworms has been submitted and is described in Addendum 1. From this study it can be concluded that the risk to earthworms can be considered acceptable.

3.2 Proposed decision

Based on these additional data, it is now proposed to include *Bacillus subtilis* (strain QST 713) in Annex I of Directive 91/414/EEC.

To Volume 3:

B.1 Identity

B.1.1 Identity of the micro-organism

B.1.1.3 Name and species description (Annex IIB 1.3)

A study in Addendum 1 to the Draft Assessment Report describes strain discrimination of *Bacillus subtilis* QST 713 by ribotyping (Lehman, 2002). This study, a comparison of two QST 713 samples with five other *B. subtilis* strains, was not considered to be comprehensive enough to confirm that ribotyping using restriction enzyme PvuII is an acceptable system for distinction among closely related *B. subtilis* strains.

Therefore, an additional ribotyping study has been carried out to address this concern (Anonymous 2004, BWS2005-13). The results are summarised in a statement by Heins (2004). Besides *B. subtilis* QST 713, a total of 13 strains were selected based on geographic location of isolation, phenotypic activity of the strain and potential relatedness. The selected strains belonged to 3 subgroups of *B. subtilis* (e. g. *B. subtilis* subsp. a*myloliquefaciens*). They comprised three competitor strains with activity as biofungicides or plant growth enhancers used in the agricultural field, strains with industrial applications such as production of enzymes and a strain used as feed supplements. Furthermore, they comprised strains isolated from soil (like *B. subtilis* strain QST 713) and from a crop plant (soybean). Using the restriction enzyme PvuII, the 14 strains separated into 12 unique ribogroups. *B. subtilis* strain QST 713 did not cluster with any of the other 13 strains. The similarity index reported by the contract laboratory compared the 13 strains to QST 713 on a scale of 0 - 1 where 1.0 is a 100 % match. The ribogroup closest to QST 713 had a similarity index of only 0.4 (40 % similarity). Therefore, this second study demonstrates that ribotyping using PvuII restriction enzyme is a suitable method for distinguishing *B. subtilis* QST 713 from strains in different subgroups and in the same subgroup, isolated from similar environments and with similar bioactivity characteristics. Together, Lehman (2002) and Anonymous (2004, BWS2005-13) studied a total of 19 strains with this method, and QST 713 was distinguished as a unique strain.

Report: Anonymous (2004)
Documentation related to sample group NG-091853 prepared for AgraQuest Inc., 1530 Drew Avenue, Davis, CA 95616, U.S.A, Lancaster Laboratories, 2425 New Holland Pike, Lancaster, PA 17605, U.S.A - unpublished

Guideline: Not stated

Acceptability: The study is considered to be acceptable.

Material and Methods:
14 strains of *Bacillus subtilis* originating from AgraQuest Inc. or the American Type Culture Collection (ATCC) were provided as agar slant samples and compared in a ribotyping analysis with the commercial RiboPrinter® System (DuPont Qualicon, Wilmington, U.S.A.) for automated rRNA operon ribotyping. Ribotyping involves Southern blotting of digested chromosomal DNA of the organism of interest, probing with the *E. coli* rRNA operon, and conducting a computer analysis of the resulting patterns. These patterns were compared to a

database for identification and to other strains for strain differentiation. The restriction enzyme PvuII was used to generate different patterns to further distinguish strains as it was found effective in separating *B. subtilis* strain QST 713 from other *B. subtilis* strains in a previous study. The following strains were subjected to the analysis (Table B.1.1.3-1):

Table B.1.1.3-1: Description of the samples

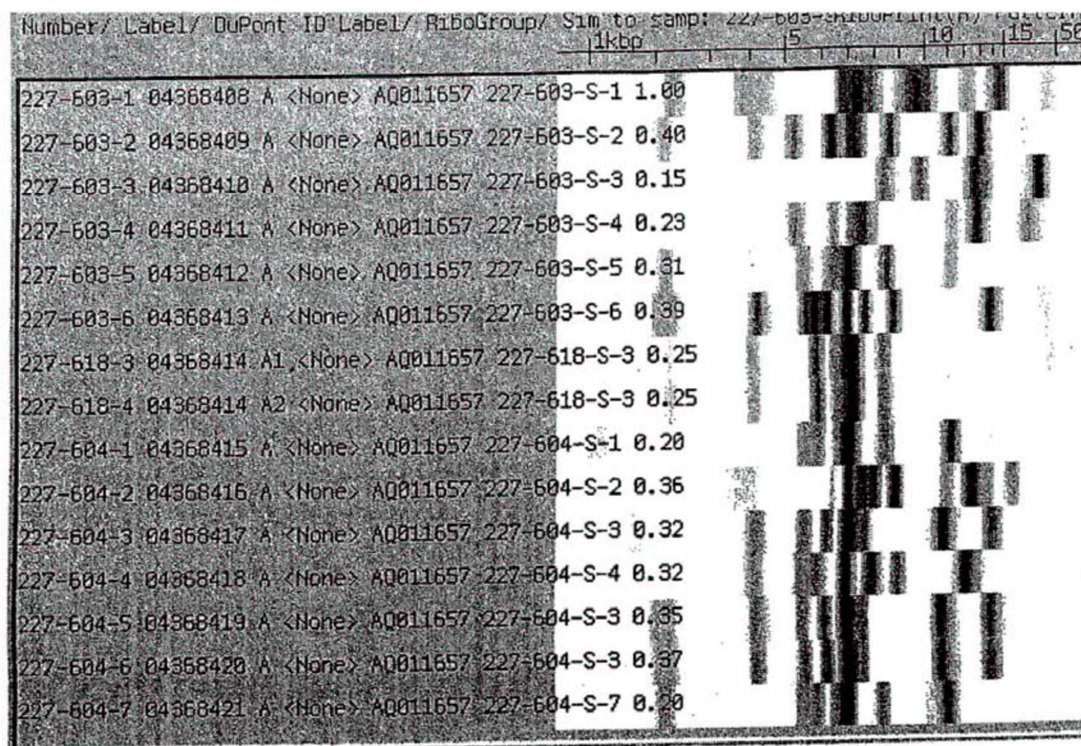
Sample description	Lancaster Lab Number	Comments / Applications
713 R2 *Bacillus subtilis*	NS-04368408	
Competitor #1 *Bacillus subtilis*	NS-04368409	
Competitor #2 *Bacillus subtilis*	NS-04368410	
Competitor #3 *Bacillus subtilis*	NS-04368411	
ATCC 7003 *Bacillus subtilis*	NS-04368412	Isolation: soil
ATCC 23059 *Bacillus subtilis*	NS-04368413	Isolation: soil, produces isoprene
ATCC 465 *Bacillus subtilis*	NS- 04368414	
ATCC 23856 *Bacillus subtilis*	NS-04368415	Produces isoprene
ATCC 10783 *Bacillus subtilis*	NS-04368416	
ATCC 27505 *B. subtilis* subsp. *amyloliquefaciens*	NS-04368417	Isolation: soybean
ATCC 13933 *Bacillus subtilis*	NS-04368418	Isolation: soil, produces aterrimin and feed supplements
ATCC 15818 *Bacillus subtilis*	NS-04368419	
ATCC 6633 *Bacillus subtilis* subsp. *spizizenzii*	NS-04368420	enzymes
ATCC 6051 *Bacillus subtilis* subsp. *subtilis*	NS-04368421	Produces isoprene

Confidential standard operating procedures (LL Test Methods 5781 and 2815) were used for organism identification which are similar to the methodology used in the previous ribotyping study (Lehman 2002, BWS2002-2).

