ECCO PEER REVIEW PROGRAMME

FULL REPORT ON **BACILLUS SUBTILIS**

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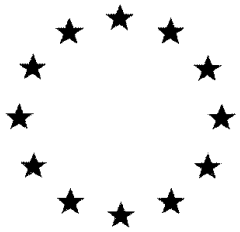
European Commission

Peer Review Programme



ECCO Peer Review Meetings

Full Report on
Bacillus subtilis

- Reporting table
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- Other documents considered at the meetings

ECCO-Team, at: Bundesamt für Verbraucherschutz und Lebensmittelsicherheit
16.09.2003

Comments on the Draft Assessment Report on Bacillus subtilis

Date	Supplier	File
12.09.2001	Finland	01_bacillus_subtilis_com_fin.doc
23.10.2001	AgraQuest	02_bacillus_subtilis_com_agraquest.doc
19.11.2001	The Netherlands	03_bacillus_subtilis_com_nl.doc
20.11.2001	Denmark	04_bacillus_subtilis_com_dk.doc
03.12.2001	Finland	05_bacillus_subtilis_com_fin2.doc
19.11.2001	Sweden	06_bacillius_subtilis_com_se.doc

Chemicals Department 20 August, 2008 (received by ECCO 12 Sept. 2001)

ECCO-Team (BBA)
Biologische Bundesanstalt für Land- und Forstwirtschaft
Abteilung für Pflanzenschuzmittel und Anvendungstechnik
Messeweg 11/12
D-38104 Braunschweig
Germany
e-mail: ecco@bba.de

Comment to the Monograph on *Bacillus subtilis* strain QST 713 prepared by Germany to the European Commission. These are Finland's comments concentrating to methods and health issues. Other topics will possibly be commented by Finnish Environment Institute (SYKE) and Plant Production Inspection Centre.

Volume 1

2.1.1 Identity of the micro-organism

The organism is not adequately identified as required in directive 2001/36/EC. By using available morphological, physiological and biochemical data one can only confirm that the particular strain belongs to *B. subtilis.* However, to identify the organism at strain level more specific methods, in practice molecular methods, should be used. Methods and/or information concerning the properties of *B. subtilis* QST 713 strain to distinguish it from other *B. subtilis* strains should be added.

2.1.2 Biological properties of the micro-organism

We agree with the RMS that more information is needed for the differentiation of *B. subtilis* from pathogenic *Bacillus* species, as well as, possible antibiotic resistance genes carried and antibiotics produced by *B. subtilis* QST 713. Additionally, all pathogenic *Bacillus* species are not mentioned in the monograph. For example, *Bacillus licheniformis*, which has been associated with food poisonings (Salkinoja-Salonen *et al.* 1999) and bovine abortions (Agerholm *et al.* 1997), was not mentioned.

2.1.4 Classification and labelling

The preparation consists of dried *B. subtilis* QST 713 strain on average 146 g/kg (5×10^{10} cfu/g). The preparation should be classified also into biological agents group 1 (unlikely to cause human disease) according to the directives 90/679/EEC and 89/391/EEC for biological agents, if requirements in point 2.1.2 are met.

2.4.1 Definition of the residues

Studies of operator exposure and residue levels in plants are required with all plant protection products. These studies should be done with *B. subtilis* QST 713 also. *B. subtilis* has been used for enzyme production on a large industrial scale and even for food production without having caused

major health hazards. However, infections caused by *B. subtilis* have been published in literature (e.g. Kiss *et al* 1988, Richard *et al* 1988, Thomas and Whittet 1991, Velasco *et al.* 1992, Oggioni *et al* 1998). Moreover, many "non-pathogenic" bacteria have a tendency to become opportunistic pathogens when they are present in large amounts and find a susceptible host.

The RMS claims that "a plant product (fruit) carrying a layer built up of *B. subtilis* can be easily washed with water". Washing with water does not remove all *B. subtilis* bacteria and its spores from the surface of fruit. Fruit, washed or non-washed, will afterwards contaminate at irregular intervals its surroundings and when consuming the fruit the consumer will ingest the bacteria. Thus, the amount of possible residues should be better studied by the notifier and assessed by the RMS.

Volume 3

B.2.1.7 Genetic stability and factors affecting it

Possible gene transfer after application must be considered. Gene transfer is a common phenomenon in soils especially with gram-negative bacteria like *Escherichia coli*. Moreover, since there is evidence of gene transfer between *B. subtilis* and a well-known pathogen *B. cereus*. The existence of such mechanism should be ruled out with *B. subtilis* QST 713.

B.5.1.1 Methods for the identification of the micro-organism (Annex IIB 4.1)

On culture media, the colour of the bacterial colonies is usually defined by the contents of the culture media. Here, the colour is described as "light cream (brownish) to cream", but the type of culture media is not mentioned.

B.5.1.2.1 Quality control measures applied to the production of QST 713 WP

The measures to maintain product quality are shortly described, but whether the manufacturer has any certified quality assurance system, which it should have (e.g. HACCP), is not presented.

B.6.3.1 Acute intravenous toxicity, pathogenicity and infectivity

B. subtilis QST 713 caused sepsis to all animals (rat) after i.v. exposure. Test substance was detected in the blood, liver, lungs, spleen and kidneys on day 0 from challenge. All these organs are usually sterile. Additionally, complete clearance from all tissues happened as slowly as 80 days from challenge. Sepsis as such is not surprising after i.v. application of micro-organism. Surprising is that clearance took as long time as 80 days.

Yours respectfully,

Markku Keskimäki, Ph. D.
Senior Officer
National Product Control Agency for Welfare and Health
Chemicals Department
P.O. Box 210
FIN-00531 Helsinki, Finland
E-mail:markku.keskimaki@sttv.fi

REFERENCES

Agerholm JS, Willadsen CM, Nielsen TK, Giese SB, Holm E, Jensen L, Agger JF. 1997. Diagnostic studies of abortion in Danish dairy herds. Zentralbl. Veterinarmed. A. 44:551-558.

Kiss TA, Gratwohl R, Frei B, Osterwalder A Tichelli and B. Speck. 1988. *Bacillus subtilis* infections. Schweiz. Rundsch. Med. Prax. 77:1219-1223.

Oggioni MR, Pozzi G, Valensin PE, Galieni P and C Bigazzi. 1998. Recurrent septicemia in an immunocompromised patient due to probiotic strains of *Bacillus subtilis*. J. Clin. Microbiol. 1998. 36:325-326.

Richard VP, Van der Auwera R, Snoeck D, Daneau and F Meunier. 1988. Nosocomial bacteremia caused by *Bacillus* species. Eur. J. Clin. Microbiol. Infect. Dis. 7:783-785.

Salkinoja-Salonen MS, Vuorio R, Andersson MA, Kampfer P, Anderssen MC, Honkanen-Buzalski T and AC Scoging. 1999. Toxigenic strains of *Bacillus licheniformis* related to food poisonings. Appl. Environ. Microbiol. 65:4637-4645.

Thomas M, and H Whittet. 1991. Atypical meningitis complicating a penetrating head injury. J. Neurol. Neurosurg. Psychiatry 54:92-93.

Velasco E, De Sousa CA, Martins, D Tabak, and LF Bouzas. 1992. *Bacillus subtilis* infection in a patient submitted to a bone marrow transplantation. Rev. Paul. Med. 110:116-117.

CC: Plant Production Inspection Centre, Finland
Finnish Environment Institute (SYKE), Finland

02_Bacillus_subtilis_com_agraquest.doc

GAB Consulting GmbH, Hinter den Höfen 24, 21 769 Lamstedt

Biologische Bundesanstalt
für Land- und Forstwirtschaft
Abt. Pflanzenschutzmittel
und Anwendungstechnik
ECCO Team

ECCO Peer review of new active substances under directive 91/414/EEC, draft assessment report on *Bacillus subtilis* submitted by Germany.

Your sign	Your letter dated	Our sign	Lamstedt,
12464/ECCO/BBA/01FH	July, 23rd, 01	CT	Oct. 22nd, 01

Comment of the applicant, AgraQuest Inc., USA, to the monograph on *Bacillus subtilis*, submitted by the representative in Europe, GAB Consulting GmbH, Germany.

Contact person:

Name:	Christina Thobor
Function:	Project Manager
Company:	GAB Consulting GmbH
Street, no.:	Hinter den Höfen 24
Location:	D-21769 Lamstedt, Germany
Phone:	(49) 4773 888913
Fax:	(49) 4773 888920
E-Mail:	Christina.thobor@GAB-consult.de

Table of Contents

Comments and assessment of submitted further information regarding the data request as outlined in the monograph, volume 1, level 4

1. Comment on point 4.1.3: Toxicology, Pathogenicity and Infectivity,

relating to Annex IIB, Section 3 of directive 91/414/EEC,
Annex point 5.2.5.1: Health effect after repeated inhalatory exposure.

Note: Documents and information referred to have been submitted to the RMS in April, 19th, 2001 and September 24th, 2001, and have not been considered within the monograph (see appendix 1 for listing of documents).

Further data requirements on toxicity of *B. subtilis* are primarily addressed to the clearance capacity of rats for spores of strain QST 713 of *B. subtilis* following repeated inhalative exposure (monograph, vol. 1, point 4.1.3, sub-point 1).

The toxicological concern regarding the potential sensitizer subtilisin, as expressed in the monograph, (monograph, vol. 1, point 4.1.3, sub-point 1) is not regarded as relevant by the BgVV in view of the lack of valid exposure limits for subtilisin in the US (RYDER FOX 2001). The official health experts at the BgVV and the BBA therefore agreed not to ask for further information on production of subtilisin (monograph, vol. 1, point 4.1.3, sub-point 2).

A first draft of a protocol for a relevant study has been submitted in April 2001 (LEUSCHNER 2001), but major changes have been implemented after discussions with experts from the BgVV (Consument's Health and Veterinary Agency). The new protocol has been submitted for review to the BgVV (September 2001).

To date the study still has not been initiated, because of new evidence provided by a literature search. The below mentioned references have been submitted to both the German BBA and the BgVV (Consument's Health and Veterinary Agency) to re-evaluate the data request as outlined in the monograph.

Relevant studies have been reported by WATSON et al. (1973) and SAUNDERS et al. (1983), applying *B. subtilis* spores on rats (intratracheally) and pigs (inhalative exposure), respectively.

Rats received a single dose of 8×10^7 cfu viable spores, and clearance from lungs was monitored over a 48h period (WATSON et al. 1973). At 48 h post-exposure no viable spore was detected, and clearance was achieved to 85% (15% of injected dose remaining). In preliminary tests inactiviation of viable spores was demonstrated to occur in lung tissue due to bactericidal substance(s) found naturally in the lungs.
Pigs were exposed to a an aerosol generated by an ultrasonic nebulizer for 15 minutes (SAUNDERS et al. 1983). This technique implies that no viable spores were applied, but for determining clearance of spores this was not required. Clearance was monitored during a 12h period based on the initial deposition of spores in lungs determined immediately after exposure. No dose rate per animal was given, since this is hard to be exactly defined for the route of inhalative exposure.

- Conclusively the results of both references indicate a fast clearance of *B. subtilis* spores from exposed tissues.

Further publications address to other Bacillus species or fungal pathogens, indicating that respiratory tract and lungs own specific defense mechanisms to eliminate even pathogenic spores.

One reference on pulmonary clearance relates to *B. thuringiensis* (TSAI et al. 1997) after intratracheal injection of a single dose of 1 x 10^8 cfu/rat. Behaviour and toxicological effects of this species cannot be compared to the species *B. subtilis*, which does not produce exotoxins.
Clearance of *Bacillus anthracis* was studied following inhalative exposure to guinea pigs (VANCURIK 1965). The authors calculated the dose rate per animal from the aerosol concentration by a special mathematical formula. The employed dose rates ranged from 1.32 x 10^5 cfu/ animal (for mice), to 2.9 x 10^5 (small guinea-pigs), and 2.43 x 10^6 (larger guinea-pigs).
Results of the toxicological investigation are not relevant for *B. subtilis*, since *B. anthracis* is a known pathogen and *B. subtilis* is innocuous to humans. Clearance of *B. anthracis* spores from lungs was determined to occur fast, with a half-life of not more than 2 days, and to be complete within 36 days. Spores infiltrating the tracheobronchial nodes were cleared less rapidly and suggested to be a cause of the noted relapse having discontinued antibiotic prophylaxis.

Clearance of fungal spores from lungs of mice was determined to be 30 days following intranasal inoculation at 5 x 10^6 cfu/ animal (WALDORF et al. 1984). Spores of the employed fungal species (*Rhizomucor pusillus*) extracted from the tissues were found to be viable and infectious, however, this result is not applicable to the non-pathogenic spores of *B. subtilis*. The studies of WALDORF et al. (1984) and more specifically WHITE (1977) indicate the importance of an active defense mechanism of the exposed tissue, since Cortison treatment did impair the defense profoundly and resulted in markedly higher germination of fungal spores.

The fate of metal stained *Aspergillus terreus* spores following inhalation was monitored by microscopy (GREEN et al. 1980). The uptake of spores by alveolar macrophages was demonstrated to be rapid virtually completed within 3 hours after exposure. This reference gives an insight into the defense mechanisms of the respiratory system towards spores in general.

Inactivation of fungal spores upon intratracheal installation in rabbit lung was demonstrated by KURUP (1984), who examined the ability of macrophages to destroy pathogenic fungal spores of different species under different conditions. The significance of the species was clearly shown. VOISIN et al. (1971) also address to the immune system response towards pathogenic fungi, following inhalation and intratracheal inoculation.

Evidence for a low health impact of *bacterial* spores in general can be delineated from an epidemiological study on 8482 farmers and spouses performed in Norway (MELBOSTAD & EDUARD 2001). Exposure to bacterial and fungal spores is accompanying manifold tasks carried out in farms. The National Institute of Occupational Health in Norway concluded from the vast data generated that work related symptoms are common in farmers and are associated with exposure to total dust, fungal spores and endotoxins. No statistical correlation was determined for bacterial spores.

- In conclusion there is an effective defense mechanism of lungs towards inhaled spores, and
- there is no epidemiological evidence for an inhalative health risk for farmers who are exposed to bacterial spores.

The current status of the official evaluation process is, that the BgVV experts offered to evaluate the data request newly, based on the submitted literature, and referred to the discussion on member state level at a future ECCO meeting to ultimately decide upon this data request.

Conclusions:

Considering the presented scientific evidence of a fast and efficient clearance of spores by the exposed respiratory tissues, the applicant concludes that the data requirement, as outlined in the monograph (volume 1, point 4.1.3: repeated dose inhalation toxicity study) is adequately addressed to by submitted references and information and therefore the performance of a repeated dose inhalation toxicity study is not justified.

Therefore the applicant did not initiate the corresponding study (see LEUSCHNER 2001b) and applies for an exemption from this data request.

In addition, it has to be taken into account that the non-pathogenic and non-infectious character of strain QST 713 of *B. subtilis* has been proven in the toxicological and ecotoxicological studies submitted within the EU Dossier. The relevant studies showed that this strain of *B. subtilis* does not produce toxins, and does not germinate or proliferate in tissues of mammals following oral, intratracheal, or inhalative exposure.

Further, the performance of the repeated inhalative toxicity study itself is a critical point, since there is no specific OECD guideline for testing micro-organisms yet, which act basically different than chemicals. The relevant test guideline OECD 412, addressing to chemical active ingredients, states a daily 6h interval for a period of 4 weeks for a repeated inhalative exposure. This exposure scenario will under no circumstances reflect real conditions, under which applicants may be exposed to the dust when preparing the spray.

So far two study protocols have been developed in an extensive discussion process with the German officials at the BgVV to meet all required data demands, especially the main task of assessing clearance (LEUSCHNER 2001a and 2001b). Still the protocol would require some discussion and adjustments, since it is technically almost impossible to ensure a pre-set dose rate per animal by inhalative exposure, which only employs a given concentration of spores in the air. Regarding determination of clearance a pre-set concentration of spores would allow the monitoring of tissue spore content as well.
Finally, determining complete clearance of spores from lungs requires a long post-exposure observation period and a high number of test animals, without yielding necessary toxicological information.

2. Comment on point 4.1.5: Acute Toxicity and/ or Pathogenicity and Infectivity to Earthworms

relating to Annex IIB, Section 6, of directive 91/414/EEC: Effects on Non-Target Organisms annex point 8.5

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.

Appendix 1:

Listing of documents and references cited in this comment, and submitted to the BBA at the stated date of submission (see 4th column).

Note: all references listed are addressing to the data request for a repeated dose inhalative toxicity study (see monograph, volume 1, level 4, point 4.1.3), relating to Annex IIB, Section 3 of directive 91/414/EEC: Toxicology, Pathogenicity and Infectivity, Annex Point 5.2.5.1: Health effect after repeated inhalatory exposure.

Author (year),	Year	Title of document, source (Type of document)	Date of submission y-m-d	Considered for monograph Y/N
EDUARD, W. & MELBOSTAD, E.	2001	Organic dust-related respiratory and eye irritation in Norwegian farmers. American Journal of Industrial Medicine VOL. 39 (2) pp. 209-217 (Reference)	01-09-24	N
GREEN, F. H. et al.	1980	SEM studies on the in vivo uptake of *Aspergillus terreus* spores by alveolar macrophages. Scan Electron Microsc NO. 3, pp. 307-14 (Reference)	01-09-24	N
KURUP, V. P.	1984	Interaction of *Aspergillus fumigatus* spores and pulmonary alveolar macrophages of rabbits. Immunobiology VOL. 166 (1) pp. 53-61 (Reference)	01-09-24	N
LEUSCHNER, J.	2001a	Draft protocol: 4-week repeated dose inhalation toxicity of *Bacillus subtilis* (QRD 713 TP) to Sprague Dawley rats. Laboratory for Pharmacology and Toxicology, LPT, Hamburg (Study plan)	01-04-19	N
LEUSCHNER, J.	2001b	2nd Draft protocol: 4-week repeated dose inhalation toxicity of *Bacillus subtilis* (QRD 713 TP) to Sprague Dawley rats (Study plan)	01-09-24	N
RYDER-FOX	2001	US OSHA limits for subtilisin (personal communication)	01-04-19	N

Author (year),	Year	Title of document, source (Type of document)	Date of submission y-m-d	Considered for monograph Y/N
SAUNDERS, J.R.; SEBUNYA, T.N.K.; OSBORNE, A.D.	1983	Pulmonary clearance of *Bacillus subtilis* spores in pigs. CAN J COMP MED; VOL. 47 (1) pp. 43-47 (Reference)	01-09-24	N
TSAI, S-F.; LIAO, J-W.; WANG, S-C.	1997	Clearance and effects of intratracheal instillation to spores of *Bacillus thuringiensis* or *Metarhizium anisopliae* in rats. Journal of the Chinese Society of Veterinary Science; VOL. 23 (6) pp. 515-522 (Reference)	01-09-24	N
VANCURIK, J.	1966	Causes of the failure of antibiotic prophylaxis of inhalation anthrax and clearance of the spores from the lungs. Folia Microbiol (Praha) VOL. 11 (6), pp. 459-64 (Reference)	01-09-24	N
VOISIN, C. et al.	1971	An experimental investigation of farmer's lung. Comparative study of the pulmonary clearance capacity for *Aspergillus fumigatus*, *Candida albicans* and *Mycro-polyspora faeni* in guinea pigs. Rev Fr Allergol VOL. 11 (2), pp. 129-36 (Reference)	01-09-24	N
WALDORF, A. R.; PETER, L.; POLAK, A.	1984	Mucormycotic infection in mice following prolonged incubation of spores in vivo and the role of spore agglutinating antibodies on spore germination. Sabouraudia; VOL. 22 (2): 101-8 (Reference)	01-09-24	N
WATSON, J. A.; AULD, J. A.; MEYER, G. C.	1973	Clearance and inactivation of the vegetative and spore forms of *Bacillus subtilis* Var niger in rat lungs. Am Rev Respir Dis; VOL. 107 (6), pp. 975-84 (Reference)	01-09-24	N
WHITE, L. O.	1977	Germination of *Aspergillus fumigatus* conidia in the lungs of normal and cortisone-treated mice. Sabouraudia; VOL. 15 (1): 37-41 (Reference)	01-09-24	N

Subject: Comments of the **Netherlands** on EU monograph ***Bacillus subtilis***
RMS: Germany
Date: 19 November 2001

Comments on the Biological properties

No comments.

Comments on Toxicology, metabolism and classification and labelling

Volume 1, Level 2 and 4

2.3 Impact on human health
In general, adjustments should be made in accordance with comments made on the summaries in chapter 6 of Annex B, volume 3.

4.1.3 Toxicology, pathogenicity and infectivity
The reviewer agrees with the requested repeated dose inhalation study.

Volume 3, Annex B

B.4 Proposals for the classification and labelling
No comments.

B.6 Toxicity, pathogenicity and infection
No comments.

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

B.6.1.2 Genotoxicity
No comments.

B.6.1.3 Cell culture studies
No comments.

B.6.1.4 Short-term toxicity
The reviewer agrees with the request for a repeated dose inhalation study.

B.6.2 Step II – Additional studies (micro-organism)
No comments.

B.6.3 Step III – Specific toxicity, pathogenicity and infectivity studies under immunosuppression (micro-organism)
The reviewer suggests to change the title of this sections into “Step III – Specific toxicity, pathogenicity and infectivity studies (micro-organism)”, since it includes studies without immunosuppression.

B.6.4 Medical data

No comments.

B.6.5 Summary of mammalian toxicity, pathogenicity and infectivity and overall evaluation

The comments made on the individual sections also apply to the overall summary.

B.6.6 Step I – Basic acute toxicity studies (preparation)

No comments.

B.6.7 Step II – Additional acute toxicity studies

No comments.

B.6.8 Data on exposure

Summary of the exposure analyses

- The described formulation of this fungicide is WP.
- For the estimation of exposure of operators, the national authority of Germany uses the German model.
- For bystanders, no assessment of exposure is made.
- For workers, no assessment of exposure is made.
- Experimental data on dermal absorption are not available.

Criticisms on the presented exposure assessment

- The EUROPOEM, UK and Dutch models for mixer/loaders and applicators are not used.
- It seems appropriate to use the available European model EUROPOEM for the operators, at least for the application. For the Dutch approach see the annex.
- Bystander exposure may be considered irrelevant if the exposure of the operators is not relevant for the risk assessment, as is stated by the rapporteur.
- For workers the exposure should also be estimated, just like it is done for operators.

Recommendation

- No specific recommendations.
- An estimation of worker exposure is relevant and should, therefore, be performed.

B.6.9 Available toxicological data relating to non-active substances

No comments.

B.6.10 Supplementary studies for combination of plant protection products

No comments.

B.6.11 Summary of mammalian toxicology and conclusion

Comments made on the individual studies also apply to the overall summary.

