Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

VKM Report 2016: 21

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Opinion of the Panel on Plant Protection Products of the Norwegian Scientific Committee for Food Safety

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Assessed and approved

The opinion has been assessed and approved by Panel on Plant Protection Products of VKM. Members of the panel are:

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Competence of VKM experts

Persons working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

Table of Contents

Summary

Bacillus subtilis are gram-positive bacteria that occur globally in soil, water, air, and decomposing plant material. The *Bacillus* bacteria are responsive to genetic alterations, and a large number of bacteria strains are part of the *Bacillus* genus. Some of the *Bacillus* bacteria strains have acquired genes for pathogenic toxins, but available techniques exist to identify and discriminate between the different strains.

Bacillus subtilis has been used for industrial enzyme and food production, and even as a probiotic to treat gastro intestinal tract symptoms and disorders, including diarrhoea in infants and young children.

Bacillus subtilis has been shown to act fungistatic and fungitoxic following contact with fungal pathogens at leaf or root surfaces, and may therefore be used as a biological fungicide in different crops. The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) has been asked by The Norwegian Agency for Food safety to assess the health and environment related aspects related to the use of the plant protection product Serenade ASO, containing the active ingredient *Bacillus subtilis* QST 713.

VKM has considered the data presented for the *Bacillus subtilus* strain QST713, and shares the opinion of EFSA, and also US Environmental Protection Agency, in that *Bacillus subtilis* "is considered a benign organism as it does not possess traits that cause disease. It is not considered pathogenic or toxigenic to humans, animals, or plants."

Bacillus subtilis is ubiquitously present in water, air and soil, also in Norway, and subject to competition by other indigenous microorganisms. Thus, the number of introduced *Bacillus subtilis* will rapidly decline and reach a natural equilibrium in nature. *Bacillus subtilis* show low or acceptable toxicity in terrestrial and aquatic non-target organisms. Effects on non-target micro-organisms cannot be excluded, but are not expected to adversely affect natural microbial communities.

The possible risk related to *Bacillus subtilis* is linked to the possibility of miss-identifying a toxic *Bacillus* strain such as Bacillus anthracis or *Bacillus cereus* for *Bacillus subtilis*. Thus, VKM emphasizes the importance of product control, to ensure and document the identity, purity and relevant contents and property of each product batch prior to marketing and use.

Bacillus subtilis is not known to produce toxins, but produce proteolytic enzymes considered to be responsible for effects observed in inhalation experiments. Thus, proper preventive equipment should be used.

Key words: VKM, risk assessment, Norwegian Scientific Committee for Food Safety, Serenade ASO, *Bacillus subtilis* QST 713

Sammendrag på norsk

Bacillus subtilis er gram-positive bakterier som finnes globalt i jord, vann, luft og råtnende plantemateriale. *Bacillus* bakterier følsomme for genetiske forandringer, og *Bacillus* slekten består derfor av et stort antall bakteriestammer med ulike egenskaper, noen av dem med gener for sykdomsfremkallende giftstoffer. Det finnes imidlertid i dag metoder til å identifisere og skille mellom de ulike *Bacillus*-stammene.

Bacillus subtilis har vært brukt til industriell enzym- og matproduksjon, og som probiotika, inkludert til behandling av diaré hos barn.

Bacillus subtilis er vist å virke fungistatisk ved kontakt med sopp-patogener på planterøtter eller blader, og kan derfor benyttes som et soppmiddel i forskjellige avlinger. Vitenskapskomiteen for mattrygghet (VKM) er bedt av Mattilsynet å vurdere helse- og miljørelaterte forhold knyttet til bruk av plantevernmiddelet Serenade ASO, som inneholder virkestoffet *Bacillus subtilis* QST 713.

VKM har vurdert tilgjengelige test-data for bakteriestammen *Bacillus subtilus* QST713, og deler oppfatningen til EFSA, og US Environmental Protection Agency; at *Bacillus subtilis* «kan ses på som en godartet organisme som ikke har egenskaper som forårsaker sykdommer. Bakterien anses ikke som patogen eller giftig for mennesker, dyr eller planter.»