Based on the resulting banding patterns, strains were divided into ribogroups due to their similarities. A similarity index value was computed to express the relative relatedness of the other 13 *B. subtilis* strains to *B. subtilis* strain QST 713. 1.0 on the index means a 100 % match on the scale of 0 - 1.

Findings:
The panel of 14 strains tested separated into 12 unique ribogroups (”RiboGroup”) using the PvuII restriction enzyme (Figure 1.1.3-1). *B. subtilis* strain QST 713 did not cluster with any of the other 13 strains tested. The calculated similarity index value (”Sim to samp”) was found to be 0.4 for the closest related strain ”Competitor #1 *Bacillus subtilis* (Lab No. 04368409)”, indicating a 40 % similarity to QST 713.

Figure B.1.1.3-1: Ribotyping analysis of 14 *Bacillus subtilis* strains with the Ribo-Printer® system for automated rRNA operon ribotyping using the restriction enzyme PvuII.



Conclusions:
The PvuII restriction enzyme in the ribotyping method demonstrates that it is a suitable tool for distinguishing *B. subtilis* strain QST 713 from strains in different subgroups as well as in the same subgroup, isolated from similar environments and with similar bioactivity characteristics.

B.1.3 References relied on

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BBA registration number	Data protection claimed Y/N	Owner
AIIB-1	Anonymous	2004	Documentation related to sample group NG-0914853 prepared for AgrarQuest. Report-no. not stated not GLP, unpublished BWS2005-13	Y	QST
AIIB-1	Heins, S.	2004	Comparison of *Bacillus subtilis* QST 713 to additional *Bacillus subtilis* strains by Ribotyping using the enzyme PvuII. Report-no. not stated not GLP, unpublished BWS2005-12	Y	QST

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BBA registration number	Data protection claimed Y/N	Owner
AIIB-1	Lehman, L.	2002	Strain discrimination of *Bacillus subtilis* QST 713 by Ribotyping. Statement Report-no. not stated not GLP, unpublished BWS2002-2	Y	QST

B.2 Biological properties of the organism – Technical properties of the preparation

B.2.1 Biological properties of the micro-organism (Annex IIB 2)

B.2.1.2 Information on target organism(s) (Annex IIB 2.2)

B.2.1.2.2 Mode of action (Annex IIB 2.2.2)

Several references addressing the issue of secondary metabolites with antimicrobial activities produced by *B. subtilis* QST 713 and their role for its mode of action, were submitted (e.g. Kunst et al., 1997, BWS2005-24, BERDY et al., 1985, BWS2005-27).

Secondary metabolites produced by strain QST 713 were characterised (Manker, 2001, BWS2005-32). The detected metabolites belong to the known class of lipopeptides and include iturins, plipastatins and surfactins. According to the CRC Handbook of Antibiotic Compounds (Berdy et al., 1985, BWS2005-27), there are 72 compounds reported to be produced by *B. subtilis*. However, many of the entries are from the 1950's and 60's and therefore have little or no spectroscopic or other characteristic data. Sometimes an entry simply refers to a documented activity (e.g. 00000-4954 lists only the producing organism, *B. subtilis*, with antifungal activity). In these cases, there is not enough information to determine whether the compounds responsible for the reported activity are identical to compounds characterised later or to those produced by the *B. subtilis* strain QST 713. However, two of the entries were reported from the fermentation broth of *B. subtilis* strain QST 713 (Manker, 2001, BWS2005-32). One is iturin-A, (43140-1750 in Berdy et al., 1985) which is also known as bacillomycin B, eumycin, bacillomycin-R, bacillomycin and fungocin. The second is surfactin (44230-1960 in Berdy et al., 1985). The iturinic compounds differ from surfactin by their lower surfactant properties. Surfactin showed no inhibitory activity against *Saccharomyces cerevisiae* however it has been reported that the antifungal activity of iturin-A was enhanced by the presence of surfactin (Thimon et al., 1992).

There are multiple modes of action of *B. subtilis,* as outlined in the monograph of strain QST 713 (B.2.1.2.2) and discussed by Alabouvette & Lemanceau (1998). They include competition, colonisation, induction of biochemical resistance responses in the plant and inhibition of plant pathogens by secondary metabolites.

An additional reference submitted summarises results of the sequencing of the full genome of *B. subtilis* strain 168 (Kunst et al., 1997). The authors identified over 4000 putative protein-coding sequences. However, using protein analysis software, only 58 % of the protein coding sequences could be assigned a known function based on at least one significant counterpart from protein databases. The other potential gene products remain unidentified. Kunst et al. (1997) also found that approximately 4 % of the genome codes for large multifunctional enzymes with similarity to those involved in the synthesis of antibiotics in other genera of Gram-positive bacteria such as *Streptomyces*. According to the authors, the production of antibiotics such as surfactin, fengycin and difficidin can be linked to the gene loci coding for the above mentioned multifunctional enzymes.

Further reports on the mode of action/activity against bacteria such as *Erwinia amylovora,* the causal agent of fire blight, were summarised by Rumbolz (2004, BWS2005-14). Farabee and

Lockwood (1958) co-isolated a yellow bacterium with *E. amylovora* from fire blight cankers. Comparison of inhibitory activity in buffered and unbuffered media showed that *Bacterium* sp. inhibited *E. amylovora* by increasing the acidity of culture media to a degree unfavourable for growth of the pathogen. Abo-el-Dahab and El Goorani (1964) co-isolated *B. subtilis* with *E. amylovora*. Sterile culture filtrates of the *B. subtilis* isolate grown on sucrose nutrient broth resulted in the production of an antibacterial substance that yielded significant inhibition of growth of *E. amylovora*. Efficacy was comparable to standard commercial antibiotics like streptomycin. More recent studies reported *in-vitro* antibacterial activities of *B. subtilis* strains against *Streptomyces scabies*. Culture filtrates of the *B. subtilis* strain FZB24 exhibited a relatively high antibacterial activity against phytopathogenic bacteria such as *Agrobacterium tumefaciens, Clavibacter* sp. and *Xanthomonas* sp. but not against *Erwinia* spp. and certain *Pseudomonas* spp. (Krebs et al., 1998). Sharga and Lyon (1998) showed *in-vitro* and *in-vivo* activity of *B. subtilis* against *E. carotovora.* The inhibitory spectrum of one *B. subtilis* strain even comprises some human pathogens such as *E. coli*, *Staphylococcus aureus* and *Salmonella typhimurium* besides phytopathogenic bacteria (Földes et al., 2000). Also in these cases, production of antibiotic compounds was considered to be responsible for the antimicrobial effect.

B. subtilis strains differ in the composition of metabolites, although some classes of compounds, such as the biologically active lipopeptides, seem to be common in this *Bacillus* species. Secondary metabolites produced by the *B. subtilis* strain QST 713 have been analysed and determined to belong to the known class of lipopeptides, including the iturins, plipastatins and surfactins (Manker, 2001, BWS2005-32).