CRITICAL ENDPOINT LIST

Hazard to humans

Pathogenicity:	No evidence of adverse effects from acute studies - except minor and transient effects after intratracheal challenge.
Infectivity:	No evidence of adverse effects from acute studies. *B. subtilis* infections are only reported from immuno-deficient patients.
Toxicity:	Rat LD50 oral: > 1.13 x 10^8 cfu/animal Rat LD50 intratracheal: > 1.2 x 10^8 cfu/animal Rabbit LD50 dermal > 2.3-2.7 x 10^{11} cfu/animal Rat LD50 intravenous: > 9.4 x 10^6 cfu/animal
Irritation, Sensitisation:	Rabbit: Very slight irritating effects (skin, eye) Based on the sensitising property of the formulation: R43
Genotoxicity:	Not relevant since no genotoxins produced
Medical reports:	Limited database: No adverse health effects observed among personnel involved in laboratory investigations. *B. subtilis* is capable of producing subtilisin which may cause allergic reactions after repeated exposure. *B. subtilis* has been reported to be associated with food poisoning and infections in immuno-deficient patients.
Formulation:	Rat LD50 oral: > 5000 mg/kg bw (~ 2.5 x 10^{10} cfu/kg bw) Rat LD50 inhalation: > 0.63 mg/l air; 4 h (~ 5 x 10^8 cfu/kg bw) Rabbit LD50 dermal > 2000 mg/kg bw (~ 1 x 10^{10} cfu/ kg bw) Skin sensitisation (Buehler test): positive (R43)

Operator exposure

Application method:	FCTM, HCTM, HCHH
Operator exposure models:	In relation to the results of the available acute toxicity studies, sufficient margins of safety exist (calculation on the basis of cfu / German model)

Classification and proposed labelling (Annex IIA, point 10)

with regard to toxicological data	R43: Based on the sensitising property of the formulation

Annex I

SHORT DESCRIPTION OF THE GENERAL APPROACH TAKEN IN THE NETHERLANDS FOR THE ESTIMATION OF OPERATOR/WORKER/BYSTANDER EXPOSURE TO PESTICIDES FOR EU MONOGRAPHS (COUNCIL DIRECTIVE 91/414/EEC)

Summary of Dutch method
If no adequate field studies are available for estimating exposure, predictive models are used.
- Operators
For mixing/loading and application the databases of the European model EUROPOEM are used only, when a database is of adequate size, i.e. sufficiently large for the choice of the 75th percentile for chronic exposure estimates. If this is not the case, the three available national (European) models are used and the results compared, considering that an estimate is required for potential exposure (no protective measures, i.e. normal work clothing) under reasonable worst case conditions, i.e. about the 90th percentile of the for Europe less accurate national exposure database sets. The relevant data are compared and the median of the three estimates is taken as surrogate for risk assessment, again for chronic exposures.
- Workers
For re-entry activities a model is used based on the scientific literature in which potential dermal exposure is directly related to the amount of dislodgeable foliar residue on the crop, a transfer factor and exposure time.
- Bystanders
For bystanders no suitable model is available. Exposure will be estimated on basis of expert judgement.

Introduction
Generally, operator exposure to pesticides occurs during mixing, loading and application of pesticides.
During some activities bystanders might be present and therewith be exposed. After application it may be necessary to handle crops or crop products in such a way that exposure to the workers may occur due to contact with pesticide residues.
For the present purpose the potential exposure will be estimated for an unprotected worker, i.e. wearing normal work clothing, without additional protective measures. The degree of protection required depends on the detailed conditions at work, which may depend on various variables, and which in the context of an EU-monograph cannot be considered in detail. For the bystander even normal work clothing may be an over-estimation of the degree of clothing.

1 Operator exposure
Representative and well-designed field studies with the compound under consideration should form the basis for an adequate exposure assessment (surrogate exposure value(s)). In case such studies are not available the level of occupational exposure must be estimated using appropriate modelling systems. Exposure estimates can be derived using the published models which reflect European conditions. For the present purpose the results of these models will be used for the above-mentioned potential exposure of mixer/loaders and applicators (operators). Presently, the European Predictive Operator Exposure Model (EUROPOEM) is operative, though not optimal for all scenario's. The databases for which the chosen surrogate value in EUROPOEM is based on the 75th percentile,

form the best available estimate for chronic exposures in Europe (EUROPOEM, 1997).
When a proper European database is not available, the considered models are the German model (Lundehn et al., 1992), the UK model (PSD, 1992) and the Dutch model (Van Hemmen, 1992). It should be noted that these models have different underlying assumptions, different underlying databases and use different statistics (about the 75th percentile for the UK, indicative 90th percentile for the Netherlands and the geometric mean for Germany) and formats.
The choice of the statistic is especially important, since the variations in actual practice for the level of exposure are large for many reasons, such as work practices, climatic conditions, variations in equipment and especially personal hygiene. For this reason the calculations with the German model will be done with the geometric mean as well as the 90th percentile. The consideration here is that the underlying studies for the UK and German model are not publicly available for consideration, have not been considered according to basic and explicit criteria, as has been done for EUROPOEM, and that the studies have especially local (national) value, which may not give the required spread for European applications. A more in-depth analysis of the use of different models, not including EUROPOEM, has been published (Van Hemmen, 1993).
For the calculations with all models it will be assumed that a person has a typical weight of 70 kg.
For inhalation exposure the models are applicable for compounds with relatively low volatility (up to 10-100 mPa) at ambient temperature, according to e.g. the Pesticide Manual. When granules have to be considered and an adequate database is not available, it is assumed that the dust content is 10%, unless evidence indicates another percentage.
For use of the various models it is important to define the reasonable worst case options that are relevant for the calculations. This refers to application rates and volume rates.

Exposure estimates with EUROPOEM (EUROPOEM 1997)
EUROPOEM has not yet considered defaults for application areas and times, nor times for mixing/loading. In the analyses, the same defaults will be used as for the Dutch model, when required (see below).
The format of exposure chosen is mg/kg as probably the best estimate, whenever possible. Only 75th percentiles are used (as far as available from the description of the surrogate values for mixing/loading and application). The model is based on studies that have been considered in detail by the EUROPOEM expert group.

Exposure estimates with the UK model (PSD, 1992)
Some assumptions that are made for the UK model are an application area of 50 ha for downward spraying, 30 ha for upward spraying and 1 ha for manual spraying per day. The format of exposure is volume of spray per unit of time. A typical work day reflects 1 hr of mixing/loading and 6 hours of application. The exposure during mixing/loading is estimated on the basis of package size, type of formulation, and number of operations. The format of exposure is weight or volume of formulation.
The model is largely based on unpublished studies, carried out in England by industry and MAFF.

Exposure estimates with the German model (Lundehn et al., 1992)
Some assumptions that are made for the German model are an application area for downward spraying of 20 ha, for upward spraying of 8 ha and for manual spraying of 1 ha per day. For mixing/loading the nature of the formulation is an important

variable. The format of the exposure is mg/kg. 90th Percentiles are calculated from the data in the model.
The model is based on unpublished studies, done by industry and all carried out in Germany.

Exposure estimates with the Dutch model (van Hemmen, 1992; van Golstein Brouwers et al., 1996)
The Dutch model assumes an application area for downward spraying of 10 ha, for upward spraying of 6 ha and for manual spraying of 1 ha. The application time is taken as 6 hr for tractor-driven applications and 3.5 hr for manual applications. The times for mixing and loading are taken as 1 hr for tractor-driven applications and 0.5 hr for manual applications. For greenhouse applications the model considers the full work shift of mixing, loading and application. Indicative 90th percentiles are deduced from the various exposure databases.
The formats of exposure are volume or weight per unit of time, for liquids and solids respectively, expressed for the spray liquid (application) or the formulation (mixing/loading).
The model is based on studies published in the scientific literature and on studies done in The Netherlands.

Discussion of the results
The basic choices are from the truly European model (EUROPOEM), when the databases are considered good enough to calculate the 75th percentiles for surrogate values. This is not done for the national databases which have not been considered according to basic and explicit criteria and which may consist of only local (national) studies, as is the case for the UK and German model. It is evident that the geometric means and 90th percentiles are quite apart from each other. In view of the requirement that reasonable worst case conditions should be estimated, and the considerations given above, the 90th percentile is the best choice for the present purpose.
If relevant surrogate exposures can be estimated by all three exposure models, the median of the assessed exposure values will be used as surrogate for the risk assessment.

2 Bystander exposure
The presence of bystanders should be kept at a minimum. This can easily be achieved in greenhouses, where no person should be allowed that is not involved in the spraying process. Outdoors, such measures cannot be taken that easily.
For field crops, the exposure to bystanders during mixing and loading will be insignificant in comparison to the mixer/loader. This is true for the inhalation exposure as well as the dermal exposure which, in many cases, is largely restricted to the hands of the mixer/loader. For downward spraying such conclusions cannot be drawn that easily, although it should be realized that the distance between bystanders and the nozzles will generally be more than a few metres. The highest levels of exposure will be encountered by a bystander when he or she is in the downwind area of the drift. This is unlikely to happen several times for a bystander walking along the edge of the field. Even for people watching the application, the distance between the edge of the field and the closest nozzle on the boom will change with every spraying swath.
For high crops the level of exposure to a bystander may get higher than in the case of the field crops. There is, however, presently no explicit means of estimating these levels for a bystander. It is expected that the levels of exposure will be small in comparison to the levels of exposure to the operator. Frequency of exposure will be incidental for bystanders.

3 Worker exposure

The exposure to workers in crops after application (re-entry) has been considered by various researchers, but this has, so far, not resulted in a formal data base that can be used for the estimation of the exposure to such crop-workers, especially harvesters. A general approach has been described by Popendorf and Leffingwell (1982) and Popendorf (1985; 1992). A more explicit approach has been described by Van Hemmen et al. (1995) for the harvesting of ornamental flowers grown in greenhouses.

For the present purpose the re-entry activities for workers is mainly considered for tree crops and/or greenhouse crops. For other relevant crops the general approaches are similar, but some general parameters may differ, especially transfer factors.

Exposure estimation for re-entry activities in greenhouse crops (van Golstein Brouwers et al., 1996)

For the estimation of exposure during work with high crops in greenhouses within 1-3 days, i.e. for pesticides with relatively stable dislodgeable foliar residues over that period, an algorithm has been developed for exposure during cutting and sorting/bundling. These activities are considered the most exposure-prone processes for many crops and are considered to be performed each for 3 hr a day.

The model is based on studies carried out in The Netherlands on behalf of the Dutch government. The format of exposure is (mg/hr)/(kg/ha).

Exposure estimates for re-entry activities in tree crops and other crops (Van Hemmen et al., 1995)

For the harvesting of fruits from tree crops the dermal exposure level can be estimated in an indirect way assuming no decay of the dislodgeable foliar residue between last application and re-entry activities. Assuming an application rate of AR kg/ha, and a leaf area index of about LAI m^2/m^2, the initial foliar dislodgeable residue is about 0.01 x (AR : LAI) µg/cm^2 (taking care for the dimensions).

If for the activities in tree crops a typical transfer coefficient is presumed of 10,000 cm^2/hr, the level of exposure per hour can be estimated as about 0.1 x (AR : LAI) mg/hr. For a working day of 6 hr, this would amount to 0.6 x (AR:LAI) mg/day. It must be emphasized that this calculation concerns workers with normal work clothing and bare hands. Furthermore, our knowledge on the various factors that are relevant for the exposure under practical conditions is still far from complete, so these data have to be considered as preliminary estimates.

The inhalation exposure cannot be estimated in a similar way due to lack of data. On the basis of expert judgement it is considered unlikely that the level of inhalation exposure is higher than that of the operators.

Discussion of the results

The exposure data must be considered relevant for the crops with the highest levels of contact with the crop and thus levels of exposure. Exposure levels will be lower when the time between application and re-entry is increased as this is largely dependent on the degree of dissipation of the pesticide residue on the crop. Exposure levels will also be lower for crops with only minor contact between crop and worker during the re-entry activities.

4 Risk management

From the exposure data, it may be concluded that the estimated level of potential dermal exposure to the operators is too high. For that reason a generic assessment of protective clothing is required to estimate the actual exposure for *protected* operators. In view of the fact that the potential exposure is assessed by taking the 75-90th percentile from the relevant exposure models or relevant field data, it is

considered appropriate to use values of about a factor 10 for the protection afforded by adequate protective gloves, protective clothing and respiratory protective equipment; this presumes that a reasonable degree of personal hygiene is taken care of by the operators. The value of a factor 10 is appropriate for generic use at the level of the putting on annex I of the active substance under consideration, i.e. for consideration at the Community level. For the registration of plant protection products in Member States, a more elaborate consideration of crops, techniques and work methods may lead to some adjustment of these values.

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Comments on Residues

Volume 1
No comments

Volume 3, Annex B

B.5 Methods of analysis

B.5.2 Analytical methods for the determination of residues in food and feed
No comments

B.7 Residue data

B.7.1 Metabolism, distribution and expression of residues in plants
No comments

B.7.2 Metabolism, distribution and expression of residues in livestock
No comments

B.7.3 Definition of the residue
No comments

B.7.4 Use pattern
No comments

B.7.5 Identification of critical GAP's
No comments

B.7.6 Residues resulting from supervised trials

Methods of analysis applied in the supervised residue trials
No comments

Supervised residue trials
No comments

Stability of residues prior to analysis
No comments

B.7.7 Effects of industrial processing and/or household preparation
Effects on the nature of the residue

Effects on residue levels
No comments

B.7.8 Livestock feeding studies
No comments

B.7.9 Residues in succeeding crops or rotational crops
No comments

B.7.10 Proposed pre-harvest intervals for envisaged uses, or withholding periods, in the case of post harvest uses

No comments

B.7.11 Community MRLs and MRLs in EU Member States
Not applicable

B.7.12 Proposed MRLs and justification for the acceptability of those residues
Not applicable

B.7.13 Estimates of potential and actual dietary exposure through diet and other means

Intakes by domestic animals
Not applicable

Intakes by humans
Not applicable

B.7.14 Summary and evaluation of residue behaviour
No comments

Comments on Fate and behaviour / Ecotoxicology

General comments on monograph Bacillus subtilis

The EPA has raised some questions for the US notification of Serenade™ WP, apparently the same product from the same company and based on the same dossier (at least respecting the ecotoxicity studies) (see http://www.epa.gov/oppbppd1/biopesticides/reds/brad_006479.htm). Therefore we are interested in the opinion of the RMS regarding the EPA comments.

In the US the product has been conditionally registered for a period of two years (until July 2002). Within that period 3 confirmatory studies — freshwater fish, freshwater invertebrates and parasitic hymenoptera — and 2 new studies — a 30-day whole bee-hive study and a 30-day study with a shrimp — must be conducted and reported to the EPA. The originally submitted studies (all first-tier) with these non-target groups were considered less reliable (*e.g.* the possible involvement of pathogenicity was not well studied). However, as the approximate $L(E)C_{50}$ values in the tests with freshwater fish, freshwater invertebrates and parasitic hymenoptera were very high, indicating adverse effects only at very high dosages, and *e.g.* the (extensive) public literature on the ecotoxicity of *Bacillus subtilis* on invertebrates or fish indicating no evidence of adverse effects, all these tests were considered acceptable, though confirmatory tests are required and recommended to be “conducted at more reasonable concentrations”. The required new study with a whole bee-hive — indicating a very high 5-day LC_{50} of 5663 ppm (corrected for control mortality) — was considered unreliable in view of the fact that all concentrations (incl. more environmentally realistic) showed a high treatment-related mortality (according to the EPA, this is difficult for us to judge as we do not have the primary source), in view of the short test duration, and the fact that the possible involvement of pathogenicity was not well studied. Therefore a 30-day whole bee-hive study was required by the EPA. The fact that no toxic standard seems to have been used is in our opinion a disadvantage as well. Finally, a 30-day test with *Paleomonetes vulgaris* (shrimp) is required as a particular strain was found in the public literature to be pathogenic to a terrestrial amphipod in New Zealand.

What to do with this information? First, the requirements for confirmatory data indicate that adverse effects to fish, aquatic invertebrates and parasitic hymenoptera are not expected but that the EPA wants to be more secure about that. This may refer to shrimps as well, though the fact that there are three species of aquatic endangered amphipods in the US may have played its part as well. Second, the low toxicity of *Bacillus subtilis* to bees in the submitted test, should in the EU not have triggered a second-tier test. However, this may be different if infectivity *c.q.* pathogenicity would have been determined, and that is difficult to judge after such a short test (30 days tests are also recommended as first-tier tests in the US). The EPA apparently focuses on the point that the possibility of infection/pathogenicity for these non-target groups cannot be ruled out *in view of the submitted tests* and *under the proposed conditions of use*, and that may be correct. On the other hand: infection *c.q.* pathogenicity does not seem to be the major mode of action of *Bacillus subtilis*, the growth of *Bacillus subtilis* is not host dependent, and we have no reports on *e.g.* naturally occurring infections of bees due to *Bacillus subtilis* (presuming the QST 713 strain is indigenous in Europe). EPA states indeed that for all these non-target groups the risk quotients — of course referring to toxicity — do not indicate environmental risks.

The reason that we address this issue is to find out how we could discuss about such issues. Another argument to focus on the possible risks to bees is that Serenade™ WP will be registered for use in blossoming pome fruit orchards against fire blight. Again we do not have the feeling that under the proposed conditions of use adverse effects to bees can be expected, but how sure should we be in this statement?
The relevant *Bacillus subtilis* strain QST 713 has been deposited at the American Type Culture Collection. However, in C.1 (Confidential information) the specific ATCC number has not been included. We propose to do so (if possible).

For specific comments on the list of endpoints referring to the environment (Appendix III.2) see next page.
Abbreviations of application methods in the list of endpoints (level 2, Appendix III.3, operator exposure) FCTM, HCTM, HCHH should be included in 2.7.1 Standard terms and abbreviations Part 1 Technical Terms. This applies to the abbreviation MAF on p. 141 in B.9.4.3 (Risk assessment for non-target terrestrial arthropods) as well (8th line).
It is interesting to note that one of the co-formulantia is 1,2-benzisothiazol-3(2H)-one: a non-agricultural pesticide (biocide) that as an active ingredient also falls under the Pesticide Act in the Netherlands. This implies that for an overall risk evaluation of Serenade WP in the Netherlands two active “substances” have to be taken into account. The non-agricultural pesticide then will be evaluated as an agricultural pesticide.
For specific comments on Volume 3 Annex B (environmental fate and behaviour B-8 and ecotoxicology B-9) see p. of these comments.

In view of the submitted data and rationales we agree with the postponed decision on inclusion *Bacillus subtilis* on Annex 1, depending on the filling of data gaps and the answers of questions by the RMS.

II Comments on the list of endpoints for Bacillus subtilis *(Appendix III.2)* (additions/improvements marked with red and underlined)

2.7.3.2 Appendix III.2: Chapter 2 (Hazard evaluation)

Fate and behaviour in the environment

PEC(soil)	Single application Actual	Multiple application Actual
Initial	10 mg product/kg (5 x 10^4 cfu/g soil)	160 mg product/kg (8 x 10^5 cfu/g soil)

Effects on non-target organisms

Effects on birds (Annex IIB, point 8.1, Annex IIIB, point 10.1)

Information on toxicity, infectivity and pathogenicity	Low toxicity to birds. No evidence of pathogenicity or replication of the QST 713 strain of *Bacillus subtilis* in birds; 5-day-LD50 (bobwhite quail): > 10^{11} cfu/kg/d
Further information	~~None~~. The oral dose is administered 5 times in 5 days. Only one concentration was tested.

Effects on aquatic organisms (Annex IIB, point 8.2, Annex IIIB, point 10.2)

Group	Test substance	Time-scale	Endpoint	Toxicity, infectivity and pathogenicity
Laboratory tests				
Oncorhynchus mykiss	Bacillus subtilis QST 713 Technical	30 d	LC50	162 mg as/L (1)(2)
Daphnia magna	*Bacillus subtilis* QST 713 Technical	48 h	EC50	108 mg as/L
Scenedesmus subspicatus	*Bacillus subtilis* QST 713 Technical	72 h	NOEC	100 mg as/L
Daphnia magna	*Bacillus subtilis* QST 713 Technical	21 d	NOEC	7.5 mg as/L

1) No signs of infection in gill, intestine or muscle tissue at test end.
2) Toxicity is probably underestimated as fish were also subjected to dietary exposure.

Effects on bees (Annex IIB, point 8.3; Annex IIIB, point 10.3):

Information on toxicity, infectivity and pathogenicity to bees	~~No evidence of toxicity of *Bacillus subtilis* QST 713 to honeybees~~; No evidence of toxicity of *Bacillus subtilis* QST 713 to honeybees at doses applied at practical use; 5-day-Dietary LC_{50}: ~ 8900 ppm, equivalent to ~ 1.8 x 108 cfu/ml diet
Further information	~~none~~ No toxic standard is used

Effects on arthropods other than bees (Annex IIB, point 8.4, Annex IIIB, point 10.4)

Test material	Species	Develop-mental stage	Sub-strate	Dosage kg/ha	Effects % lethal	sublethal
Predatory mites						
Bacillus subtilis (Serenade WP)	*T. pyri*	Protonymphs	I	16	30.7	13. 04
Parasitoids						
Bacillus subtilis (Serenade[1)])	*A. rhopalosiphi*	Adults	I	16	5.13	25.3
Bacillus subtilis (Serenade WP[1)])	*N. vitripennis*	Adults	I	0.9	46.15	none
Bacillus subtilis (Serenade WP[1)])	*N. vitripennis*	Adults	I	9	19.23	negligible
Bacillus subtilis (Serenade WP[1)])	*N. vitripennis*	Adults	I	90	65.39	negligible
Plant dwelling species						
Bacillus subtilis (Serenade WP[2)])	*C. carnea*	Larvae	I	0.9	0.5	none
Bacillus subtilis (Serenade WP[2)])	*C. carnea*	Larvae	I	9	47.6	none
Bacillus subtilis (Serenade WP[2)])	*C. carnea*	Larvae	I	90	26.3	none
Bacillus subtilis (Serenade WP[2)])	*H. convergens*	Adults	I	0.9	11.8	negligible
Bacillus subtilis (Serenade WP[2)])	*H. convergens*	Adults	I	9	4.7	negligible
Bacillus subtilis (Serenade WP[2)])	*H. convergens*	Adults	I	90	2.4	negligible

I = Inert substrate,
1) 10^8 CFU/g (nominal)
2) $2.0 * 10^{10}$ CFU/g

Effects on earthworms

Information on toxicity, infectivity and pathogenicity to earthworms	No data available
Reproductive toxicity	

Additional studies

Bacillus subtilis is a micro-organism of ubiquitous occurrence but primarily a soil inhabitant. As vegetative growth declines as the nutrient sources declines this species does not seem to compete well for limited resources and *B. subtilis* population will be subject to competition in the natural microflora.

2.7.7.3 Appendix III.3: Chapter 3 (Exposure assessment and risk evaluation)

Exposure of the environment

Natural occurrence, background level:	*B. subtilius* is a ubiquitous -not geographically restricted- inhabitant of the soil, from which it is spread to associated environments, including plants and plant materials (straw, composts), foods (cereals, esp. dried spices), animals and their faeces (by ingestion of spores) and is also naturally found in aquatic environments (fresh water, estuarine and coastal waters). Although *B. subtilis* is commonly found in soil it occurs in almost any environment, including niches in kitchen and bathrooms. The magnitude of occurrence of *B. subtilis* in the soil is not definitely stated in the supplied literature. Indications for their general prevalence can be derived from high levels of presumably soil-born *Bacillus* spp. spores in straw approaching 10^5 cfu/g and from the high numbers of *Bacillus* spp. found in coastal waters (where they constitute up to 20 % of total bacterial population) and from the major contribution of their endospores in estuarine and coastal sediments (achieving up to 80 % of the heterotrophic flora).

