BASELINE DOSSIER

Bacillus subtilis QST 713

Microbial pest control agent against plant pathogenic fungi and bacteria

Dossier according to OECD guidance for industry data submissions for microbial pest control products and their microbial pest control agents – August 2006

Summary documentation, Tier II

Annex IIM, Section 6

Point IIM 8; Effects on non-target organisms

revised

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Applicant

Bayer CropScience AG

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Introduction

This document summarizes all data submitted for the initial evaluation of *Bacillus subtilis* QST 713 as an active substance under Directive 91/414. Data provided in the initial dossier and in subsequent additional submissions are listed chronologically under the respective data point according to the OECD dossier guidance (2006).

This document is further named as **"Baseline Dossier"** since it presents all data previously submitted.

IIM 8 Effects on non-target organisms

IIM 8.1 Effects on birds

EU-Dossier: Doc M-IIB, Point 8.1

Report : IIM 8.1/01 [redacted]. (1998): *Bacillus subtilis*: An avian oral pathogenicity and toxicity study in the northern bobwhite; [redacted]., unpublished; Project No. 489-101; dates of experimental work: May 15, 1998 – August 18. 1998.

Document No: M-473475-01-1

Guideline: EPA Microbial Pesticide Test Guidelines OPPTS 885-4050.
Corresponds generally to SETAC – Society of Environmental Toxicology and Chemistry, 1995: Procedures for Assessing the Environmental Fate and Ecotoxicity of Pesticides

GLP: Yes (self certification by the laboratory)

Materials and Methods: QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No. 8AQ07D6; titer: 2×10^{10} cfu/g)
Test substance was suspended in reverse-osmose water and administered directly into the crop or proventriculus of male and female northern bobwhite (30 birds) at a total volume of 10 mL/kg b.w. and a daily dosage of 1×10^8 cfu/g of b.w. for 5 days. Observations were recorded twice daily.

Findings: One treatment related mortality occurred within the treatment group of 30 birds, being noticed on Day 1. Necropsy findings were non-specific, normal progression of autolysis due to enduring exposure to relatively high room temperature over night.
During the dosing period (5 days) additional 7 of the 30 birds temporarily showed acute clinical signs, including depression, loss of coordination, inability to stand, a ruffled appearance, reduced reaction to external stimuli, slight wing droop, shallow and rapid respiration. One bird continued to display intermittant or persisting clinical signs like feather loss and subcutaneous emphysema – partly ascribed to a head injury.
Post-dosing another four birds showed symptoms - mainly gaping and coughing (3 birds of the control group temporarily displayed these symptoms as well), one bird exhibited a ruffled appearance, wing droop and lethargy, later ventral head curl appeared.
There were no treatment related effects on body weight or feed consumption and no evidence for pathogenicity or replication of *B. subtilis* at gross necropsy.
LD_{50}: > 1×10^8 cfu/g b.w. per day (for 5 days). The LD_{50} could not be calculated since 50% mortality was not obtained.
The **NOEL** could not be calculated due to above mentioned signs of toxicity/mortality.

Conclusions: High oral doses of *B. subtilis* can cause unspecific clinical symptoms and exceptionally mortality in susceptible individual bobwhite, without evidence for pathogenicity. Relating to Serenade™ WP, on a weight basis the oral LD_{50} corresponds to > 2000 mg a.i./kg b.w.

IIM 8.2 Effects on fish

EU-Dossier: Doc M-IIB, Point 8.2.1

Report : IIM 8.2/01 [redacted]. (1998a): *Bacillus subtilis*: a five-concentration toxicity and pathogenicity test with the rainbow trout (*Oncorhynchus mykiss*); [redacted], unpublished; Project No. 489A-101; dates of experimental work: May 5, 1998 – August 18, 1998.

Document No: M-473642-02-1

Guideline: EPA-Pesticide Assessment Guidelines, OPPTS 885.4200 and ASTM (American Society for Testing and Materials) Standard E729-88a
Corresponds generally to EEC C1, Directive 92/69/EEC (deviations: exposure duration 30 days instead of 4, *additional* dietary exposure) and to OECD guideline 204 (applying to chemical substances).

GLP: Yes (self certification by the laboratory)

Materials and Methods: QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No. 8AQ07D6; reported titer: 2 × 10^{10} cfu/g)
The test substance was added to well water and 10 rainbow trout per treatment group were exposed to initial concentrations of 52, 86, 144, 240 and 400 mg/L (corresponding to ~1 × 10^9- 8 × 10^9 cfu/L) – test solutions were renewed 3 times/week during the 30 day exposure. Additionally, fish in all treatment groups received a diet of trout chow containing 736 mg test substance/kg (~ 1,47 × 10^{10} cfu/kg). Observations were recorded daily.
Findings: After 30 days of exposure rainbow trout mortality in the 144, 240 and 400 mg/L treatment groups was 30, 100 and 100% respectively.
LC_{50} = 162 mg/L corresponding to 3,24 × 10^9 cfu/L (with 95% confidential limits of 86 and 240 mg/L or 1,72 × 10^9 and 4,8 × 10^9 cfu/L)
No-Mortality Concentration and NOEC were: 86 mg/L or in terms of *c*olony *f*orming *u*nits: 1,72 × 10^9 cfu/L
Gross necropsy at the end of the test showed no signs of infection in gill, intestine or muscle tissue.
Conclusions: The LC_{50} value exceeds ~ 160 times the limit value for toxic or adverse effects (according to the EC directive 67/548/EEC). Therefore QST 713 TP can be evaluated as non- toxic to rainbow trout and there are no classification or labelling requirements.

Included under 3rd Additional Submission

Report:	KIIM 8.2/02; [redacted]; 2001; M-473492-01-1
Title:	QST 713 technical: A five-concentration toxicity and pathogenicity test with the rainbow trout (Oncorhynchus mykiss)
Report No.:	489A-108
Document No.:	M-473492-01-1
Guideline(s):	U.S. Environmental Protection Agency Series 885 - Microbial Pesticide Test Guidelines OPPTS Number 885.4200
Guideline deviation(s):	not specified
GLP/GEP:	**yes**

Report:	KIIM 8.2/03; [redacted]; 2001; M-473476-01-1
Title:	QST 713 technical powder - Infectivity and pathogenicity to grass shrimp (Palaemonetes pugio) during a 30-day static renewal test
Report No.:	13759.6101
Document No.:	M-473476-01-1
Guideline(s):	OPPTS Guideline 885.4280
Guideline deviation(s):	not specified
GLP/GEP:	**yes**

Report:	KIIM 8.2/04; [redacted]; 2001; M-473458-02-1
Title:	QST 713 technical: A 21-day life-cycle toxicity and pathogenicity test with the cladoceran (Daphnia magna)
Report No.:	489A-107A
Document No.:	M-473458-02-1
Guideline(s):	U.S. Environmental Protection Agency Series 885 - Microbial Pesticide Test Guidelines OPPTS Number 885.4240
Guideline deviation(s):	not specified
GLP/GEP:	**yes**

Studies on acute toxicity and/or pathogenicity and infectivity to freshwater fish ([redacted] et al., 2001*a*), acute toxicity to the freshwater invertebrate *Palaemonetes pugio* ([redacted], 2001), chronic (21 d) toxicity to *Daphnia magna* ([redacted] et al. 2001*b*) were submitted in June 2002 and are cited

in Addendum 1 to the Monograph (date of issue: 04.12.2002) and a risk assessment was performed. In conclusion, the overall risk to aquatic organisms is considered to be acceptable.

As a conclusion of the ECCO Working Group Evaluation Meeting on 26.03.2003, it was stated that these data requirements are fulfilled.

IIM 8.3 Effects on aquatic invertebrates

EU-Dossier: Doc M-IIB, Point 8.2.2.1 and Point 8.2.2.2

Acute toxicity study

Report : IIM 8.3/01 [redacted]. (1998b): *Bacillus subtilis*: a 48 hour static acute toxicity test with the cladoceran (*Daphnia magna*[redacted]., unpublished; Project No. 489A-103; dates of experimental work: July 10, 1998 – Aug. 18, 1998

Document No: M-473465-01-2

Guideline: EPA Series 72 of Pesticide Assessment Guidelines, FIFRA Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms; EPA 540/9-82-024
EPA Standard Evaluation Procedure, Acute Toxicity Test for Freshwater Invertebrates. Hazard Evaluation Division. Office of Pesticide Programs. EPA 540/9-85-005
ASTM (American Society for Testing and Materials) Standard E 729-88a (1994) Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians.
Corresponds to EEC C2 – Directive 92/69/EEC, and to OECD guideline 202, Part I (EC 50 acute immobilisation test) (applying to chemical substances).