Surfactin, a cyclic lipopeptide antibiotic and biosurfactant produced by *B. subtilis* strains including QST 713, is well-known for its interactions with artificial and biomembrane systems (e.g., bacterial protoplasts or enveloped viruses). A study by Vollenbroich et al. (1997) determined the cytotoxicity of surfactin and observed an improvement in proliferation rates and changes in the morphology of mycoplasma-contaminated mammalian cells after treatment with this drug. Disintegration of the mycoplasma membranes as observed by electron microscopy indicated the mode of action of surfactin. Disintegration is obviously due to a physicochemical interaction of the membrane-active surfactant with the outer part of the lipid membrane bilayer, which causes permeability changes and at higher concentrations leads finally to disintegration of the mycoplasma membrane system by a detergent effect. The low cytotoxicity of surfactin for mammalian cells permits specific inactivation of mycoplasmas without significant deleterious effects on cell metabolism and the proliferation rate in cell culture.

Similar to the mode of action of surfactin, a general mechanism for the antibacterial action of peptides was proposed by Lin et al. (2001). These authors cloned a *B. subtilis* gene encoding for a peptide that inhibits growth of *Xanthomonas oryzae pv. oryzae* and postulated that many microbial peptides share two unique characteristics in that they are polycationic and amphipatic. This enables these molecules to bind to negatively charged lipopolysaccharides, insert into the negatively charged cytoplasmic membrane of bacteria, permeabilise the outer and cytoplasmic membranes and cause bacterial cell death. Due to the structural similarities (see Manker, 2001, BWS2005-32), iturins and plipastatins as the other subgroups of cyclic lipopeptides produced by *B. subtilis* strain QST 713 may act as biomembrane-active molecules in a similar fashion and account for the activity of the micro-organism against *E. amylovora*.

B.2.1.8 Information on the production of metabolites (especially toxins) Annex IIB 2.8)

Secondary metabolites produced by *B. subtilis* strain QST 713 were characterised (Manker, 2001, BWS2005-32). The detected metabolites belong to the known class of lipopeptides and include iturins, plipastatins and surfactins. For details regarding the structure of the lipopeptides and method of analysis see confidential addendum.

The species *B. subtilis* has been reported to produce subtilisin, which may cause allergic or hypersensitive reactions. The QST 713 strain was tested for production of this compound (Manker, 2002, BWS2002-6). The content of subtilisin was < 1 µg/mL. The analysis was performed with only one production batch. For details of the method see confidential addendum.

Additional studies
In order to assess effects on yeasts and lactobacilli, additionally several fermentation trials done with grapes treated with the preparations Serenade WP and Serenade AS were submitted. The results of these studies indicate, that the preparations and the potentially secondary metabolites of *B. subtilis* QST 713 had no adverse effects on the fermentation kinetics in which yeasts and sometimes lactobacilli (*Leuconostoc oenos*) were involved (Bloy, 2002; Grivot, 2000; Anonymous, 2001, BWS2005-33; Anonymous, 2002, BWS2005-34).

B.2.1.9 Antibiotics and other anti-microbial agents (Annex IIB 2.9)

A susceptibility profile of antibiotics was established for *Bacillus subtilis* **strain QST 713** (Lehmann, 2001). Nine antibiotics were used representative of multiple structural classes including ß-lactams, aminoglycosides, macrolids, glycopeptids, peptides, sulfonamides and of multiple modes of action (inhibition of cell wall synthesis, interference of protein synthesis and inhibition of folic acid synthesis). QST 713 proved susceptible to all antibiotics tested except bacitracin. The susceptibility especially to ampicillin, erythromycin and tetracyclin is very important since these antibiotics can be used in case of an human infection with the anthrax bacillus, *B. anthracis*. The lack of susceptibilty to bacitracin is not a surprising result, since bacitracin is a peptide antibiotic produced by *Bacillus licheniformis*, a closely related organism to *B. subtilis*, and strain QST 713 produces similar peptide secondary metabolites. Therefore, medically relevant antiobitic resistance caused by the intended use of *B. subtilis* is not anticipated.

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

Table B.2.2: Summary of the physical, chemical and technical properties of the plant protection product

Section (Annex point)	Study	Method	Results	Comment/ Conclusion	Reference
B.2.2.2.1 (IIIB 2.2)	Effects of light, temperature and humidity		The test substance QST 713 WP was determined to be stable for 3 years when stored at warehouse (ambient) conditions. The titer of QST 713 WP was between 1.2×10^{10} to 4.7×10^{10} on TSA medium.	Acceptable	Gingras (2001) PHY2002-297

B.2.3 References relied on

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BBA registration number	Data protection claimed Y/N	Owner
AIIB-2.2.2	Abo-El-Dahab and El-Goorani, M.A.	1964	Antagonistic effect of a *Bacillus subtilis* strain upon *Erwinia amylovora*, Vol. 54, 1285-1286. Report-no. not stated not GLP, published BWS2005-16	N	-
AIIB-2.2.2 AIIB-2.8 AIIB-2.9	Berdy et al.	1985	CRC Handbook of Antibiotic Compounds. CRC Press, Boca Raton, FL, U.S.A. not GLP, published BWS2005-27	N	-
AIIB-2.2.2	Farabee, G.J. and Lockwood, J.L.	1958	Inhibition of *Erwinia amylovora* by bacterium sp. isolated from fire blight cankers. Phytopathology, Vol. 48, 209-211 not GLP, published BWS2005-15	N	-
AIIB-2.2.2	Földes, T., Banhegyi, I., Herpai, Z., Varga, L. and Szigeti, J.	2000	Isolation of *Bacillus subtilis* strains from the rhizosphere of cereals and *in vitro* screening for antagonism against phytopathogenic, food-borne pathogenic and spoilage micro-organisms. Journal of Applied Microbiology, 89, 840-846 not GLP, published BWS2005-19	N	-

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BBA registration number	Data protection claimed Y/N	Owner
AIIB-2.2.2	Krebs, B., Höding, B., Kübart, S., Alemayehu Workie, M., Junge, H., Schmiede-Knecht, G., Grosch, R., Bochow, H. and Hevesi, M.	1998	Use of *Bacillus subtilis* as biocontrol agent. I. Activities and characterisation of *Bacillus subtilis* strains. Journal of Plant Diseases and Protection, 105 (2), 181-197 not GLP, published BWS2005-17	N	-
AIIB-2.2.2	Kunst, F. et al.	1997	The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature, Vol. 390, 249-256 not GLP, published BWS2005-24	N	-
AIIB-2.2.2	Lin, D., Qu, L.-J., Gu, H. and Chen, Z.	2001	A 3.1-kb genomic fragment of *Bacillus subtilis* encodes the protein inhibiting growth of *Xanthomonas oryzae pv. Oryzae*. Journal of Applied Microbiology 91, 1044-1050 not GLP, published BWS2005-21	N	-
AIIB-2.2.2 AIIB-2.8	Manker, D.C.	2001	Chemical Characterisation of QST 713. not GLP, unpublished BWS2005-32	Y	QST
AIIB-2.2.2	Rumbolz, J.	2004	Serenade WP (*Bacillus subtilis*, strain QST 713) - Expert Statement - not GLP, unpublished BWS2005-14	Y	QST
AIIB-2.2.2	Sharga, B.M. and Lyon, G.D.	1998	*Bacillus subtilis* BS 107 as an antagonist of potato blackleg and soft rot bacteria. Canadian Journal of Microbiology (44), 777-783 not GLP, published BWS2005-18	N	-
AIIB-2.2.2	Thimon, L., Maget-Dana, R. and Michel, G.	1992	Surface active properties of antifungal lipopeptides produced by *Bacillus subtilis*. JAOCS, Vol. 69 (1), 92-93 not GLP, published BMF2000-144	N	-
AIIB-2.2.2	Vollenbroich, D., Pauli, G., Özel, M. and Vater, J.	1997	Antimycoplasma properties and application in cell culture of surfactin, a lipopeptide antibiotic from *Bacillus subtilis*. Applied and Environmental Microbiology, 63(1), 44-49 not GLP, published BWS2005-20	N	-

