Vitenskapskomiteen for mattrygghet
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VKM Report 2016: 20

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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:

Torsten Källqvist (chair), Katrine Borgå, Hubert Dirven, Ole Martin Eklo, Merete Grung, Jan Ludvig Lyche, Marit Låg, Asbjørn M Nilsen, Line Emilie Sverdrup

Acknowledgment

The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) has appointed a working group consisting of VKM members from different panels to answer the request from the Norwegian Food Safety Authority. Project manager from the VKM secretariat has been Edgar Rivedal. The members of the working group Torsten Källqvist (Panel on Plant Protection Products), Hubert Dirven (Panel on Plant Protection Products), Tor Gjøen (Panel on Microbial Ecology), Jørgen Lassen (Panel on Biological Hazards), Richard Meadow (Panel on Genetically Modified Organisms), Line Emilie Sverdrup (Panel on Plant Protection Products), Arne Tronsmo (Panel on Microbial Ecology), Siamak Yazdankhah (Panel on Biological Hazards) are acknowledged for their valuable work on this opinion.

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.

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Summary

Bacillus thuringiensis are anaerobic, gram-positive bacteria that produce parasporal crystalline protein inclusions, δ-endotoxin, which are toxic to certain invertebrates, especially larvae belonging to the insect orders *Coleoptera*, *Diptera* and *Lepidoptera*. Different strains of *Bacillus thuringiensis* have therefore a long standing history as plant protective insecticides in many countries, but have not been approved for use in Norway.

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 Turex 50 WG, containing the active ingredient *Bacillus thuringiensis* ssp. *aizawai* CG-91.

VKM has considered the data material obtained from The Norwegian Agency for Food safety as well as available published research articles and has concluded as follows:

Identity and analysis of the active ingredient

Because of the close relationship with toxin-producing bacterial strains, and the possibility for gene transfer between bacterial strains, each manufactured product batch should be analysed and documented for relevant parameters including number of spores determined as Colony Forming Units per gram (CFU/g); activity (IU/mg) and content (g/kg) of δ-endotoxin; level of enterotoxin produced by the vegetative cells.

Health risk – mammalian toxicology

It is the opinion of VKM that there are more quantitative than qualitative differences between different strains of *Bacillus cereus* and *Bacillus thuringiensis* with regard to some of the aspects of importance for possible effect on human health, especially the formation of enterotoxins. The general consideration of *Bacillus cereus* as being pathogenic, and *Bacillus thuringiensis* being unproblematic, seems not to be supported by available data. Also non-rodent species should be considered as test organisms. Existing data should be supplemented with toxicological characterization with now available methods, to form a better basis for assessing possible risk to human health from the use of *Bacillus thuringiensis* as insecticide.

Health risk – residues in crops

It is the opinion of VKM that it cannot be ruled out that intake of *Bacillus thuringiensis* spores as residues in food items sprayed with plant protection products, or vegetative cells from improperly stored food may under certain conditions cause intestinal human illness resulting from the production of enterotoxins by vegetative *Bacillus thuringiensis* cells. It is recommended to generate data on this using the conditions of use in Norway (Nordic countries)

Health risk – drinking water

VKM considers that the prescribed use of *Bacillus thuringiensis* as an insecticide is unlikely to pose a threat to human health via drinking water.

Transfer of genetic material

It is the opinion of VKM that the potential for harmful effects caused by transfer of genetic material in the environment is low. The fact that such gene transfer may take place highlights however the importance of strict procedures for analysis and control of purity, genotypic and phenotypic properties of the active ingredients.

Groundwater and soil contamination

VKM find it unlikely that the spores or the protoxins/toxins will be translocated to groundwater, and that the use of Turex 50 WG will result in permanently increased density of *Bacillus thuringiensis* in Norwegian soils.

Ecotoxicology

VKM concludes that the use of Turex 50 WG according to GAP will not pose an unacceptable risk to the environment.

Antimicrobial resistance

There is a need for more data regarding this topic, including antimicrobial susceptibility testing (MIC-values) of *Bacillus thuringiensis* ssp. *aizawai*, strain GC-91 against different antimicrobial agents, and clarification of the intrinsic and acquired resistance properties.