Bacillus subtilis er til stede over alt i vann, luft og jord, også i Norge, i samspill og konkurranse med andre mikroorganismer. Det forventes derfor at antall tilførte *Bacillus subtilis* raskt vil avta og nå en naturlig likevekt i naturen. *Bacillus subtilis* viser lav eller akseptabel toksisitet i terrestriske og akvatiske organismer utenfor målgruppen. Effekter på andre mikroorganismer enn skadegjørerne kan ikke utelukkes, men ventes ikke å medføre betydelig påvirkning av naturlige mikroorganismesamfunn.

Mulig risiko av *Bacillus subtilis* er knyttet til muligheten for å feil-identifisere en giftig *Bacillus* stamme, for eksempel *Bacillus anthracis* eller *Bacillus cereus,* som *Bacillus subtilis*. VKM understreker viktigheten av produktkontroll, for å sikre og dokumentere identitet, renhet og egenskaper før omsetning og bruk.

Bacillus subtilis produserer ikke toksiner, men proteolytiske enzymer som anses å være årsak til de effekter som observeres i inhalasjons-eksperimenter. Tilpasset beskyttelsesutstyr bør derfor brukes når det er fare for inhalasjon.

Abbreviations

ADI	Acceptable Daily Intake
AOEL	Acceptable Operator Exposure Level
Bta	*Bacillus thuringiensis* ssp. *aizawai*
Btk	*Bacillus thuringiensis* ssp. *kurstaki*
CFU	Colony Forming Units
DAR	Draft Assessment Report
DG SANCO	Directorate General for Health and Consumer Affairs
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
GAP	Good Agricultural Practices
HBL	*Bacillus* haemolytic enterotoxin
HQ	Hazard quotient
In vitro	Experiment outside an organism – in test tube
IU	International Units
LC50	50% Lethality Concentration
LOAEL	Lowest-observed-adverse-effect level
Mattilsynet	Norwegian Agency for Food Safety
MIC	Minimum inhibitory concentration
NHE	Non-haemolytic enterotoxin
NZRR	Northern Zone Registration Report
NZRMS	Northern Zone Reporting Member State
OECD	Organisation for Economic Co-operation and Development
PCR	Polymerase chain reaction

PECgw	Predicted Environmental Concentrations in ground water
PECsw	Predicted Environmental Concentrations in surface water
PER	Predicted Environmental Rate
POEM	Predictive Operator Exposure Model
SCFCAH	Standing Committee on the Food Chain and Animal Health
TER	Toxicity Exposure Ratio
VKM	Norwegian Scientific Committee for Food Safety/Vitenskapskomiteen for mattrygghet
ZRMS	Zonal Rapporteur Member State

Background as provided by the Norwegian Food Safety Authority

Active substance	*Bacillus subtilis* QST 713
Product name	Serenade ASO
Applicant	Bayer CropScience
Manufacturer	AgraQuest, Inc., 1540 Drew Avenue, Davis, CA 95618, U.S.A.
Active substance	*Bacillus subtilis* QST 713
Order:	*Bacilliales*
Family:	*Bacillaceae*
Genus:	*Bacillus*
Species:	*Bacillus subtilis*
Strain:	QST 713, identical with strain AQ 713
Concentration of active substance (micro-organism)	14 g/L *Bacillus subtillis* QST 713
Formulation type	Suspension concentrate, Code:SC
Packaging	1, 5 and 10 L HDPE bottle
Function	Biological fungicide
Application Background	This is a new product containing a new active substance, a new micro-organism.
Application date	23.09.2014
Status in Norway	Plant protection products with a.o. *Bacillus subtilis* QST 713 have not been evaluated in Norway.