III. Specific comments on Volume 3 Annex B (environmental fate and behaviour B-8 and ecotoxicology B-9) Bacillus subtilis

VOLUME 3. Annex B: summary, evaluation and assessment

B.8 Environmental fate and behaviour

B.9 Ecotoxicology

B.9.1 Effects on birds

B.9.1.1

We wonder why the toxicity to birds has been classified as “low to moderate”, as the data seems to indicate a low toxicity rather than a moderate toxicity. ‘mg/kg’ should be ‘mg/kg bw’.

B.9.1.2 Risk assessment for birds.

The highest dose was 5000 mg/kg/d which is equivalent to 10^{11} CFU. The LD_{50} was >10^{11} CFU/kg/d. Serenade WP has an activity of 5 * 10^9 CFU/g. According to the RMS this is 2.5 times higher compared to the test material in the bobwhite quail test. In our opinion the test material is 2*10^{10}/5 * 10^9= 4 times higher than Serenade WP. Expressed in mg/kg bw the LD_{50} of Serenade WP is 5000 x 4 is 20,000 mg/kg bw/d. The acute TER is >20,000/17 = 1170.

B.9

B.9.2.4

In table B.9.2-1 the TER of *D. magna* is 730 and not 723

On the basis of a maximum application rate of 15 kg/ha 1.5 kg as/ha is used. 1 g contains 5 * 10^{10} CFUs. Consequently, 1.5 kg/ha is equivalent to 1.5 * 5 * 10^{10} = 7,5 * 10^{10} CFU/ha.

De initial PEC is calculated for water adjoining the field is calculated as : dosage * drift * D (depth of the ditch).

$PEC_{surface\ water}$= 7.5 * 10^{10} CFU/10.000 m^2 * 0.269* 0.3m= 6.1 *10^5 CFU/m^3=

610 CFU/L.

610 CFU equals 610/5 * 10^{10}= 1.22 *10^{-5} mg.

This PEC is much lower than the PEC calculated by the RMS. This has no consequences for the TER. Nevertheless the RMS is asked to explain the PEC_{sw}

B.9.5

In the second paragraph of this section the last sentence seems to have been mistakenly included as it refers to mice rather than to earthworms.

MILJØ & ENERGI
MINISTERIET

Mr. J. R. Lundehn
Biologische Bundesanstalt für Land- und Forstwirtschaft
Abteilung für Pflanzenschutz und Anwendungstechnik
Messeweg 11/12
D-38104 Braunschweig
Germany

MINISTRY OF ENVIRONMENT AND ENERGY

DANISH FOREST AND NATURE AGENCY

J.no. SN 2001-4111-0016

Danish comments to the Monograph on *Bacillus subtilis* strain QST 713

20. November 2001

Dear Mr. Lundehn

The Danish Forest and Nature Agency have carefully examined the *Bacillus subtilis* strain QST 713 Monograph. Our general opinion is that it is a thorough and well-written Monograph and we agree with the rapporteur that:

- More information is needed on how *B. subtilis* can be differentiated from the closely related *B. cereus*, *B. thuringiensis* and *B. anthracis*
- More information is needed on the resistance of *B. subtilis* QST 713 to antibiotics and the production of antibiotics by *B. subtilis* QST 713
- Documentation of the clinical importance of the presumed resistance of *B. subtilis* QST 713 to a number of antibiotics is needed
- A supplementary repeated inhalation dose study must be performed due to the unusual long residence time
- *B. subtilis* QST 713 has to classified as sensitising.
- Data on acute toxicity, pathogenicity, and infectivity to earthworms must be submitted.
- Decision on annex I inclusion of *B. subtilis* strain QST 713 should be postponed until the required information has been submitted.

We received additional data and an amendment to Document M submitted by the notifier in order to address these questions less than two weeks ago. Our examination of this material gives rise to the following comments:

- We find that the new data satisfactorily gives information on how *B. subtilis* morphologically can be differentiated from *B. cereus*, *B. thuringiensis*, and *B. anthracis.*

Miljø- og Energiministeriet
Skov- og Naturstyrelsen
Haraldsgade 53
DK-2100 København Ø

Phone: +45 39 47 20 00
Fax: +45 39 27 98 99
E-mail: sns@sns.dk

- We find that the new data satisfactorily gives information on the resistance of *B. subtilis* QST 713 to antibiotics and the production of antibiotics by *B. subtilis* QST 713.
- We still want the notifier to elaborate on the clinical importance of the presumed resistance of *B. subtilis* QST 713 to a number of antibiotics.
- An examination of the new references submitted by the notifier as an argument not to perform a repeated dose inhalation study does not exclude a cumulative effect of repeated administration of *B. subtilis* QST 713. This assessment is based on the fact that all the submitted studies concern single dose administration and in several cases they were not even performed with *B. subtilis*. We therefor still require a repeated dose inhalation study performed with *B. subtilis* QST 713.
- The comments made by notifier regarding subtilisin all relate examination and threshold values to American conditions. We still want to see information on absence / quantity of subtilisin produced under relevant conditions and how these quantities relate to possible set European threshold values for the contents of subtilisin in the air.

Even we find the Monograph to be thoroughly prepared it gives rise to the following further remarks and questions:

1. We do not find any experimental evidence in the Monograph regarding whether *B. subtilis* QST 713 occur as vegetative cells or endospores (or both) in the technical product. It is important to distinguish between the two stages, as their effects are quite different. Additional, we would like to see documentation for the absence of the closely related species, *B. cereus*, *B. thuringiensis*, and *B. anthracis* in the product.
2. The mode of action of *B. subtilis* QST 713 is described in rather broad terms. It is important for a final risk assessment of *B. subtilis* QST 713 to differentiate between the general action of *B. subtilis* and the specific action of *B. subtilis* QST 713. *B. subtilis* QST 713 has been selected among other bacteria and other *B. subtilis* for its superior ability to protect plants against several plant pathogens. So, *B. subtilis* QST 713 differs from other *B. subtilis* by this ability. Thus, information elucidating the following questions are needed for a final risk assessment:
    - How do *B. subtilis* QST 713 differ from other *B. subtilis* strains with regard to protection against plant pathogens?
    - *B. subtilis* is known to produce a number of secondary metabolites with antimicrobial activities (according to Berdy et al. (1980-1985) “Handbook of Antibiotic Compounds” approximately 70 different compounds are produced by different *B. subtilis* strains). Which of these compounds are produced by *B. subtilis* QST 713? And what role do these compounds have for the mode of action?
    - Further, the full gene sequence of *B. subtilis* 168 is known (Kunst et al., 1997 (Nature 390, 249-256)) and therefor the gene products of this strain are very well described. Which implications does this information have for the current understanding of the mode of action of *B. subtilis* for plant protection? Have this knowledge further implications for the risk assessment of *B. subtilis* QST 713?
3. *B. subtilis* is not characterised by a distinct host specificity. Is the selected strain *B. subtilis* QST 713 characterised by a distinct host specificity? Is *B. subtilis* QST 713 more effective than other *B. subtilis* strains? We would like the notifier to elaborate on the effects of *B. subtilis* QST 713 on the general, not pathogenic microflora of soils.

4. We would like to see documentation concerning the genetic stability of *B. subtilis* QST 713 under the environmental conditions for the proposed use, including whether possible antibiotic resistance genes are transmittable.
5. The notifier specifies that *B. subtilis* QST 713 do not produce toxins at relevant levels. We do not find any experimental evidence for the non-production of toxins by *B. subtilis* QST 713. We would like to see documentation for this statement; especially we would like to see documentation for absence of subtilisin.
6. Information is missing on specific agricultural, plant health or environmental conditions under which the active organism, *B. subtilis* QST 713 may or may not be used, when this in the light of the test results might be necessary.
7. *B. subtilis* is identified by a number of general microbiological methods. It appears not unambiguously whether it is possible by these methods to differentiate between *B. subtilis* QST 713 and other *B. subtilis* strains. Normally, such a differentiation has to be based on very specific molecular methods. An evaluation of the methods for this differentiation is needed. If this evaluation shows that a differentiation at this level is impossible, development of a new differentiating method is required. This is mainly needed for control purposes, including documentation that the active organism is identical with the parent strain *B. subtilis* QST 713. Further we would like to see documentation on variability and viability of *B. subtilis* QST 713.
8. We would like to information on specific methods to determine viable and non-viable residues in or on treated products (food).
9. The basic test for mutagenicity is missing.
10. The annex point regarding toxic effects on livestock and pets have not been answered. We would like the notifier to elaborate on this point.
11. Information is missing on residues in or on treated products (food). It is important to notice that *B. subtilis* several times have caused food borne disease. Likewise it has been demonstrated that *B. subtilis* is able to produce compounds with cytotoxic effect on cell cultures. Therefor it cannot be excluded that some strains of *B. subtilis* can produce toxins and cause food borne diseases. It should be considered to require a test for the cytotoxic properties of *B. subtilis* QST 713 against cell cultures.
12. The study referred to in the Monograph on viable colonies on pepper leaf surfaces shows a highly increased number of colony forming units on the crop until seven days after treatment. In order to minimise the amount of viable colonies on the harvested crop we suggest a pre-harvest interval of seven days.
13. No data on the persistence of *B. subtilis* QST 713 in soil is presented. The predicted load of *B. subtilis* QST 713 to the soil surface is really uncertain. Risk assessment has partly to be based on knowledge about the persistence. It is impossible to predict the persistence without specific knowledge about:
    - growth, survival and endospore formation in phylloplane and on fruits. Dispersal from these sites to the soil by rain and litter
    - fate in the soil
    - the implications of several applications on fate at these sites
14. The tests on effects on bees are carried out with dried *B. subtilis* QST 713, so the cells are most likely present as endospores. The cells seem to be dividing for a few days after application to leaves or fruits (and perhaps flowers); thus bees are exposed to vegetative cells. The effect of vegetative cells on bees might be quite different from the effects of spores. Are the cells in the diet present as endospores or vegetative cells? Do endospores and vegetative cells have the same effects on bees?

15. The comments and questions regarding effects on bees (14) also apply to the effects on non-target arthropods other than bees.
16. As for the active organism, information is missing on specific agricultural, plant health or environmental conditions under which the product containing *B. subtilis* QST 713 may or may not be used, when this in the light of the test results might be necessary.
17. The instructions for use should be looked at again when all Member States have had the opportunity to evaluate the efficacy data.
18. Denmark has not yet received any efficacy data. We have therefor not had any opportunity to evaluate whether the product, Serenade effectively can control the plant pathogens claimed by the notifier.
19. We would like to see information concerning percutaneous absorption of the product.

Yours sincerely

Lene Thomsen

c.c.: ECCO-TEAM (BBA)

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FINNISH ENVIRONMENT INSTITUTE

[COMMENT1]

Helsinki 19 November 2001

Our ref. SYKE-2001-P-163-042
Your ref. *Bacillus subtilis*

Mr J-R- Lundehn
Biologische Bundesanstalt für Land- und Forswirtschaft
Abteilung für Pflanzenschutzmittel und Anwendungstechnik (AP)
Messeweg 11/12
D-38104 Braunschweig
Germany

Subject COMMENTS ON *BACILLUS SUBTILIS* (DRAFT MONOGRAPH AND PROPOSED DESICION) - FATE AND BEHAVIOUR IN THE ENVIRONMENT AND ECOTOXCOLOGY

Dear Mr Lundehn,

Please find enclosed the Finnish comments on the sections environmental fate and behaviour and ecotoxicology of the draft monograph of *Bacillus subtilis*. Other sections of the monograph are commented by the respective authorities, if necessary.

In our opinion the draft monograph is well prepared and transparent. The studies on environmental fate and behaviour and ecotoxicology have been reported in detail and the conclusions are clearly presented.

We agree with the Rapporteur Memeber State that decision of accepting *B. subtilis* in the Appendix I should be postponed until information on the impact of *B. subtilis* on earthworms is presented.

However, we still would like to draw your attention to three items, where in our opinion further data is necessary:

1. The monograph states that the number of viable cells of *B. subtilis* declines rapidly after the addition of the strain into soil. However, the product containing *B. subtilis* can be used up to 16 times in a growing season for fruits and in 5 to 7 days intervals for lettuce. Therefore, results on the environmental fate of *B. subtilis* in soil after multiple additions should be addressed.

Furthermore, the number of cells of *B. subtilis* declines after it has been introduced into the soil, but sporulation occurs. It would be interesting to know, what happens if conditions for *B. subtilis* later become more favourable, and whether proliferation of the strain can then be excluded.

Postal address	Visiting address	Telephone	Telefax	E-Mail	Home page
P.O. Box 140	Kesäkatu 6	international	international	kirjaamo.syke@vyh.fi	http://www.vyh.fi/syke/syke.html
FIN-00251 Helsinki Finland	FIN-00260 Helsinki Finland	+358 9 403 000	+358 9 4030 0190		

2. All ecotoxicity tests reported in the monograph are single dose tests. However, the *B. subtilis* containing product can be used multiple times during a growing season. Therefore information on ecotoxicological effect of multiple use of *B. subtilis* containing product on the studied species should be required.

3. In the non-target arthropod tests, no results for controls were reported, *i.e.* the tests with zero grams addition. Inclusion of the control results would make the results easier to interpret.

Yours sincerely

Division Manager Esa Nikunen

Senior Advisor Kimmo Suominen

CC: ECCO-team BBA
Plant Production Inspection Centre, Finland
National Product Control Agency for Welfare and Health, Finland

KS/ks

Postal address	Visiting address	Telephone	Telefax	E-Mail	Home page
P.O. Box 140	Kesäkatu 6	international	international	kirjaamo.syke@vyh.fi	http://www.vyh.fi/syke/syke.html
FIN-00251 Helsinki Finland	FIN-00260 Helsinki Finland	+358 9 403 000	+358 9 4030 0190		

KEMI
KEMIKALIEINSPEKTIONEN
NATIONAL CHEMICALS INSPECTORATE

Pesticides Division
Astrid Mårtenson
☎ +46 8 783 1447
e-mail: astridm@kemi.se

COMMENTS

2001-11-19

Registration number

F-2019-298-00

Rapporteur Member State Germany
Mr Herbert Koepp
h.koepp@bba.de/
ECCO-Team ECCO@bba.de

06_bacillus_subtilis_com_se

Swedish comments on the monograph prepared in the context of the possible inclusion of the following active substance in Annex I of Council Directive 91/414/EEC, *Bacillus subtilis strain QST 713* - Volume 1 and 3 Annex B - Doc. 12463/ECCO/BBA/01

Please find below some general and specific comments on the report.

1) **Monograph (vol. 1) overall**: To make the monograph more transparent it would be appropriate to state the complete scientific names along with the pest names as their common names may differ between countries. In the current draft this is done in an inconsequent manner.

2) **Monograph (vol 1), p. 15, Genetic stability:** See comment under B.2.1.7.

3) **Monograph vol. 1, p. 17, "Residues" (2.2.3):** It is stated that no residues relevant to the safety of consumers occur. Please state why the reported food poisoning cases (p. 20, "Medical data") are not relevant, as the product is intended for use on fruits and lettuce. Does this strain lack food poisoning properties?

4) **Monograph (vol. 1) Appendix p. 29 standard terms:** To use the term dna for designated national authority is questionable, since it could be confused with the established term DNA. For example "designated NA" or similar could be used.

LEVEL 4
4.1.1 We suggest that there should be more information on the differentiation between the strain *QST 713* and the indicated pathogenic *Bacillus* species (*B. anthracis, B. cereus and B. thuringiensis*).

4.1.3 We suggest that there should be a genetic confirmation on the absence of food poisoning traits.

Monograph (vol 3)

B.1.1.3.2. and B.1.1.3.3 The methods and tests used for identification should be described. We also think that modern molecular biological methods should have been used for identification.

B.1.1.4.1, B.1.1.4.2 and B.1.1.4.3 There must be some information that is not confidential. Why not inform about the formulation of the product? What does the product consists of more than the a.i.? We understand that the quantities of ingredients have to be confidential, but still we would like to know a bit more about the product.

B.2.1.1.1 Historical background If it is clear that groups exists within the genus, it is important for us to know which one *B. subtilis strain QST 713* belongs to.

B.2.1.1.2 Origin and natural occurrence In the last part of B.2.1.1.2 it is written "The QST 713 strain was screened and fungicidal activity was confirmed." Please, clarify this statement on the following issues:

- Which species of fungus have been tested?
- For which species does *QST 713* show fungicidal activity?
- Which fungus g species are none-sensitive to *QST 713*?

B.2.1.3. Effect on non-target organisms. Micro-organisms. What effect did Iturin give on mycorrhiza under field conditions? **Plants.** Avoid conclusions that generalize. Numbers and facts provided by applicant should be supported by reference to some sort of data.

B.2.1.6. Relationship. The relationship between *B. anthracis*, *B. cereus*, and *B. thuringiensis* is very close – *B. cereus* and *B. thuringiensis* are more or less the same species. They show small variations in plasmid content and some virulence factors, but are very similar (or even indistinguishable) at rRNA and DNA level. *B. subtilis* is the "type species" in the *subtilis* group of *Bacillus*. Similarities are found by "classic" classification and also by rRNA classification, but at genome and DNA similarity level, the species differ much more. See: Okstad OA, Hegna I, Lindback T, Rishovd AL, Kolsto AB in Microbiology (1999)145 Pt 3:621-31 for more information. Detection of pathogens is possible, utilising the pathogenic traits. This should be clarified further in the monograph.
The reference in the monograph is to U.S. Food and Drug Administration. Maybe also Codex Alimentarius has information of interest for the monograph.

B.2.1.7. Genetic stability. Gene transfer. Last par. We suggest to change to the following text: "Taken together, available knowledge indicates that gene transfer within *B.subtilis* or between *B. subtilis* and related species under natural conditions may be a rare event. However, gene transfer is a naturally occurring event, even if it may be difficult to detect in a natural environment with existing methods. *B. subtilis* is a known natural transformer and several phages are present in the environment. Transfer rate is limiting, but this will depend on factors belonging to indigenous bacteria/phages.

Fermentation starts with pure cultures of QST 713 and therefore gene transfer between bacteria during that stage should be of minor importance. QST 713 is not considered to have any undesirable traits why gene transfer to the background flora should be insignificant The question is rather if the strain QST 713 can achieve pathogenic traits from other bacteria present, such as *B. cereus*, and if this would cause any problems."

Due to the last sentence it is necessary to discuss around the possibility of achieving pathogenic traits from other bacteria and the consequences of that.

B.2.1.8 Information on the production of metabolites It is supposed that *B. subtilis QST 713* does not produce subtilisin. We are of the opinion that this assumption should be established by measurements as well as measurements of which metabolites and exo-enzymes that is produced by *QST 713*. We propose that this is done in the fermentation medium.

B.2.1.9 Antibiotics and other anti-microbial agents Se comments at B.2.1.8.

B.3.1.4.2, B.3.1.4.3 and B.3.1.4.6 We are of the opinion that all information can not be confidential. It must be possible to give some information of importance for the risk assessment thus making the monograph more transparent.

B.3.2.3 Details of intended use We are of the opinion that a minimum pre-harvest interval is necessary.

B.3.2.6 Method of application We would like to have more information about the application. How high pressure is possible to use without causing any damage to QST 713? Is it possible to use the same nozzle as with spraying chemical plant protection products? Can an ordinary sprayer be used or is there a risk of sedimentation?

B.4.1 Proposals including justification of the … As far as we know bacteria cannot be classified according to existing rules for classification of chemicals.

B.5.1.1 Methods for the identification of the micro-organism We would like to have a summary of what is written in the ATCC report 1997, so that we do not have to go back to the ATCC report for the information.
We think modern molecular biological methods should have been used for analysis instead of those used here. We also think more information about the analysis should be accessible in the monograph.

B.5.1.1 Methods for the identification of the micro-organism c-d) Test for microbial contaminants and detection of human pathogens We would appreciate clearer information and more references about the test methods used.

B.5.1.2 Methods for the analysis of the preparation Simple methods have been used, and we think that modern molecular biological methods should have been in parallel.

B.5.3.2 Residue analysis We are of the opinion that methods for residue analysis are necessary.

B.6 Toxicity, pathogenicity and infectivity Data requirements for micro-organisms now exists (Commission Directive 2001/36/EEC of 16 May 2001).

B.6.1.5 Pathogenicity and infectivity under immunosuppression 3rd indent, line 4: Please change dissipating to disseminating.

B.6.4.3 Direct observation…. The reference DONIZ et al. (1988) is missing in the reference list.

B.9.2.1 Acute toxicity and/or pathogenicity and infectivity to freshwater fish
The LC50 value was determined to 162 mg/l and NOEC to 86 mg/l. The gross necropsy showed no signs of infection. It was noted in material and methods that “After mixing, the test solutions appeared tan in colour and were cloudy”.
Furthermore,it was noted in the results that “Due to the cloudiness of the 240 and 400 mg/l treatment groups, biological observation of survival were difficult to make. Observations of survival were made using a dip net to look for dead organisms. Evaluations of clinical signs of toxicity were made on the organisms which could be seen.”
Comment: A remark about the uncertainty of the values would be useful when effects on fish are discussed. The effects could be caused by “mechanical effects” instead of the micro-organism.

Conclusions of the Working Group 'Plant Protection Products' (evaluation)
24-25 March 2003

Peer Review Programme under Directive 91/414/EEC

Subject: **Bacillus subtilis strain QST 713**

Rapporteur Member State: **DE**

Co-Rapporteur Member State: **SE**

The substance will be discussed in a special Evaluation meeting on March 26. Separate minutes are available from that meeting.

DE suggests a repeated dose inhalation toxicity / pathogenicity study. A protocol for such a study was already discussed with the applicant, but the study was not conducted so far. Instead, several publications were submitted to address the inhalative toxicity / pathogenicity with a motive to avoid further testing.

DE confirms the view that the repeat study should be required.