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BBA registration number	Data protection claimed Y/N	Owner
AIIB-2.8	Anonymous	2001	Side effects of Serenade (as *B. subtilis* QST 713) on Fermentation and Sensorial Properties of Wine - Translation and summary of studies performed in 1999 - 2001. GLP, unpublished BWS2005-33	Y	QST
AIIB-2.8	Anonymous	2002	Side effects of Serenade (as *B. subtilis* QST 713) on Fermentation and Sensorial Properties of Wine - Translation and summary of studies performed in 2000 - 2002. GLP, unpublished BWS2005-34	Y	QST
AIIB-2.8	Bloy, C.	2002	Study of unintentional effects of experimental fungicide compounds "Serenade WP" and "Serenade AS" on production and quality of must and wines. Report-no. VCE 0001 GBI not GLP, unpublished BWS2005-35	Y	QST
AIIB-2.8	Grivot, S.	2000	Etude des effets non intentionnels de produits phytopharmaceutiques (fongicides) sur lè laboration et la qualite des vins. not GLP, unpublished BWS2005-36	Y	QST
AIIB-2.8	Manker, D.C.	2002	Analysis of Serenade for Presence of Subtilisin. not GLP, unpublished BWS2005-6	Y	QST
AIIB- 2.9.	Lehman, L.	2001	Antibiotic Susceptibility Testing of *Bacillus subtilis* QST 713. AgraQuest, Inc., 1530 Drew Avenue, Davis, CA 95616, U.S.A Report-no. not stated not GLP, unpublished	yes	QST
AIIIB-2.2	Gingras	2001	Storage stability of Qst 713 strain of dried *Bacillus subtilis* with residual fermentation media identified as Qst 713 WP. Final Report Project No. L08726 SN9 GLP, unpublished PHY2002-297	Y	QST

B.6 Toxicity, pathogenicity and infectivity

B.6.1 Step I – Basic studies (micro-organism) (Annex IIB 5.1)

B.6.1.2 Genotoxicity testing (Annex IIB 5.2.3)

Bacillus subtilis produces several different secondary metabolites and two entries were found in the fermentation broth of *B. subtilis* strain QST 713: Surfactin and Iturin compounds are cyclic lipoheptapeptides, which contain a ß-hydroxy fatty acid and a ß-amino fatty acid, respectively, as lipophilic moiety and a heptapeptide as hydrophilic component. Both are amphiphilic membrane-active biosurfactants and peptide antibiotics with potent antimicrobial activities.
No genotoxicity studies have been performed. In the opinion of the RMS, there is no need to investigate mutagenicity of these secondary metabolites based on following facts:
Taking into account its ubiquitous distribution, even in foods, genotoxicity testing appears to be dispensable.
There are no structural moieties of Surfactin and Iturin lipopeptides which suggest that these lipopeptides may induce direct mutagenicity, e.g. point mutations, frameshift mutations, or clastogenicity.
Furthermore, no public literature report are available on this well-studied Bacillus species indicating a genotoxic or carcinogenic hazard, whereas several reports demonstrated the harmlessness of *B. subtilis*: neither toxic nor pathogenic actions against higher organisms were seen (Boer & Diderichsen, 1991, U.S. EPA, 1997). According to the U.S. EPA, *B. subtilis* does not appear to have specialised attachment mechanisms typically found in organisms capable of colonising the human body.
Medical data confirms the low human health risk attributed to *B. subtilis*. Reviewing clinical cases and referring to GRAS petitions, which couldn´t demonstrate any invasive properties of B. subtilis it was concluded that *B. subtilis* is a safe host for the production of harmless products (Boer & Diderichsen, 1991).
Furthermore, this species is listed in Class 1 Containment of European Federal Law of Biotechnology (Frommer et al., 1989).
Recently Daptomycin (Cubicin), a lipoproteine which is structurally closely related to the lipopeptides found in *B. subtilis* QST 713, was approved by U.S. FDA as a human therapeutic for the treatment of complicated skin and skin structure infections.

Cytotoxicity
The cytotoxic potential of another strain of *B. subtilis* (MB 1252) has been assessed in Chinese hamster ovary (CHO-K1) cells (Pedersen et al., 2002). The strain was used in the production of industrial enzyme products. The *B. subtilis* strain tested was nontoxic to CHO-K1 cells. Additionally it was demonstrated that industrially used strain of *B. subtilis* did not react with antibodies against *B. cereus* enterotoxins.

B.6.1.4 Short-term toxicity, pathogenicity and infectivity (Annex IIB 5.2.5.1)

Bacillus subtilis has been isolated in some cases of food poisoning and from human infections in very few patients with a compromised immune status (B.6.4.3). Given the production of toxicologically relevant substances and the slow clearance of *Bacillus subtilis* spores from some rat tissues after intratracheal and intravenous administration, a repeated dose inhalation study was required in this special case.

Report: Arts, J.H.E. and Tap, S.H.M. (2004): Sub-acute (4-week) inhalation toxicity study, including an 8-week recovery study, with Serenade Biofungicide in rats
TNO, Location Zeist, Utrechtseweg 48, 3700 AJ Zeist, The Netherlands. Report No. V 5435 (Dates of experimental work: 12/18/2003 to 04/29/2004), unpublished.

Guideline: OECD Guideline for Testing of Chemicals, No. 412 (May 1981); EC Guideline B.8, EEC Directive 92/69/EEC; OPPTS Guideline No. 885.3600

Deviations: None

GLP: Yes

Acceptability: The study is considered to be acceptable.

Materials and methods:
Test item: Serenade Biofungicide (technical powder, approx. 1.4 x 10^{10} cfu of *B. subtilis* strain QST 713/g, batch 8AQ07D20.
The inhalation toxicity of Serenade Biofungicide was studied in groups of 16 male and 16 female, 7-9 weeks-old Sprague Dawley rats (Charles River Deutschland, Sulzfeld, Germany), exposed in nose-only inhalation chambers to control air or to a nominal concentration of 0.35 mg/L test item for six hours a day on five days a week during 28 days, with a total of 20 exposure days. A control group of 16 male and female rats was exposed to clean air.

The concentration level of 0.35 mg/L was based on a preceding 5-day pilot test with a exposure of 0.65 mg/L to a group of 2 male and 2 female rats who showed reduced body weight/body weight gain, changes in breathing pattern and other clinical signs (hunched back posture, reduced activity, tremor) during the exposure period. No abnormalities were observed at necropsy one week after the last exposure. Based on the clinical signs it was decided to reduce the target concentration in the main study to 0.35 mg/L (or 350mg/m³). Taking into account the concentration of the test substance (i.e. approximately 1.4 x 10^{10} cfu/g), a number of 0.35 x 1.4 x 10^{10} = 0.49 x 10^{10} cfu/m³ air was generated. In 6 hours an animal will inhale about 100 litres of air (taking into account a ventilation of 250 mL/min), indicating a number of about 5 x 10^8 cfu (external dose) per animal per day. This is above the required dose of 1 x 10^8 units of MCPA (microbial pest control agent) administered per day according to the OPPTS 885.3600 guideline.