Status in the EU	*Bacillus subtilis* QST 713 was included on Annex I of Directive 91/414 EC by the Commission Directive 2007/6/EC of 14 February 2007. There is applied for the product in the EU. Slovenia acts as Rapporteur Member State. In the Northern Zone there is applied for the authorisation of this product in SE, EE, LV and LT.
Product uses	
Crops	Strawberry grown in greenhouse and plastic tunnel, carrot on the field, lettuce on the field and tomato, pepper, aubergine in greenhouse.
Target organisms	Plant pathogenic fungi: *Botrytis cinerea* in strawberry in greenhouse and plastic tunnel *Alternaria dauci*, *Erysiphe heraclei* in carrot on the field *Botrytis cinerea* and *Sclerotionia sclerotiorum* in lettuce on the field *Botrytis cinerea*, *Alternaria* spp. in tomato, pepper, aubergine
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* may induce systemic resistance response in the plant, indicated by enhanced peroxidase production. (SANCO/10184/2003 - rev. final, 14 July 2006)
Pre-harvest interval	Due to the low toxicity and the fast degradation of *B. subtilis* by UV light, a pre-harvest interval is not required

Max. number of applications per crop/ season

6 times

Min. interval between applications

5-14 days

Dosage and time of application

The maximum application rate of Serenade ASO is 8 L/ha. In terms of units of micro-organism per ha this is 0.112 kg/ha corresponding to 8.34×10^{12} CFU/ha. Max. total rate per crop/season is 48 L/ha.

Application equipment

The Norwegian Institute of Bioeconomy Research (NIBIO) will provide the information about the application equipment. In the RR ZRMS Slovenia, 2013 is stated that the product is applied by foliar or high vegetation spraying, depending on the crop. This may be done e.g. by tractor mounted boom or by knapsack sprayers. The volume of diluent spray amounts to 200 - 1000 L water/ha in field use and 400 to 1000 L/ha in indoor use.

Annexes

REVIEW REPORT, Commission working document, *Bacillus subtilis*, SANCO/10184/2003 - rev. final, 14 July 2006

REGISTRATION REPORT, Part A and B, SERENADE ASO, *Bacillus subtilis* QST 713, ZRMS: SLOVENIA, December 2013

ANNEX II/III dossier

Terms of reference as provided by the Norwegian Food Safety Authority

Serenade ASO is a new product containing the new active substance/organism *Bacillus subtilis* QST 713. The intended use is as a biological fungicide in strawberry in greenhouse and plastic tunnels, carrot in field, lettuce in field and tomato, pepper, aubergine in greenhouses.

In this regard, The Norwegian Food Safety Authority would like an assessment of the following:

- The human health risk by using *B. subtilis* QST 713 as a plant protection product. The committee is particularly asked to evaluate if it is necessary to set reference values for operators, workers and bystanders, and reference values in food.
- The fate and behaviour in the environment and the eco-toxicological effects and risks with regard to the use of Serenade ASO as a plant protection product. More specific, the committee is asked to make an assessment on the occurrence of this organism in Norway. If this organism is not found naturally in Norway, an assessment must be made on the potential for this organism to survive and spread in the environment under Norwegian conditions. The committee is also asked to make an assessment on the target species specificity under Norwegian conditions. The risk to non-target organisms under Norwegian conditions should be assessed.

1 Hazard Assessment of *Bacillus subtilis* QST 713

1.1 Introduction

The background information is taken from the documents supplied by the Norwegian Safety Authority (Se Appendix I).

The active ingredient in the plant protection product Serenade, *Bacillus subtilis* strain QST 713, was assessed by the EUROPEAN COMMISSION, HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL in July 2006 (European Commission, 2006). The Commissions assessment was based on the draft assessment report prepared by Germany, as rapporteur Member State and Sweden, as co-rapporteur Member State.

The Commission states that the review of *Bacillus subtilis* did not reveal any open questions or concerns and that the overall conclusion from the evaluation is that it may be expected that plant protection products containing *Bacillus subtilis* will fulfil the safety requirements laid down in Directive 91/414/EEC.

1.1.1 Identity and mode of action

The *Bacillus subtilis* strain QST713 is a naturally occurring strain that was isolated in 1995 by AgraQuest Inc. from soil in a California peach orchard. The strain has been identified as *Bacillus subtilis* by microscopy using classical morphological (cellular and colonial morphology) criteria and by using physiological and biochemical criteria.

The endospore plays an important role in the biology and the life-cycle of *Bacillus subtilis*. The spores are dormant structures which enables the micro-organism to survive when under unfavourable conditions for vegetative growth. *Bacillus* is assumed to have a global distribution and is not characterised by distinct host specificity since growth is not dependent upon a host.