GLP: Yes (self certification by the laboratory)

Materials and Methods: QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No. 8AQ07D6; titer: 2×10^{10} cfu/g).
Spray dried filtrate without *B. subtilis*, a tan powder, identified as 32-45-14B, lot # 812-0919.
The test substance was added to well water and daphnids (2 × 10 per control group and per concentration in treatment group) were exposed to nominal concentrations of 13, 25, 50, 100 and 200 mg/L for 48 hours. One control group was exposed to a spray dried filtrate of fermentation material without *B. subtilis*. Observations were recorded at ~ 5 , 24 and 48 hours.

Findings: Daphnids in the control group exposed to the spray dried filtrate without *B. subtilis* appeared as normal and healthy as daphnids in the negative control group.
24 h EC_{50}-for *Daphnia magna* was 147 mg/L
48 h EC_{50}-for *Daphnia magna* was 108 mg/L (calculated from mortality/ immobility data; 95% confidence limits were 50 and 200 mg/L).
The No Mortality/ Immobility Concentration and the NOEC were 13 mg/L

Conclusions: The 48 h **EC_{50}** exceeds ~ 100 times the limit value for toxic or adverse effects (according to the EC directive 67/548/EEC). Thus, no classification or labelling of *B. subtilis* strain QST 713 is required.

Chronic (21-day) toxicity

Report : IIM 8.3/02 [redacted](1998c): *Bacillus subtilis*: a 21-day life-cycle toxicity and pathogenicity test with the cladoceran (*Daphnia magna*); [redacted]., unpublished; Project No. 489A-102B; dates of experimental work: June 9, 1998 – Aug. 24, 1998

Document No: M-473638-02-1

Guideline: EPA Microbial Pesticide Test Guidelines OPPTS 885.4240
ASTM Standard E 1193-87 Standard Guide for Conducting Renewal Life-Cycle Toxicity Tests with *Daphnia magna*.
Corresponds to OECD guideline 202, Part II (reproduction test), applying for chemical substances (applying to chemical substances).
Deviations: Slightly lower test concentration applied compared to OPPTS guideline (recommended minimum concentration in test water: 1×10^6 cfu/mL).

Justification: selection of test concentrations were based upon the results of a range-finding toxicity test (in consultation with the sponsor)

GLP: Yes (self certification by the laboratory)

Materials and Methods: QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No. 8AQ07D6; titer: 2×10^{10} cfu/g)
The test substance was added to well water and daphnids (4 × 5 per control group and per concentration in treatment group) were exposed to nominal concentrations of 1,9; 3,8; 7,5; 15 and 30 mg/L (corresponding to $3,8 \times 10^7$, $7,6 \times 10^7$, $1,5 \times 10^8$, 3×10^8 and 6×10^8 cfu/L respectively). Test solutions were renewed 3 times/ week. Observations were recorded daily.

Findings: None of the tested concentrations caused ≥ 50% mortality or immobility.
21-day EC_{50}: > 30 mg/L.
Test substance concentrations up to 7,5 mg/L did not cause significant reduction in survival, reproduction or growth. *Daphnia magna* exposed to 15 mg/L showed significant reduction in reproduction, mean length and dry weight.
NOEC= 7,5 mg/L (= $1,5 \times 10^8$ cfu/L)
LOEC (*l*owest-*o*bserved-*e*ffect-*c*oncentration): 15 mg/L (= 3×10^8 cfu/L).
The MATC (*m*aximum *a*cceptable *t*oxicant *c*oncentration) was calculated to be 10,6 mg/L (= $2,1 \times 10^8$ cfu/L) - as the geometric mean of the NOEC and the LOEC.

Conclusions: The chronic NOEC exceeds by far the limit value of 1 mg/L for potential long-term adverse effects (according to the EC directive 67/548/EEC). Thus, no classification or labelling of *B. subtilis* strain QST 713 is required.

Included under 3rd Additional Submission

Report: KIIM 8.3/02; [redacted]; 2001; M-473492-01-1
Title: QST 713 technical: A five-concentration toxicity and pathogenicity test with the rainbow trout (Oncorhynchus mykiss)
Report No.: 489A-108
Document No.: M-473492-01-1
Guideline(s): U.S. Environmental Protection Agency
Series 885 - Microbial Pesticide Test Guidelines
OPPTS Number 885.4200
Guideline deviation(s): not specified
GLP/GEP: **yes**

Report: KIIM 8.3/03; [redacted]; 2001; M-473476-01-1
Title: QST 713 technical powder - Infectivity and pathogenicity to grass shrimp (Palaemonetes pugio) during a 30-day static renewal test
Report No.: 13759.6101
Document No.: M-473476-01-1
Guideline(s): OPPTS Guideline 885.4280
Guideline deviation(s): not specified
GLP/GEP: **yes**

Report:	KIIM 8.3/04; [redacted].; 2001; M-473458-02-1
Title:	QST 713 technical: A 21-day life-cycle toxicity and pathogenicity test with the cladoceran (Daphnia magna)
Report No.:	489A-107A
Document No.:	M-473458-02-1
Guideline(s):	U.S. Environmental Protection Agency Series 885 - Microbial Pesticide Test Guidelines OPPTS Number 885.4240
Guideline deviation(s):	not specified
GLP/GEP:	**yes**

Studies on acute toxicity and/or pathogenicity and infectivity to freshwater fish ([redacted] et al., 2001*a*), acute toxicity to the freshwater invertebrate *Palaemonetes pugio* ([redacted], 2001), chronic (21 d) toxicity to *Daphnia magna* ([redacted] et al. 2001*b*) were submitted in June 2002 and are cited in Addendum 1 to the Monograph (date of issue: 04.12.2002) and a risk assessment was performed. In conclusion, the overall risk to aquatic organisms is considered to be acceptable.

IIM 8.4 Effects on algal growth and growth rate

<u>**EU-Dossier: Doc M-IIB, Point 8.2.3**</u>

Report : **IIM 8.4/01** [redacted] (2000): Testing of toxic effects of QST 713 TP on the single cell green alga *Scenedesmus subspicatus*; [redacted].
Unpublished. Study code: 99431/01-AASs; dates of experimental work: November, 29 – December, 2nd 1999

Document No : M-473469-01-1
Guideline: OECD 201: "Alga growth inhibition test"
Corresponding to EC directive C. 3
Deviations: The range-finding test and the main test were combined to a limit-test, since there was evidence for no inhibitory effects of QST 713 TP at any test concentration
GLP: Yes
Materials and Methods: QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No. 8AQ07F1 / Drum 20, actual cfu-content: 3.3×10^{9} cfu/g of QST 713 TP)
Growth inhibition test: during several generations the algae were exposed to a concentration range between 0.01 and 100 mg/L of test substance (spaced by a dilution factor of 10). Algae cell numbers were counted after 24, 48 and 72 hours of exposure. The inhibition (EC, effect concentration) of cell multiplication was evaluated by calculating the ErC_{50}, EbC_{10}, EbC_{50}, LOEC and NOEC values. (The indices r and b refer to "growth rate" and "biomass" respectively).
Findings: Concentrations of test substance (cfu in test solutions) maintained sufficiently stable during the test.
No adverse effects of QST 713 TP were observed at any test concentration. Therefore, no EC values could be calculated.
Growth stimulation was observed at test substance concentrations of 1 and 10 mg/L.
NOEC ≥ 100 mg/L
LOEC ≥ 100 mg/L
Conclusions: The NOEC exceeds by far the limit value of 1 mg/L for potential long-term adverse effects (according to the EC directive 67/548/EEC). Thus, no classification or labelling of *B. subtilis* strain QST 713 is required.

IIM 8.5 Effects on aquatic plants

Not stated.

IIM 8.6 Effects on terrestrial plants

Not stated.