Key words: VKM, risk assessment, Norwegian Scientific Committee for Food Safety, Turex 50 WG, *Bacillus thuringiensis* ssp. *aizawai* GC-91

Sammendrag på norsk

Bacillus thuringiensis er anaerobe, gram-positive bakterier som produserer krystallinske protein-komplekser av δ-endotoksin, som er giftig for enkelte virvelløse dyr, særlig larver som hører til insekt-ordenene *Coleoptera*, tovinger og *Lepidoptera*. Ulike stammer av *Bacillus thuringiensis* har derfor lenge vært brukt som plantevernmidler mot insekter i mange land, men har ikke vært godkjent for bruk i Norge.

Vitenskapskomiteen for mattrygghet (VKM) er bedt av Mattilsynet å vurdere helse- og miljørelaterte forhold knyttet til bruk av plantevernmiddelet Turex 50 WG, som inneholder virkestoffet *Bacillus thuringiensis* ssp. *aizawai* CG-91.

VKM har vurdert datamaterialet mottatt fra Mattilsynet samt tilgjengelige publiserte forskningsartikler, og har konkludert som følger:

På grunn av at det finnes flere typer toksinproduserende *Bacillus* bakterier som *Bacillus anthracis* og *Bacillus cereus*, og muligheten for genoverføring mellom ulike bakteriestammer, bør hvert produkt før det sendes ut på markedet analyseres for relevante egenskaper, som antall sporer angitt som kolonidannende enheter per gram (CFU/g); aktivitet som insektmiddel (IU/mg); innhold (g/kg) av δ-endotoksin; nivå av enterotoksin som produseres av vegetative celler.

VKM er av den oppfatning at det er mer kvantitative enn kvalitative forskjeller mellom ulike stammer av *Bacillus cereus* og *Bacillus thuringiensis* med hensyn til egenskaper som kan ha betydning for effekt på menneskers helse, spesielt dannelse av enterotoksiner. Oppfatningen av *Bacillus cereus* som sykdomsfremkallende, og *Bacillus thuringiensis* som uproblematisk, synes ikke å bli støttet av det tilgjengelige data-grunnlaget. Det har også skjedd en betydelig utvikling av nye metoder til karakterisering av mikrober. Eksisterende data bør suppleres med en mer oppdatert genetisk og toksikologisk karakterisering for å gi et bedre grunnlag for å vurdere og kontrollere mulig helserisiko ved bruk av *Bacillus thuringiensis* som insektmiddel.

Det er VKMs oppfatning at det ikke kan utelukkes at inntak av sporer av *Bacillus thuringiensis* som rester i matvarer sprayet med plantevernmidler, eller som vegetative celler fra mat som har vært oppbevart for lenge ved for høy temperatur, kan gi tilfeller av matforgiftning som følge av enterotoksiner fra *Bacillus thuringiensis* bakterier. Her bør datagrunnlaget forbedres med fokus på bruksforhold i Norge/Norden.

VKM anser at det er usannsynlig at den foreskrevne bruk av *Bacillus thuringiensis* som insektmiddel vil utgjøre en trussel mot menneskers helse via drikkevann.

Det er videre VKMs oppfatning at potensialet for skadelige effekter forårsaket av overføring av genetisk materiale i miljøet er lav. Det faktum at en slik genoverføring kan finne sted

understreker imidlertid betydningen av strenge prosedyrer for analyse og kontroll av renhet, samt genotypiske og fenotypiske egenskaper til de virksomme komponentene.

VKM finner det lite sannsynlig at sporer eller toksiner vil bli overført til grunnvann, og at bruk av Turex 50 WG ikke vil føre til vedvarende økt tetthet av *Bacillus thuringiensis* i norsk jordsmonn.

VKM konkluderer med at forskriftsmessig bruk av Turex 50 WG ikke vil utgjøre en uakseptabel risiko for miljøet.

Det er behov for mer data om antibiotikaresistens, inkludert antimikrobiell resistenstesting (MIC-verdier) av *Bacillus thuringiensis* ssp. *aizawai* GC-91 mot forskjellige antimikrobielle midler, og avklaring av iboende og ervervede resistensegenskaper.

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

Bacillus thuringiensis ssp. *aizawai* strain CG-91 is the active organism in the biological plant protection product Turex 50 WG which has been sought approval for use as a microbiological insecticide in a wide range of crops.