The mode of action of *Bacillus subtilis* is fungistatic and fungitoxic by disruption of hyphae following contact with the fungal pathogen at the leaf surface (Elliott et al., 2001). According to the manufacturer of the GB03 strain (Kodiak ®), *Bacillus subtilis* delivers extended protection against disease pathogens through three distinct modes of action:
1. Colonies of *Bacillus subtilis* take up space on the roots, leaving less area or source for occupation by disease pathogens.
2. *Bacillus subtilis* feeds off plant exudates, which also serve as a food source for disease pathogens. Because it consumes exudates, *Bacillus subtilis* deprives disease pathogens of a major food source, thereby inhibiting their ability to thrive and reproduce.

3. *Bacillus subtilis* combats pathogenic fungi through the production of a chemical (an iturin) that inhibits the pathogen's growth (Gustafson LLC, 2004). Backman et al. (Backman, 1997) reported that 60-75% of the seed used for the US cotton crop was treated with *Bacillus subtilis* for suppression of *Fusarium* and *Rhizoctonia* Pathogens.

1.1.2 Toxicity and health risk

Bacillus subtilis produces enzymes which contribute to decay of organic matter. Subtilisin is such an enzyme which has low toxigenic properties but has been shown to cause allergic or hypersensitive reactions in individuals following repeated exposure. *Bacillus subtilis* is generally considered to have a low degree of virulence to humans.

The risk of developing resistance is considered low since its effect is based on a broad range of types of action.

1.1.2.1 Production control

The seed cells used to initiate fermentation is stated to be checked for purity both microscopically and by streak plating. The fermentation product of each product batch is tested for colony forming units (CFU) of *Bacillus subtilis*, microscopic examination, optical density and tested for contaminating human pathogens by plating analysis. The test results are stated to show no detectable levels of human pathogens or other contaminants.

1.1.2.2 Toxicity test results (Taken from SANCO/10184/2003 – rev. final 2006)

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

Rat 28 d inhalation (nose-only): no clinical effects of 5 x 10^8 cfu/animal

Rabbit: Slight irritating effects on skin and eye

No mutagenicity of secondary metabolites

1.1.2.3 Operator risk

Calculations on the basis of CFU using German model suggest that sufficient margins of safety exist.

1.1.2.4 Consumer risk

Residues of *Bacillus subtilis* strain QST 713 on crops are not expected at concentrations of relevance for health impacts. *Bacillus subtilis* has been used for industrial enzyme production and even for food production, with no observed health effects, since it does not produce toxins.

1.1.3 Environmental distribution and ecology

The European Commission review report on *Bacillus subtilis* QST 713 (SANCO, 2006) includes the following information as regards the distribution of the species: "*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)". The presence of *B. subtilis* in Norway has been documented by Østensvik et al. (Ostensvik, From, Heidenreich, O'Sullivan, & Granum, 2004).

In soil *B. subtilis* is mostly found as spores, but vegetative cells have been found in association with decaying organic material (Siala, Hill, & Gray, 1974). Liang et al. (Liang, Sinclair, Mallory, & Alexander, 1982) showed that the number of viable cells introduced to natural, unsterilized soil declined rapidly without formation of spores. *B. subtilis* can also grow in close association with plant root surfaces and can be isolated, in greater numbers than most other spore-forming bacteria, from the rhizosphere of a variety of plants (Earl, Losick, & Kolter, 2008). There is evidence that through these associations *B. subtilis* may promote plant growth.

The ability of *Bacillus subtilis* to form biofilms seems to be an important property for its interactions with plants as well as for its use as a plant protection product. The formation of biofilms involves production of extra-cellular polymeric substances including lipopeptides with anti-fungal action (Kim, Ryu, Kim, & Chi, 2010). Among the lipopeptides produced by *Bacillus subtilis*, especially iturines and fengcyines are known to have antibiotic effects on a broad spectrum of fungi (Nagorska, Bikowski, & Obuchowski, 2007; Ongena & Jacques, 2008) and bacteria (Ramachandran, Chalasani, Lal, & Roy, 2014). Another lipopeptide, surfactin, triggers the formation of biofilm and has a synergetic antibiotic effect in combination with fengcyin and iturin (Zeriouh, de Vicente, Perez-Garcia, & Romero, 2014).