Two male and two female rats, each of the control and treated group, were necropsied prior to exposure, on day 1 after the last exposure and every four weeks later up to 8 weeks after exposure. As *B. subtilis* was not detected in any organs/tissues eight weeks after the last exposure, the recovery period lasted eight instead of 24 weeks in deviation of the study plan. Clinical signs, body weights, food consumption and food conversion efficiency were determined prior, during and after exposure. In addition, a full necropsy was performed and selected organs (lungs, spleen, thymus, lymph nodes draining the respiration organs and the intestines) were removed for organ weight determinations and for microbiological examinations.

Analytical results:

In the main study, the mean actual concentration (± standard deviation) of the test item in the test atmospheres was 350 ± 28 mg/m³. The mean nominal concentration was 769 ± 73 mg/m³, indicating a generation efficiency of 46 %. The average mass Median Aerodynamic Diameter and the geometrical standard deviation of the particles was 2.6 µm ± 2.1.

Microbiological analysis:

Microbiological analysis was performed using the spread plating on triplicates. The microbiological purity of the test substance was determined in triplicates on TSA and SDA. An average colony count of 1.8 x 10^{10} cfu/g on TSA was found. Neither bacterial, nor fungal contaminants were detected. The test substance suspension was homogeneous on TSA/P plates. The recovery of the test substance (number of viable bacteria) was determined on TSA and TSA/P. The rate was depended on the amount of inoculum. High inoculum (estimated at 7 x 10^3 cfu/mL suspension) revealed a mean recovery rate of 33 % for viable colonies in the lung suspensions (n = 4) compared with obtained colony counts of samples without organ before blending of the samples, whereas post-blended organ suspensions produced a recovery rate of 16 %. It cannot be excluded, that blending of the organ tissues exerted an negative effect on the viability of the spores. In contrast, low inoculum (ca. 70 cfu/mL) showed recovery rates of 25 % in pre-blended organ suspensions and 22 % in samples after blending.

Findings:

Clinical findings:

No treatment-related clinical signs were observed before, during and after exposure. Mean body weight gain was statistically significantly reduced only in male animals during the 4-week exposure period but recovery was seen thereafter (Table B.6.1.4-1).

Table B.6.1.4-1: Mean body weights (g)

	Males		Females	
	control	test	control	test
Day 0	292.2	288.5	209.6	208.6
Day 7	316.4	304.3*	216.7	217.2
Day 14	341.8	324.0*	230.7	227.3
Day 28	374.0	345.3**	237.2	232.4
Day 56	477.1	470.3	274.6	273.4
Day 84	535.8	531.5	291.6	289.5

* $p < 0.05$
** $p < 0.01$

Food consumption was significantly increased in males on day 49; food conversion efficiency was statistically significantly decreased in males on day 7 and 14 but increased on days 35 and 42 (Table B.6.1-2). No significant changes were observed in females.

Table B.6.1.4-2: Mean food conversion efficiency (g weight gain/g food consumed)

	Males		Females	
	control	test	control	test
Day 7	0.13	0.08	0.03	0.04
Day 14	0.16	0.13*	0.11	0.08
Day 28	0.07	0.02	0.00	-0.01
Day 35	0.17	0.25**	0.12	0.12
Day 42	0.16	0.18**	0.06	0.08
Day 56	0.11	0.11	0.04	0.05
Day 84	0.07	0.08	0.06	0.03

* $p < 0.05$
** $p < 0.01$

Necropsy:
No treatment-related macroscopic changes were observed at any of the necropsy periods.

Organ weights:
At week 4 (day 28), rats were necropsied one day after the last exposure. Compared with the control animals, the test groups showed increased absolute and relative weights of the lungs, mediastinal lymph nodes in both sexes (each n = 2) and of the mesenterial and jugular lymph nodes in males. The increases of the lungs were more pronounced in males than in females. No conspicuous changes were seen in thymus and spleen weights.
Four weeks later (day 56), the absolute weights of the lungs in male and females rats were still increased when compared with the control rats killed at the same time. However, this effect is not obvious when compared with the control animals at day 84. Additionally, the relative weight was not affected.
Weights of mediastinal lymph nodes were almost similar in control and test animals, but the relative weights of the cervical lymph nodes were significantly increased in males of the test group.
Finally, eight weeks after the 28-day exposure period (day 84), the absolute lung lobe weight of one male was increased and the mesenterial lymph node of each one male and female revealed higher values compared with the control.
The organ weights are listed in Table B.6.1.4-3 and Table B.6.1.4-4. Since only 2 animals of each sex were sacrificed and examined at each scheduled time, all changes must be considered with some reservation and any statistical evaluation was seen by the RMS as irrelevant.

Table B.6.1.4-3: Organ weights (absolute(relative)) in male rats (single data)

	Rat No.	Body weight (g)	Mes.Ln g (%)		Med.Ln g (%)		Jug.Ln g (%)		Cerv.Ln g (%)		Lungs g (%)	
		g	g	%	g	%	g	%	g	%	g	%
Day 0	Control 2	274.1	0.149	0.544	0.015	0.055	0.028	0.102	0.08)	0.274	1.74	6.35
	Control 4	264.2	0.129	0.488	0.012	0.045	0.015	0.057	0.08	0.310	1.58	5.99
	Test 34	275.9	0.082	0.297	0.011	0.040	0.012	0.043	0.06	0.217	1.51	5.46
	Test 36	256.7	0.158	0.616	0.011	0.043	0.013	0.051	0.08	0.3	1.32	5.13
Day 28	Control 6	357.4	0.163	0.456	0.013	0.036	0.012	0.034	0.08	0.227	1.27	3.55
	Control 8	333.1	0.123	0.369	0.014	0.042	0.023	0.069	0.05	0.138	1.26	3.79
	Test 38	348.0	0.204	0.586	0.059	0.170	0.042	0.121	0.1	0.290	2.13	6.12
	Test 40	313.4	0.210	0.670	0.046	0.147	0.085	0.271	0.15	0.491	2.55	8.13
Day 56	Control 10	429.9	0.173	0.402	0.015	0.035	0.029	0.067	0.06	0.140	1.47	3.42
	Control 12	451.7	0.160	0.354	0.025	0.055	0.040	0.089	0.07	0.153	1.40	3.10
	Test 42	427.2	0.250	0.585	0.032	0.075	0.025	0.059	0.09	0.215	1.80	4.22
	Test 44	465.3	0.126	0.271	0.011	0.024	0.033	0.071	0.09	0.191	1.81	3.90
Day 84	Control 14	450.6	0.183	0.406	0.024	0.053	0.034	0.075	0.08	0.173	1.81	4.01
	Control 16	563.9	0.181	0.321	0.021	0.037	0.049	0.087	0.10	0.179	1.91	3.39
	Test 46	507.9	0.290	0.571	0.035	0.069	0.040	0.079	0.11	0.219	1.85	3.64
	Test 48	508.9	0.178	0.350	0.011	0.022	0.027	0.053	0.06	0.124	2.26	4.44

Table B.6.1.4-4: Organ weights (absolute(relative)) in female rats (single data)