Product Status

Our reference	2012/2755
Active substance	*Bacillus thuringiensis*, ssp. *aizawai* strain CG-91
Product name	Turex 50 WG
Applicant	Certis USA L.L.C, Columbia, MD,21046
Importer	Profilering AS
Active substance	*Bacillus thuringiensis* ssp. *aizawai* strain CG-91 Family: *Bacillaceae* Genus: *Bacillus* Species: *Bacillus thuringiensis*. Subspecies: *aizawai* Serotype: H-7 StrainGC-91 *Bacillus thuringiensis* ssp*. aizawai* strain CG-91 is a trans-conjugant of *Bacillus thuringiensis* ssp. *aizawai* strain HD 135-S4 (recipient strain), with a *Bacillus thuringiensis* ssp. *kurstaki* strain HD 191-A2 (donor strain). The new trans-conjugant strain GC-91 is a product of a natural crossing (conjugation) between the two strains. (See Statement regarding Turex/Agree WP, March 19, 2012)
Concentration of active substance	500 g/kg *Bacillus thuringiensis* ssp. *aizawai* CG-91
Formulation	Water dispersible granules (WG). Profilering AS has submitted the application of 5.1.2012 for registration of plant protection product Turex 50 WP (*Bacillus thuringiensis* ssp. *aizawai* CG-91). Profilering AS has applied for the formulation change on 16.8.2013. The formulation WG shall replace WP.
Packaging	1 kg package (plastic bag in a carton box), 10 box of 1 kg in a master carton box
Type of pesticide	Microbiological insecticide.

Application Background	This is a new product containing a new active substance, a new micro-organism.
Application date	05.01.2012

Active Substance Status

Identity	*Bacillus thuringiensis* (*Bt*) is a facultative anaerobic, gram-positive bacterium that forms characteristic protein inclusions adjacent to the endospore. Bt subspecies can synthesize more than one parasporal inclusion. *Bt* is genetically indistinguishable from *Bacillus cereus (Bc)*, except for the ability of *Bt* to produce parasporal crystalline inclusions, which are toxic for certain invertebrates, especially species of insect larvae belonging to the insect orders *Coleoptera*, *Diptera* and *Lepidoptera*. The basic phenotypic taxon is the subspecies, identified by the flagellar (H) serotype. By 1998, 67 subspecies had been described.
Status in Norway	Plant protection products with a.s. *Bacillus thuringiensis* var. *aizawai* CG-91 has not been evaluated in Norway. The species *Bacillus thuringiensis* was evaluated in Norway (product Vectobac), but was not approved due to insufficient documentation.
Status in the EU	*Bacillus thuringiensis* subsp. *aizawai* strains ABTS-1857 and GC-91 Authorizations at national level: BE, CY, DE, EL, ES, FI, FR, IT, LU, NL, PT, SE. *Bacillus thuringiensis* subsp. *israeliensis* (serotype H-14) strain AM65-52 Authorizations at national level: ES, SE. *Bacillus thuringiensis* subsp. *kurstaki* strains ABTS 351, PB 54, SA 11, SA12 and EG 2348. Authorizations at national level: AT, BE, CY, CZ, DE, DK, EL, ES, FR, HU, IT, LT, LU, MT, NL, PL, PT, RO, SI, SK, UK. *Bacillus thuringiensis* subsp. *tenebrionis* strain NB 176 (TM 14 1). Authorizations at national level: AT, DE, EL, ES, FR, HU, IT, PL
Other countries:	*Bacillus thuringiensis* have been used as biopesticides for the last 35 years.

Efficacy

The evaluation is based on the summary of efficacy evaluation carried out by the Norwegian Institute for Agricultural and Environmental Research (Now: The Norwegian Institute of Bioeconomy Research (NIBIO)) and draft label from the applicant. Please see the efficacy

evaluation carried out by the Norwegian Institute for Agricultural and Environmental Research and draft label from the applicant for further information.

Product uses and effect

Crops

Vegetables in greenhouse and field, ornamentals in greenhouse, fruit trees in field and plastic tunnels, berries (incl. strawberry), plant nurseries, urban landscape and forestry. The Norwegian Institute for Agricultural and Environmental Research suggest specifying of intended use of the product. The crops shall be listed on the label. (See the efficacy evaluation carried out by the Norwegian Institute for Agricultural and Environmental Research).