1.1.3.1 Fate and behaviour in soil

The fate and behaviour of *Bacillus subtilis* from field application of Serenade is discussed in the Registration Report for the Central Zone, prepared by Slovenia (CZRMR, 2013). The report states that the survival of the endospores of *Bacillus subtilis* in soil is very likely for a period of a few months during which time a natural breakdown begins and gradually reduces the numbers of spores remaining. In a dry state endospores can remain viable for several years, vegetative cells, however, are far more rapidly degraded. It is generally accepted that population densities decline with increasing soil depth indicating that the level of translocation to deeper layers is negligible and proliferation in ground water is not likely to occur.

The predicted environmental concentration of *B. subtilis* in soil after application of Serenade ASO at the maximum application rate (of 8 L/ha) has been calculated to 1,0 x 10^5 CFU/g soil (without interception), or 5,0 x 10^4 CFU/g soil (with 50 % interception) in the Registration Report (CZRMR, 2013).

1.1.3.2 Fate and behaviour in water

Bacillus subtilis is frequently occurring in different aquatic environments, but does not find optimal conditions for growth. Therefore, proliferation is not likely to occur. Bacterial cells and especially endospores may survive, but will be subject to natural competition in the diverse micro-flora and to natural physical and chemical degradation in natural waters.

Aquatic organisms may be exposed to Serenade ASO through spray drift from the application site into adjacent water bodies. The predicted environmental concentration in surface water (PECSW) has been calculated in the Registration Report (CZRMR, 2013). The worst case situation is based on multiple applications with single use drift value. Due to the PECSW calculation in that case, the initial concentration of Serenade ASO in 30 cm depth in surface waters is 2003.53 µg/L (26.92 µg *Bacillus subtilis*/L) corresponding to 2 × 10^6 CFU/L.

1.1.4 Environmental Risk Characterization

The risk for adverse effects on terrestrial and aquatic non target organisms as reported in the Registration report (CZRMR, 2013) is outlined below.

1.1.4.1 Birds

Table 1.1.4.1.1. Summary of avian toxicity endpoint for *B. subtilis* QST 713

Study type	Test substance	Species	Endpoint
Short-term dietary toxicity	*B. subtilis* QST 713 Technical	*Colinus virginianus* (Northern bobwhite)	$LD_{50} > 10^{11}$ CFU/kg bw/d

Birds are typically exposed to dry residues on their food items following the dilution and spraying of the formulated product. During these processes, much of the formulation constituents are likely to be lost by volatilisation. Therefore, where oral exposure is the main route of exposure, toxicity data for the active substance are used in preference to data from tests with the formulated material. Exposure to Serenade ASO via dermal and inhalation routes is considered unlikely, since at the time of application and for a short period thereafter, most wild birds will leave the immediate vicinity of spray operations in response to the human disturbance.

The exposure level in birds was calculated for a scenario where a bird consumes the spray liquid as the only source of water (i.e. water intake of a 10 g bird: 2.70 x 10^{-3} L/d). This results in an exposure density of 2.2x10^{9} CFU/kg bw/d. The LD50 is >10^{11} spores (CFU)/kg b.w. /d. which is more than 10 times higher than exposure via water uptake.

1.1.4.2 Terrestrial mammals

Table 1.1.4.2.1. Summary acute toxicity to mammals. (For references – see (CZRMR, 2013)).

Substance	Species	Endpoint	Value (mg/kg bodyweight)
Serenade AS[a)]	Rat	LD_{50}	> 5000 mg/kg b.w. (> 5 x 10^{9} CFU/kg bw)
QST 713 WP	Rat	LD_{50}	LD_{50} > 5000 mg/ kg bw (> 2.5 × 10^{10} CFU/kg bw corresponding to 25,000 mg Serenade ASO/kg bw)

Similarly formulated as Serenade ASO, containing the same amount of active substance: 1.34% w/w *B. subtilis* QST 713

Mammals are typically exposed to dry residues on their food items following the dilution and spraying of the formulated product. During these processes, much of the formulation constituents are likely to be lost by volatilisation. Therefore, oral uptake by feeding on contaminated food is the main route of exposure. Dermal and inhalation routes of exposure to Serenade ASO are considered unlikely, since at the time of application and for a short period thereafter, most wild mammals will leave the immediate vicinity of spray operations in response to human disturbance.