	Rat No.	Body weight	Mes.Ln g (%)		Med.Ln g (%)		Jug.Ln g (%)		Cerv.Ln g (%)		Lungs g (%)	
		g	g	%	g	%	g	%	g	%	g	%
Day 0	Control 1	177.9	0.090	0.506	0.012	0.067	0.015	0.084)	0.06	0.360	1.23	6.89
	Control 3	195.4	0.226	1.157	0.009	0.046	0.016	0.082	0.07	0.358	1.50	7.66
	Test 33	179.1	0.101	0.564)	0.011	0.061	0.017	0.095	0.09	0.480	0.94	5.24
	Test 35	188.1	0.148	0.787	0.009	0.048	0.027	0.144	0.06	0.340	1.03	5.48
Day 28	Control 5	206.1	0.165	0.801	0.010	0.049	0.014	0.068	0.08	0.364	0.99	4.81
	Control 7	229.1	0.215	0.938	0.016	0.070	0.023	0.100)	0.07	0.297	0.99	4.34
	Test 37	219.3	0.130	0.593	0.071	0.324	0.025	0.114	0.09	0.397	1.78	8.10
	Test 39	209.7	0.116	0.553	0.051	0.243	0.026	0.124	0.05	0.253	1.55	7.37
Day 56	Control 9	270.4	0.160	0.592	0.027	0.1	0.029	0.107	0.12	0.433	1.23	4.55
	Control 11	249.2	0.198	0.795	0.011	0.044	0.031	0.124	0.07	0.256	1.15	4.62
	Test 41	276.1	0.117	0.424	0.022	0.080	0.026	0.094	0.10	0.359	1.51	5.46
	Test 43	250.9	0.152	0.606	0.016	0.064	0.026	0.104	0.09	0.339	1.54	6.16
Day 84	Control 13	269.0	0.173	0.643)	0.023	0.086	0.059	0.219	0.07	0.253	1.45	5.41
	Control 15	303.7	0.140	0.461	0.018	0.059	0.030	0.099	0.09	0.286	1.50	4.94
	Test 45	273.0	0.203	0.744	0.028	0.103	0.035	0.128	0.12	0.440	1.42	5.21
	Test 47	259.6	0.150	0.578	0.028	0.108	0.022	0.085	0.06	0.250	1.41	5.45

Microbiological findings:

No *B. subtilis* was detected in organs or tissues of the control or test animals before the start of exposure. On the day after the 4-week exposure period, the numbers of *B. subtilis* in the lungs of the test animals were ca. 1×10^5 - 7×10^5 cfu/mL organ suspension (Table B.6.1-5). In the mediastinal lymph nodes, amounts of *B. subtilis* were ca. 100 - 280 cfu/mL tissue suspension. In the remaining organs/tissues (cervical, internal jugular plus posterior, superior mesenteric lymph nodes, and spleen and thymus), no or low numbers of colonies of *B. subtilis* were detected. In control animals, colonies with the typical colony morphology of *B. subtilis* in a considerably lower amount compared with the lungs of the test group animals were observed.

Four weeks after the last exposure, a low number of *B. subtilis* colonies (ca. 20 cfu/mL organ suspension) were detected only in the lungs of one of four test group animals. *B. subtilis* was not detected in any of the other organs/tissues.
Eight weeks after the last exposure, *B. subtilis* was not detected in any of the organs/tissues of the test group animals. Therefore, no measurements were carried out at the other scheduled time points after the last exposure.

Table B.6.1.4-5: The presence of viable micro-organisms* (*Bacillus subtilis* QST 713) in organ suspensions of the control and test group after the 4-week exposure period (Wk 4).

TNO Codes Animals	Colony counts (cfu [1)]/mL organ suspension)							Colony counts (cfu[1)]/mL organ suspension) after pasteurisation [3)]						
	SPL [2)]	THY	MED	IJP	MES	CER	LL	SPL	THY	MED	IJP	MES	CER	LL
Group A - control group														
5	< 10	< 10	< 10	< 10	< 10	< 10	< 10	Not determined						
6	< 10	< 10	< 10	< 10	ca. 10-200	< 10	< 10							
7	< 10	< 10	< 10	< 10	< 10	< 10	10^2-10^3							
8	< 10	< 10	< 10	< 10	< 10	< 10	+ [4)]							
Group B - test group														
37	+ [5)]	+ [5)]	1.4x 10^2	< 10	< 10	+ [5)]	5.7x 10^5	< 10	+ [5)]	ca. 80	< 10	< 10	ca. 40	7.0x 10^5
38	+ [5)]	< 10	1.5x 10^2	ca. 10	+ [5)]	ca. 20	4.4x 10^5	ca. 20	< 10	+ [5)]	ca. 20	< 10	ca. 20	5.2x 10^5
39	+ [5)]	ca. 20	2.8x 10^2	ca. 20	< 10	+ [5)]	1.5x 10^5	ca. 20	ca. 50	1.8x 10^2	ca. 10	ca. 10	ca. 60	6.6x 10^5
40	< 10	< 10	1.0x 10^2	+ [5)]	< 10	ca. 20	7.2x 10^5	ca. 40	+ [5)]	1.5x 10^2	ca. 20	ca. 10	ca. 10	5.6x 10^5

* viable micro-organism: counts of colonies which had the typical colony morphology of the *Bacillus subtilis* test substance

1) cfu = colony forming units

2) SPL = Spleen, THY = Thymus, MED = Mediastinal lymph nodes, IJP = Internal jugular plus posterior lymph nodes, MES =Mesenteric lymph nodes, CER = Cervical lymph nodes. LL = Lung lobes

3) Pasteurisation at 65 °C for 30 minutes

+ [4)] colonies with typical colony morphology of the *Bacillus subtilis* test substance were present, but the outcome of the decimal dilution series was irregular, possibly caused by clumping factors. Therefore, no (range of) colony count(s) could be calculated, but the numbers found in the lungs of the animal of the control group were considerably lower than the numbers found in lung lobes of the animals from the test group.

+ [5)] colonies with typical colony morphology of the *Bacillus subtilis* test substance were present, but the outcome of the decimal dilution series was irregular, possibly caused by clumping factors. Therefore, no (range of) colony count(s) could be calculated, but the numbers found in the examined organ were considerably lower than the numbers found in lung lobes of the animal.

Discussion:
The nose-only inhalation of Serenade Biofungicide evoked presence of viable spores in lungs and draining lymph nodes. The increases in organ weights indicated a physiological, immunological reaction to the spores accompanied by a decreased body weight gain in males, but no other clinical signs. Eight weeks after the last exposure, viable spores could not longer been detected in lungs and draining lymph nodes. Therefore, the study was finished. However, minor observations on organ weights at day 84 could indicate residual immunological reactions to a low level of spores which were might still present in the organs but could not grow on TSA. This assumption could support the slow clearance seen in the acute intratracheal toxicity study.

Conclusion:
In this study, minor clinical signs and organ weight increases caused by the nose-only inhalation of Serenade biofungicide showed, that *Bacillus subtilis* is of low health risk by inhalation and is eliminated from the body at least after 8 weeks.