IIM 8.7 Effects on bees

EU-Dossier: Doc M-IIB, Point 8.3

Report : IIM 8.7/01 [redacted] (1998a); *Bacillus subtilis*: a dietary pathogenicity and toxicity study with the honey bee (*Apis mellifera*); [redacted] unpublished; Project No.: 489-102 C; dates of experimental work: June 5, 1998 – Aug. 27, 1998
Document No: M-473639-01-2
Guideline: EPA Microbial Pesticide Test Guidelines OPPTS 885.4380
Corresponds generally to OECD guideline 213 (applying to chemical substances)
Minor deviations: Shortened observation period (5 days instead of recommended *30* days after exposure) is justified by > 20% mortality in the negative control group (complying with OPPTS 885.4340 Nontarget Insect Testing (5)).
GLP: Yes (self certification by the laboratory)
Materials and Methods: QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No. 8AQ07D6; reported titer: 2×10^{10} cfu/g)
5 day feeding test: the test substance was administered in a honey/ water diet ad libitum to honey bees (6 × 20 per control group and per concentration in treatment group) for a period of 5 days.
Applied test substance concentrations in the diet were 600, 6000 and 60 000 ppm (relating to factor 1, 10 and 100 of EEC (Estimated Environmental Concentration) – equivalent to $1{,}2 \times 10^7$ – $1{,}2 \times 10^8$ and $1{,}2 \times 10^9$ cfu/mL of diet.
Observations were made twice within the first 4 h of test initiation and then daily until negative control mortality exceeded 20% on Day 5 of the test. Mortality in treatment groups was adjusted for control mortality.
Findings: Clinical signs as immobility, lethargy or loss of equilibrium were exhibited by a few bees in all treatment groups (starting on Day 0 in the higher dosed groups and on Day 3 in the lowest concentration group).
Treatment related mortality was dose responsive. Considerable increase in mortality occurred by Day 2 in the highest dosage group (receiving 60 000 ppm).
Dietary LC_{50}: ~ 8900 ppm (equivalent to ~ $1{,}8 \times 10^8$ cfu/mL diet) – corresponding to approximately *15 times* the reported EEC (Estimated Environmental Concentration).
Conclusions: No hazard to honey bees is to be expected from exposure to *Bacillus subtilis* strain QST 713, the active ingredient of Serenade™ WP.

Included under 2nd Additional Submission:

Report: KIIM 8.7/02; [redacted]; [redacted]; 1978; M-528225-01-1
Title: Diagnosis of honeybee diseases, parasites and pests
Report No.: M-528225-01-1
Document No.: M-528225-01-1
Guideline(s): not specified
Guideline deviation(s): not specified
GLP/GEP: no

Report: KIIM 8.7/03; [redacted]; [redacted]; 1983; M-528223-01-1
Title: Studies of aspergilosis and other pathogens of honeybees
Report No.: M-528223-01-1
Document No.: M-528223-01-1
Guideline(s): not specified
Guideline deviation(s): not specified
GLP/GEP: no

Report: KIIM 8.7/04; [redacted]; 1994; M-356399-01-1
Title: Bacillus DNA in fossil bees: an ancient symbiosis?
Report No.: M-356399-01-1
Document No.: M-356399-01-1
Guideline(s): not specified
Guideline deviation(s): not specified
GLP/GEP: **no**

Report: KIIM 8.7/05; [redacted]; 1976; M-370074-01-1
Title: Bacteria isolated from the intestinal contentsof foraging worker honey bee, Apis mellifera: The Genus bacillus
Report No.: M-370074-01-1
Document No.: M-370074-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: **no**

Report: KIIM 8.7/06; [redacted]; 1978; M-528862-01-1
Title: Bacteria belonging to the genus Bacillus isolated from honey bees, Apis mellifera, fed 2,4-D and antibiotics
Report No.: M-528862-01-1
Document No.: M-528862-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: **no**

Report: KIIM 8.7/07; [redacted]; 1990; M-528218-01-1
Title: Bacteria belonging to the genus Bacillus associated with three species of solitary bees
Report No.: M-528218-01-1
Document No.: M-528218-01-1
Guideline(s): not specified
Guideline deviation(s): not specified
GLP/GEP: **no**

Report: KIIM 8.7/08; [redacted]; 1985; M-528217-01-1
Title: Microbes from apiarian sources: Bacillus spp. in frass of the greater wax moth
Report No.: M-528217-01-1
Document No.: M-528217-01-1
Guideline(s): not specified
Guideline deviation(s): not specified
GLP/GEP: **no**

Report: KIIM 8.7/09; [redacted]; 2004; M-528612-01-1
Title: Inhibition of growth of Ascospaera apis by Bacillus and Paenibacillus strains isolated from honey
Report No.: M-528612-01-1
Document No.: M-528612-01-1
Guideline(s): not specified
Guideline deviation(s): not specified
GLP/GEP: **no**

Report: KIIM 8.7/11; [redacted]; [redacted]; 1988; M-528212-01-1
Title: On the potential of some bacterial biocides against root-knot and cyst nematodes
Report No.: M-528212-01-1
Document No.: M-528212-01-1
Guideline(s): not specified
Guideline deviation(s): not specified
GLP/GEP: **no**

Report: KIIM 8.7/12; [redacted]; [redacted] D. C.;; [redacted]; [redacted]; [redacted]; [redacted]; 2001; M-528209-01-1
Title: United states patent - Strain of Bacillus for controlling plant diseases and corn rootworm - Patent no. US 6,291,426 B1
Report No.: M-528209-01-1
Document No.: M-528209-01-1
Guideline(s): not specified
Guideline deviation(s): not specified
GLP/GEP: **no**

Report: KIIM 8.7/12; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; 2002; M-528188-01-1
Title: Insecticide activity of surfactins and iturins from a biopesticide Bacillus subtilis Cohn (S499 strain)
Report No.: M-528188-01-1
Document No.: M-528188-01-1
Guideline(s): not specified
Guideline deviation(s): not specified
GLP/GEP: **no**

Report: KIIM 8.7/13; [redacted]; 2004; M-486885-01-1
Title: Safety of the Bacillus subtilis-based biofungicide Serenade, to the honeybee, Apis mellifera L.
Report No.: M-486885-01-1
Document No.: M-486885-01-1
Guideline(s): not specified
Guideline deviation(s): not specified
GLP/GEP: **no**

Report: KIIM 8.7/14; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; 1992; M-528186-01-1
Title: Effects of chlortetracycline of honey bee worker larvae reared in vitro
Report No.: M-528186-01-1
Document No.: M-528186-01-1
Guideline(s): not specified
Guideline deviation(s): not specified
GLP/GEP: **no**

Report:	KIIM 8.7/15; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; 1996; M-528180-01-1
Title:	Field evaluation of the EPA (Kenaga) nomogram, a method for estimating wildlife exposure to pesticides residues on plants
Report No.:	M-528180-01-1
Document No.:	M-528180-01-1
Guideline(s):	not specified
Guideline deviation(s):	not specified
GLP/GEP:	**no**

It is important to recognize the difference between commensal organisms and pathogens, and to understand the value of the normal microflora in the honeybee *Apis mellifera* L. *B. subtilis*, like *B. cereus*, will appear in association with many disease states, but neither is considered pathogenic, nor does the literature attribute specific disease to these bacteria. The most common bacterial pathogens of honeybees include the gram-positive *Bacillus larvae* (American foulbrood, AFB), and the gram-negative *Melissicoccus pluton* (European foulbrood, EFB; previously *Streptococcus pluton*). *Bacillus alvei*, *B. laterosporus*, *B. pulvifaciens* , and *B. euridice* are frequently found in diseased larvae, and used to assist with the diagnosis of EFB ([redacted] & [redacted], 1978). Chalk brood and Aspergillosis, or stone brood, are common fungal diseases of bees caused by *Ascophaera apis, Aspergillus flavus,* and other species. A number of commensal bacteria including *B. subtilis* and *B. cereus* can be isolated from honeybees displaying fungal or bacterial disease ([redacted] and [redacted], 1983).

In 1973, Aloysius Kreig documented the effect of whole, nonsporulated cultures of *Bacillus cereus* (strain c-47) and *Bacillus thuringiensis* (strains I/5 and III/36) against honeybees. Two fractions were found to be toxic to adult honeybees following oral administration. The toxicity was attributed to thermostable beta-exotoxin, and thermolabile alpha-exotoxin. However, unlike the vegetative cultures, sporulated cultures of *B. cereus* and *B. thuringiensis* strain III/36, serotype H3, lack insecticidal toxins and were considered “not harmful to bees”. *Bacillus thuringiensis* (B.t.) biopesticides are based on exotoxin-free strains that produce a variety of proteinaceous delta-endotoxins. It is the specificity of these toxins, and the presence of others virulence factors that allow B.t. to invade the midgut of susceptible lepidoptera, diptera, and coleopteran hosts. The specificity of these toxins and their relative safety to honeybees is accepted fact.