The worst case scenario for exposure, where the mammals was assumed to consume spray liquid as their sole water source was used to calculate an exposure density of 1.3x10^{9} CFU/kg bw/d. The acute oral LD50 is > 2.5 x 10^{10} CFU/kg bw (more than 10 times higher than exposure via water uptake).

This would mean that if mammals would consume the spray liquid as their daily water intake they would not be at risk.

1.1.4.3 Aquatic organisms

Aquatic organisms may be exposed to Serenade ASO and Bacillus subtilis QST 713 through spray drift. Exposure of aquatic organisms from this route was estimated by calculating Predicted Environmental Concentration in surface water (PECSW) at 2 × 10^6 CFU/L.

Table 1.1.4.3.1. Summary of toxicity and Toxicity Exposure Ratios (TER) for Daphnia, Fish (*Oncorynchus mykis*s) and algae (*Scenedesmus subspicatus*)

Test organism	Test substance	48 hour EC_{50}	PEC_{SW}	TER_A	Trigger value
Daphnia magna	*B. subtilis* QST 713 Technical	2.16 × 10^9 CFU/L	2 × 10^6 CFU/L	1080	100
Oncorhynchus mykiss	*B. subtilis QST 713 Technical*	1.72 × 10^9 CFU/L	2 × 10^6 CFU/L	860	10
Scenedesmus subspicatus	*B. subtilis QST 713 Technical*	3.3 × 10^8 CFU/L	2 × 10^6 CFU/L	165	10

The acute TER value for *B. subtilis* QST 713 is above the Annex VI trigger values for Daphnia, fish and algae, indicating that GAP directed use of Serenade ASO poses no risk to aquatic organisms.

1.1.4.4 Bees

Table 1.1.4.4.1. Honey bee toxicity endpoints for Serenade ASO

Test substance	Endpoint	Tested value	Application rate of Serenade ASO/B. subtilis QST 713
***B. subtilis* QST 713 Technical**	LC_{50}	1.8 × 10^8 CFU/mL diet	4.17 × 10^7 CFU/mL spray solution

According to the GAP directed use of Serenade ASO concentration of *B. subtilis* in the spray solution is calculated for application in carrots, as here the highest concentration is expected: Per hectare 8 L Serenade ASO, corresponding to 8.336 × 10^{12} CFU are used, suspended in a water volume of 200 L water. Assuming 200 L as worst case, the concentration of B. subtilis in the spray solution will be 4.17 × 10^{10} CFU/L The dietary LC50 is 4.3 times higher than the maximum concentration of *B. subtilis* in the tank mix suspension, indicating that application of Serenade ASO does not pose risk to honey bees.

1.1.4.5 Arthropodes other than bees

Studies on dietary toxicity are considered the most relevant to investigate potential infectivity and pathogenicity towards non-target arthropods. A study assessing the dietary toxicity and pathogenicity of *B. subtilis* on the non-target arthropods, *Hippodamia convergens*, *Chrysoperla carnea* and *Nasonia vitripennis* was conducted (CZRMR, 2013). The LC50 value was determined to be 9 × 10^{11} CFU/L diet. Hence, the LC50 value is 21 times higher than the maximum concentration of *B. subtilis* in the spray solution (4.17× 10^{10} CFU/L), indicating that application of Serenade ASO does not pose risk to non-target arthropods.

1.1.4.6 Earthworms

The potential acute risk of Serenade ASO to earthworms was assessed by comparing the maximum instantaneous PEC_S with the 14-day LC_{50} value to generate the acute TER value.