Target organisms

The following pests or group of pests against which the product is to be used belong to the following Lepidopteran families: *Geometridae*, *Plutellidae*, *Noctuidae*, *Pieridae*, *Crambidae*, *Lymantriidae*, *Lasiocampidae*, *Yponomeutidae*, *Tortricidae*, and *Gelechidae*.

Mode of action

Upon ingestion of *Bacillus thuringiensis* by the larvae, the crystalline inclusions dissolve in the larval midgut, releasing insecticidal crystal proteins (δ-endotoxins). Most of the crystal proteins are protoxins, converted proteolytically into smaller toxic polypeptides under the alkaline conditions in the insect midgut. The activated Cry toxins interact with the midgut epithelium cells of susceptible insects. After binding to specific midgut receptors, they are inserted into the apical membrane to create ion channels, or pores, disturbing the osmotic balance, permeability and the regulation of the trans-membrane electric potential. This results in colloid-osmotic lysis of the cells. Spore germination and proliferation of the vegetative cells into the haemocoel may result in septicaemia, contributing to mortality of the insect larvae.

Impact on beneficial organisms

Turex 50 WP is reported to be gentle against beneficial organisms; including biological control agents (macro and micro-organisms) and pollinating insects.

Resistance

The development of resistance in target pests against *Bacillus thuringiensis* ssp. *aizawai* is possible. Standard resistance management strategies should be implemented to reduce the risk of development of resistance in the pest population against Turex 50 WG.

Due to the different mode of action of *Bacillus thuringiensis* ssp. *aizawai* compared to conventional insecticides, the risk of pest populations developing cross resistance is very low. *Bacillus thuringiensis* ssp. *aizawai* can be considered a valuable part in resistance management strategies. (Please see the efficacy evaluation carried out by the Norwegian Institute for Agricultural and Environmental Research for further information.)

Dosage and time of application

Turex 50 WG is applied at a dose rate of 1.0- 2.0 kg/ha. The Norwegian Institute for Agricultural and Environmental Research used the Norwegian draft label and GAP-table for Turex 50 WG to summarize the recommended dosage and time of application. (See table in the efficacy evaluation carried out by the Norwegian Institute for Agricultural and Environmental Research).

The number of application is not included on the draft label. The Norwegian Institute for Agricultural and Environmental Research is recommending 6 applications in floriculture crops, tree nursery crops, perennials and Solanaceous and Cucurbitaceous fruiting vegetables. In all other crops 3 applications is the maximum.

Pre-harvest interval: Due to the low toxicity and the fast degradation of *Bacillus thuringiensis* ssp. *aizawai* by UV light, a pre-harvest interval is not required.

Standardized Area Dose – (Normert Arealdose – NAD)

Based on the proposed use against caterpillars in fruits the standardized area dose is set to 2 kg per hectare.

Application equipment

Turex 50 WG is applied by foliar spray.

Recommendations by the The Norwegian Institute of Bioeconomy Research (NIBIO)

NIBIO recommends registration of Turex 50 WG in all crops mentioned on the Norwegian label and in the GAP table with clear instructions about the conditions needed for good efficacy with regards to temperature, UV-light and the target species ingesting the product as young larvae. The different European and Norwegian efficacy trails and experiences show that Turex WG will provide good control of small/ young (0,5-1 cm) caterpillars in different crops. No phytotoxicity has occurred in the trials for any of the recommended dosages. Turex will be a valuable addition to very few products registered for use against caterpillars. Standard resistance management strategies should be implemented.

Residues data

According to the DAR written by Italy in 2007, *Bacillus thuringiensis* spores or crystal proteins are not toxic to man or domestic animals. Persistence of *Bacillus thuringiensis* products on aboveground leaves and fruits is low. Half-life of viable spores is about 1 day. Applied as a spray, the δ-endotoxins are rapidly degradable and endospores are rapidly inactivated when exposed to UV radiation. Thus, residue data are not required.

EFSA concludes that the active components of commercial *Bacillus thuringiensis aizawai* strains GC-91 preparations are not toxic or pathogenic to humans. According to EFSA, the only remaining issue for consumer exposure is that *Bacillus thuringiensis* species carry the genetic material that encodes for the *Bacillus cereus* enterotoxin, and it is not known if this can be expressed, and if so under what conditions. In a 2005 EFSA opinion on *Bacillus cereus* it was presented that food poisoning incidents in rare cases were caused by levels of 10^3 CFU/g of food.