Numerous authors have shown a variety of Bacillus to be some of the oldest and most common bacteria associated with honeybees ([redacted] *et al*., 1994). The digestive tract of healthy foraging adult worker bees contains a variety of bacteria including *B. polymyxa, B. macerans, B. brevis, B. pulvifaciens, B. circulans, B. pantothenticus, B. subtilis, B. firmus, B. alvei, B. laterosporus, B. coagulans, B. cereus, B. pumilus and B. licheniformis* ([redacted] & [redacted], 1976). Queen honeybees are inhabited by similar micro flora. In one study, *B. cereus* and *B. megaterium* were the most common bacteria isolated from the internal organs of queen bees. Like workers, the queens were host to *B. subtilis, B. brevis, B. licheniformis*, and *B. coagulans* ([redacted], 1978). These organisms are ubiquitous in *A. mellifera* and a variety of other bees including *Anthophora sp*., *Centris pallida*, *Melipona fasciata*, and a necrophage of the genus Trigona. The Bacilli are common associates of many Apoidea, participating both in metabolic conversions of food and in the control of competing microorganisms ([redacted] *et al*., 1990).

Macropredators of honeybees, including the greater wax moth (*Galleria mellonella*) and the acarid mite (*Varroa jacobsoni*) harbor similar microorganisms. *B. cereus* was the most common organism found in wax moth frass from managed honeybee colonies. *B. sphaericus* was the most frequent isolate in the frass of a laboratory culture of the wax moth, whereas *B. megaterium* and organisms belonging to the *B. alvei/B. thiaminolyticus* spectrum were the most frequent isolates in the frass from feral honeybee colonies ([redacted], 1984).

The antagonism associated with a pathogenic microbe is dependent on virulence factors, or attributes that allow the organism invade a susceptible host though direct colonization or displacement of the normal micro flora. In the case of *B. subtilis* and *B. cereus*, recent work has shown that the normal flora from honey in Argentina has an inhibitory effect on the fungus *Ascosphaera apis*, the causative agent of chalkbrood disease in honeybee larvae. Bacterial strains isolated from honey showed antagonistic effects to *A. apis* in laboratory disk-diffusion assays. *B. cereus*, *B. circulans*, *B. megaterium, B. pumilus, B. subtilis,* and *Paenibacillus alvei* were all inhibitory to *A. apis*. The best antagonists were *B. subtilis* and *B. megaterium* ([redacted] *et al*., 2004).

B. subtilis is known to produce subtilisin, and a variety of lipopeptides. The *B. subtilis* in Serenade® (AQ713, QST713, NRRL B21661) does not produce subtilisin as documented by [redacted] (2001; see document J). On the other hand, several authors have noted insecticidal activity associated with *B. subtilis*. Gotke and [redacted] (1988) reported nematicidal activity from whole broths of *B. subtilis*, *B. pumilus*, and *B. cereus*. Non-cellular extracts from *B. subtilis* gave 90% mortality against four species of nematode. [redacted] *et al*. (2001), patented the insecticidal activity of *B. subtilis* against western spotted cucumber beetles (corn rootworms), but saw no activity against beet armyworm, fruit fly, or German cockroach. [redacted] *et al*. (2002) have shown a dose response with purified C-15 surfactin from *B. subtilis* Cohn (strain S499) against fruit fly. C-14 surfactin was less active than C-15, and C-14 iturin was not active at all. The broad specificity of AQ713 against fungal pathogens, and its relatively weak activity against insect targets is attributed to the specific mixture of lipopeptides produced during the manufacture of Serenade® (see document J).

To address the potential toxicity and pathogenicity of Serenade® against honeybees, four separate studies have been conducted using a variety of AQ713 preparations. On January 9, 2004, AgraQuest Inc. submitted a document to the US EPA titled, "Discussion of the Results of Honeybee Studies Conducted with QST 713 Technical and Serenade® Products". To fulfil one of the data requirements for the registration of Serenade Wettable Powder (WP), AgraQuest Inc. initiated a dietary pathogenicity and toxicity study against honeybees, in June 1998. The objective of this study was to estimate the toxic and pathogenic effects of QST 713 technical powder to the honeybee during a 30-day exposure period. Bees were caged and fed honey water solutions containing 3 geometric serial dilutions of QST 713: 60,000, 6,000, and 600 ppm. Observations of mortality were made twice within the first four hours of test initiation and then daily until the test was terminated prematurely because negative control mortality exceeded 20% on day five. Based on control corrected mortality the dietary LC_{50} was estimated at 8,900 µg/g (1.8×10^8 CFU/mL). After adjusting for control mortality, the USEPA estimated an LC_{50} of 8,919 ppm using the binomial method. The estimated environmental concentrations (EEC) from the Kenega nomogram appropriate for honeybees in leaves and leafy crops, or forage, alfalfa, and clover, are 250 and 115 ppm respectively. Because of the limitations of this test and its premature termination, it was concluded that the risk to honeybees from the end-use product Serenade might be significant. It was recommended that a 30-day whole hive study be conducted to help quantify the risk to honeybees.

A field study with Serenade Wettable Powder and free-flying honeybees was conducted on a blooming alfalfa field between June 26 and July 27, 2000. This whole hive study included 6 applications of Serenade WP at 10 lb/acre in 5 gallons of water/acre in a 30-day period. This protocol was used to simulate a worst-case scenario and was approved by the Biopesticide and Pollution Prevention Division of USEPA. The results of this study demonstrated that Serenade WP was non-toxic to honeybees, even under these severe conditions. Honeybees in Serenade-treated plots behaved similarly to those in the water-treated control plots as evaluated by adult and immature mortality and foraging activity. These results were contrasted to those in the dimethoate-treated plots where mortality was so great that no evaluations were possible after the second treatment. Upon review of this study (April 2001), EPA agreed "that the use of Serenade at the 10 pound per acre rate ... with a five day treatment interval is not likely to present a significant risk to honeybees". Because of two anomalous data, EPA requested that AgraQuest conduct another study to assess the potential for acute toxicity.

The protocol, "Evaluation of the Potential Acute Toxicity to the Honey Bee of Serenade® Biofungicide WP in a Semi-Field Study," was submitted by AgraQuest Inc. and approved by US EPA in 2002. This study was conducted with the organic formulation, Serenade WPO, because it had replaced the original formulation in the marketplace. The purpose of this study was to investigate the potential toxicity and pathogenicity of Serenade® to honeybees when standard-size colonies were fed a controlled dose during a part of the season when little natural forage is available.

Within the first block of 12 hives, three colonies were treated with 336 g of autoclaved Serenade WPO/colony in a total volume of two liters of 50% sucrose solution (attenuated control). Each of three standard size colonies was treated with 336g of Serenade WPO in a total volume of two liters of 50% sucrose solution (168,000 ppm). Only one colony was treated with 159 mL Dimethoate 4E in a total volume of two liters of 50% sucrose, and three colonies were fed two liters of untreated 50% sucrose syrup as negative controls. The colonies selected for treatment were fed within 30 to 90 minutes after mixing the test substance with the sucrose syrup using one in-hive feeder in each colony. The amount of syrup consumed by each colony was evaluated until completely consumed, or the residual was recorded periodically. The numbers of frames with brood in each colony were estimated and the numbers of frames of adult bees in each colony (hive strength) were recorded. Todd Dead Bee Traps were attached to each of the colonies and the number of dead larvae, pupae,

and adult bees were recorded for 75 days. A second block of 12 hives were initiate two weeks after the first block with the same amount of Serenade diluted in a final volume of 3.8 L.