Table 1.1.4.6.1. Acute toxicity and Toxicity Exposure Ratios (TER) for Earthworms

Compound referred to	LC_{50}	Maximum PEC_S for Serenade ASO	TER_A	Trigger value
Serenade ASO	>5.07×10^9 CFU /kg dry weight soil	1.0003×10^8 CFU/kg dry weight soil	> 51	10

The acute TER value is much higher than the Annex VI acute trigger value of 10, indicating that GAP directed application of Serenade ASO poses no acute risk to earthworms. Due to the absence of acute toxicity no adverse effects on earthworms are to be expected even upon prolonged exposure to Serenade ASO or *B. subtilis* QST 714.

1.1.4.7 Soil microflora

No studies of effects on the soil microflora were conducted for the registration of Serenade ASO in the Central Zone. Studies on the effects on micro-organisms were not considered to be necessary due to the following generally accepted aspects in the ecology and environmental behaviour of *Bacillus subtilis*, derived from open literature:

The active substance *Bacillus subtilis* is a member of the natural micro-flora in soils and occurs without geographical restriction in almost any environmental niche, including the immediate human environment. It is an autochthonous soil micro-organism and has originally been isolated from soil in a peach orchard in the U.S.A. Therefore its possible multiplication in this natural habitat does not disturb the natural micro-flora. Although it is commonly found in soil, it occurs in almost any environment, including niches in kitchens and bathrooms (de Boer & Diderichsen, 1991).

As vegetative growth declines with declining nutrient source this species does not seem to compete well for limited resources and *B. subtilis* populations will be subject to competition in the natural micro-flora on ecological basics (Campbell, 1989).

In conclusion, negative effects to the soil microflora following application of Serenade ASO according to GAP directed uses are not expected.

1.2 VKM assessment

Bacillus subtilis is extremely common and found globally in soil, water, air, and decomposing plant material due to its ability to form hardy endospores. *Bacillus subtilis* has been shown to act fungistatic and fungitoxic following contact with fungal pathogens at leaf or root surfaces, and has been reported used in biological strategies to reduce the impact of mycotoxins in various crops (Chulze et al., 2015). VKM has been asked by The Norwegian

Agency for Food safety to assess the health and environment related aspects related to the use of the plant protection product Serenade ASO, containing the active ingredient *Bacillus subtilis* QST 713. The *Bacillus* strain QST713 is a naturally occurring strain that was isolated from the soil in a peach orchard in California in 1995.

The *Bacillus* bacteria are highly responsive to genetic mutation, for instance by the acquiring of plasmids from other bacterial strains. This has resulted in a large number of bacteria strains in the *Bacillus* genus. Many of them are closely related, and this has earlier made the identification of the exact type of strain challenging, but with the new fast and reliable sequencing-techniques this is now possible. Some of the *Bacillus* bacteria strains have acquired genes for pathogenic toxins, such as *Bacillus anthracis* and *Bacillus cereus*. This is described in more detail in a parallel assessment report on the use of *Bacillus thuringiensis* as plant production product (VKM, 2016). On the other hand, *Bacillus subtilis* has been used as a probiotic to treat gastro intestinal tract symptoms and disorders, including diarrhoea in infants and young children.

The possible risk related to *Bacillus subtilis* is linked to the possibility of miss-identifying *Bacillus anthracis* or *Bacillus cereus* for *Bacillus subtilis*. As of today, however, this should not be a problem since methods have been developed where the disease-causing *Bacillus* species are easily distinguished from *Bacillus subtilis*. It is however vital that such methods are used in a proper manner to prevent that the products with *Bacillus subtilis* contain pathogenic *Bacillus strains*.

Due to the relative ease with which *Bacillus* bacteria are able to have its genetic content changed, this should also be kept in mind when using *Bacillus* genus bacteria, and when assessing possible health and environmental risks.

Bacillus subtilis is not known to produce toxins, but produces protease enzymes including subtilisin, used in detergents, and also considered to be responsible for effects observed in inhalation experiments. Thus proper preventive equipment should be used.

Bacillus subtilis is a ubiquitously present organism, also in Norway. It plays an important role in the soil microorganism community. Application of *B. subtilis* as plant protection agent will cause a local and temporal increase in density of bacteria. In soil, *Bacillus subtilis* are subject to competition by the indigenous bacteria and fungi and affected by other infectious agents. Thus, the number of introduced *Bacillus subtilis* is expected to decline rapidly and reach a natural equilibrium.