Appendix 1: Evaluation table rev. 1-1: Bacillus subtilis strain QST 713

Appendix 2: complete list of end points: Bacillus subtilis strain QST 713

WORKING DOCUMENT – DOES NOT NECESSARILY REPRESENT THE VIEWS OF THE COMMISSION SERVICES

1. Identity, Physical and chemical properties, Details of uses and further information, Methods of analysis

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
				Section 1: Data requirements : 13 Open points : 6
1.1	Further identification method with respect to strain differentiation has to be submitted. Modern molecular biological methods are preferred. IIB, 1 A	A relevant study is submitted to the RMS: Lehman, L. (2002): "Strain Discrimination of *Bacillus subtilis* QST 713 by Ribotyping". AgraQuest Inc. March 2002. The strain differentiation protocol has been 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 computer analysis of the resulting patterns. These patterns may be compared to a database for identification or to other strains for strain differentiation. The database identified QST 713 as *B. subtilis*. The Riboprinter protocol was developed in association with the scientists from Accugenix, a commercial strain identification laboratory. Seven Bacillus strains were studied including two	22.07.02 New information received on identification method of Bacillus subtilis strain QST 713. With the new molecular biological identification method it is possible to distinguish between *Bacillus subtilis* strain QST 713 and other *Bacillus subtilis* strains. The RMS considers that the requirement has been fulfilled.	Evaluation meeting 26 March 2003: One MS is not satisfied with the explanation. Too few strains were so far investigated in the comparison. The specificity of the method is, therefore, still insufficiently documented. Other MS are invited to comment.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		replicates of QST713 and a commercial strain of *B. subtilis*, Kodiak. Several enzyme digests were tested in an effort to find a pattern or ”fingerprint” that would allow us to differentiate QST 713 from other strains. We were able to find an appropriate enzyme, PvuII, that separated QST713 from other strains in the test group, which included *B. subtilis* and other closely related strains from the *B. subtilis* grouping. This enzyme choice can be reconsidered if continuing developmental work uncovers a better choice.		
1.2	Applicant to provide documentation on variability and viability of *B. subtilis* QST 713. IIB, 4.1 A	The storage of production strains such as QST 713 requires that the strain remains viable and that the metabolic activity of the strain remains unchanged. For QST 713 the opportunity for strain variability is reduced by producing a master seed lot from which all subsequent working seed lots are produced. Storage conditions for the master seed lot prevent variability from occurring and each lot is streaked on a plate to determine that the culture is pure and viable. Continuous need for subculturing of the strain is eliminated with this method. Normally one working seed lot is produced each year to meet all production requirements. *Viability* and purity of the strain QST 713 is ensured at each transfer point in the production seed chain process by microscopic examination of the culture, and plating onto Nutrient Broth Agar and	16.07.02 The RMS considers that the requirement has been fulfilled.	Evaluation meeting 26 March 2003: Data requirement fulfilled.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		examining these cultures after growth for the appropriate micro and macro morphologies. For *B. subtilis* strain QST 713, the *viability*, taken as the germination ability of spores in the preparation, can be documented by a 3-year storage stability study (Gingras 2001: "Storage Stability of QST 713 Strain of Dried Bacillus subtilis With Residual Fermentation Media Identified as QST 713 WP". AgraQuest. Inc., Project ID: L08726 SN9).. Results & conclusions: For all time intervals through year 3, the titers of QST 713 WP were between $1.2x10^{10}$ to $4.7x10^{10}$ cfu/g on TSA (Trypticase Soy Agar). Comparable growth was seen on BA (Blood Agar) medium. The test substance was determined to be stable for at least three years when stored at warehouse (ambient) conditions.		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
1.3	In relation to the content of the micro-organism in the technical product the applicant has to clarify whether *B. subtilis* occur as vegetative cells or as endospores. It is recommended not to propose this information as confidential IIB, 1.4.1 A	In both technical and end use product *B. subtilis* predominantly occurs in the endospore form, due to the conditions set to induce sporulation during the fermentation process (depletion of nutrients). In addition the subsequent drying process is limiting the occurrence of vegetative cells. The fermentation broth is comprised of approximately 80% endospore and 20% vegetative cells determined by microscopic examination. An analysis of total CFU/g vs. counts of spores of the end product reveals that the spore count and the total CFU count are in the same log, indicating the end product is almost entirely spores. This is to be expected, as vegetative cells would be more susceptible to loss during the drying process than spores.	16.07.02 The RMS considers that the requirement has been fulfilled.	Evaluation meeting 26 March 2003: Data requirement fulfilled.
1.4	The applicant has to provide the information about the group to which *B. subtilis* belongs to. IIB, 2.1.1 A	The traditional genus *Bacillus* contained a number of discrete groups and has recently been split into several additional genera, including *Paenabacillus* and *Brevibacillus*.. As the neotype strain of the genus *Bacillus*, however, *B. subtilis* has remained in the genus *Bacillus*. *B. subtilis* is traditionally grouped with the closely related strains *B. amyloliquifaciens*, *B. atropheus*, *B. pumilus*, and *B. licheniformis*. QST713 was identified as a *B. subtilis* species by traditional biochemical methods by ATCC (which was state-of-the-art in 1997 when	16.07.02 The RMS considers that the requirement has been fulfilled.	Evaluation meeting 26 March 2003: Data requirement fulfilled.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		this organism was isolated) and by molecular methods using the Riboprint technology (see statement under point 1.1)		
1.5	In relation to the screening (fungicidal activity) of *B. subtilis* strain QST 713 the applicant has to clarify: - Which species of fungus have been tested? - For which species does QST 713 show fungicidal activity? - Which fungus species are none-sensitive to *QST 713* IIB, 2.2.2 A	*B. subtilis* strain QST 713 has been tested against numerous plant pathogenic fungi and has shown activity in the laboratory and in the field against many of these including: *Botrytis cinerea, Alternaria solani, Erysiphe cichoracearum, Venturia inaequalis, Sphaerotheca fuliginea, Sclerotinia minor, Leveillula turica*.. All of these uses for either control or suppression of these organisms have been labelled by the U.S. EPA. No commercial activity has been found when it was tested against *Fusarium*, *Rhizopus*, several species of *Pythium* and *Geotrichum*.	16.07.02 The RMS considers that the requirement has been fulfilled.	Evaluation meeting 26 March 2003: Data requirement fulfilled.
1.6	The applicant has to provide information about secondary metabolites of *B. subtilis* strains with antimicrobial activities (according to Berdy et al. (1980-1985) and which of these compounds are produced by *B. subtilis* QST 713? And what role do these compounds have for the mode of action? IIB, 2.2.2 A	Secondary metabolites produced by strain QST 713 have been characterized according to the German data request and submitted to all member states within the file of further submitted data, in October 2001 (MANKER 2001, annex point 2.8, also see Amendment to Document M, IIB 2.8). The detected metabolites belong to a known class of lipopeptides, including iturins, plipastatins and surfactins. These findings had not been considered in the monograph. According to the Handbook of Antibiotics (Berdy et al., 1980-85), there are 72 compounds reported to be produced by	30.07.02 The RMS considers that the requirement has been fulfilled. An addendum including the data about secondary metabolites will be provided by the RMS (See open point 1.1).	Evaluation meeting 26 March 2003: Data requirement tentatively fulfilled. The RMS is invited to provide the addendum

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		Bacillus subtilis.. Each entry was accessed and analysed to determine whether it is present in Serenade. Many of the entries were reported in the 1950's and 60's and therefore had little or no spectroscopic or other characteristic data. In some cases they simply listed one activity (e.g. 00000-4595 lists only the producing organism with antifungal activity). In these cases, there is not enough information to determine whether they are the same as compounds characterized later or as the ones produced by our strain. However, we can confirm the presence of two entries in our product. One is Iturin-A, (43140-1750 in Berdy et al.) which is also known as Bacillomycin B, Eumycin, Bacillomycin-R, Bacillomycin and Fungocin. A second entry, 44230-1960 or Surfactin is also present in our product. From our experiments, we have shown that surfactin is not active by itself against plant pathogenic agents. However, it has been reported to synergize the antifungal activity of iturins (Thimon, et al., JAOCS, 69, pg 92-93.) In addition, it is widely reported that iturins have antifungal activity (Besson et al., 1976, Journal of Antibiotics, vol. 29, pg 1043-1049). With respect to the implications of this information on the mode of action of *B. subtilis* QST 713 for plant protection, there is not a definitive answer. There are		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		multiple modes of action of *B. subtilis* strain QST 713 as outlined in the monograph (B. 2.1.2.2) and in the EU dossier (Doc. M-IIB, 2.2.2) and discussed by Alabouvette and Lemanceau (1998) including: competition, colonization, induction of systemic acquired resistance and inhibition of plant pathogens by secondary metabolites. Addressing to the MS comment of Denmark in the Reporting Table (B.2.1.2.2 Mode of action), regarding the full genome sequence of *B. subtilis* 168 as described in Kunst et al. (1997; Nature vol. 390: 249-256): the authors identified over 4000 punitive 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 coded for large multifunctional enzymes, which the authors likened to those involved in antibiosis synthesis in Streptomyces and Gram-positive bacteria. The identified genes did not include those coding for the lipopeptides found in QST 713.		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
	Open point 1.1 The monograph has to be amended by an Addendum (Manker, 2001).	Applicant agrees	30.07.02 Secondary metabolites produced by B. subtilis strain QST 713 belong to the class of lipopeptides, including iturins, plipastatins and surfactins. The RMS will provide an addendum about "Chemical characterization of QST 713" (Manker 2001) and "Analysis of Serenade for presence of subtilisin" (Manker 2002) when the additionally required toxicological information is available.	Evaluation meeting 26 March 2003: The RMS is invited to provide the addendum.
1.7	What effect did Iturin give on mycorrhiza under field conditions? IIB, 2.3 A	Preliminary laboratory data collected by the notifier in 1998 indicated that there were no adverse effects on endomycorrhiza when inoculated tomato seedlings were treated with a drench of QST 713 whole broth. There is no indication for a different performance in the field, since the applied *in vivo* conditions employing the tomato host plant are close to field conditions and inhibition was only observed *in vitro* (CITERNESI et al. 1994; submitted within EU dossier, IIB, 2.1.1/03) Iturins are the best-investigated and most common antimicrobial compounds formed by *B. subtilis.* But the potential action of iturin cannot be regarded separately from the beneficial effect on mycorrhiza development and plant growth of the whole organism, which is relevant to the environment. Despite their	22.07.02 The RMS considers that the requirement has been fulfilled.	Evaluation meeting 26 March 2003: Data requirement fulfilled.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		prevalency, *B. subtilis* is known to have a beneficial effect on plant health and crop yield, and is being applied as a growth stimulator - as stated in the EU dossier (Doc M-IIB, 2.1.1) and the monograph (B. 2.1.1.1). *B. subtilis* also has been found to act as a mycorrhiza-enhancing bacterium TORO et al. 1997: ”Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability (32P) and nutrient cycling”, Applied and Environmental Microbiology, vol. 63(11): 4408-4412 (= reference #20 listed in THOBOR 2000, submitted to all member states in 10/2001). Therefore, the notifier feels that the beneficial aspects of *B. subtilis* have been shown and adequately address the question relating to iturin.		
1.8	Notifier has to clarify on which plant species information is available. IIB, 2.3	Field trials are conducted once activity in the lab is confirmed. The data are used to help define target pests/crops and use rates that will be used on the label both in the E.U. and elsewhere. The U.S. EPA approved label uses are based on efficacy and phytotoxicity data collected by the notifier for the crops specified on the label.. The crops stated on the U.S. label include: apples, pears, broccoli, carrots, cherries, cucurbits, grapes, hop, leafy vegetables	16.07.02 The RMS considers that the requirement has been fulfilled.	Evaluation meeting 26 March 2003: Data requirement fulfilled.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		(e.g. lettuce, celery and spinach), onion, garlic, peanut, pepper, potato, tomato, walnut. A biological file will be submitted in case of application for national registration in any EU member state, and can then be reviewed. In addition, there is a published reference, reporting that *B. subtilis* was not phytotoxic to young apple trees when applied as a root-dip (abstract available): Utkhede, RS & Smith, EM (1993): ”Evaluation of biological and chemical treatments for control of crown gall on young apple trees in the Kootenay Valley of British Columbia”. J PHYTOPATHOL (BERL); 137 (4). 1993. 265-271. Further, Kneusel et al. (1990) have shown that *B. subtilis* detoxified a certain compound involved in phytotoxicity caused by Alternaria leaf blight disease (abstract available): Kneusel RE, Matern U, Wray V, Kloppel KD: ”Detoxification of the macrolide toxin brefeldin A by *Bacillus subtilis”*. *FEBS Lett* 1990 Nov 26;275(1-2):107-10		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
	Open point 1.2: Monograph has to be amended by an Addendum. (Heins 2001)	agrees	16.07.02 The closely related species *B. cereus*, *B. thuringiensis* and *B. anthracis* can be distinguished from B. subtilis based on size, motility, spore location, maximum growth temperature and biochemical trests (Heins, 2001). dendum including these data will be provided by the RMS.	Evaluation meeting 26 March 2003: Data requirement tentatively fulfilled. The RMS is invited to provide the addendum.
1.9	The applicant has to provide information to distinguish *B. subtilis* and *B. licheniformis.* IIB, 2.6 A	QST 713 is distinguished from *B. licheniformis* based on their different morphologies. QST 713 does not occur in long chains whereas *B. licheniformis* does. 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). Biochemically *B. licheniformis* can be differentiated from other species in the genus by the use of API diagnostic test kits (Logan and Berkeley, 1981). In addition, *B. licheniformis* was also easily distinguishable from other closely related members of the genus using pyrolysis gas-liquid chromatography (O'Donnell et al., 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	30.07.02 The two species *B. subtilis* and *B. licheniformis* can be distinguished by morphological and biochemical characteristics. An addendum including these data will be provided by the RMS.	Evaluation meeting 26 March 2003: Data requirement tentatively fulfilled. The RMS is invited to provide the addendum.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		Systems".. International Journal of systematic Bacteriology, vol. 30: 448-459). *B. licheniformis* is a member of the *B. subtilis* group of bacilli. According to the EPA Final Risk Assessment of *Bacillus licheniformis* (February 1997), is a ubiquitous bacterium. It is the most commonly isolated bacillus in milk and milk products. A survey of the literature by Edberg (1992) failed to reveal any toxigenic substances produced by *B. licheniformis.* The authors of the Risk Assessment stated, "while not innocuous, *B. licheniformis* appears to have a low degree of virulence." Most of the medical literature on this organism deals with immunocompromised or traumatized individuals. Addressing to the comment of Finnland under point Vol. 1, 2.1.2; B.2.1.6 of the Reporting Table, the cited reference on food poisoning: Salkkinoja-Salonen et al. (1999) isolated a compound from *B. licheniformis* based on the inhibition of boar spermatozoan motility, an assay that was previously used to measure the emetic toxin of *B. cereus*.. While this assay led to the partial isolation of a compound, the effect of this compound on the boar spermatozoa differed from the effects seen when emetic-toxin-producing	Indeed, there is no sufficient evidence to support the assumption that *B. subtilis* QST 713 may actually cause food poisoning incidents.	

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		B. cereus extracts were assayed. Additionally, the *B. licheniformis* extracts were assayed at 1000x greater concentration than those of the *B. cereus.* It is not clear that the compound described can be considered a food poisoning toxin without an emetic assay. Regardless of the existence of pathogenic *Bacillus* species the species *B. subtilis* is classified by the U.S. National Institute of Health (NIH; view: www.nih.gov/od/orda/apndxb.htm) as RISK GROUP 1 Agent, i.e. agents that are not associated with disease in healthy adult humans. Further, B. *subtilis* is listed as a GRAS organism (generally recognized as safe) by the U.S. Food and Drug Administration (FDA), as cited within the EU dossier (e.g. see Doc. M-IIB, 2.6).		
1.10	Applicant to provide information on toxins produced by *B. subtilis* QST 713. IIB, 2.8 A See open point 1.1	Secondary metabolites produced by strain QST 713 have been characterized according to the German data request and submitted to all member states within the file of further submitted data, in October 2001 (MANKER 2001, Annex point 2.8, also see Amendment to Document M, IIB 2.8). The detected metabolites belong to a known class of lipopeptides, including iturins, plipastatins and surfactins. Strain QST 713 does not produce toxins or metabolites of	From a toxicological point of view, the submitted information is considered sufficient for comprehensive assessment. 30.07.02 An addendum including these data will be provided by the RMS (see open point 1.1).	Evaluation meeting 26 March 2003: Data requirement fulfilled.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		significance in human medicine. These findings had not been considered in the monograph. In addition all submitted toxicological studies evidenced that strain QST 713 does not produce toxins, since any adverse impact resulting from the presence of toxic metabolites would have been recorded in the complete toxicological series of studies conducted by the notifier. *B. subtilis* has been reported to produce subtilisin, however the QST 713 strain was tested for production of this compound and no detectable levels were determined, as shown by a new study: Denise C. Manker, Ph.D.: Analysis of Serenade® for Presence of Subtilisin AgraQuest, Inc., May 2002 An ELISA assay was developed for subtilisin and samples of the QST 713 whole broth were tested for the presence of this enzyme for product stewardship purposes by the notifier. The assay was active in the µg/ml range and no subtilisin was detected in a production batch of the technical material used to make Serenade WP. There is no European threshold level for subtilisin.. Germany withdrew their concerns and request of data on subtilisin production based on the further submitted		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		information on U.S. regulations, proving that there is no valid limit for subtilisin in the air of the industrial setting (see RYDER FOX 2001, further data submitted in 10/2001, under point IIB, 5.2.5.1). The EPA Registration Eligibility Decision does not state a specific risk derived from subtilisin. Regarding potential impacts of secondary metabolites formed by strain QST 713 of *B. subtilis* on beneficial micro-organisms it was determined that during the course of several fermentation trials done with the preparation Serenade WP and Serenade AS, no adverse effects were found. The reports on side effects on fermentation and sensorial properties of wine will now be submitted (within the complete file of further documents cited in this table), for trials conducted in France and in Germany: - France: Viti R&D (Viticulture Recherche et Development), 101, Impasse des capitelles, F-34400 Vielletelle (report April 2001 : ”Study on unintentional effects of experimental fungicide compounds Serenade WP and Serenade AS on production and quality of musts and wine”.) - Germany: Amtliche Versuchseinrichtung Rheinland-Pfalz für Weinbau, Staatliche Lehr- und Versuchsanstalt, FB		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		Phytomedizin, D- 67 435 Neustadt, Westphalen (Englsih summary and data sheets (German) of fermentation trials in 1999 and 2000 with subsequent testing of sensorial properties for two consecutive years)		
	Open point 1.3: Monograph has to be amended by an Addendum (concerning Antibiotics and other anti-microbial agents)	Agrees. An extended testing of antimicrobial agents, as required within a national application process in 2001, can now be submitted: Lehman, L. (2002): ”Antibiotic Susceptibility Testing of Bacillus subtilis QST 713”; AgraQuest Inc.. Susceptibility testing was performed with eighteen commonly used human antibiotics, representative of multiple structural classes of antibiotics, including the B-lactams, aminoglycosides, macrolides, tetracyclines, and quinolines, and of multiple modes of action. While inhibition zone standards do not exist for *B. subtilis*, comparisons to the inhibition zones of control organisms, *P. aeruginosa* and *S. aureus*, suggest that *B. subtilis* QST713 is susceptible to all the antibiotics at the concentrations tested.	An addendum including these data will be provided by the RMS when the additionally required toxicological information is available.	Evaluation meeting 26. March 2003: RMS is invited to provide the addendum. Point tentatively closed.
	Open point 1.4: MS to decide whether additional documentation for the absence of the closely	For information on seed stock purity determination and maintenance refer to point B.5.1.1, in vol. 3 of the monograph. Notifier agrees to RMS comment in	16.07.02 The RMS considers that the requirement has been fulfilled.	Evaluation meeting 26 March 2003: Data requirement fulfilled.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
	related species, *B. cereus*, *B. thuringiensis*, and *B. anthracis* in the product is necessary.	Reporting Table to point B.5.1 ”Analytical methods for formulation analysis”, referring to the documentation of absence of microbial contaminants and human pathogens, as submitted in the EU Dossier within confidential papers (GINGRAS 1998a and 1998b). The applicant further agrees to RMS comment given under point B.5.1.1 c-d) of Reporting Table ”Test for microbial contaminants and detection of human pathogens”, stating that the employed procedures are given in detail, including media and solutions and interpretation of test results for detection of human pathogens and microbial contaminants by the confidential document (BELLET 1998a, submitted in Doc. J of EU Dossier, IIB, 1.4/01). It is important to note that these tests are performed by a third-party laboratory according to the approved Bacteriological Analytical Manual. Authorities have access to the confidential papers (Appendix C of monograph, respectively Document J of EU dossier). The applicant does not want to disclose this confidential business information within the monograph since it is contained in the reference noted above. In conclusion, the absence of *B. cereus*, *B. thuringiensis*, and *B. anthracis* or other contaminants in the product has been well	See also open point 1.2	

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		documented by the applicant.		
	Open point 1.5: MS to decide whether a minimum pre-harvest interval is necessary. (see open point 5.1)	A pre-harvest interval as set for chemical agents is not required, since there is no health risk imposed by the active ingredient or formulation components in Serenade. To the contrary, there is some evidence from published literature on a rather beneficial than detrimental health effect of long-term oral intake of *B. subtilis* spores: Novelli, A.; Ulivelli, A.; Reali, E. F.; Mannelli, F.; Trombi Belcari, L.; Spezia, R.; Periti, P: "*Bacillus subtilis* spores as a natural pro-host oral agent. Preliminary data in children". Chemioterapia; VOL. 3; NO. 3; 1984 Jun; PP. 152-5) . The authors report on a therapeutic use of *B. subtilis* spores as a biological response modifier to improve the immune response of children suffering from recurrent infectious diseases of the respiratory tract. The results show that long-term *B. subtilis* spore therapy significantly reduced the frequency of respiratory tract infections in treated children. Also please refer to statement given under point 1.13, open point 1.6 regarding the relevance of residues of this benign micro-organism, and its classification .	02-08-12 RMS agrees with the argumentation of the notifier	Evaluation meeting 26 March 2003: Data requirement fulfilled.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
1.11	Applicant to provide additional information about the application (pressure, nozzles). IIIB, 3.6 A	Experiences from field trials show that any ordinary sprayer can be used. No restrictions on technical devices are necessary, since *B. subtilis* occurs mainly as endospores in the product. Serenade is used commercially in four countries under a variety of nozzle pressures ranging from 40 to 250 pounds per square inch.	30.07.02 The RMS considers that the requirement has been fulfilled.	Evaluation meeting 26 March 2003: Data requirement fulfilled.
1.12	Applicant to clarify whether the three media TSA, BA and SDA (Gingras, 1998) or a Nutrient Broth Agar is used for identification and whether the colour of the colonies is in all cases ”light cream”. IIB, 4.1 A	For colony identification and enumeration nutrient broth agar (NBA) is used, yielding a ”light cream” colour of *B. subtilis* colonies, as mentioned in the AQ report on determination of aerobic colony forming units (EU dossier, IIB, 4.1/03). The base colour of the colonies of *B. subtilis* is ”cream”, but may vary slightly (light cream (white) to cream (brownish); see 5 batch analysis by Gingras 1998 (EU Dossier IIB, 1.4/03). This slight variation in colour is due to the source of carbon, i.e. agar type and eventually also brand of the same agar type. Enumeration of spores has been compared between plating *B. subtilis* on NBA verus TSA (Trypticase Soy Agar , giving comparable results: Van KOPENHAGEN 2002: Content of Active Ingredient of Serenade® Biofungicide Wettable Powder (EPA Reg. No. 69592-4) NBA versus TSA Plates).	30.07.02 The RMS considers that the requirement has been fulfilled.	Evaluation meeting 26 March 2003: Data requirement fulfilled.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
1.13	Applicant to provide information whether the manufacturer has any certified quality assurance system (e.g. HACCP) IIIB, 5.1 A	Manufacturing is under GMP-like conditions (working under SOPs complying with principles from US-Food and Drug Administration provisions). An EPA establishment number for the manufacturing plant is available, i.e. plant is subject to audit by EPA.	30.07.02 The RMS considers that the requirement has been fulfilled.	Evaluation meeting 26 March 2003: Data requirement fulfilled.
	Open point 1.6: MS to decide whether specific methods to determine viable and non-viable residues in or on treated products (food) are necessary	Residue analysis should not be required, as a waiver for consideration of residues has been requested based on the benign microbial composition of the product. Residues are not regarded as relevant, since the active ingredient is a benign micro-organism, which is prevalent in many environmental compartments, has not been genetically altered, and the active ingredient of the preparation is suggested to be classified into biological agents group 1 according to directive 89/391/EEC, as suggested within the reporting table (see SF comment under point xxiii, Vol. 1, 2.1.4; the other suggested directive 90/679/EEC is not in force, to our knowledge). We agree that the QST 713 strain of *B. subtilis* should be classified as: 'unlikely to cause human disease'. This classification is supported by the U.S. National Institute of Health, which has classified *B. subtilis* as RISK GROUP 1 Agent, i.e. agents that are not associated with disease in healthy adult humans (view: www.nih.gov/od/orda/	26.06.02 If MRLs are set then methods will be required.	Evaluation meeting 26 March 2003: Position of the RMS is confirmed but it is unlikely that MRL's are needed. No data are required for the time being.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		apndxb.htm). Also see statement under point 1.9 of this table). Further, *B. subtilis* is listed as a GRAS organism (generally recognized as safe) by the U.S. Food and Drug Administration (FDA), as cited in the EU dossier (e.g. Doc. M-IIB, 2.5). However, the active ingredient of the product can be determined by counting colony forming units (CFU) employing serial dilution plating. For grapes the notifier can submit a relevant residue study: Ryder Fox (2001): "Serenade WP Residue on Wine Grapes". Also see summary given under point 2.1 of this table.		
1.14	NEW DATA REQUIREMENT The applicant is invited to comment on the mode of activity against *Erwinia amylovora*			Evaluation Meeting 26. March 2003: It appears that the applicant has so far addressed only the fungicidal activity of the organism. The activity against the bacterium *Erwinia amylovora* (which is claimed as one of the target pests) must also be explained.