To assess acute toxicity it is imperative that a known dose be administered in a reasonably short time, and that meters of growth or reproduction be monitored for at least one complete life cycle following administration of the test substance. At 336 g in 2 or 3.8 L of 50% sucrose, adult honeybees did not rapidly assimilate Serenade WPO. In a single block with three replicates per treatment, honeybee colonies consumed two liters of 50% sucrose per colony in three days. Serenade WPO treatments were not completely consumed after 11 weeks of exposure. However, the results of this study suggest that chronic exposure to the 10-lb/acre rate of Serenade WPO was no more toxic than 50% sucrose, or an autoclave-attenuated sample of Serenade WPO. Todd dead bee counts, colony weight loss, and brood cell numbers were not impacted by long-term exposure. Dilution of the Serenade WPO in twice the original volume of sugar solution did not improve the palatability of Serenade WPO to honey bees in a second block of twelve colonies ([redacted], 2004a).

To address the issues of acute toxicity and chronic pathogenicity required a method that insured that the administered dose was consumed, and that growth parameters could be accurately monitored. Once it became obvious that the in-hive feeding system failed to deliver the prescribed dose, we initiated studies using methods to feed honeybee larvae minute quantities of chemical fungicides.

Our modified method delivered ten-µL doses of test substance directly into the brood cells of recently hatched honeybee eggs. We held the frame in an incubator for 30 minutes to allow the larvae to consume the aliquot, before the frame was returned to the nest. This insured that the dose was delivered, and consumed completely by the target organisms. In our first pilot scale experiments it became obvious that timing was critical to larval capping and adult survival. If the larvae were held outside of the nest too long, they starved, and were rejected by attendant nurse bees. If the frames were returned before the larvae had consumed the entire dose, residual *Bacillus* in the larval cells triggered house-cleaning behaviors. This caused larvae to be ejected or cannibalized, and the cells to cleaned or destroyed.

Larvae found highly concentrated *Bacillus* suspensions unpalatable. When QST 713 was delivered in water, or 50% sucrose, at 116,000 ppm, the larvae rejected the food, and the cells were cleaned out completely within hours of re-installation in the nest. We found that Javelin® WDG, or a *Bacillus thuringiensis* primary powder, caused the same response. Hive bees may have perceived the Bacillus solutions to be indications of a disease state, which released protective behaviors. When QST 713 was fed at 100,000 ppm in the artificial larval bee diet (LBD: royal jelly, sugar and yeast extract, [redacted] *et al*., 1992) most of the larvae survived, entered a prepupal state, were capped by attendant nurse bees, and emerged as healthy adult bees. Emergence occurred within a time frame equal to larvae fed LBD only, and the unfed larvae that were mapped as untreated controls.

When first instar honeybee larvae were dosed with ten-µL of a 100,000-ppm solution of QST 713 technical powder, they consumed 1 mg of *Bacillus subtilis*, or five to ten times the average weight of a newly emerged first instar. Combined actual data in Volume 3 of our US EPA submission show that 100,000-ppm of QST 713 technical powder only resulted in 38.3% mortality at capping (61.7% survival). Adult emergence of the honeybees that survived this acute dose mirrored the capping data. No evidence of behavior abnormality or delayed eclosion was noted. Mean separation by ANOVA and DMRT indicate that this treatment is the only rate that differs significantly from the mapped-only control (UTC = 86.1% survival to adult). At 10,000-ppm, survival at capping and eclosion increased to 71.0%. ANOVA indicates that this mean is not significantly different from the untreated control, nor is it different from 84% survival at 1,000 ppm. These “no observable effect levels” (NOEL) greatly exceed appropriate EEC’s of concern.

Based on the current label for QST 713, the 10-lb/acre-label rate translated to 116,000 ppm. Currently the highest label rate for Serenade WPO is 8 lbs/acre for hops (per pending label amendment submitted 10/17/03). The label rate in vegetable crops, where bees are used, is 2-6 lbs per acre. The label rate recommended for tree crops, where bee exposure is likely to occur, is similar to vegetable crops. The EEC’s of concern in our first study (leaves and leafy crops) were in the range of 250 to 100 ppm. The EECs for pod containing seed and fruit crops are even lower (120-70 ppm at 10 lb/acre – 80-15 ppm at 2- 6 lb/acre). These estimates of residual were originally written by Kenega for persistent chemicals, and revisited by [redacted] *et al.* (1996) using similar compounds. With a NOEL between 10,000 and 100,000 ppm for immature honeybee larvae, the toxicity and pathogenicity of *Bacillus subtilis* in Serenade is considered negligible.

The overall exposure level and effect on 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 micro flora. From the work presented in this review, and the study submitted in Volume 3, it should be concluded that Serenade® poses an insignificant risk to honeybees when applied at appropriate field rates. Our compiled body of work demonstrates a minimal threat to honey bees when larvae are presented with an acute dose and when whole colonies are presented with a chronic dose.

Included under 3rd Additional Submission:

Report:	KIIM 8.7/16; [redacted]; 2000; M-473494-01-1
Title:	Honey bee field study of Serenade biofungicide wettable powder in Alfalfa
Report No.:	00-001
Document No.:	M-473494-01-1
Guideline(s):	OPPTS 850.3040 (Draft, 1996) and OPPTS 855.4380 (Draft, 1996)
Guideline deviation(s):	not specified
GLP/GEP:	**yes**

A 30-d field study on honey bee ([redacted], 2000) was submitted in June 2002 and was evaluated in Addendum 1 to the Monograph (date of issue: 04.12.2002). It was concluded that honeybees will not be set at risk by a practical use of *Bacillus subtilis*-containing products.

In order to address potential pathogenicity of *B. subtilis* and to exclude a *B. cereus* like activity, the results of an additional study ([redacted] & [redacted], 2003), a discussion statement summarising all bee studies on Serenade ([redacted], 2004*a*) and an expert statement on existing literature on bacterial pathogens ([redacted], 2004*b*) were submitted in November 2004.

[redacted] (2004a) summarizes four studies conducted with Serenade formulations or Serenade Technical Powder, respectively. Among these, new studies not yet reported within the application for inclusion of *B. subtilis* QST 713. These are the Third Study Attempted on Honey Bees and the Fourth Study on Honey Bees ([redacted] & [redacted], 2003, see below).

In the Third Study Attempted on Honey Bees, which was conducted in the Central Valley of Northern California, a feeding test was designed to develop data on the honeybee hazard of Serenade WPO under whole-colony, controlled dose feeding conditions. Honeybee colonies were fed for 11 weeks to a controlled dose of Serenade WPO which was in excess of the worst-case exposure. This was done in an area of limited natural foraging, during a part of the season when little natural forage was available. An amount of 336 g Serenade WPO as test treatment was fed to colonies in 2.0-3.8 L sucrose syrup. The same amount of autoclaved Serenade WPO was fed as attenuated controls. Colonies fed with 2.0-3.8 L of 50 % w/w sucrose syrup served as negative controls. Dimethoate 4E or Iprodione served as toxic standard. Adult and brood mortality and reproductive fitness were assessed as relevant endpoints.

Assessment of acute toxicity was hampered by the fact that the administered dose of Serenade WPO was not taken up over a short period. Treatments were not completely consumed after 11 weeks of exposure. However, the results of this study suggest that chronic exposure to the 10-lb/acre (11.2 kg/ha) rate of Serenade WPO was no more toxic than 50% sucrose, or an autoclave-attenuated sample of Serenade WPO. Todd dead bee counts, colony weight loss, and brood cell numbers were not impacted by long-term exposure. Dilution of the Serenade WPO in twice the original volume of sugar solution did not improve the palatability of Serenade WPO to honey bees in a second block of twelve colonies.