Test with various terrestrial and aquatic species has not shown toxic effects of Bacillus subtilis or the preparation Serenade ASO on birds, mammals, fish, crustaceans, algae, arthropods including bees and earthworms, and considering the expected environmental concentrations no adverse effects of the natural populations of these organisms is forseen.

Bacillus subtilis produces a number of extracellular metabolites with antibiotic effects on several species of fungi and bacteria. It is therefore not possible to exclude that certain microorganism species other than the four fungi target organisms specified in the Norwegian application for Serenade ASO may be affected. However, considering that *Bacillus subtilis* is ubiquitously present in the Norwegian environment such effects is not expected to have severe effects on natural microbial communities. Since most studies on the antibiotic effect of *Bacillus subtilis* has focussed on pest species, not much is known on possible effects on other species in the microbial flora. Recently Wei et al. reported results of a field study where effects of application of Serenade ASO with *Bacillus subtilis* QST713 on strawberries were investigated (Wei, Hu, & Xu, 2016). They found that application of *Bacillus subtilis* caused only minor effects on overall microbial composition and abundance of individual microbes in the phyllosphaere, indicating that the effects were selective. However, the specific effects on the target-organism (*Botrytis cinerea*) were not reported.

2 Conclusion

VKM shares the opinion of EFSA, and also US Environmental Protection Agency, in that *Bacillus subtilis* “is considered a benign organism as it does not possess traits that cause disease. It is not considered pathogenic or toxigenic to humans, animals, or plants.”

Bacillus subtilis is ubiquitously present in water, air and soil, also in Norway, and subject to competition by other indigenous microorganisms. Thus, the number of introduced *Bacillus subtilis* is likely to rapidly decline and reach a normal equilibrium in nature.

Bacillus subtilis show low or acceptable toxicity in terrestrial and aquatic non-target multicellular organisms and no adverse ecological effects of application of Serenade ASO according to the proposed application scheme is foreseen.

The antibiotic effect of lipopeptides produced by *Bacillus subtilis* is, however, broad spectrum and is likely to affect also non-target species of fungi and bacteria in microbial communities exposed to *Bacillus subtilis* from application of Serenade ASO. However, considering that *Bacillus subtilis* is ubiquitously present in the Norwegian environment such effects is not expected to have severe effects on natural microbial communities.

Finally, and importantly, VKM emphasizes the importance of product control, to ensure and document the identity, purity and relevant contents and property of each product batch prior to marketing and use.

3 Appendix I

Reports obtained from Mattilsynet:

1. Terms of reference
2. *Bacillus_subtilis*_report_final: Report from EC SANCO
3. IIIM_7.1.1_01_dossier: Acute oral rat (test data)
4. IIIM_7.1.2_01_dossier: Acute dermal rat (test data)
5. IIIM_7.1.3_01_dossier: Acute inhalation rat (test data)
6. IIIM_7.1.4_01_dossier: Acute dermal irritation rabbit (test data)
7. IIIM_7.1.5_01_dossier: Acute eye irritation rabbit (test data)
8. IIIM_7.1.6_01_dossier: Dermal sensitization guinea pig (test data)
9. IIIM_9_01_dossier: Published article: Liang, L.N et al, «Fate in Model Ecosystems of Microbial Species of Potential Use in Genetic Engineering", Applied and Environmental Microbiology, 44 (3), 708-714, 1982
10. RR Part A national Serenade ASO indoor: Registration Report all zones (Slovenia) (2013)
11. RR PART A national Serenade ASO field use: Registration Report all zones (Slovenia) (2013)
12. RR Part B3 Serenade ASO field use (Mammalian toxicology)
13. RR Part B3 Serenade ASO indoor use (Mammalian toxicology)
14. RR Part B5 Serenade ASO field and indoor use (Fate and Behaviour in the environment)
15. RR Part B6 Serenade ASO field use (Eco-toxicological Studies)
16. RR Part B6 Serenade ASO indoor use (Eco-toxicological Studies)

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