2. Environmental fate and behaviour

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
				Section 2 : Data requirements : 1 Open points : 0
2.1	The applicant should provide further information about the environmental fate of *B. subtilis* in soil after multiple applications. IIB, 7.1.1 A	Strain QST 713 of *B. subtilis* has been isolated from soil, and has not been genetically modified. This strain has no toxigenic properties towards any of the tested non-target organisms. It is a natural part of the soil microflora and will be exposed to the antagonistic actions of many competing saprophytic microbes. From published references stated below it can be inferred that upon application *B. subtilis* levels will decline gradually, but may establish at a moderate level , as shown for *B. subtilis* , strain NB22 (TOKUDA et al. 1993, reference data given below). As for all bacilli the endospore is the survival propagule of strain QST 713, meant to persist unfavourable environmental conditions, such as lack of nutrients, drought and heat. Evidence from literature supports that vegetative growth of *B. subtilis* applied to soil is dependent on supply of organic matter, and upon application of vegetative cells to soil cell numbers decline followed by sporulation (see monograph, B. 8.1, respectively EU dossier, Doc. M-IIB, 7.1.1). Therefore, the	14-08-02 New published literature have been submitted to the RMS. Different references have shown, that after application of *Bacillus subtilis* the number of cfus declines in soil to an equilibrium and introduced *B. subtilis* cells will be subject to competition with the indigenous microflora. The RMS considers that the requirement has been fulfilled.	Evaluation meeting 26 March 2003: Data requirement fulfilled.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		predominant form in which *B. subtilis* exists in the soil is the endospore, independent from a single or multiple applications. Assuming conditions turned favourable for germination of spores, by, e.g., supply of organic matter, there still is no risk for unlimited growth of strain QST 713 of B. *subtilis*, a naturally occurring saprophytic soil inhabitant, because it is subject to natural competition within the saprophytic soil microflora. But even establishment of *Bacillus subtilis* strain QST 713 in the soil ecosystem would not present an unacceptable risk for any of the potential soil inhabitants, as proven by lack of adverse effects when tested with: - Aquatic invertebrates, birds, fish and non-target arthropods (see monograph, B.9.2-9.4) - Earthworms (as requested within the EU evaluation process for *B. subtilis* under point 3.5) - A literature search revealed no evidence for any adverse effect of *B. subtilis* on other soil micro-organisms (see THOBOR 2000, submitted with further documents in October 2001; also refer to submitted 'Amendment to Document M', annex point IIB, 8.6). It should be considered that any data generated on population dynamics of a micro-organism are always dependent		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		on the study conditions, e.g. the soil type, the temperature, and background microflora etc. For the highly variable conditions given under agricultural practise conditions in the field, the growth rate of any micro-organism introduced into the soil cannot exactly be predicted. But this may be of concern for genetically modified micro-organims or for micro-organism which are producing toxins, or have other detrimental effects on other organisms. None of these concerns is applicable to *B. subtilis* strain QST 713. Therefore, we see no justification for stating this data gap. In order to provide more substantial information on the ability and potential of *B. subtilis* to colonize the soil environment, a new literature search in the Agricola database has been performed. Now more references on population dynamics of *B. subtilis* can be submitted, together with references already stated in THOBOR 2000. List of available references: - Bochow, H.; Gantcheva, K. EDITOR- Vanachter, A. ”Soil introductions of *Bacillus subtilis* as biocontrol agent and its population and activity dynamic”.. Acta Horticulturae		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		NO. 382; 1995; PP. 164-172 (listed as #4 in THOBOR 2000 reference list submitted in 10/2001) This reference shows that *B. subtilis* was able to colonize roots of maize and peas for at least 2 months up to the 2 year study duration, when added directly to the soil. Higher soil temperatures, and sufficient soil moisture enhanced growth and phytosanitary activities of *B. subtilis* against *Rhizoctonia solani*.. Dormant spores of *B. subtilis* reacted by outgrowth when the soil was heat shocked or watered, raising the cfu levels from log 4 cfu/g soil to > log 8. The importance of natural competition among introduced *B. subtilis* and the authochthone soil microflora was stressed. - 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”. APPL. ENVIRON. MICROBIOL. vol. 54, no. 9, pp. 2161-2166 In this reference *B. subtilis* spores were added to soil to serve as prey for bacterial predators, as one factor to study the effect of different environmental conditions, including excess copper, on survival of these predators in soil.		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		- Milus, E. A.; Rothrock, C. S. ”Rhizosphere colonization of wheat by selected soil bacteria over diverse environments”. Canadian Journal of Microbiology, VOL. 39; NO. 3; 1993: pp. 335-341 (listed as #12 in THOBOR 2000 reference list submitted in 10/2001). Reference showing that the level of two *B. subtilis* strains ranged from $10^{3.7}$ to $10^{7.1}$ cfu/ g dry weight of roots in wheat in falls and spring. - Pandey, A.; Palni, LMS; 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, Vol. 156: 377-382 This reference shows the saisonal variation in the composition of the rhizosphere microflora, and the antagonism between fungi and bacteria as an important factor. - Pantastica-Caldas, M; Duncan, KE; Istock, CA (1992): ”Population dynamics of bacteriophage and *Bacillus subtilis* in soil”. Ecology, Vol. 73(5), 1992: pp. 1888-1902 (listed as #15 in THOBOR 2000 reference list submitted in 10/2001). Reference proves that *B. subtilis* is subject to		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		predators. - Tokuda, Y.; Ano, T.; Shoda, M.: ”Survival of *Bacillus subtilis* NB22 and its transformant in soil”. Applied soil ecology : a section of Agriculture, Ecosystems & Environment June 1995. v. 2 (2) p. 85-94 (listed as #19 in THOBOR 2000 reference list submitted in 10/2001) This reference introduces the complex ineractions among *B. subtilis* derived from a desert soil, and a temperate bacteriophage. Addressing to comments of Denmark to the e-fate of *B. subtilis* spores on fruits and phylloplane (see point 8.1 of reporting table), related to this open data point: *Survival on fruits:* a recent residue study on grapes can be provided: Ryder Fox (2001): ”Serenade WP Residue on Wine Grapes”. AgraQuest inc. Within the third week after application of 5 kg/ha, and 10 kg/ha Serenade WP, respectively, the cfu level had declined in 28 days by approximately 93% (one log) to a level of ~10^3 cfu/g (several thousand spores/g grape). *Survival on phylloplane*: a glasshouse study		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		on pepper has been submitted within the EU dossier (YUAN & HEINS 2000; see monograph B.7.1). This study showed a decline of ~ 99% of the cfus in the 21-day period of the glasshouse study, with an approximate 93% decline of cfu-counts by the first 8 days of the study. Further, spores are subject to photodegradation when exposed on the phylloplane under field conditions. Basically, the leaf surface is regarded a stressed micro-habitat restricting growth of saprophytic micro-organisms, and thereby impeding the desired establishment of a protective layer of biocontrol agents on the foliage (CAMPBELL 1989: ”Biological Control of Microbial Plant Pathogens”, chapter 3: ‘Biocontrol on Leaf Surfaces’, Cambridge University Press 1989; cited in both monograph – B. 7.1 – and EU dossier, Doc. M-IIB, 6.1). In conclusion, the possible spread of this *Bacillus subtilis* strain to the environment will not be hazardous since this species is a naturally occurring bacteria with widespread geographical distribution and will be subject to competition with the indigenous microflora.		

3. Ecotoxicology

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
				Section 3 : Data requirements : 5 Open points : 1
3.1	The two confirmatory studies (freshwater fish/invertebrates) as well as the new 30-d-shrimp study required by US-EPA should be submitted when finished. IIB, 8.2 MS	All ecotoxicological studies submitted to the U.S. EPA will be submitted to the EU and member states. Following extended studies on aquatic organisms are available: 1. Rainbow trout: Drottar et al. 2001: " QST 713 Technical : A Five Concentration Toxicity and Pathogenicity Test With the Rainbow Trout (*Oncorhynchus mykiss*)" GLP: YES. U.S. EPA Series 885 - Microbial Pesticide Test Guidelines OPPTS No. 885.4200 Fish were exposed to test substance concentrations of 2,3 x 10^6, 4,6 x 10^6, 9,2 x 10^6, 1,84 x 10^7, and 3,68 x 10^7 cfu/mL for 30 days. Potential effects not related to pathogenicity of the a.s. were assessed by a sterile filtrate with heat-killed spores at the highest employed test concentration. A broth concentrate (at 5,3 x 10^6 cfu/mL) was employed as additional control, besides the negative control. LC_{50} = 1,4 x 10^7.. NOEC = 1,7 x 10^6 cfu/mL.	2002-07-23 The respective studies have been submitted to the RMS. . A detailed evaluation will be presented in an addendum to the DAR. The data requirement is considered to be fulfilled	Evaluation meeting 26 March 2003: Data requirement fulfilled. RMS is invited to update entry in Column C and provide a brief summary of the results.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		No signs of pathogenicity were observed in any QST 713 Technical treatment group. After 30 days mortality in the 3,68 x 10^7 cfu/mL group was 90%, however, in the attenuated control group mortality was 100%. Therefore, mortality most likely was due to the physical nature of the test solution, rather than pathogenicity of this strain. 2. Grass shrimp: Machado, MW (2001): ” QST 713 Technical Powder – Infectivity and Pathogenicity to Grass Shrimp (*Palaemonetes pugio*) During a 30-Day Static Renewal Test”. GLP: YES. U.S. EPA Series 885 - Microbial Pesticide Test Guidelines OPPTS No. 885.4280 The estuarine shrimp were exposed to test substance dietary doses of 3,7 x 10^6 cfu/g, determined to be stable and adhere to the food in a preliminary test, and confirmed for the main test.. Measured concentrations of the dietary dose and the actual aqueous exposure due to some diffusion of cfu into the exposure solution are given in the report. At study termination no mortalities, abnormal physical appearance or behaviour was observed among shrimp of any treatment group. Molting was observed in		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		all replicates during the study, indicating growth of exposed organisms. The LC50 was not calculated. The NOEC can be determined to be 3,7 x 106 cfu/g diet, or 100 times the Expected Environmental Concentration (EEC), based on an aplicattion rate of 10 lbs/acre (= 11,2 kg/ha). - Daphnids: Drottar et al. (2001): "QST 713 Technical – A 21-Day Life Cycle Toxicity and Pathogenicity Test With the Cladoceran (*Daphnia magna*)" GLP: YES. Guideline: U.S. EPA Series 885 - Microbial Pesticide Test Guidelines OPPTS No. 885.4240 Neonate Cladocerans were exposed to mean measured test concentrations of 7,9 x 10^5, 1,8 x 10^6, 3,4 x 10^6, 1,84 x 10^7, and 3,68 x 10^7 cfu/mL. Potential effects not related to pathogenicity of the a.s. were assessed by a sterile filtrate with heat-killed spores at the highest employed test concentration. A broth concentrate (at 2,4 x 10^6 cfu/mL) was employed as additional control, besides the obligatory negative control. 21-day EC50 = 1,6 x 10^6 cfu/mL, NOEC = 7,9 x 10^5 cfu/mL		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		LOEC = 1,8 x 10^6 cfu/mL Mortality of *Daphnia magna* exposed to the attenuated control was 100%. Conclusively the mortality observed was most likely due to the physical nature of the test solutions, rather than pathogenicity of strain QST 713. All reports will be provided to the RMS. These studies prove lack of a significant risk to any of the aquatic species tested. Further, *B. subtilis* has no host specific infectivity or virulence to non-target organisms but is a naturally occurring predominant saprophyte. In conclusion, there is no need for investigating effects of repeated exposure on further species.		
3.2	In relation to the effects on bees the applicant has to clarify if *B. subtilis* occur as vegetative cells or as endospores. IIB, 8.3 A	Dietary exposure of bees to the ai is mainly to endospores but there may also be a negligible amount of vegetative cells. Exposure to **vegetative cells** can be assumed for the 30 d honey bee field study, with multiple applications of 10 pounds/acre at a 5-d interval on alfalfa. The study will be provided and shows that there was no significant effect of Serenade treatment on honey bee mortality, foraging behaviour or brood during a 30-day field study when exposed to Serenade at 10 lb/a every 5 days for the duration of the study.	2002-08-06 The respective studies have been submitted to the RMS. The RMS considers that the requirement has been fulfilled. An addendum including these data will be provided by the RMS.	Evaluation meeting 26 March 2003: Data requirement fulfilled. RMS is invited to update entry in Column C and provide a brief summary of the results.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		See summary given under point 3.3. Two field studies on side-effects of multiple applications of Serenade WP on *Typhlodromus pyri* are available. In these studies exposure to vegetative cells also can be assumed for the study period of several months. Ipach, R. (2000): ”Effects of Serenade WP on predatory mites (*Typhlodromus pyri*) under typical vine culture condition son grape vines, Germany 2000”. Two reports with same title, but report no. GAB01 for location Ruppertsberg, and GAB02 for location Edenkoben, both in vine region Pfalz. GLP: YES. Guideline: BBA VI, 23-2.3.4.Variety: Riesling. Application of Serenade WP: 0,75% (equivalent to dose rates of 7,5 to ~12kg/ha), applied 4x starting at ~BBCH stage 68 (June) to BBCH stage 81 (August). The population density was assessed before the 1st application, 1 week after the 2nd application and 1, and 4 weeks after the last application. For all assessments populations of the predatory mites were only slightly lower in treated compared to control plots. The effect was < trigger value of 40% (namely 16 to 24%). The author concluded		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		that under the conditions of this study Serenade WP is harmless to field populations *T. pyri*.		
3.3	The new 30-day whole bee-hive study has to be submitted. IIB, 8.3 MS	The 30 d field study on honey bee will be submitted to the RMS. Mayer, DF (2000): ”Honey Bee Field Study of Serenade Biofungicide Wettable Powder in Alfalfa”. GLP: YES. Guidelines employed: EPA Ecological Effects Test Guidelines, Field Testing for Pollinators OPPTS Guideline No. 850.3040 Draft Doc. (4/1996); and EPA Microbial Pesticide Test Guidelines, Honey Bee Testing, TIER I (TIER IV per OPPTS 885.0001), OPPTS 885.4380, Draft Doc. (2/1996). Based on mean no. of dead honey bees per day a hazard rating was established using the method of Johansen & Mayer (1990): ”Pollinator Protection: A Bee and Pesticide Handbook”. Wicwas Press, Cheshire, CT: 212 pp. Location: Irrigated Agricultural Research & Extension Center, Washington State University, Prosser, WA. For 30 days honeybees were exposed to Serenade WP when visiting treated flowers of alfalfa. Serenade was applied 6 times in an interval of 5±1 day, at 11,22 kg/ha.. Toxic standard: Dimethoate 4E. Plot size: 5 acres. Observations: number of pollinating	2002-07-23 The respective study has been submitted to the RMS. The new study is representing a suitable addition to the former laboratory tests. The test is confirming the results of the laboratory tests: Honeybees will not be set at risk *by the practical use of Bacillus subtilis*-containing products. A detailed evaluation will be presented in an addendum to the DAR.	Evaluation meeting 26 March 2003: Not all MS are convinced about the outcome of the test as it is reported here. It should be confirmed that the study would have detected effects similar to those caused by *Bacillus cereus*, which is an important pathogen for bees. A direct comparison to dimethoate may not be appropriate to detect slow, pathogenic responses. The notifier or RMS should confirm this point or use other sources of information address potential pathogenicity of *B. subtilis* and exclude a *B. cereus* like activity.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		bees, number of dead bees in Todd Dead Bee Traps, brood mortality and reproductive fitness. Results: no significant different numbers in foraging bees, no significant differences in numbers of frames of adult bees and brood in uncaged colonies in Serenade treated compared to colonies in untreated control plot, and no significant differences in overall number of dead adult honey bees in the Todd Dead Bee Traps compared to untreated control. On one observation date the numbers of dead bees were higher in the Serenade treated plot, but this difference was evaluated as being due to biological variability and not treatment related.		
3.4	To confirm the risk assessment, the applicant has to submit the study with a parasitic hymenoptera when finished IIB, 8.4 MS	The relevant study is available and proves lack of pathogenicity of *B. subtilis*, strain QST 713 to parasitic Hymenoptera. Bryan et al. (2000) : ”*Bacillus subtilis* strain QST 713 : A Dietary Pathogenicity and Toxicity Study With the Parasitic Hymeniopteran (*Nasonia vitripennis*)” GLP: YES. Guideline: U.S. EPA OPPTS No. 885.4340. For 10 days 25 wasps per treatment group were exposed to 4 dietary concentrations of QST 713 Technical Powder: 295 ppm (=1,5 x 10^7cfu/mL),	2002-08-06 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. MS considers that the requirement has been fulfilled. An addendum including these data will be provided by the RMS.	Evaluation meeting 26 March 2003: Data requirement fulfilled.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		1730 ppm (=9,1 x 10^7cfu/mL), 10200 ppm (=5,4 x 10^8cfu/mL), 60000 ppm (=3,4 x 10^9 cfu/mL). Control groups: negative control, attenuated control and sterile filtrate control. 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. LC_{50} = 24739 ppm (corrected for negative control mortality), NOEC = 1730 ppm. 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. Two field studies on side-effects of multiple applications of Serenade on the predatory mite *Typhlodromus pyri*, performed in Germany in 2000 are available. See summary under point 3.2 of this table.		
3.5	Data on acute toxicity, infectivity and pathogenicity of *Bacillus*	An acute toxicity , infectivity and pathogenicity of *Bacillus subtilis* study was	16.07.02 New information received on toxicity,	Evaluation meeting 26 March 2003:

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
	subtilis to earthworms must be submitted. IIB, 8.5 A	finalized in May 2002: Stäbler, D. (2002): "Acute Toxicity of QST 713 WP (Serenade WP) on Earthworms, *Eisenia fetida* Using an Artificial Soil Test". GLP: YES, Guideline: OECD 207 (acute toxicity study); no guideline exists for the histopathological investigation, required for assessing infectivity and pathogenicity (Directive 2001/36/EC), therefore the aim of this investigation was defined in discussion with the UBA (Environmental Protection Agency, Berlin). Based on a range finding test employing a toxic standard (2-chloroacetamide) as reference ten earthworms per group were exposed to Serenade WP at 100, 178, 316, 562 and 1000 mg/kg soil for 14 days.. Body weight change, behavioural effects and mortality in treatment groups was compared to untreated control. Body weight loss was 9% in untreated and 11 to 16% in treated groups. At the highest test rate of 1000 mg/kg the earthworms showed behavioural effects, i.e. lethargy and weak reaction to a mechanical stimulus. During the study performance no mortality occurred in the Serenade treated groups and the LC_{50} was determined to exceed 1000 mg/kg soil dry weight. For histopathology 3 worms per group	infectivity and pathogenicity of *B. subtilis* to earthworms. In the acute test the LC_{50}(14 d) was determined to exceed 1000 mg Serenade/kg soil dry weight. However, within the higher concentrations tested some sublethal symptoms were observed. In addition to that, the histopathological investigation showed some remarkable anomalies of the tissues. The detailed evaluation and risk assessment are included in the addendum provided by the RMS. The RMS considers that the requirement has been fulfilled.	Data requirement fulfilled.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		were prepared for lightmicroscopic examinations of tissues belonging to: - the cerebral ganglion (segment 3, cross section), - the genital region (segment 9 to 15, frontal section) and - the Clitellum (segments 34 to 36, cross section). One animal of the 562 mg/kg treatment group had died after study termination, and was investigated separately. The main observation is a bacterial colonization in animals of test groups 562 and 1000 mg/kg soil. This colonization is polymorph and therefore appears to be caused by different bacteria. Further, no penetration of bacteria through the epithelium of the body wall was found, and even in the control group bacterial colonization was observed, to a lesser extent though. The increase in bacterial colonization is discussed to be a secondary effect on the immune system by extremely high doses of *B. subtilis*, impeding the normal function of controlling the internal microflora of the earthworm. The critical level is assumed to lie between 316 and 562 mg/kg soil, but may also be higher for earthworms with a better nutritional status, compared to the earthworms in this study, who were not fed for a period of 18 days.		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		Malnutrition also has been suggested as possible cause for the observed degeneration in the testes and fine granular glands in the clitellum region in all groups.. Further, a reference shows that earthworms are repeatedly exposed to *B. subtilis* in their guts, as evidenced by the fact that many of the Actinomycetes found to inhabit the earthworm gut are producing antibiotics, mainly active against gram positive bacteria (representative species tested against: *B. subtilis*), but no antibiotics were formed by gut isolates towards gram negative bacteria. Kristufek, -V; Ravasz, K; Pizl, V (1993): Actinomycete communities in earthworm guts and surrounding soil; Pedobiologia, Vol. 37(6), 1993: pp. 379-384		

4. Mammalian Toxicology

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
				Section 4 : Data requirements : 2 Open points : 1
4.1	An inhalation study with repeated administration including the determination of clearance rate from selected organs and tissues must be performed	The waiver for the repeated dose inhalation toxicity study was submitted with further data (mainly literature) in October 2001 (see 'Amendment to document M' of EU dossier, point 5.2.5.1; also see comment to ECCO team). The technical aspect of performing such a study also needs to be considered: there is no valid guideline for the performance with a microbial agent, and the technical aspect of ensuring a constant concentration of spores in the air for weeks poses problems, as does the method of exposing the animals for 28 days.. The notifier strongly feels that this repeated-dose test could produce results that are simply a consequence or an artifact of the method of exposure and would not reflect the actual exposure to the product. It is expected that these effects will be negligent, based on other toxicological data observed previously for this organism. Further this scenario (6h/day during 4 weeks) is not realistic for operator exposure. In February 2002 a new literature search related to inhalation exposure potential of microbial products was	As previously, it is considered necessary to conduct a repeated dose inhalation study. The objectives of this additional study have been given before and do not need to be repeated once more. Furthermore, this new data requirement was supported by other MS when the monograph was peer-reviewed. The publications referred to may contribute to a more reliable assessment of the results obtained in a subacute inhalation study with regard to human health but are not appropriate to justify waiving of experimental data.	Evaluation meeting 26 March 2003: Data requirement confirmed. Without a clear undertaking to conduct the study the further review must be stopped.