B.6.12 References relied on

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not	Data protection claimed Y/N	Owner
AIIB-5.2.3	Pedersen, P.B., Bjornvad, M.E., Rasmussen, M.D. and Petersen, J.E.	2002	Cytotoxic potential of industrial strains of *Bacillus SP.* not applicable Regul. Toxicol. Pharmacol., Vol. 36, pp. 155-161 Report-no. not applicable not GLP, published	N	-
AIIB-5.2.3	Boer de, A.S. and Diderichsen, B.	1991	On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: A review. not applicable Appl. Microbiol. Biotechnol., Vol. 36, pp. 1-4 Report-no. not applicable not GLP, published	N	-
AIIB-5.2.3	Frommer, W., Archer, L. and Brunius, G.	1989	Safe Biotechnology III. Safety precautions for handling Microorganisms of different classes. not applicable Appl. Microbiol. Biotechnol., Vol. 30, pp. 541-552 Report-no. not applicable not GLP, published	N	-
AIIB-5.2.3	U.S.EPA	1997	Final decision document, TSCA section 5 (H) (4) exemption for Bacillus subtilis+ Attachment 1. not applicable EPA-TOX Report-no. not applicable not GLP, published	N	

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not	Data protection claimed Y/N	Owner
AIIB-5.2.5.1	Arts, J.H.E. and Tap, S.H.M.	2004	Sub-acute (4-week) inhalation toxicity study, including an 8-week recovery study, with Serenade Biofungicide in rats. TNO, AJ Zeist, Netherlands AgraQuest, Inc., 1530 Drew Avenue, Davis, CA 95616, U.S.A Report-no. 5435 GLP, unpublished	Y	QST

B.9 Ecotoxicology

B.9.3 Effects on bees (Annex IIB 8.3; Annex IIIB 10.3)

Report: Jimenez, D.R. and Richards, K.B. (2003): Evaluation of the Dietary Effect(s) of QST 713 Technical Powder on Larval Honeybee Development (*Apis mellifera* L.). Study Number: CAR 158-03.

BVL-Reg.-No.: BIE2005-76

Testguideline: EPA Assessment Guidelines, Series 885 - Microbial Pesticide Testguidelines, Group D - Non-target Organisms and Environmental Expression Test Guidelines, OPPTS Number 885 4380, Honeybee Testing, Tier I

GLP compliance: Yes

valid: Yes

Test design:
First study:

Treatment	Concentration (ppm)	Volume per cell (µL)
1. QST 713 TP 004 with LBD *	100 000	10
2. QST 713 TP 004 with LBD *	10 000	10
3. QST 713 TP 004 with LBD *	1000	10
4. control: LBD	-	10
5. reference substance dimethoate with LBD *	5	10
6. untreated control	mapped only	-

* LBD = Larval Bee Diet

Second study:

Treatment	Concentration (ppm)	Volume per cell (µL)
1. QST 713 TP 004 with LBD *	100 000	10
2. reference substance dimethoate in combination with LBD *	100	10
3. reference substance dimethoate in combination with LBD *	5	10
4. control substance: LBD * only	-	10
5. untreated control	mapped only	-

*LBD = Larval Bee Diet

Test procedure:
A preliminary range finding test was performed with 5 concentrations of the test substance including as reference substance the microbial insecticide *Bacillus thuringiensis* var. kurstaki and an untreated control. Test substance and reference substance were applied in the same concentration. The survival rates for the test substance at the day of capping were 90 % at 10 ppm, 95 % at 100 ppm, 95 % at 1000 ppm, 90 % at 10 000 ppm and 75 % at 100 000 ppm. The test was performed in two runs (first study and second study) using 4 replicates per concentration with 20 larvae per replication.

Findings:
First study:

Treatment	Number capped cells day 6	Survival day 6 (%)	Number emerged day 24	Survival day 24 (%)
1. QST 713 TP 004 with LBD * at 100 000 ppm	13.50	67.50	13.00	65.00
2. QST 713 TP 004 with LBD * at 10 000 ppm	13.25	66.25	13.25	66.25
3. QST 713 TP 004 with LBD * at 1000 ppm	16.25	81.25	16.25	81.25
4. control: LBD	19.25	96.25	19.00	95.00
5. dimethoate with LBD * 5 ppm	19.25	96.25	19.25	96.25
6. untreated control	16.75	83.75	16.75	83.75

* LBD = Larval Bee Diet

Second study:

Treatment	Number capped cells day 7	Survival day 7 (%)	Number emerged day 22	Survival day 22 (%)
1. QST 713 TP 004 with LBD * at 100 000 ppm	10.50	52.50	10.50	52.50
2. dimethoate with LBD * at 100 ppm	1.75	8.75	1.75	8.75
3. dimethoate with LBD * 5 ppm	18.25	91.25	18.25	91.25
4. control: LBD	19.50	97.50	19.50	97.50
5. untreated control	17.0	85.0	17.0	85.0

It is impossible to estimate accurate LD_{50} values as the mortality rates never exceed 50 %. Moreover it is impossible to find out the exact weight of the tested larvae. Based on the analysis of the collected data the predicted LD_{50} will be 94 000 ppm. A calculation of an LD_{50} in mg/kg based on an average weight of 0.2 µg and the final pupae weight of 140 mg would produce values between 4 700 000 and 6714 mg/kg.

Conclusion:
The tests show that *Bacillus subtilis* is not acting as pathogen in honeybees. No behavioural or morphological abnormalities were observed in any treatment in all tests. The study confirmed that there will be no risk for honeybees when *Bacillus subtilis* products are applied as intended.

Report: Jimenez, D.R. (2004): Safety of the *Bacillus subtilis* - based biofungicide, Serenade, to the honeybee, *Apis mellifera* L. - A report about published studies regarding the possible effects of *Bacillus subtilis* and other species of the genus *Bacillus* to honeybees.

BVL-Reg.-No.: BIE2005-77

Former investigations have shown that different Bacillus species (i.e. *B. subtilis, B. cereus , B. polymyxa, B. macerans, B. brevis, B. pulvifaciens, B. circulans, B. pantothenticus, B. firmus, B. alvei. B. alterosporus, B coagulans, B. pumilus and B. licheniformis*) are commonly associated with honeybees. The mentioned *Bacillus* species have been found in the digestive tract of healthy workers and a similar microflora is inhabiting queens. It can assumed that also the greater wax moth (*Galleria mellonella*) and Varroa mite are inhabited by similar micro organisms.
On the other hand some authors have described an insecticidal activity associated with B. subtilis and a nematicidal activity from broths if *B. subtilis, B. pumilus and B. cereus*. To address the potential toxicity and pathogenicity of Serenade (*B. subtilis*, variety A Q 713) to honeybees several studies have been conducted.
The objective of a laboratory study was to estimate the toxic and pathogenic effects of QST 713 technical powder to honeybees during a 30-day exposure period. In this test 3 concentrations of QST 713 technical powder (60 000, 6000 and 600 ppm) were administered to the bees. The test was terminated prematurely as the untreated control exceeded 20 % mortality on day 5. The LC_{50} was estimated at 8900 µg.
Because of the premature termination of the feeding test a 30-day field study with Serenade wettable powder was conducted on a blooming alfalfa field. The test was designed as a worst case situation: Serenade WP was applied 6 x at 10 lb/ acre (corresponds to 11.2 kg/ha) in the 30-day period. The results of the study demonstrated that Serenade WP was non-toxic to honeybees, even under these severe conditions.
Another study was conducted to assess a potential acute toxicity. The study was performed as a semi-field study. Colonies of standard size were administered a 50 % sucrose solution containing 336 g Serenade WPO (186 000 ppm) in an in-hive feeder. One colony was treated with 50 % sucrose solution containing 159 mL dimethoate 4E and 3 colonies received 2 L uncontaminated sucrose solution. The test was performed with a 2nd run 2 weeks later using a volume of 3.8 L instead of 2 L. For the assessment of dead bees and larvae all colonies had dead bee traps over a period of 75 days. The results of the test suggest that Serenade WPO is not more toxic to bees than 50 % sucrose.