A fourth study on honey bees was conducted based on an improved protocol ([redacted] & [redacted], 2003):

Report : **IIM 8.7/17** [redacted]. (2003): “Evaluation of Dietary Effects(s) of QST713 Technical Powder on Larval Honeybee Development (Apis mellifera L.)” [redacted]. – published: no, Report No. CAR 158-03 (Dates of work: 10/02/2003 to 12/18/2003)

Document No:	M-473477-01-1
Guideline:	EPA Microbial Pesticide Test Guidelines, Honey Bee Testing, Tier 1, OPPTS Guideline Number 885.4380 Draft Document (February 1996) Deviations: none
GLP:	Yes

Materials and Methods: Location: California Agricultural Research, Kerman, CA
Test item: Serenade Technical Powder (QST713 TP004), Lot no. 3309162045.
Range-finder test: QST 713 technical powder was applied to 20 larvae per treatment in combination with larval bee diet (LBD) at 10 µL per cell. LBD was used as a carrier to increase palatability of the *Bacillus* powders. QST 713 was administered at a rate of 100 000, 10 000, 1 000, 100 and 10 ppm. *Bacillus thuringiensis* var kurstaki technical powder (BBQP 0712) was mixed and administered at the same rates.
First study: QST 713 technical powder was administered at a rate of 100 000, 10 000, and 1 000 ppm to honeybee larvae in combination with larval bee diet (LBD) to increase palatability of the *Bacillus* powders. Control A: LBD only. Control B: untreated, mapped only. Toxic standard: technical Dimethoate at 5 ppm in LBD.
Second study: QST 713 technical powder was administered at a rate of 100 000 ppm to honeybee larvae in combination with larval bee diet (LBD) to increase palatability of the *Bacillus* powders. Control A: LBD only. Control B: untreated, mapped only. Toxic standard: technical Dimethoate at 5 and 100 ppm in LBD.
Test substance treatments for both studies were compared to each of the respective study controls substance treatments using ANOVA and Duncan's Multiple Range Test (DMRT).
Findings:
Observations: Survival to capping of honeybee larvae and adult emergence (1.+2. study only)
Range-finder test: Minimal detrimental effect at all rates of QST 713 was found. In contrast the high rate of BBQP 0712 only allowed 45% of the treated larvae to survive capping.

First study: Survival to capping at day 6 was 67.5%, 66.25%, and 81.25% for honeybee larvae treated with 10 µL of 100 000, 10 000, and 1 000 ppm of QST 713 technical powder in LBD, respectively. Survival in treatments of 5 ppm Dimethoate in combination with LBD was 96.25%, while survival in LBD only and the untreated control was 96.25% and 83.75%, respectively.
At caging on day 12, three larvae had emerged prior to caging, which were not included in the statistical analysis.
Survival from dosing to emergence at day 24 was 65.00%, 66.25%, and 81.25% in treatments with 100 000, 10 000, and 1 000 ppm of QST 713 technical powder in LBD, respectively. Survival from dosing to emergence in the LBD-only treatment was 95.00%. The reference Dimethoate at 5 ppm showed 96.25% survival from dosing to emergence. In the untreated control survival from dosing to emergence was 83.75%. As Dimethoate at 5 ppm revealed little mortality, a second study was conducted using 5 ppm and the higher rate of 100 ppm. The 5 ppm rate was repeated to confirm the ineffectiveness of Dimethoate at 5 ppm.

Second study: Percentage of survival to capping (day 7) was 52.5 % for honeybee larvae treated with 10 µL of 100 000 ppm QST 713 in LBD. Survival was 8.75% and 91.25 at 100 and 5 ppm Dimethoate, respectively. The LBD-only treatment yielded a survival of 97.5% and the untreated control a percentage of 85.00%.
Survival percentages from dosing to emergence (day 22) were the same as the survival to capping results.
There were no behavioural or morphological abnormalities observed in any treatment of either study.

Combined actual data show that 100 000 ppm of QST 713 technical powder only resulted in 38.3% mortality at capping (61.7% survival) (Table 8.3-1). Adult emergence of the honeybees that survived this acute dose mirrored the capping data. No evidence of behaviour abnormality or delayed eclosion was noted. Mean separation by ANOVA and DMRT indicate that this treatment is the only rate that differs significantly from the mapped-only control (UTC = 86.1% survival to adult). At 10 000 ppm, survival at capping and eclosion increased to 71.0%. ANOVA indicates that this mean is not significantly different from the untreated control, nor is it different from 84% survival at 1 000 ppm.

Treatment	Dose (ppm)	N	% Survival	Control Corrected Mortality	Control Corrected Survival
QST 713	100 000	9	61.7	36.4	63.6
QST 713	10 000	5	71.0	26.7	73.3
QST 713	1 000	5	84.0	13.3	82.6
QST 713	100	1	95.0	2.0	98.0
QST 713	10	1	90.0	7.1	92.9
BBQP0712	100 000	1	45.0	53.6	46.4
BBQP0712	10 000	1	95.0	2.0	98.0
BBQP0712	1 000	1	90.0	7.1	92.9
BBQP0712	100	1	95.0	2.0	98.0
Dimethoate	5	8	93.8	3.3	96.7
Dimethoate	100	4	8.8	91.0	9.0
LBD-only	0	8	96.9		
Untreated, mapped-only	0	9	86.1		

Conclusions: Results of ANOVA and DMRT indicate that 100 000 ppm of QST 713 technical powder reduced survival of early instar honeybee larvae by 38%. Larvae that survived, eclosed on time, and showed no indication of behavioural or physical abnormality. Lower doses were not significantly different from the untreated control.

The statement of [redacted] (2004*b*) discusses main bacterial diseases in apiculture, i.e. American Foulbrood caused by *B. larvae* and European Foulbrood, caused by *Melissicoccus pluton*. Although effects of both *B. cereus* and *B. thuringiensis* against honeybees were reported in the literature, sporulated cultures of both species lack insecticidal toxins and are regarded "not harmful to bees". In addition numerous authors have shown association of *Bacillus* species with honeybees as commensal organisms, including *B. subtilis* and *B. cereus*. The latter and other species showed strong antagonism against common fungal diseases of bees. The lipopeptides produced by *B. subtilis* QST 713 have only weak insecticidal properties, attributed to non specific detergent-like activity.

All four studies on toxicity to honeybees are briefly summarised. Due to a NOEL between 10 000 and 100 000 ppm for immature honeybee larvae ([redacted] & [redacted], 2003), the toxicity and pathogenicity of *B. subtilis* in Serenade is considered negligible. The estimated environmental concentrations (EEC) from the Kenega nomogram appropriate for honeybees in leaves and leafy crops, or forage, alfalfa, and clover, are 250 and 115 ppm respectively. These values are greatly exceeded by the NOEL established for honeybee larvae. It is therefore concluded that Serenade poses an insignificant risk to honeybees when applied at appropriate field rates.

Report: KIIM 8.7/18; [redacted], 2004; M-473455-01-1
Title: Discussion of the results of honeybee studies conducted with QST 713 technical and Serenade products
Report No.: M-473455-01-1
Document No.: M-473455-01-1
Guideline(s): Guideline OPPTS 885.4380
Guideline deviation(s): not specified
GLP/GEP: **no**

IIM 8.8 Effects on terrestrial arthropods other than bees

EU-Dossier: Doc M-IIB, Point 8.4 (Subpoints 8.4.1 – 8.4.5)

Leafdwelling predators: ladybird beetle

Report : IIM 8.8/01 [redacted] (1998b): *Bacillus subtilis*: a dietary pathogenicity and toxicity study with the ladybird beetle (*Hippodamia convergens*); [redacted]., unpublished; Project No.: 489-103B; dates of experimental work: June 5, 1998 – Aug. 18, 1998

Document No: M-473489-01-2

Guideline: EPA Microbial Pesticide Test Guidelines OPPTS 885.4340
No OECD guideline applicable

GLP: Yes (self certification by the laboratory)

Materials and Methods: QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No. 8AQ07D6; reported titer: 2×10^{10} cfu/g)

The test substance was administered in a honey/ ladybird beetle diet to ladybird beetles (3 × 25 per control group and per concentration in treatment group). Applied test substance concentrations in the diet were 600, 6000 and 60 000 ppm (relating to factor 1, 10 and 100 of EEC (Estimated Environmental Concentration) – equivalent to $1{,}2 \times 10^7$ – $1{,}2 \times 10^8$ and $1{,}2 \times 10^9$ cfu/mL of diet

Observations were made twice within the first 4 h of test initiation and then daily for 30 days.

Findings: The results did not indicate treatment related mortality; the mortality in the treatment groups did not occur in a dose-responsive manner and was not significantly different from the control except for one replicate group within the lowest dosed group that showed a marked increase in mortality. Occasional beetles appeared lethargic and/ or immobile in all groups during the test.

NOEC: 60 000 ppm ($1{,}2 \times 10^9$ cfu/mL).

Dietary LC_{50}: > 60 000 ppm ($1{,}2 \times 10^9$ cfu/mL), exceeding the reported EEC by a factor of 100.

Conclusions: No adverse effects on ladybird beetles being exposed to *B. subtilis* strain QST 713 are anticipated from this study.