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		performed and it mostly revealed references on pathogenic micro-organisms. No study on clearance of *Bacillus subtilis* spores following repeated exposure was found. However, the effect of repeated exposure to bacterial spores has been addressed by an epidemic survey (see MELBOSTAD & EDUARD 2001, submitted to all member states in 10/2001), in which a health survey among farmers revealed that **respiratory** and eye irritation in Norwegian farmers correlated to the **long-term exposure to fungal spores**, but **not to bacterial spores**, which have been found to be prevalent in the inhaled air of farm areas (evaluation based on ~300 measurements!). For *oral* intake another reference supports a positive rather than negative health effect of *B. subtilis* spores on humans (NOVELLI et al. 1984). The authors report on a therapeutic use of long-term oral intake of *B. subtilis* spores as a biological response modifier to improve the immune response of children suffering from recurrent infectious diseases of the respiratory tract. The results show that *B. subtilis* spore therapy significantly reduced the frequency of respiratory tract infections in treated children. Although this study refers to the oral route, it should be considered that *B. subtilis* spores apparently have a positive effect on respiratory infections.		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		New references: - Novelli, A.; Ulivelli, A.; Reali, E. F.; Mannelli, F.; Trombi Belcari, L.; Spezia, R.; Periti, P: Bacillus subtilis spores as a natural pro-host oral agent. Preliminary data in children. Chemioterapia; VOL. 3; NO. 3; 1984 Jun; PP. 152-5 - Jericho, K. W.; O'Connell, D. C. ”Deposition in the respiratory tract of cattle of spores of Bacillus subtilis var niger by inhalation and by nasal instillation.” Can J Comp Med Vet Sci ; VOL. 38; NO. 3; 1974 Jul; PP. 260-5 In this study *B. subtilis* was chosen as a micro-organism causing NO adverse effects, since the aim of the study was to clarify any difference in spore exposure due to the application method. Lillie, L. & Thomson, R.G.:" The Pulmonary Clearance of Bacteria by Calves and Mice", Can. J. comp. Med., vol. 36: 129-136. The technique of determining clearance of spores following inhalative exposure is described. The fast rate of clearance in mice and calves demonstrates an efficient respiratory defense mechanism inactivating		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
		the two tested bacteria (*Staphylococcus aureus*, and *Pasteurella haemolytica*).. Further, it should be noted that the EPA Registration Eligibility Decision is waiving the requirement of a repeated dose inhalation study, based on positive results of acute studies and considering that the particle size of the wettable powder poses only a low risk of inhalation exposure. Further, any risk is mitigated due to standard personal protective equipment employed in the manufacturing plant. Finally, in the submitted study on clearance of spores following intravenous challenge of rats (Harrington, K.A. 1998c; IIB. 5.1.4/01) no sign of toxicity, infectivity or germination was observed throughout the study in any organ. No dissemination to other organs occurred. The benign character of these spores must be considered.		

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
	Open point 4.1: Basic test for mutagenicity - This open point should be addressed when the production of metabolites had been subject to further clarification.	The standard Salmonella testing is not an appropriate system for micro-organisms. Guideline tests for mutagenicity were developed for chemicals not microbial products. The secondary metabolites formed by QST 713 of B. subtilis are described (MANKER 2001; submitted with further data to all member states in October 2001) and none of these metabolites is a toxin or has mutagenic activity. Further, nothing from the vast available literature on this well-studied species indicates any genotoxic or carcinogenic potential of B. subtilis..	The RMS agrees that it is not necessary to perform further mutagenicity testing with this microorganism. However, at least basic in vitro data on mutagenicity of the metabolites Iturin A and Surfactin which are present in the product Serenade should be provided. Published data, if available, may be used.	Evaluation meeting 26 March 2003: Assessment of the RMS supported. No further data are required for Bacillus subtilis.
4.2	NEW DATA REQUIREMENT Information on mutagenicity of the metabolites Iturin A and Surfactin is required.		At least basic in vitro data on mutagenicity of the metabolites Iturin A and Surfactin which are present in the product Serenade should be provided. Published data, if available, may be used.	Evaluation meeting 26 March 2003: New data requirement. The question may be addressed by literature data.

5. Residues

No.	Column A Conclusions of the Accelerated ECCO-Peer Review	Column B Comments from the applicant	Column C Rapporteur Member State and Co-rapporteur Member State comments on applicant comments	Column D Conclusions of the evaluation group
				Section 5 : Data requirements : 0 Open points : 2
	Open point 5.1: MS to decide whether a pre-harvest interval is necessary. (see open point 1.5)	See statement given under point 1.10 of this table (open point 1.5) In conclusion, a PHI is not regarded necessary for Serenade.	02-08-12 RMS agrees with the argumentation of the notifier.	Evaluation meeting 26 March 2003: RMS is supported. No pre-harvest interval is needed.
	Open point 5.2: MS to decide whether it is necessary to conduct studies on the residues level in plants.	See statement given under point 1.13 of this table (open point 1.6), and point 2.1. For grapes the notifier will now submit a residue study (see brief summary under point 2.1).	02-08-12 Study received The study show the amount of residual *Bacillus subtilis* strain QST 713 on grapes after application of the plant protection product Serenade WP. Populations of *Bacillus subtilis* strain QST 713 decline within 28 days 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.	Evaluation meeting 26 March 2003: In view of the low toxicity of the organism, no further residue studies are required and no MRL have to be set. However, the results of the repeated dose inhalation study and the mutagenicity information on the metabolites are still pending.

Conclusions Working Group Evaluation 26 March 2003:

A repeated dose inhalation toxicity / pathogenicity study is required. COM takes the position that without a clear undertaking to conduct the study the further review must be stopped and the completeness decision repealed.

Information on the mutagenicity of the metabolites Iturin A and Surfactin should be provided. Literature data are sufficient.

Applications for provisional authorisations were made in DE, NL and IT, but not granted so far.

COM to send a letter to the applicant and set a deadline for the submission of the subacute inhalation study. It should be made clear that the Completeness Decision will be repealed in June if there is no unmistakable commitment to do the study.

APPENDIX 1

LIST OF USES SUPPORTED BY AVAILABLE DATA (date: 23.07.2002)

Active substance: **Bacillus subtilis strain QST 713**

Crop and/ or situation (a)	**Member State or Country**	**Product name**	**F G or I (b)**	**Pests or Group of pests controlled (c)**	**Formulation**		**Application**			**Product application rate per treatment (g)**			**PHI (days) (h)**	**Remarks (i)**
					Type (d)	**Conc. of as (e)**	**method kind**	**growth stage (f)**	**number min max**	**kg/100 L**	**water l/ha min max**	**kg/ha min max**		
Orchards, Apple, Pear	North- and South-Europe	Serenade™ WP	F	*Venturia inaequalis* (scab) *Erwinia amylovora* (fire blight)	WP	5 x 10^9 cfu/g	spraying	BBCH 10 to 72 during blossom	1 - 16 4	1 (i.e. 10^{12} cfu)	500 - 1.500	5 - 15	---	*
Orchards, stone fruits		Serenade™ WP	F	*Monilia*	WP	5 x 10^9 cfu/g	spraying	BBCH 55 to 69 BBCH 70 to 84 BBCH 85 to 89	4 4 2	1 (i.e. 10^{12} cfu)	500 - 1.500	5 - 15	---	* **

Crop and/ or situation (a)	Member State or Country	Product name	F G or I (b)	Pests or Group of pests controlled (c)	Formulation		Application			Product application rate per treatment (g)			PHI (days) (h)	Remarks (i)
					Type (d)	Conc. of as (e)	method kind	growth stage (f)	number min max	kg/100 L	water l/ha min max	kg/ha min max		
Grapevines	Middle- and South-Europe	Serenade™ WP	F	*Uncinula necator (Oidium)* *Botrytis cinerea*	WP	5 x 10^9 cfu/g	spraying	BBCH 55 to 75 BBCH 68 to 81	1 - 8 1 - 4	1 (i.e. 10^{12} cfu)	400 – 1.200	4 - 12	---	*
Lettuce	North- and South-Europe	Serenade™ WP	F, G	*Bremia lactucae*	WP	5 x 10^9 cfu/g	spraying	after planting	--	1 (i.e. 10^{12} cfu)	400 – 1.200	4 - 12	---	**

(a) EU and Codex classification
(b) Outdoor or field use (F), glasshouse application (G) or indoor application (I)
(d) internationally (GIFAP) agreed codes
(e) cfu = colony forming units
(f) according to BBCH scale; grapevines: 55 inflorescences swelling, flowers closely pressed together; 75: berries pea-sized, bunches hang; 68: 80% of flowerhoods fallen; 81: beginning of ripening: berries begin to develop variety- specific colour;
Application timing: exact timing depends on local conditions: spray when infestation will occur or according to local extension service
(g) minimum pre-harvest interval not relevant, no residues
(h) product stated as active substance expressed in colony forming units (cfu)
* spray interval max. 5 days; use product in spraying sequence with other fungicides
** spray interval 5 to 7 days, up to the day of harvest

Complete List of Endpoints Bacillus Subtilis

Appendix III.1: Chapter 1 (Identity and biological properties of the micro-organism)

Intended uses:	Biocontrol of plant pathogenic fungi in viticulture and horticulture

1. Identity and biological properties of the micro-organism

Known or new organism:	Bacterium *Bacillus subtilis* (Cohn 1872)
Taxonomy:	The genus *Bacillus* belongs to the family Bacillaceae among the group gram-positive eubacteria.
Species, subspecies, strain:	Strain QST 713, identical with strain AQ 713
Identification / detection:	Using the available morphological, physiological and biochemical data, the strain QST 713 was clearly identified as *Bacillus subtilis*. Besides the basically relevant positive Catalase reaction inherent to all *Bacillus* species, further biochemical key parameters identifying strain QST 713 as *B. subtilis* are e.g.: positive Voges-Proskauer reaction and growth in 7 % NaCl.
Methods of analysis:	The species is identified by microscopy using classical morphological (cellular and colonial morphology) criteria and by using physiological and biochemical criteria.
Mode of action:	The mode of action of *B. subtilis* is fungistatic and fungitoxic by disruption of hyphae following contact with the fungal pathogen at the leaf surface. Besides antagonism nutrient competition is involved in the mode of action and more importantly *B. subtilis* induces systemic resistance response of the plant, indicated by enhanced peroxidase production.
Life cycle:	All spore-formers, including members of the Genus *Bacillus*, undergo a cycle consisting of several discernible phases: germination, outgrowth, multiplication, and sporulation. The primary cell formed at the end of outgrowth can, under some conditions, such as insufficient nutrients, divide asymmetrically and proceed directly to sporulation or, in time of favourable conditions, such as sufficient nutrients, can divide symmetrically and proceed through many divisions before sporulating. The endospore plays a dominant role in the biology and the life-cycle of *B. subtilis* and relatives. It is a dormant structure which enables the micro-organism to survive when environmental conditions turn unfavourable for vegetative growth and is a vehicle for dispersal by dust and air streams, as it is easily blown up. The global distribution of *Bacillus* spp. may largely be derived from the endospore-forming capability. Basically the endospore is the most heat tolerant bacterial life-form, enduring temperatures >80°C or even >100°C. The endospore does not present an obligate stage in the life-cycle, vegetative growth by cell-division may be predominant - or even the norm, unless e.g. lack of nutrients occurs. In a dry state endospores can remain viable for several

	years.
Host specificity:	*B. subtilis* is not characterised by a distinct host specificity since growth is not dependant upon a host but upon supply with decomposable organic matter. Moreover the endospore is prevalent in all environmental compartments and *B. subtilis* is not geographically restricted.
Known opportunist:	*B. subtilis* is considered an opportunist with no pathogenic potential. In some cases *B. subtilis* was isolated from surgical wounds or tumour drainages; only highly immunosuppressed patients were reported to have suffered from dissipating infections.
Toxin production:	*B. subtilis* produces different exo-enzymes contributing to the decay of organic matter. The extracellular enzyme *subtilisin* is known to elicit allergic or hypersensitive reactions in individuals repeatedly exposed to it however its toxigenic properties are assessed to be very low. *B. subtilis* does not produce significant quantities of extracellular enzymes or toxins and is generally considered to have a low degree of virulence to humans.
Resistance:	Up to now there is no indication of decreasing efficacy of the *Bacillus subtilis* strain in Serenade^{TM} WP against fungal pathogens to be controlled. The mode of action of strain QST 713 of *Bacillus subtilis* has been demonstrated to rely on a broader base than single site action, since it includes diverging mechanisms not easily to overcome by pathogens. The risk on the occurrence of development of resistance is to be classified as low.
Resting stages:	The endospore of *B. subtilis* is a dormant structure which enables the micro-organism to survive when environmental conditions turn unfavourable for vegetative growth and is a vehicle for dispersal by dust and air streams, as it is easily blown up.
Production control:	Each “seed”(liquid media with suspended cells) transfer is checked for purity both microscopically and by streak plating. The completed fermentation material (broth) of each fermentation run (batch) is tested by counts of colony forming units (cfu) of *B. subtilis*, microscopic examination, optical density and is tested for contaminants by plating analysis, esp. with regard to human pathogens. Content of cfu and contaminants may additionally be determined for the Technical Powder. The test results showed no detectable levels of human pathogens or other contaminants.

Appendix III.2: Chapter 2 (Hazard evaluation)

Hazard to humans

Pathogenicity:	No evidence of adverse effects from acute studies - except minor and transient effects after intratracheal challenge.
Infectivity:	No evidence of adverse effects from acute studies. *B. subtilis* infections are only reported from immuno-deficient patients.
Toxicity:	Rat LD50 oral: > 1.13 x 10^8 cfu/animal Rat LD50 intratracheal: > 1.2 x 10^8 cfu/animal Rabbit LD50 dermal > 2.3-2.7 x 10^{11} cfu/animal Rat LD50 intravenous: > 9.4 x 10^6 cfu/animal
Irritation, Sensitisation:	Rabbit: Very slight irritating effects (skin, eye) Based on the sensitising property of the formulation: R43
Genotoxicity:	Not relevant since no genotoxins produced
Medical reports:	Limited database: No adverse health effects observed among personnel involved in laboratory investigations. *B. subtilis* is capable of producing subtilisin which may cause allergic reactions after repeated exposure. *B. subtilis* has been reported to be associated with food poisoning and infections in immuno-deficient patients.
Formulation:	Rat LD50 oral: > 5000 mg/kg bw (~ 2.5 x 10^{10} cfu/kg bw) Rat LD50 inhalation: > 0.63 mg/l air; 4 h (~ 5 x 10^8 cfu/kg bw) Rabbit LD50 dermal > 2000 mg/kg bw (~ 1 x 10^{10} cfu/ kg bw) Skin sensitisation (Buehler test): positive (R43)

Effects on non-target organisms

Effects on birds (Annex IIB, point 8.1, Annex IIIB, point 10.1)

Information on toxicity, infectivity and pathogenicity	No evidence of pathogenicity or replication of the QST 713 strain of *Bacillus subtilis* in birds; 5-day-LD50 (bobwhite quail): > 10^{11} cfu/kg/d
Further information	none

Effects on aquatic organisms (Annex IIB, point 8.2, Annex IIIB, point 10.2)

Group	Test substance	Time-scale	Endpoint	Toxicity, infectivity and pathogenicity
Laboratory tests				
Oncorhynchus mykiss	Bacillus subtilis QST 713 Technical	30 d	LC_{50}	162 mg as/L [(1)]
Daphnia magna		48 h	EC_{50}	108 mg as/L
Scenedesmus subspicatus		72 h	NOEC	100 mg as/L

[(1)] *No signs of infection in gill, intestine or muscle tissue at test end.*

Effects on bees (Annex IIB, point 8.3; Annex IIIB, point 10.3):

Information on toxicity, infectivity and pathogenicity to bees	No evidence of toxicity of *Bacillus subtilis* QST 713 to honeybees; 5-day-Dietary LC_{50}: ~ 8900 ppm, equivalent to ~ 1.8 x 108 cfu/ml diet
Further information	none

Effects on arthropods other than bees (Annex IIB, point 8.4, Annex IIIB, point 10.4)

Test material	Species	Develop-mental stage	Sub-strate	Dosage kg/ha	Effects % lethal	Effects % sublethal
Predatory mites						
Bacillus subtilis (Serenade WP)	*T. pyri*	Protonymphs	I	16	30.7	13. 04
Parasitoids						
Bacillus subtilis (Serenade)	*A. rhopalosiphi*	Adults	I	16	5.13	25.3
Bacillus subtilis (Serenade WP)	*N. vitripennis*	Adults	I	0.9	46.15	
Bacillus subtilis (Serenade WP)	*N. vitripennis*	Adults	I	9	19.23	
Bacillus subtilis (Serenade WP)	*N. vitripennis*	Adults	I	90	65.39	
Plant dwelling species						
Bacillus subtilis (Serenade WP)	*C. carnea*	Larvae	I	0.9	0.5	
Bacillus subtilis (Serenade WP)	*C. carnea*	Larvae	I	9	47.6	
Bacillus subtilis (Serenade WP)	*C. carnea*	Larvae	I	90	26.3	
Bacillus subtilis (Serenade WP)	*H. convergens*	Adults	I	0.9	11.8	
Bacillus subtilis (Serenade WP)	*H. convergens*	Adults	I	9	4.7	
Bacillus subtilis (Serenade WP)	*H. convergens*	Adults	I	90	2.4	

I = Inert substrate,

Effects on earthworms

Information on toxicity, infectivity and pathogenicity to earthworms	No data available
Reproductive toxicity	

Additional studies

Bacillus subtilis is a micro-organism of ubiquitous occurrence but primarily a soil inhabitant. As vegetative growth declines as the nutrient sources declines this species does not seem to compete well for limited resources and *B. subtilis* population will be subject to competition in the natural microflora.

Appendix III.3: Chapter 3 (Exposure assessment and risk evaluation)

Operator exposure

Application method:	FCTM, HCTM, HCHH
Operator exposure models:	In relation to the results of the available acute toxicity studies, sufficient margins of safety exist (calculation on the basis of cfu / German model)

Exposure of the environment

Natural occurrence, background level:	*B. subtilius* is a ubiquitous -not geographically restricted- inhabitant of the soil, from which it is spread to associated environments, including plants and plant materials (straw, composts), foods (cereals, esp. dried spices), animals and their faeces (by ingestion of spores) and is also naturally found in aquatic environments (fresh water, estuarine and coastal waters). Although *B. subtilis* is commonly found in soil it occurs in almost any environment, including niches in kitchen and bathrooms. The magnitude of occurrence of *B. subtilis* in the soil is not definitely stated in the supplied literature. Indications for their general prevalence can be derived from high levels of presumably soil-born *Bacillus* spp. spores in straw approaching 10^5 cfu/g, and from the high numbers of *Bacillus* spp. found in coastal waters (where they constitute up to 20 % of total bacterial population) and from the major contribution of their endospores in estuarine and coastal sediments (achieving up to 80 % of the heterotrophic flora).

Consumer exposure:

Residues:	Residues of *B. subtilis* strain QST 713 on crops, feedingstuffs or foodstuffs are not expected at relevant concentrations: - With regard to its natural global distribution and non-pathogenic character *B. subtilis* cells left on the surface of treated areas or plant products do not imply health or environmental impacts. - *B. subtilis* has been used for enzyme production on a large industrial scale, and is even used for food production without having caused health or environmental hazards or damages. - *B. subtilis* does not produce toxins. - *B. subtilis* has no special attachment ability to plants or plant products, i.e. there is no compatibility comparable to host-pathogen interactions.

- The unfavourable environmental conditions prevailing on the leaf surface and the dependence of *B. subtilis* on organic matter supply are restricting its growth. In addition, in processing of raw products no growth or sporulation of *B. subtilis* is expected to occur.

List of uses supported by available data

Crop and/ or situation (a)	Member State or Country	Product name	F G or I (b)	Pests or Group of pests controlled (c)	Formulation		Application			Product application rate per treatment (g)			PHI (days) (h)	Remarks (i)
					Type (d)	Conc. of as (e)	method kind	growth stage (f)	number min max	kg/100 L	water l/ha min max	kg/ha min max		
Orchards, Apple, Pear	North- and South-Europe	Serenade™ WP	F	*Venturia inaequalis* (scab) *Erwinia amylovora* (fire blight)	WP	5 x 10^9 cfu/g	spraying	BBCH 10 to 72 during blossom	1 - 16 4	1 (i.e. 10^{12} cfu)	500 - 1.500	5 - 15	---	*
Orchards, stone fruits		Serenade™ WP	F	*Monilia*	WP	5 x 10^9 cfu/g	spraying	BBCH 55 to 69 BBCH 70 to 84 BBCH 85 to 89	4 4 2	1 (i.e. 10^{12} cfu)	500 - 1.500	5 - 15	---	* **
Grapevines	Middle- and South-Europe	Serenade™ WP	F	*Uncinula necator (Oidium)* *Botrytis cinerea*	WP	5 x 10^9 cfu/g	spraying	BBCH 55 to 75 BBCH 68 to 81	1 - 8 1 - 4	1 (i.e. 10^{12} cfu)	400 - 1.200	4 - 12	---	*
Lettuce	North- and South-Europe	Serenade™ WP	F, G	*Bremia lactucae*	WP	5 x 10^9 cfu/g	spraying	after planting	--	1 (i.e. 10^{12} cfu)	400 – 1.200	4 - 12	---	**

(a) EU and Codex classification
(b) Outdoor or field use (F), glasshouse application (G) or indoor application (I)
(d) internationally (GIFAP) agreed codes

(e) cfu = colony forming units
(f) according to BBCH scale; grapevines: 55 inflorescences swelling, flowers closely pressed together; 75: berries pea-sized, bunches hang; 68: 80% of flowerhoods fallen; 81: beginning of ripening: berries begin to develop variety- specific colour;
Application timing: exact timing depends on local conditions: spray when infestation will occur or according to local extension service
(g) minimum pre-harvest interval not relevant, no residues
(h) product stated as active substance expressed in colony forming units (cfu)
* spray interval max. 5 days; use product in spraying sequence with other fungicides
** spray interval 5 to 7 days, up to the day of harvest

List of studies which were submitted during the evaluation process and were not cited in the draft assessment report: *Bacillus subtilis*

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
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

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 8.4, 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

15750/ECCO/BVL/03
16 September 2003

CONCISE OUTLINE REPORT (Co-Rapporteur System)

Peer Review Programme under Directive 91/414/EEC

Subject: **Bacillus subtilis strain QST 713**

Rapporteur Member State: **DE**

Co-Rapporteur Member State: **SE**

The following comments were submitted:

Date	Supplier	File
12.09.2001	Finland	01_bacillus_subtilis_com_fin.doc
23.10.2001	AgraQuest	02_bacillus_subtilis_com_agraquest.doc
19.11.2001	The Netherlands	03_bacillus_subtilis_com_nl.doc
20.11.2001	Denmark	04_bacillus_subtilis_com_dk.doc
03.12.2001	Finland	05_bacillus_subtilis_com_fin2.doc
19.11.2001	Sweden	06_bacillius_subtilis_com_se.doc

1. Definition of the residues

Residues:	Residues of B. subtilis strain QST 713 on crops, feedingstuffs or foodstuffs are not expected at relevant concentrations: - With regard to its natural global distribution and non-pathogenic character B. subtilis cells left on the surface of treated areas or plant products do not imply health or environmental impacts. - B. subtilis has been used for enzyme production on a large industrial scale, and is even used for food production without having caused health or environmental hazards or damages. - B. subtilis does not produce toxins. - B. subtilis has no special attachment ability to plants or plant products, i.e. there is no compatibility comparable to host-pathogen interactions. - The unfavourable environmental conditions prevailing on the leaf surface and the dependence of B. subtilis on organic matter supply are restricting its growth. In addition, in processing of raw products no growth or sporulation of B. subtilis is expected to occur.