Conclusion:
One finding of the test was that high concentrations of *B. subtilis* make the feeding suspensions unpalatable for the larvae. At concentrations of 116 000 ppm the larvae rejected the food and the cells were completely cleaned out. This indicates that the reduction of surviving larvae/bees in feeding tests is not depending on toxic properties but on the changing behaviour of the larvae (refuse of food uptake).
With a NOEL between 10 000 and 100 000 ppm for honeybee larvae the toxicity and pathogenicity of *B. subtilis* is considered negligible. The overall exposure level and effect in honeybee colonies is dependent on biopesticide deposition rates on the crop flowers, on colony foraging, and reproductive dynamics at the time of exposure. It is unlikely that foraging adults will transport sufficient contaminated nectar and pollen back to the colony and expose adults, juveniles, or the queen through trophyllaxis. Attendant workers will process stored nectar and pollen - a process that functions as a biological filter to protect subsequent generations. *B. subtilis* is known to exist in this ecological niche as a part of the natural

microflora. From the work presented in this review it should be concluded that Serenade® poses an insignificant risk to honeybees when applied at appropriate field rates.

Report: Jimenez, D.R. (2004): Discussion of the Results of Honeybee Studies Conducted with QST 713 Technical and Serenade Products.

BVL-Reg.-No.: BIE2005-91

In this paper the same studies are discussed as in the report BIE2005-77. The compiled studies demonstrate a minimal threat to honeybees when larvae are offered acute doses and when whole colonies are presented a chronic dose.

Several additional studies were submitted (Shimanuki, H. et al., 1978; Shabanov, M. et al., 1983; Krieg, A., 1973; Cano, R.J. et al., 1994; Gilliam, M. et al., 1976; Gilliam, M., 1978; Gilliam, M. et al., 1990; Gilliam, M., 1985; Reynaldi, F.J. et al, 2004; Gokte, N. et al, 2004; Heins, S.D. et al., 2004; Assie, L.K., et al., 2002; Peng, C.Y.-S., et al., 1992; Pfleeger, T.G. et al, 1996). These studies are not of relevance for assessing the risk of Bacillus subtilis to honeybees.

B.9.2 References relied on

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BBA registration number	Data protection claimed Y/N	Owner
IIB, 8.3 IIIB, 10.3	Jimenez, D.R. and Richards, K.B.	2003	Evaluation of the dietary effect(s) of QST 713 technical powder on larval honeybee development (*Apis mellifera L.*). CAR 158-03 GLP, unpublished BIE2005-76	Y	QST
IIB, 8.3 IIIB, 10.3	Jimenez, D.R.	2004	Safety of the *Bacillus subtilis* - based biofungicide, Serenade®, to the honeybee (*Apis mellifera L.*). not GLP, unpublished BIE2005-77	Y	QST
IIB, 8.3 IIIB, 10.3	Jimenez, D.R.	2004	Discussion of the results of honeybee studies conducted with QST 713 technical and Serenade® products. not GLP, unpublished BIE2005-91	Y	OST
IIB, 8.3	Shimanuki, H. and Cantwell, G.	1978	Diagnosis of honeybee diseases, parasites and pests. ARS-NE 87, pp 1-3 not GLP, published BIE2005-78	N	-

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BBA registration number	Data protection claimed Y/N	Owner
IIB, 8.3	Shabanov, M. and Balabanov, V.A.	1983	Aspergilosis in bees in Bulgaria and it's causes. Acta Microbiologica, Vol. 12, pp 77-82 not GLP, published BIE2005-79	N	-
IIB, 8.3	Krieg, A.	1973	Über die toxische Wirkung von *Bacillus cereus*- und *Bacillus thuringiensis*-Kulturen auf die Honigbiene (*Apis mellifera*). Zeitschrift f. Pfl.krankh. und Pfl.sch., Vol. 80, pp 483-486 not GLP, published BIE2005-80	N	-
IIB, 8.3	Cano, R.J. et al.	1994	Bacillus DNA in fossil bees: An ancient symbiosis?. Applied and Enviromental Entomology, Vol. 60, pp 2164-2167 not GLP, published BIE2005-81	N	-
IIB, 8.3	Gilliam, M. and Valentine, D.K.	1976	Bacteria isolated from the intestinal contents of foraging worker honey bees *Apis mellifera*: The genus *Bacillus*. Journal of Invertebrate Pathology, Vol. 28, No. 2, pp 275-276 not GLP, published BIE2005-82	N	-
IIB, 8.3	Gilliam, M.	1978	Bacteria belonging to the genus *Bacillus* isolated from selected organs of queen honey bees, *Apis mellifera*. Journal of Invertebrate Pathology, Vol. 31, pp 389-391 not GLP, published BIE2005-83	N	-
IIB, 8.3	Gilliam, M., Buchmann, SL., Lorenz, B.J. and Schmalzel, R.J.	1990	Bacteria belonging to the genus *Bacillus* associated with three species of solitary bees. Apidologie (1990) 21, 99-105 not GLP, published BIE2005-193	N	-
IIB, 8.3	Gilliam, M.	1985	Microbes from Apiarian Sources: *Bacillus* spp. in frass of the greater wax moth. Journal of Invertebrate Pathology, Vol. 45, pp 218-224 not GLP, published BIE2005-84	N	-

Annex point/ reference number	Author(s)	Year	Title source (where different from company) report no. GLP or GEP status (where relevant), published or not BBA registration number	Data protection claimed Y/N	Owner
IIB, 8.3	Reynaldi, F.J., De Giusti, M.R. and Alippi, A.M.	2004	Inhibition of growth of *Ascospaera apis* by *Bacillus* and *Paenibacillus* strains isolated from honey. Revista Argentina de Microbiologia, Vol. 36 no. 1, pp 52-55 not GLP, published BIE2005-85	N	-
IIB, 8.3	Gokte, N. and Swarup, G.	2004	On the potential of some bacterial biocides against root-knot and cyst. Indian Journal of Nematology, Vol. 18, pp. 152-153 not GLP, published BIE2005-86	N	-
IIB, 8.3	Heins, S.D., Manker, D.C., Jimenez, D.R., McCoy, R.J., Marrone, P.G. and Orjala, J.E.	2004	Strain of *Bacillus* for controlling plant diseases and corn rootworm. US Patent 6,291425 B1 not GLP, published BIE2005-87	Y	-
IIB, 8.3	Assie, L.K., Deleu, M., Arnaud, L., Paquot, M., Thonart, P., Gaspar, C. and Haubruge, E.	2002	Insecticide activity of surfactins and iturins from from a biopesticide *Bacillus subtilis* Cohn (S499 Strain). Med. Fac. Landbouww Univ. Gent 67/3 not GLP, published BIE2005-88	N	-
IIB, 8.3	Peng, C.Y.-S., Mussen, E., Fong, A., Montague, M.A. and Tyler, T.	1992	Effects of chlortetraycline of honey bee worker larvae reared *in vitro*. Journal of Invertebrate Pathology Vol. 6 pp, 127-133 not GLP, published BIE2005-89	N	-
IIB, 8.3	Pfleeger, T.G., Fong, A., Hayes, R., Ratsch, H. and Wickliff, C.	1996	Field evaluation of the EPA (Kenaga) nomogram, a method for estimating wildlife exposure to pesticides residues on plants. Environmental Toxicology and Chemistry Vol. 15, pp. 535-543 not GLP, published BIE2005-90	N	-

Addendum 3

to the Draft Assessment Report

of 15 May 2001

(relating to Volume 4)

Bacillus subtilis

confidential

30 September 2005

<u>Rapporteur Member State: Germany</u>

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