Leafdwelling predators: lacewing larvae

Report : IIM 8.8/02 [redacted] (1998c): *Bacillus subtilis*: a dietary pathogenicity and toxicity study with green lacewing larvae (*Chrysoperla carnea*); [redacted]., unpublished; Project No.: 489-104; dates of experimental work: June 5, 1998 – Aug. 18, 1998

Document No: M-473488-01-2

Guideline: EPA Microbial Pesticide Test Guidelines OPPTS 885.4340
No OECD guideline applicable

GLP: Yes (self certification by the laboratory)

Materials and Methods: QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No. 8AQ07D6; reported titer: 2×10^{10} cfu/g)

The test substance was administered in a moth egg diet to lacewing larvae (30 individually housed larvae in the control group and per concentration in the treatment group). Applied test substance concentrations in the diet were 600, 6000 and 60 000 ppm (relating to factor 1, 10 and 100 of EEC (Estimated Environmental Concentration) – equivalent to $1{,}2 \times 10^7$ – $1{,}2 \times 10^8$ and $1{,}2 \times 10^9$ cfu/mL of diet.

Observations were made once within the first four h of test initiation and then continued daily through day 13 of the test.

Findings: The results did not indicate treatment related mortality: mortality in the treatment groups did not occur in a dose-responsive manner and was comparable to negative control group. No signs of toxicity were noted in the treatment groups.

Reduction in pupation rates indicated treatment related effects (not dose-responsive): this rate decreased in the 6000 ppm and 60 000 ppm treatment groups compared to the control group.

NOEC: 600 ppm ($1{,}2 \times 10^7$ cfu/mL of diet).

Dietary LC_{50}: > 60 000 ppm ($1{,}2 \times 10^9$ cfu/mL), exceeding the EEC (Estimated Environmental Concentration) - based on the reported maximum application rate - by a *factor of 100* in minimum.

Conclusions: No adverse effects of strain QST 713 of *B. subtilis* on exposed lacewing larvae are anticipated from this study.

Parasitic hymenoptera: Nasonia vitripennis

Report : IIM 8.8/03 [redacted] (1998d): *Bacillus subtilis*: a dietary pathogenicity and toxicity study with the parasitic Hymenoptera (*Nasonia vitripennis*) (*Chrysoperla carnea*); [redacted]., unpublished; Project No.: 489-105A; dates of experimental work: June 5, 1998 – Aug. 18, 1998

Document No: M-473640-01-2

Guideline: EPA Microbial Pesticide Test Guidelines OPPTS 885.4340
No OECD guideline applicable

GLP: No

Materials and Methods: QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No. 8AQ07D6; reported titer: 2×10^{10} cfu/g)

The test substance was administered in a honey/ water diet to parasitic hymenoptera (3 × 25 wasps in each treatment and control group). Applied test substance concentrations in the diet were 600, 6000 and 60 000 ppm (relating to factor 1, 10 and 100 of EEC (Estimated Environmental Concentration) – equivalent to $1{,}2 \times 10^7$ – $1{,}2 \times 10^8$ and $1{,}2 \times 10^9$ cfu/mL of diet

Observations were made twice within the first four h of test initiation and then continued daily until day 15 (when mortality exceeded 20% in the negative control group).

Findings: Mortality rates differed in the 3 treatment groups:

A marked increase in the lowest treatment group (primarily in one replicate group) was not regarded as occurring in a dose-responsive manner and was not considered to be treatment related, since values in the mean concentration group (6000 ppm) remained on a level comparable to the control. A high mortality rate occurred in the 60 000 ppm group, considered to be treatment related.

Dietary LC_{50} (15 days): ~ 30 000 ppm (~ 6×10^8 cfu/mL)

NOEC: 6000 ppm ($1{,}2 \times 10^8$ cfu/mL)

(provided that the observed mortality in the 600 ppm treatment group was not treatment related, see conclusion).

Conclusions: Remarks: validity of LC_{50} is limited due to vague data base: calculation evidently based upon 2 values merely, since 1 (the lowest) out of 3 tested concentrations created inconsistently high mortality values (primarily in one replicate group*) – the lacking dose-response among the 2 lower-level concentrations was evaluated as an indication for lacking treatment relation.

Considering the low mortality rate in the 6000 ppm treatment group, exposed to 10 times the reported EEC, no adverse effects of strain QST 713 of *B. subtilis* on the tested species are anticipated.

* Generally comparable phenomenon occurred in study IIB, 8.4./01 with ladybird beetle as test organism

Parasitic hymenoptera: Aphidius rhopalosiphi

Report : IIM 8.8/04 [redacted] (2000): QST 713 TP: Acute toxicity to the Aphid Parasitoid *Aphidius rhopalosiphi* (Hymenoptera, Braconidae); [redacted]; unpublished; Study code: 99431/01-NLAp; dates of experimental work: Oct., 5 1999 – Dec. 21, 1999

Document No: M-473472-01-2

Guideline: Guidance Document on Regulatory Testing Procedures for Pesticides With Non-Target Arthropods (BARRETT et al. 1994)
Ring-test method by MEAD-BRIGGS (1992), a further development of a.m. guidance document

GLP: Yes (according to OECD principles and law of chemicals, attachment 1, Federal Republic of Germany)

Materials and Methods: QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No. 8AQ07F1/Drum 20, analyzed cfu-content: > 10^6)

10 adults (5 male and 5 female) per replicate were exposed to a freshly applied dry layer of test substance on glass-plates. Deionized water served as negative control and a toxic standard yielding 100% mortality (dimethoate) was applied as positive control. Each treatment group included 4 replicates. Mortality of the wasps was assessed after 30 min., 2 h, 24 h and 48 h. 11 days later the reproduction rate of the surviving test animals was determined as numbers of mummies produced within 24 h.

Applied test substance concentration corresponds to an application rate of 16 kg/ha

Findings: The table lists the results, showing that adult *A. rhopalosiphi* exposed to QST 713 TP were not affected by either a significantly increased mortality or decreased fertility rate compared to the control group:

Treatment group	Control	QST 713 TP (16 kg/ha)	Toxic standard
Mortality (%)	2.5	7.5	100
Corrected mortality (%)	-	5.13	100
Mummies per female	11.1	8.29	n.a.
Reproduction factor	-	0.75	n.a.

n.a.= not assessed

Conclusions: It is assumed that QST 713 strain of *B. subtilis* will cause no detrimental effects (increased mortality or decreased fertility) on *A. rhopalosiphi*, since test results comply with the limit values set by EC directive 91/414/EEC.

Predatory mites: *Typhlodromus pyri*

Report : **IIM 8.8/05** [redacted] (2000): QST 713 TP: Toxicity to the predatory mite, *Typhlodromus pyri* SCHEUTEN (Acari, Phytoseiidae) in the laboratory); [redacted]; unpublished; Study code: 99431/01-NLTp; dates of experimental work: Nov. 29, 1999 – Dec. 13, 1999

Document No: M-473491-01-2

Guideline: Louis/Ufer (1995) based on Overmeer (1988) and Guidance Document on Regulatory Testing Procedures for Pesticides With Non-Target Arthropods(BARRETT et al, 1994)

GLP: Yes (according to OECD principles and law of chemicals, attachment 1, Federal Republic of Germany)

Materials and Methods: QST 713 Technical (dried *Bacillus subtilis* with residual fermentation media; Lot No. 8AQ07F1/Drum 20, cfu-content: $> 10^{6}$)

Protonymphs of *T. pyri* were exposed to a freshly applied dry layer of test substance on glass cover slides in laboratory exposure units. Each treatment variant (test substance (at 16 kg/ha), toxic standard (dimethoate at 0.04 L/ha) and control) included 5 replicates, each containing 20 mites. Mortality of the nymphs was assessed after 7 days by counting surviving, dead, missing and affected animals. The reproduction rate was evaluated in a fertility test afterwards by counting the total number of offspring (eggs and larvae).

Applied test substance concentration corresponds to an application rate of 16 kg/ha.

Findings: Protonymphs of *T. pyri* exposed to QST 713 TP were affected by an increased mortality, which was significantly different from the control group (see table below). No adverse effect on the fertility rate occurred in the group exposed to QST 713 TP.