2. Classification and labelling:

Physical and Chemical properties	No proposal
Fate and Behaviour	No proposal
Mammalian Toxicology	No proposal
Ecotoxicology	No proposal

Appendix 1: Reporting table: Bacillus subtilis strain QST 713

List of end points: see Rep_0(WGeval)_Bacillus_subtilis.doc

Reporting table Bacillus subtilis (Fu) *EU RESTRICTED* 12711/ECCO/BBA/01, rev. 2 (02.04.2002) 4/40
section 1

1. Identity; Biological properties of the organism; Physical/Chemical Properties of the preparation; Details of Uses and Further Information; Methods of Analysis

No.	Column 1 Data point based on draft assessment report or comments from MS	Column 2 Comments from Member States or applicant	Column 3 Evaluation by (i) Co-rapporteur, and (ii) Rapporteur	Column 4 Data requirement or Open Point (if data point not addressed or fulfilled) (Annex point)
(i)	Vol.1, 2.1.1; B.1.1 Identity of the micro-organism	SF/DK: The organism is not adequately identified as required in directive 2001/36/EC. By using available morphological, physiological and biochemical data one can only confirm that the particular strain belongs to *B. subtilis.* However, to identify the organism at strain level more specific methods, in practice molecular methods, should be used. Methods and/or information concerning the properties of *B. subtilis* QST 713 strain to distinguish it from other *B. subtilis* strains should be added. DK: The method is mainly needed for control purposes, including documentation that the active organism is identical with the parent strain *B. subtilis* QST 713. Notifier to provide documentation on variability and viability of *B. subtilis* QST 713.	(i) Agree (ii) RMS aggrees. Further method with respect to strain differentiation will be amended by end of April 2002 (ii) RMS aggrees	1.1 Further identification method with respect to strain differentiation has to be submitted. Modern molecular biological methods are preferred. IIB, 1 A 1.2 Applicant to provide documentation on variability and viability of *B. subtilis* QST 713. IIB, 4.1 A

No.	Column 1 Data point based on draft assessment report or comments from MS	Column 2 Comments from Member States or applicant	Column 3 Evaluation by (i) Co-rapporteur, and (ii) Rapporteur	Column 4 Data requirement or Open Point (if data point not addressed or fulfilled) (Annex point)
(ii)	Vol.1, 2.1.1; B.1.1 Identity of the micro-organism	NL: The relevant *Bacillus subtilis* strain QST 713 has been deposited at the American Type Culture Collection. The specific ATCC number has to be included.	(i) The deposition number is included in B.1.13.2. It could also be mentioned in Vol 1. (ii) The strain QST 713 has been added to the internationally accepted Agriculture Research Culture Collection (NRLL), Illinois, USA, code number NRLL B-21661 as stated in the monograph Vol. 1, point 1.3.3 Name and species description.	-
(iii)	B.1.1.3.2 and B.1.1.3.3	SE: The methods and tests used for identification should be described. We also think that modern molecular biological methods should have been used for identification.	(i) – (ii) RMS aggrees. Further method with respect to strain differentiation are amended by end of April 2002. See above, point (i)	See data requirement 1.1
(iv)	B.1.1.4.1 Content of the micro-organism continued: B.1.1.4.1 Content of the	DK: We do not find any experimental evidence in the Monograph regarding whether *B. subtilis* QST 713 occur as vegetative cells or endospores (or both) in the technical product. It is important to distinguish between the two stages, as their effects are quite different.	(i) Concerning hole B.1.1: The identity is not sufficiently described. Modern molecular biological methods should be used. The methods should be described. The difference between *B. subtilis* and other *B. sp* need to be demonstrated. Make clear if *B. subtilis* occur as vegetative cells or as endospores.	See data requirement 1.1 1.3 In relation to the content of the micro-organism in the technical product the applicant has to clarify whether *B. subtilis* occur as vegetative cells or as endospores. It is recommended

Reporting table Bacillus subtilis (Fu) *EU RESTRICTED* 12711/ECCO/BBA/01, rev. 2 (02.04.2002) 6/40
section 1

No.	Column 1 Data point based on draft assessment report or comments from MS	Column 2 Comments from Member States or applicant	Column 3 Evaluation by (i) Co-rapporteur, and (ii) Rapporteur	Column 4 Data requirement or Open Point (if data point not addressed or fulfilled) (Annex point)
	micro-organism	SE: There must be some information that is not confidential. Why not inform about the formulation of the product? What does the product consists of more than the a.i.? We understand that the quantities of ingredients have to be confidential, but still we would like to know a bit more about the product.	Too much of the information is classified as confidential. (ii) The notifier has to provide the information. (ii) In the confidential part of the monograph, or EU dossier, respectively, where all ingredients of the fermentation media and the formulants of the product are listed. Besides a natural mineral and fermentation media there are some minor formulants that do not impose a health or environmental risk. All formulation ingredients have been cleared for food use by the U.S. EPA.	not to propose this information as confidential IIB, 1.4.1 A
(iv-a)	B.2.1.1.1 Historical background	SE: It is clear that groups exists within the genus, it is important for us to know which one *B. subtilis* strain QST 713 belongs to.		1.4 The applicant has to provide the information about the group to which *B. subtilis* belongs to. IIB, 2.1.1 A

Reporting table Bacillus subtilis (Fu) *EU RESTRICTED* 12711/ECCO/BBA/01, rev. 2 (02.04.2002) 7/40
section 1

No.	Column 1 Data point based on draft assessment report or comments from MS	Column 2 Comments from Member States or applicant	Column 3 Evaluation by (i) Co-rapporteur, and (ii) Rapporteur	Column 4 Data requirement or Open Point (if data point not addressed or fulfilled) (Annex point)
(v)	B.2.1.1.2 Origin and natural occurrence	SE: In the last part of B.2.1.1.2 it is written "The QST 713 strain was screened and fungicidal activity was confirmed." Please, clarify this statement on the following issues: • Which species of fungus have been tested? • For which species does *QST 713* show fungicidal activity? • Which fungus g species are none-sensitive to *QST 713*?	(i) – (ii) The notifier has to provide the information	1.5 In relation to the screening (fungicidal activity) of *B. subtilis* strain QST 713 the applicant has to clarify: - Which species of fungus have been tested? - For which species does QST 713 show fungicidal activity? - Which fungus g species are none-sensitive to *QST 713* IIB, 2.2.2 A
(vi)	B.2.1.2.2 Mode of action	DK: How do *B. subtilis* QST 713 differ from other *B. subtilis* strains with regard to protection against plant pathogens?	(i) Agree, the identity is not sufficiently described. Modern molecular biological methods should be used. (ii) The notifier has to provide the information	See data requirement 1.1 and 1.5

Reporting table Bacillus subtilis (Fu) *EU RESTRICTED* 12711/ECCO/BBA/01, rev. 2 (02.04.2002) 8/40

section 1

No.	Column 1 Data point based on draft assessment report or comments from MS	Column 2 Comments from Member States or applicant	Column 3 Evaluation by (i) Co-rapporteur, and (ii) Rapporteur	Column 4 Data requirement or Open Point (if data point not addressed or fulfilled) (Annex point)
(vii)	B.2.1.2.2 Mode of action	DK: *B. subtilis* is known to produce a number of secondary metabolites with antimicrobial activities (according to Berdy et al. (1980-1985) "Handbook of Antibiotic Compounds" approximately 70 different compounds are produced by different *B. subtilis* strains). Which of these compounds are produced by *B. subtilis* QST 713? And what role do these compounds have for the mode of action? Further, the full gene sequence of *B. subtilis* 168 is known (Kunst et al., 1997 (Nature 390, 249-256)) and therefor the gene products of this strain are very well described. Which implications does this information have for the current understanding of the mode of action of *B. subtilis* for plant protection? Have this knowledge further implications for the risk assessment of *B. subtilis* QST 713?	(i) To be clarified by the notifier. (ii) The notifier has to provide the information about secondary metabolites with antimicrobial activities (according to Berdy et al. (1980-1985) "Handbook of Antibiotic Compounds". The notifier submitted to all member states a chemical characterisation of QST 713 (Manker, 2001). Therefore the monograph has to be amended.	1.6 The applicant has to provide information about secondary metabolites of *B. subtilis* strains with antimicrobial activities (according to Berdy et al. (1980-1985) and which of these compounds are produced by *B. subtilis* QST 713? And what role do these compounds have for the mode of action? IIB, 2.2.2 A Open point 1.1 The monograph has to be amended by an Addendum (Manker, 2001).
(viii)	B.2.1.3 Host specificity range and effects on species other than the target harmful organism Continued:	DK: Is the selected strain *B. subtilis* QST 713 characterised by a distinct host specificity? Is *B. subtilis* QST 713 more effective than other *B. subtilis* strains?	(i) Modern molecular biological methods should be used for more information about *B. subtilis* QST 713. SE find the question unimportant. It is the efficiency of the product that is important. (ii) The strain B. subtilis QST 713 is not characterised by a distinct host specificity.	See Data requirement 1.1

Reporting table Bacillus subtilis (Fu) *EU RESTRICTED* 12711/ECCO/BBA/01, rev. 2 (02.04.2002) 9/40

section 1

No.	Column 1 Data point based on draft assessment report or comments from MS	Column 2 Comments from Member States or applicant	Column 3 Evaluation by (i) Co-rapporteur, and (ii) Rapporteur	Column 4 Data requirement or Open Point (if data point not addressed or fulfilled) (Annex point)
	B.2.1.3 Host specificity range and effects on species other than the target harmful organism		Strain QST 713 is a common saprophyte able to live on organic matter, as stated in the monograph (B. 2.1.3, and 2.1.4) and in the EU dossier	
	Micro-organisms	SE: What effect did Iturin give on mycorrhiza under field conditions?	(ii) The notifier has to provide the information	1.7 What effect did Iturin give on mycorrhiza under field conditions? IIB, 2.3 A
	Plants	SE: Avoid conclusions that generalize. Numbers and facts provided by applicant should be supported by reference to some sort of data.		1.8 Notifier has to clarify on which plant species information is available. IIB, 2.3 A
(ix)	Vol. 1, 2.1.2; B.2.1.6 Relationships to known plant or animal or human pathogens	DE/SF/DK/SE: More information is necessary for the morphological differentiation of B. subtilis and the indicated pathogenic Bacillus specie (*B. anthracis*, *B. cereus* and *B. thuringiensis*).	(ii) New information from the notifier (Heins, 2001) show that morphological and physiological parameters give the possibility to distinguish *B. subtilis* QST 713 from pathogenic *B. antracis*, *B. cereus* and *B. thuringiensis*). No further data required	Open point 1.2: Monograph has to be amended by an Addendum.
	continued: Vol. 1, 2.1.2; B.2.1.6 Relationships to known	SF: All pathogenic *Bacillus* species are not mentioned in the monograph. For example, *Bacillus licheniformis*, which has been	(i) Information should be added and also the relationship.	1.9 The applicant has to provide information to distinguish *B. subtilis* and *B. licheniformis.*

No.	Column 1 Data point based on draft assessment report or comments from MS	Column 2 Comments from Member States or applicant	Column 3 Evaluation by (i) Co-rapporteur, and (ii) Rapporteur	Column 4 Data requirement or Open Point (if data point not addressed or fulfilled) (Annex point)
	plant or animal or human pathogens	associated with food poisonings (Salkinoja-Salonen *et al.* 1999) and bovine abortions (Agerholm *et al.* 1997), was not mentioned.	(ii) Many species that are considered low risk (e.g. *B. thuringiensis*, *B. circulans*, *B. coagulans*, *B. licheniformis*, *B. megaterium*. *B. pumilis*, *B. sphaericus*, *B. subtilis*) have in single cases been suspected to be pathogenic. But because these are single reports, all the above mentioned species are still in risk group 1 (not pathogenic). The notifier has to submit information to distinguish *B. subtilis* and *B. licheniformis.*	IIB, 2.6 A
(x)	B.2.1.7 Genetic stability and factors affecting it	SF/DK/SE: Possible gene transfer after application must be considered and whether possible antibiotic resistance genes are transmittable. Gene transfer is a common phenomenon in soils especially with gram-negative bacteria like *Escherichia coli*. Moreover, since there is evidence of gene transfer between *B. subtilis* and a well-known pathogen *B. cereus*. The existence of such mechanism should be ruled out with *B. subtilis* QST 713. SE: Fermentation starts with pure cultures of QST 713 and therefore gene transfer between bacteria during that stage should be of minor importance. QST 713 is not considered to have any undesirable traits why gene transfer to the background flora should be insignificant The question is	(i) – (ii): According to the current knowledge, gene transfer cannot be completely excluded and there are no methods available which could unequivocally prove that such a transfer will not occur. However, practical relevance of such an event, if occurring, must be taken into account. *B.subtilis* is not a human pathogen, and thus, the gain of a certain resistance genes would not result in detrimental health effects since usually there will be no infection to be cured. Because there is no antibiotic pressure on a non-pathogenic micro-organism, *B. subtilis* carrying a resistance gene would not have a selective advantage and, thus, it is likely that	-

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		rather if the strain QST 713 can achieve pathogenic traits from other bacteria present, such as *B. cereus*, and if this would cause any problems." Due to the last sentence it is necessary to discuss around the possibility of achieving pathogenic traits from other bacteria and the consequences of that.	the gene would readily disappear from the bacterial population. Furthermore, there would be a sufficient number of antibiotics from different classes remaining effective. See also comments below (xiii). No further data required	
(xi)	B.2.1.8 Information on the production of metabolites (especially toxins)	DK: The comments made by notifier regarding subtilisin all relate examination and threshold values to American conditions. Notifier to provide information on absence / quantity of subtilisin produced under relevant conditions and how these quantities relate to possible set European threshold values for the contents of subtilisin in the air.	(i) Agree (ii) no further data required, see comment section 4, point (ii)	-
	continued: B.2.1.8 Information on the production of metabolites (especially toxins)	DK/SE: Experimental evidence for the non-production of toxins by *B. subtilis* QST 713.	(ii) The notifier has to provide the information see also point (vii)	1.10 Applicant to provide information on toxins produced by *B. subtilis* QST 713. IIB, 2.8 A See open point 1.1

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(xii)	B.2.1.9 Antibiotics and other anti-microbial agents	DE/SF/DK/SE: More detailed information is necessary regarding a resistance of strain QST 713 against antibiotics and about antibiotics produced by B. subtilis strain QST 713. DK: Notifier to elaborate on the clinical importance of the presumed resistance of *B. subtilis* QST 713 to a number of antibiotics.	(ii) The notifier provided two new studies. In the first one, susceptibility of *B.subtilis* towards a number of important antibiotic drugs was investigated proving that this micro-organism was resistant to Bacitracin only as it is typical for *Bacillus* species. In contrast, *B. subtilis* QST 713 was effectively inhibited by 8 antibiotics from different classes. In a second report, a number of secondary metabolites of *B. subtilis* are described. Some of these substances do have fungicidal or fungistatic properties but there is no structural similarity to the azole fungicides or other drugs. Thus, occurrence or spread of relevant resistances is not to be expected. No further data required (i) Agree, supplement the information.	Open point 1.3: Monograph has to be amended by an Addendum.
(xiii)	B.2.2.3.1 Explosive properties; Lower dust explosive limit	DE: The study according to EEC method A 14 has to be submitted.	(i) – 28.01.02 (ii): Minimum explosible concentration (MEC) using the Kuhner-Siwek 20-Liter Sphere: 440 g/m³ Comment: The study according to EEC method A 14 has not been conducted but the submitted data for MEC are acceptable for this type of formulation (WP).	-

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(xiv)	B.2.2.4.1 Flammability	DE: The study according to EEC method A 10 has to be submitted.	(i) – 28.01.02: (ii) Result: No burning or glowing spreaded over the length of the pile. Thus the product is not highly flammable. Comment: Acceptable.	-
(xv)	B.2.2.7.3.1 Suspensibility	DE: The applied method is not indicated.	(i) – 28.01.02: (ii) CIPAC method MT 15.1 has been applied. Comment: The requirement is fulfilled.	-
(xvi)	B.3.1.4.2 Description of the production process	SE: All information can not be confidential. It must be possible to give some information of importance for the risk assessment thus making the monograph more transparent.	(i) – (ii) It depends on the Notifier. The RMS also proposes more transparency for the monograph. However, the inert ingredients (see vol. 4) pose no health or environmental risk, which has been demonstrated the testing of the formulated end use product in ecotoxicological and toxicological studies.	-
(xvii)	B.3.1.4.3 Quality and purity control	DK: Documentation for the absence of the closely related species, *B. cereus*, *B.*	(i) Agree	Open point 1.4: MS to decide whether additional

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	B.5.1 Analytical methods for formulation analysis	*thuringiensis*, and *B. anthracis* in the product.	(ii) Precautionary measures for contamination prevention include storage of seed stocks of strain QST 713 at -80°C, with any seed transfer performed under aseptic conditions during fermentation, application of good manufacturing practice throughout the production process, and during formulation process, addition of antimicrobials. Quality control is employed for each fermentation batch to ensure absence of human pathogens and any other contaminating micro-organisms, (see monograph, B.5.1.2.1), including the closely related *Bacillus* species mentioned by Denmark. The reports on 5 batch analysis, respectively Lot Characterization of *Bacillus subtilis* QST 713, as submitted within the EU dossier confirmed the lack of contaminating microorganisms in both, the technical and the formulated product, see GINGRAS 1998a and 1998b, respectively. No further data required	documentation for the absence of the closely related species, *B. cereus*, *B. thuringiensis*, and *B. anthracis* in the product is necessary.
(xviii)	B.3.1.6 Methods to prevent loss of virulence of seed stock of the	SE: All information can not be confidential. It must be possible to give some information of importance for the risk assessment thus making the monograph more transparent.	(i) – (ii) The seed stock is stored frozen at –80 °C, so	-

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	micro-organism		that no vegetative growth is possible.	
(xix)	B.3.2.3 Details of intended use	SE: A minimum pre-harvest interval is necessary.	(i) – (ii) A pre-harvest interval is not necessary since there is no health risk.	Open point 1.5: MS to decide whether a minimum pre-harvest interval is necessary.
(xx)	B.3.2.6 Method of application	SE: We would like to have more information about the application. How high pressure is possible to use without causing any damage to QST 713? Is it possible to use the same nozzle as with spraying chemical plant protection products? Can an ordinary sprayer be used or is there a risk of sedimentation?	(i) – (ii) The notifier has to provide the information	1.11 Applicant to provide additional information about the application (pressure, nozzles). IIIB, 3.6 A
(xxi)	B.3.2.9 Proposed instructions for use	DK: Information is missing on specific agricultural, plant health or environmental conditions under which the active organism, *B. subtilis* QST 713 may or may not be used, when this in the light of the test results might be necessary. The instructions for use should be looked at again when all Member States have had the opportunity to evaluate the efficacy data.	(i) See information at B.3.2.3 in the monograph (ii) The efficacy data of Serenade are still under evaluation. .	-

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(xxii)		DK: Denmark has not yet received any efficacy data. We have therefor not had any opportunity to evaluate whether the product, Serenade effectively can control the plant pathogens claimed by the notifier.	(i) Efficacy data is not essential for inclusion in Annex I. Later on each Member State will have to decide whether Serenade is effective enough or not. (ii) see point (xxi)	-
(xxiii)	Vol.1 2.1.4 Classification and labelling	SF: The preparation consists of dried *B. subtilis* QST 713 strain on average 146 g/kg (5 x 10^{10} cfu/g). The preparation should be classified also into biological agents group 1 (unlikely to cause human disease) according to the directives 90/679/EEC and 89/391/EEC for biological agents, if requirements in point 2.1.2 are met.	(i) If the classification in the mentioned directives is relevant it could be used. (ii) It is not the preparation, but the species that is classed in risk groups. *B. subtilis* is already in risk group one.	-
(xxiv)	B.4.1 Proposal including justification of the proposals for the classification and labelling of the active substance in accordance with directive 67/548/EEC	SE: As far as we know bacteria cannot be classified according to existing rules for classification of chemicals.	(i) – (ii) There is no approved system for a special classification and labelling of biological plant protection products for health effects. In case of *B.subtilis*, skin sensitising properties must be indicated for operator safety reasons. Thus, the risk phrase R 43 is used.	-
(xxv)	B.5.1.1 Methods for the identification of the micro-organism	DK: Documentation on variability and viability of *B. subtilis* QST 713.	(i) Agree	See data requirement 1.1 and 1.2

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	continued: B.5.1.1 Methods for the identification of the micro-organism	SE: We would like to have a summary of what is written in the ATCC report 1997, so that we do not have to go back to the ATCC report for the information. We think modern molecular biological methods should have been used for analysis instead of those used here. We also think more information about the analysis should be accessible in the monograph.	(ii) RMS agrees. See point (i) (ii) The ATCC report is a list of morphological and physiological criteria employed to determine the species of *B. subtilis*. See point (i), Further method with respect to strain differentiation are amended by end of April 2002.	
(xxvi)	B.5.1.1 Methods for the identification of the micro-organism	SF: On culture media, the colour of the bacterial colonies is usually defined by the contents of the culture media. Here, the colour is described as “light cream (brownish) to cream”, but the type of culture media is not mentioned.	(i) Agree (ii) Three different culture media are tested (Gingras 1998, Lot characterisation), where the colour is light cream: - TSA = Trypticase Soy Agar (BBL, Cockeysville, MD, Cat. No. 11043) - BA= Blood Agar (Remel, Racine, WI, Cat. No. 01-2000) - SDA= Sabouraud Dextrose Agar (Difco, Detroit, MI, Cat. No. 0109) The EU dossier mentions that Nutrient Broth Agar is the culture medium used.	1.12 Applicant to clarify whether the three media TSA, BA and SDA (Gingras, 1998) or a Nutrient Broth Agar is used for identification and whether the colour of the colonies is in all cases “light cream”. IIB, 4.1 A

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(xxvii)	B.5.1.1 c-d) Test for microbial contaminants and detection of human pathogens	SE: We would appreciate clearer information and more references about the test methods used.	(i) – (ii) All information stated in the monograph under this point is based on a confidential document (BELLET 1998a) that describes the employed procedures in detail, including media and solutions and interpretation of test results for detection of human pathogens and microbial contaminants. These tests are performed by a third-party laboratory according to the approved Bacteriological Analytical Manual. Authorities have access to this paper (Volume 4 of monograph, respectively Document J of EU dossier). The notifier does not want to disclose this confidential business information within the monograph since it is contained in the reference noted.	-

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(xxviii)	B.5.1.2 Methods for the analysis of the preparation	SE: Simple methods have been used, and we think that modern molecular biological methods should have been in parallel.	(i) – (ii) The strain identification work is pending, as stated under point (i); B. 1.1. of this table, but for the purpose of quality control of the formulated product, which is checked for the content of active ingredient and for bioassay efficacy of every production lot, the molecular approach is far beyond the scope of what needs to be determined. No further data required.	See data requirement 1.1
(xxix)	B.5.1.2.1 Quality control measures applied to the production of QST 713 WP	SF: The measures to maintain product quality are shortly described, but whether the manufacturer has any certified quality assurance system, which it should have (e.g. HACCP), is not presented.	(i) If any system exist it should be used. (ii) The notifier has to provide the information	1.13 Applicant to provide information whether the manufacturer has any certified quality assurance system (e.g. HACCP) IIIB, 5.1 A
(xxx)	B.5.2 Analytical methods to determine and quantify viable and non-viable residues	DK: Information on specific methods to determine viable and non-viable residues in or on treated products (food).	(i) Agree (ii) *B. subtilis* is considered to be a non-pathogen for humans and it was neither possible nor considered necessary to establish an ADI and no relevant viable residues will be expected, therefore no specific methods are necessary.	Open point 1.6: MS to decide whether specific methods to determine viable and non-viable residues in or on treated products (food) are necessary.

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(xxxi)	B.5.3.2 Residue analysis	SE: We are of the opinion that methods for residue analysis are necessary.	(i) – (ii) see point (xxx, B.5.2)	See open point 1.6

2. Environmental fate and behaviour

No.	Column 1 Data point based on draft assessment report or comments from MS	Column 2 Comments from Member States or applicant	Column 3 Evaluation by (i) Co-rapporteur, and (ii) Rapporteur	Column 4 Data requirement or Open Point (if data point not addressed or fulfilled) (Annex point)
(i)	Vol. 1, 2.7.3.2 Appendix III.2 Listing of endpoints, Hazard evaluation: Fate and behaviour in the environment	NL: Proposal to amend list of endpoints. (see comment)	(i) Should be considered. (ii) This proposal is agreed with.	-
(ii)	B.8.1 Persistence and multiplication in soil	SF: Results on the environmental fate of *B. subtilis* in soil after multiple additions should be addressed. Furthermore, the number of cells of *B. subtilis* declines after it has been introduced into the soil, but sporulation occurs. It would be interesting to know, what happens if conditions for *B. subtilis* later become more favourable, and whether proliferation of the strain can then be excluded.	(i) Agree (ii) The fate of introduced *B subtilis* is strongly depending on the environmental surroundings. Under favourable conditions the spores might germinate, and proliferation might occur until it might again limited by environmental (temperature, water potential, pH, nutrients) or biotic (competition by other soil micro-organisms) factors. The mechanisms are the same as for every other member of the soil microbiocenosis.	2.1 The applicant should provide further information about the environmental fate of *B. subtilis* in soil after multiple applications. IIB, 7.1.1 A

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(iii)	B.8.1 Persistence and multiplication in soil	DK: No data on the persistence of *B. subtilis* QST 713 in soil is presented. The predicted load of *B. subtilis* QST 713 to the soil surface is really uncertain. Risk assessment has partly to be based on knowledge about the persistence. It is impossible to predict the persistence without specific knowledge about: - growth, survival and endospore formation in phylloplane and on fruits. Dispersal from these sites to the soil by rain and litter - fate in the soil - the implications of several applications on fate at these sites	(i) Agree (ii) It is likely that the strain, once introduced, will persist in the soil. But its population will be limited, as the populations of all other strains, including those that are frequently being displaced over wide distances by air currents or via soil particles sticking to the heals of travelling tourists.	See data requirement 2.1

3. Ecotoxicology

No.	Column 1 Data point based on draft assessment report or comments from MS	Column 2 Comments from Member States or applicant	Column 3 Evaluation by (i) Co-rapporteur, and (ii) Rapporteur	Column 4 Data requirement or Open Point (if data point not addressed or fulfilled) (Annex point)
(i)	Vol. 1, 2.7.3.2 Appendix III.2 Listing of endpoints, Hazard evaluation: Effects on non-target organisms: birds, aquatic organisms, bees, arthropods other than bees	NL: Proposal to amend list of endpoints. See comment.	(i) Should be considered. (ii) - RMS agrees; see corrected list for birds, NTAs and bees. - With respect to the fish study, toxicity is not considered to be underestimated due to the additional (!) dietary exposure. The results of the chronic study with *D. magna* have been included in the list of end points.	-
(ii)	B.9 Ecotoxicology continued:	SF: All ecotoxicity tests reported in the monograph are single dose tests. However, the *B. subtilis* containing product can be used multiple times during a growing season. Therefore information on ecotoxicological effect of multiple use of *B. subtilis* containing product on the studied species should be required.	(i) Discuss and develop the reasoning. Extrapolate a repeated exposure. More information is needed if any metabolite exist. (ii) - Multiple applications are sufficiently addressed for NTAs by oral administration of up to 90 kg/ha over several weeks. - In the avian test birds have been exposed on 5 consecutive days with the overall dose clearly above exposure under field conditions. - With respect to aquatic organisms results from prolonged toxicity tests are available.	-

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	B.9 Ecotoxicology		The toxicity values are far above the expected exposure concentrations and indicate no risk, also from multiple exposure.	
(iii)	B.9.1.1 Effects on birds	NL: Why the toxicity to birds has been classified as "low to moderate", as the data seems to indicate a low toxicity rather than a moderate toxicity. 'mg/kg' should be 'mg/kg bw'.	(i) There are no classification rules for microorganisms. If the toxin is tested it might be possible to use classification rules for chemicals. (ii) agreed	-
(iv)	B.9.1.2 Risk assessment for birds	NL: The highest dose was 5000 mg/kg/d which is equivalent to 10^{11} CFU. The LD_{50} was $>10^{11}$ CFU/kg/d. Serenade WP has an activity of $5 * 10^9$ CFU/g. According to the RMS this is 2.5 times higher compared to the test material in the bobwhite quail test. In our opinion the test material is $2*10^{10}/5 * 10^9$= 4 times higher than Serenade WP. Expressed in mg/kg bw the LD_{50} of Serenade WP is 5000 x 4 is 20,000 mg/kg bw/d. The acute TER is >20,000/17 = 1170.	(i) – (ii) The TER calculation is based on the active substance (1.5 kg as/ha); consequently the activity should be $5*10^{10}$ instead of $5*10^9$; then the TER of 117 should be correct.	-

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(v)	B.9.2 Effects on aquatic organisms	NL: In the US the product has been conditionally registered for a period of two years (until July 2002). Within that period 2 confirmatory studies - freshwater fish and freshwater invertebrates - and 1 new study - a 30-day study with a shrimp - must be conducted and reported to the EPA. see general comment from NL	(i) When available, such information should be taken into account. (ii) The studies required by the US-EPA are no standard EU-data requirements. The studies submitted for Annex I-inclusion are valid and sufficient for a risk assessment. Therefore no further data are necessary. However, to confirm the risk assessment, the notifier has to submit the studies when finished.	3.1 The two confirmatory studies (freshwater fish/invertebrates) as well as the new 30-d-shrimp study required by US-EPA should be submitted when finished. IIB, 8.2 MS
(vi)	B.9.2.1 Acute toxicity and/or pathogenicity and infectivity to freshwater fish	SE: The LC50 value was determined to 162 mg/l and NOEC to 86 mg/l. The gross necropsy showed no signs of infection. It was noted in material and methods that "After mixing, the test solutions appeared tan in colour and were cloudy". Furthermore, it was noted in the results that "Due to the cloudiness of the 240 and 400 mg/l treatment groups, biological observation of survival were difficult to make. Observations of survival were made using a dip net to look for dead organisms. Evaluations of clinical signs of toxicity were made on the organisms which could be seen." Comment: A remark about the uncertainty of	(i) – (ii) The comment is gratefully acknowledged. When looking at the specific results of the study (Appendix VII), 100 % mortality is already observed on the second day in the 400 mg/l treatment group. "Mechanical effects" therefore are considered to be unlikely. Even if the LC50 is biased by mechanical effects or the cloudiness (the fish might not find their food), this would not change the risk assessment with respect to breaching of a trigger value.	-

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		the values would be useful when effects on fish are discussed. The effects could be caused by "mechanical effects" instead of the micro-organism.		
(vii)	B.9.2.4 Exposure and risk assessment for aquatic organisms	NL: In table B.9.2-1 the TER of *D. magna* is 730 and not 723 On the basis of a maximum application rate of 15 kg/ha 1.5 kg as/ha is used. 1 g contains $5 * 10^{10}$ CFUs. Consequently, 1.5 kg/ha is equivalent to $1.5 * 5 * 10^{10} = 7{,}5 * 10^{10}$ CFU/ha. The initial PEC is calculated for water adjoining the field is calculated as : dosage * drift * D (depth of the ditch). $PEC_{surface\ water}$= $7.5 * 10^{10}$ CFU/10.000 m^2 * 0.269* 0.3m= $6.1 * 10^5$ CFU/m^3= 610 CFU/L. 610 CFU equals $610/5 * 10^{10} = 1.22 * 10^{-5}$ mg. This PEC is much lower than the PEC calculated by the RMS. This has no consequences for the TER. Nevertheless the RMS is asked to explain the PEC_{sw}	(i) DE have to explain how the calculation was performed. (ii) Correct, the TER for *D. magna* is 730. PEC_{sw} has been calculated as follows: 1.5 kg as/ha with a drift rate of 29.6% equal 0.444 kg as/ha or 44.4 mg as/m^2 in a distance of 3 m. This amount is diluted in the volume of the standard water body (1 x 1 x 0.3 m = 0.3 m^3 = 300 L) and consequently gives an initial PEC_{sw} of 0.148 mg as/L.	-

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(viii)	B.9.3 Effects on bees	DK: The tests on effects on bees are carried out with dried *B. subtilis* QST 713, so the cells are most likely present as endospores. The cells seem to be dividing for a few days after application to leaves or fruits (and perhaps flowers); thus bees are exposed to vegetative cells. The effect of vegetative cells on bees might be quite different from the effects of spores. Are the cells in the diet present as endospores or vegetative cells? Do endospores and vegetative cells have the same effects on bees?	(i) Agree, make clear if *B. subtilis* occur as vegetative cells or as endospores. (ii) The tests on effects on bees were carried out with dried *B. subtilis* QST 713 (Technical) mixed into the diets, which were prepared for 5 days. So bees were exposed to vegetative cells, most likely. However, the composition of the mixtures were not fully reported, and effects of e.g. honey to *B. subtilis* remains unknown.	3.2 In relation to the effects on bees the applicant has to clarify if *B. subtilis* occur as vegetative cells or as endospores. IIB, 8.3 A
(ix)	B.9.3 Effects on bees	NL: In the US the product has been conditionally registered for a period of two years (until July 2002). Within that period 1 new study - a 30-day whole bee-hive study must be conducted and reported to the EPA. See general comment from NL	(i) When available the information should be taken into account. (ii) The study has to be submitted	3.3 The new 30-day whole bee-hive study has to be submitted. IIB, 8.3 MS

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(x)	B.9.4 Effects on non-target arthropods other than bees	DK: The comments and questions regarding effects on bees (14) also apply to the effects on non-target arthropods other than bees. Are the cells in the diet present as endospores or vegetative cells? Do endospores and vegetative cells have the same effects on non-target arthropods?	(i) Agree (ii) The tests on effects on NTAs are carried out with dried *B. subtilis* QST 713 mixed into the diets, which were prepared weekly. So NTAs were exposed to vegetative cells, most likely. However, the composition of the mixtures were not fully reported, and effects of e.g. honey to *B. subtilis* remains unknown.	-
(xi)	B.9.4 Effects on non-target arthropods other than bees	SF: In the non-target arthropod tests, no results for controls were reported, *i.e.* the tests with zero grams addition. Inclusion of the control results would make the results easier to interpret.	(i) Agree (ii) All results were corrected for control mortality and other effects, of course.	-
(xii)	B.9.4 Effects on non-target arthropods other than bees	NL: In the US the product has been conditionally registered for a period of two years (until July 2002). Within that period 1 confirmatory study - parasitic hymenoptera - must be conducted and reported to the EPA. See general comment from NL	(i) When available the information should be taken into account. (ii) The studies required by the US-EPA are no standard EU-data requirements. The studies submitted for Annex I -inclusion are valid and sufficient for a risk assessment. Therefore no further data are necessary. However, to confirm the risk assessment, the notifier has to submit the study when finished.	3.4 To confirm the risk assessment, the applicant has to submit the study with a parasitic hymenoptera when finished IIB, 8.4 MS

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(xiii)	B.9.5 Effects on earthworms	DE/SF/DK: Data on acute toxicity, infectivity and pathogenicity of *Bacillus subtilis* to earthworms must be submitted	(i) Estimate how much *B. subtilis* that is expected to be find in the soil after spraying and compare with background level. (ii) This information has been requested from the notifier and will be evaluated by the RMS when submitted.	3.5 Data on acute toxicity, infectivity and pathogenicity of *Bacillus subtilis* to earthworms must be submitted. IIB, 8.5 A
(xiv)	B.9.5 Effects on earthworms	NL: In the second paragraph of this section the last sentence seems to have been mistakenly included as it refers to mice rather than to earthworms.	(i) Needs to be clarified. (ii) The meaning is, that other strains of *B. subtilis* are known as pathogenic and there is no evidence that *B. subtilis* strain QST 713 is non-pathogenic to earthworms. Therefore information has to be submitted concerning effects of *B. subtilis* QST 713 on earthworms.	-
(xv)	B.9.6 Additional studies Continued:	DK: Notifier to elaborate on the effects of *B. subtilis* QST 713 on the general, not pathogenic microflora of soils.	(i) It should be possible to use TRFLP (Terminal Restriction Fragment Length Polymorphism) to see an impact. However, unless there is a strong impact it might be difficult to interpret the results in relation to impact in the ecosystem. (ii) Additional literature search from the notifier in 6 databases (Biosis, BBA: Phytomed select, BBA: Phytomed, Online Contents, PubMed, Toxline). Keys: *Bacillus subtilis* &	-

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	B.9.6 Additional studies		(effects on) soil micro-flora None of the relevant appears to investigate detrimental effects of *B. subtilis* on other micro-organisms, as far as the title or abstract shows.	

4. Mammalian toxicology

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(i)	Vol. 1, 4.1.3	Notifier: The additional requirement of a repeated dose inhalation study is refused with regard to a number of publications suggesting both a fast clearance of spores following intratracheal or inhalative exposure and the existence of specific defence mechanisms in the respiratory tract to eliminate even pathogenic spores.	(i) The operator exposure should be described. (ii): The publications have been submitted to the Rapporteur indeed, however, *B.subtilis* is adressed in only two of these papers. The data obtained with other, more or less related microorganims are applicable to the species of interest to a very limited degree only. Furthermore, in all these experiments, there was only single exposure included. Data obtained after repeated administration is completely lacking. An addendum summarising the additional information will be prepared by the RMS since it may be useful for interpretation of the results of the required study. However, there is no sufficient evidence from the submitted publications to support the notifiers proposal not to ask for the additional inhalation study. This requirement is also explicitly agreed with in the comments from the Netherlands and Denmark.	4.1 An inhalation study with repeated administration including the determination of clearance rate from selected organs and tissues must be performed

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(ii)	Vol. 1, 4.1.3	Notifier: Additional data on subtilisin production not relevant because of the lack of valid exposure limits in the U.S.A.	(i) Does the strain *B. subtilis* QST 713 produce subtilisin? The operator exposure should be described. (ii): To our knowledge, there are no exposure limits set for subtilisin neither in Europe nor in the U.S. In principle, the lack of exposure limits in the EU or in a country outside does not justify to waive the required data on subtilisin production by the strain QST 713. However, since allergenicity of subtilisin is the point of concern, a possible sensitising potential would be covered by respective classification and labelling of the product *Serenade* (R 43) on the basis of experimental data. Thus, it is not necessary to provide additional data on subtilisin.	
(iii)	Vol. 1, 4.1.3	DK: Information on absence / quantitiy of subtilisin production under relevant conditions and on the relation of these concentrations to European threshold values for subtilisin content in the air.	(i) Agree (ii): See comment above (ii)	

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(iv)	B.6 Toxicity, pathogenicity and infectivity	SE: Data requirements for micro-organisms now exists (Commission Directive 2001/36/EEC of 16 May 2001).	(i) – (ii) The statement of the Co-Rapporteur is absolutely correct, however, data requirements had not been adopted yet when the dossier was submitted and the monograph written. Furthermore, the currently required experimental data are or (in case of the repeated administration study) will be available. An acceptable justification for not performing mutagenicity studies was given.	-
(v)	B.6.1.2 Genotoxicity testing	DK: The basic test for mutagenicity is missing.	(i) The production of metabolites has to be discussed/investigated further. If there are relevant metabolites produced and left in the product, studies on genotoxicity could be considered. In the data requirements (Dir. 2001/36/EC mutagenicity testing is a subtitle to genotoxicity. (ii): As stated in the monograph, in this special case, testing for mutagenicity is not considered necessary. However, this issue will be subject to a discussion with the Co-Rapporteur.	Open point 4.1: Basic test for mutagenicity - This open point should be addressed when the production of metabolites had been subject to further clarification.

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(vi)	B.6.1.5 Pathogenicity and infectivity under immunosuppression	SE: 3rd indent, line 4: Please change dissipating to disseminating.	(i) – (ii) The proposed wording might be more appropriate but usually the monographs are not re-written.	-
(vii)	B.6.3 Specific toxicity, pathogenicity and infectivity studies under immunosuppression	NL: The title of this section should be changed since it does not include studies under immunosuppressive conditions.	(i) Agree (ii): This proposal is agreed with.	-
(viii)	B.6.3.1 Acute intravenous toxicity, pathogenicity and infectivity	SF: Long-lasting clearance from the body following i.v. application is surprising.	(i) It is surprising, however it does not seem to mean anything. (ii): I.v. application does not reflect realistic exposure conditions. However, since long clearance period may indeed give rise to concern, a repeated inhalation study is required.	-

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(ix)	B.6.4.3 Direct observation, e.g. clinical cases	SE: The reference DONIZ et al. (1988) is missing in the reference list.	(i) – (ii) The original reference is: Donzis, P.B.; Mondino, B.J.; Wiessman, B.A. (1988) *Bacillus* keratitis associated with contaminated contact lens care systems : Am J Ophthalmol, 105, 195-197. This publication has been referred to by de Boer and Diderichsen (1991; included in the reference list).	-
(x)	B.6.8 Data on exposure	NL: For the estimation of exposure of operators RMS uses the German model. It seems appropriate to use the available European model EUROPOEM for the operators, at least for the application. Bystander exposure may be considered irrelevant. For worker the exposure should also be estimated.	(i) Agree. (ii): The German model was used to give a rough range for a possible operator exposure. Because *B. subtilis* is considered to be a non-pathogen for humans and it was neither possible nor considered necessary to establish an ADI and/or an AOEL, a refined estimation of the exposure of operators, bystanders or workers would not result in an improved risk assessment	-

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(xi)	B.6.8 Data on exposure	SF: A study on operator exposure should be required.	(i) Agree (ii): See comment above (x). In the lack of an AOEL, data on exposure could not be compared to any reference doses. Furthermore, there is no general guidance how to perform such studies with microbiological plant protection products.	-
(xii)	No special annex point: Percutaneous absorption.	DK: Information concerning percutaneous absorption is required.	(i) DK ask for percutaneous absorption of the product. Please clarify if this means percutaneous absorption of chemicals in the formulation. (ii) Usually, such data are not required for microorganisms. For *B.subtilis*, there is no evidence that percutaneous absorption could be a point of concern	-

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(xiii)	No special annex point: Cytotoxic properties.	DK: It should be considered to require a test for the cytotoxic properties of *B.subtilis* QST 713 against cell cultures since it cannot be excluded that some strains of *B.subtilis* can produce toxins and cause food borne diseases.	(i) Step 1 should be to make an assumption of the possibility for *B.subtilis* QST 713 to produce cytotoxins. Step 2 should be to identify the possibilities for exposure of food with *B.subtilis* QST 713. Depending on the outcome of such discussions a third step might be to design an *in vitro* test for cytotoxicity. It seems however very difficult to design a relevant test. (ii) It is equivocal whether a test for cytotoxicity *in vitro* might be actually predictive for the potential to cause food borne diseases. However, this issue will be discussed with the Co-Rapporteur.	-

5. Residues

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	General comment	DK: The study referred to in the Monograph on viable colonies on pepper leaf surfaces shows a highly increased number of colony forming units on the crop until seven days after treatment. In order to minimise the amount of viable colonies on the harvested crop we suggest a pre-harvest interval of seven days.	(i) The notifier should take this into account and consider a recommendation of a pre-harvest interval. (ii) A pre-harvest interval is not necessary since there is no health risk. Nevertheless there is no need to use this active ingredient one week before harvest.	Open point 5.1: MS to decide whether a pre-harvest interval is necessary.
(i)	Vol.1, 2.2.3 Methods to determine and quantify residues …	SE: It is stated that no residues relevant to the safety of consumers occur. Please state why the reported food poisoning cases (p. 20, "Medical data") are not relevant, as the product is intended for use on fruits and lettuce. Does this strain lack food poisoning properties?	(i) – (ii) There are no reports on isolation of *B.subtilis QST 713* from food poisoning incidents. With regard to the toxicological profile of this strain as indicated by the acute studies, no such effects are to be expected.	-

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(ii)	Vol.1, 2.4.1; B. 7 Definition of residues	SF: Studies of operator exposure and residue levels in plants are required with all plant protection products. These studies should be done with *B. subtilis* QST 713 also. Infections caused by *B. subtilis* have been published in literature (e.g. Kiss *et al* 1988, Richard *et al* 1988, Thomas and Whittet 1991, Velasco *et al.* 1992, Oggioni *et al* 1998). Moreover, many "non-pathogenic" bacteria have a tendency to become opportunistic pathogens when they are present in large amounts and find a susceptible host. SF/DK: The amount of possible residues should be better studied by the notifier and assessed by the RMS.	(i) Agree (ii) *B. subtilis* is considered to be a non-pathogen for humans and it was neither possible nor considered necessary to establish an ADI. Therefore there is no need to conduct studies on the residues level in plants.	Open point 5.2: MS to decide whether it is necessary to conduct studies on the residues level in plants.
(iii)	Vol. 1, 2.7.1 Standard terms and abbreviations	SE: To use the term dna for designated national authority is questionable, since it could be confused with the established term DNA. For example "designated NA" or similar could be used.	(i) – (ii) Generic point relevant for all EU monographs Proposal: Commission/ECCO to revise standard list of abbreviations	

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(iv)	Vol. 1, 2.7.1 Standard terms and abbreviations 2.7.2 Specific terms and abbreviations	NL: Abbreviations of application methods in the list of endpoints (level 2, Appendix III.3, operator exposure) FCTM, HCTM, HCHH should be included in 2.7.1 Standard terms and abbreviations Part 1 Technical Terms. This applies to the abbreviation MAF on p. 141 in B.9.4.3 (Risk assessment for non-target terrestrial arthropods) as well (8th line).	(i) Agree (ii) Generic point relevant for all EU monographs Proposal: Commission/ECCO to revise standard list of abbreviations	