Treatment group	Control	QST 713 TP (16 kg/ha)	Toxic standard
Mortality (%)	12.0	39.0	72.0
Corrected mortality (%)	-	30.7	68.2
Mean no. of offspring per female	11.5	10.0	n.a.
Reproduction factor	-	0.87	n.a.

n.a.= not assessed

Conclusions: No effects of QST 713 strain of *B. subtilis* on the fertility of *T. pyri* are anticipated. Assessing the increased mortality of mites exposed to QST 713 TP two aspects are of concern: 1) The corrected mortality only slightly exceeds the limit value of 30% set by EC directive 91/414/EEC. 2) A possible physical effect of the test substance: for 3 days glass plates showed a greasy layer, which might have impaired mobility and feed consumption of the mites.

Included in the addendum 1 to the Monograph, 04 December 2002

##M-473490-01-1

Materials and Methods:

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 test substance
Exposure duration: 10 d
Test Substance: QST 713 Technical Powder
Lot # 54-63-4TP
5,25 x 10^{10} CFU/g
Test Concentrations:
295 ppm (=1,5 x 10^7 cfu/mL),
1730 ppm (=9,1 x 10^7 cfu/mL),
10200 ppm (=5,4 x 10^8 cfu/mL),
60000 ppm (=3,4 x 10^9 cfu/mL).

Control groups: negative control,
attenuated control and
sterile filtrate control.

Findings: For 10 days 25 wasps per treatment group were exposed to 4 dietary concentrations of QST 713 Technical powder, additional 10 wasps per group for pathogenicity observations.

All surviving wasps were normal in appearance and behaviour during the course of the study, except for incidental clinical signs, that were not dose-responsive. No apparent clinical signs indicative for a disease process.

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

LC50 = 24739 ppm (corrected for negative control mortality),
NOEC = 1730 ppm.

Conclusions: 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 Mortality in the attenuated control was comparable to mortality in 10200 ppm group, therefore, and because of lack of pathogenicity symptoms, strain QST 713 of *B. subtilis* was evaluated to be non-pathogenic to the parasitic hymenoptera.

IIM 8.9 Effects on other terrestrial invertebrates

IIM 8.9.1 Effects on earthworms

EU-Dossier: Doc M-IIB, Point 8.5

Report: KIIM 8.9.1/01; [redacted]; [redacted]; [redacted]; 1996; M-528072-01-1
Title: Persistence of Bacillus subtilis RB14 and its derivative strains in soil with respect to the LPA-14 gene
Report No.: M-528072-01-1
Document No.: M-528072-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: **no**

Report: KIIM 8.9.1/02; [redacted]; [redacted]; [redacted]; [redacted]; 1986; M-153650-01-1
Title: Survival of Pseudomonas fluorescens and Bacillus subtilis introduced into two soils of different texture in field microplots
Report No.: M-153650-01-1
Document No.: M-153650-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: **no**

Report: KIIM 8.9.1/03; [redacted]; [redacted]; [redacted]; [redacted]; 1990; M-528278-01-1
Title: Characteristics of Bacillus subtilis isolated from composts suppressing phytopathogenic microorganisms
Report No.: M-528278-01-1
Document No.: M-528278-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: **no**

Report: KIIM 8.9.1/04; [redacted]; [redacted]; 1973; M-497617-01-1
Title: Growth of Bacillus subtilis and spore germination in soil observed by a fluorescent-antibody technique
Report No.: M-497617-01-1
Document No.: M-497617-01-1
Guideline(s): not applicable
Guideline deviation(s): not applicable
GLP/GEP: **no**

Report: KIIM 8.9.1/05; [redacted]; 1997; M-528163-01-1
Title: Final decision document: TSCA section 5 (H)(4) exemption for Bacillus subtilis
Report No.: M-528163-01-1
Document No.: M-528163-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: **no**

Report: KIIM 8.9.1/06; [redacted]; 1994; M-001914-01-1
Title: Guidance document on regulatory testing procedures for pesticides with non-target arthropods
Report No.: Lit. 6841
Document No.: M-001914-01-1
Guideline(s): --
Guideline deviation(s): --
GLP/GEP: **no**

The following reference was submitted during the Annex I review but was not submitted in the Baseline dossier. At the request of the RMS, the document is submitted herein.

KIIM 8.9.1 / 10 [redacted]; 1993; Actinomycete communities in earthworm guts and surrounding soil; M-529395-01-1

Considering the natural distribution of *B. subtilis* as an autochthonous micro-organism of the soil any effects on earthworms can be excluded. Therefore no relating studies were conducted.

Serenade™ WP is applied to the foliage at a rate of 15 kg/ha in maximum and contains 5×10^9 cfu/g. An amount of 15 kg/ha will thus correspond to 7.5×10^{13} cfu/ha. Assuming the whole amount would reach the soil surface uniformly, the resultant surface load would approximate 7.5×10^9 cfu/m^2 or 7.5×10^5 cfu/cm^2. Considering references on the persistance of introduced *B. subtilis* it can be expected that part of the cells reaching the soil will not survive and the residual cells will form endospores, unless fresh organic matter is supplied ([redacted] et al., 1996; [redacted] et al., 1986; van Elsas et al., 1987; [redacted] et al., 1990; [redacted] & [redacted], 1974; EPA, 1997).

This still overestimated value can be regarded as low in view of the overall distribution of Bacilli in general, which occur at levels of 10^6 to 10^7 cfu/g (EPA, 1997) and considering the predominance of *B. subtilis* in all kinds of soils.

Employing a more realistic scenario under consideration of drift results in even lower levels of surface load:

According to Barret et al. (1994) a rate of 40% of the applied amount of product will reach the soil surface in three-dimensional crop, as orchards. Thus, one square cm of surface will receive a theoretical load of 3×10^5 cfu.

Included under 1st Additional Submission:

The performance of the required study has been discussed with German officials regarding an integrated histopathological examination. Now, in October 2001, the relevant study plan will be amended to initiate the study. The final report will presumably be available by December 2001.

IIM 8.9.2 Effects on other terrestrial invertebrates

No EC data requirement.

IIM 8.10 Effects on soil micro-organisms

Included under 1st Additional Submission:

Report:	KIIM 8.10/01; [redacted]; 2000; M-528850-01-1
Title:	Literature search - Bacillus subtilis and soil microflora
Report No.:	M-528850-01-1
Document No.:	M-528850-01-1
Guideline(s):	Further data for registration according to Directive 91/414/EEC
Guideline deviation(s):	not specified
GLP/GEP:	**no**

Note: data not requested in monograph; a literature search was performed to meet this new data request, according to the finalized directive for microbiological plant protection products (2001/36/EEC, amending Council directive 91/414).

[redacted] (2000): Literature Search

Amendment not included in monograph, since none of the submitted references specifically addressed to this data request. Date of submission: 10-16, 2001

To evaluate any potentially detrimental effects of *B. subtilis* spores introduced into the natural soil microflora a literature search was performed. The protocol of employed data bases and search terms as well as available abstracts are provided by [redacted] (2000).

A low significance of this ecological question is indicated by the fact that apparently none of the relevant articles appears to investigate detrimental effects of *B. subtilis* on other micro-organisms. On the contrary many articles focus on the *beneficial* effects of introduced *B. subtilis*, e.g. as mycorrhiza-helper-bacteria or with regard to antagonism towards soil pathogens.

In conclusion the lack of studies on adverse effects together with the vast evidence of beneficial effects indicate that *B. subtilis* does not present a risk for the native soil micro-flora.

Another issue to be considered is the fact that the relevant strain of *B. subtilis* (i.e. QST 713) is not intended to be directly applied to the soil, but onto the foliage of the crop. Therefore there is little potential for direct exposure to soil microorganisms.

Referring to the EU Dossier, the submitted information on the fate and behaviour of *B. subtilis* in the environment and the evaluation of the environmental impact prove that *B. subtilis* is a naturally

prevalent soil micro-organism, mostly existing in the endospore form, and that application of strain QST 713 of *B. subtilis* can be regarded to introduce low additional cfu-levels to the soil, according to the predicted environmental load. Further, the submitted report on testing effects on algae growth demonstrates that *B. subtilis* does not inhibit growth of this unicellular organism, neither the rate nor the biomass is adversely affected (NOEC= 100 mg/L).

Cross-references to relevant Annex-Points of the submitted dossier:

- Doc. M-IIB, Section 5, Point 7: Fate and Behaviour in the Environment
- Doc. M-IIB, Section 6, Point 8.2.3: Effects on algae growth
- Doc. M-IIB, Section 6, Point 9: Summary and Evaluation of Environmental Impact

IIM 8.11 Other/special studies

Not stated.