The species *Bacillus thuringiensis* has been recently discussed by the SCFCAH pesticide residues. There is no agreement among member states about the inclusion of the microorganism on the Annex IV of the Regulation (EC) 396/2005.

Mammalian toxicology

Turex 50 WG with the active substance/organism *Bacillus thuringiensis aizawai* GC-91 has been applied for registration in Norway. The notifier has submitted studies on acute toxicity, irritation and sensitization together with studies, regarding pathogenicity and infectivity of *Bacillus thuringiensis* ssp. *aizawai* CG-91, conducted on rats.

Bacillus thuringiensis ssp. *aizawai* CG-91 has been evaluated by the European Food Safety Authority (EFSA) in 2013. The evaluation was based on in the EU Draft Assessment Report (DAR) prepared by Italy in 2007. The DAR and the conclusion from EFSA are enclosed.

EFSA concludes that there was no evidence of toxicity, pathogenicity and infectivity in a 90 day oral study in rats. They conclude however that the microorganism may cause sensitization reactions and eye irritation.

EFSA highlights the potential of food-borne poisoning, related to *Bacillus cereus* type toxins (enterotoxins) susceptible to be produced by *Bacillus thuringiensis* ssp. *aizawai*. There is also a potential for exposure, after application, to toxins that could be produced by *Bacillus thuringiensis* ssp. *aizawai*.

The studies conducted on *Bacillus thuringiensis aizawai* CG-91, as described in the EU Draft Assessment Report (DAR), are summarized below:

Table 1: Toxicity studies conducted on *Bacillus thuringiensis aizawai* CG-91PEC$_{SW}$ reported in DAR.

Study type	Test item	Dose level	Findings	Conclusions	Report
Acute oral **rat**	Bta CGA-237218 technical FL 910331:	5050 mg per kg b.w. 1.1 x 10^{10} CFU per kg b.w.	One of ten animals died	LD_{50}>5050 mg per kg b.w.	IIM 5.3.2/01: Kuhn (1991)
Acute oral **rat**	Bta CGA-237218 technical	9.4 x 10^8 CFU per kg b.w.	No adverse effects, no infectivity	LD_{50}> 9.4 x 10^8 per kg b.w.	IIM 5.3.2/02: Hossack et al. (1990a)
Acute intratracheal **Rat**	Bta CGA-237218 technical	3.76 x 10^8 CFU/kg b.w.	2 of 36 animals died transient signs of toxicity	LD50 > 3.76 x 10^8 per kg b.w.	IIM 5.3.3/01: Hossack et al. (1990b)
Acute inhalation **Rat**	CGA-237218 WP FL-910986	0.526 and 3.16 mg/L 5.6 and 37.7 x 10^6 CFU /L	No mortalities, transient clin. signs	LC50> 3.16 mg/L 37.7x10^6 CFU /L	IIM 5.3.3/02: Holbert (1992)
Acute intraperitoneal **Mouse**	Bta CGA-237218 technical 91-7288	1.16 x 10^6 CFU/ mouse	No mortalities	NOAEL is 1.16 x 10^6 CFU per mouse	IIM 5.3.4/01: Marshall (1992a)
Acute intraperitoneal **Mouse**	Bta CGA-237218 technical 911445	2.55 x 10^6 CFU/mouse	No toxicity, no infectivity	NOAEL is 2.55 x 10^6 CFU per mouse	IIM 5.3.4/02: Marshall (1992b)
Acute intraperitoneal **Mouse**	Bta CGA-237218 FL-901966 FL-910039 FL-910040 FL-910041 FL-910042	10^8, 10^7, 10^6 CFU/animal	10^8 CFU/mouse: 82% mortality 10^7 CFU/mouse: 10% mortality 10^6 CFU/mouse: no mortality , no toxicity	LD50 > 10^7 CFU per mouse	IIM 5.3.4/03: Vlachos (1991)
Acute intravenously **Rat**	Bta CGA-237218 Technical	7.6 x 10^7 CFU per rat	No infectivity, no toxicity	NOAEL 7.6 x 10^7 CFU per rat	IIM 5.3.4/04 Hossak et al. (1992)

Study type	Test item	Dose level	Findings	Conclusions	Report
Dermal toxicity **rat**	CGA-237218 technical FL 891267	2020 mg /kg b.w. for 24 h	No systemic effects, Slight to well defined oedema and erythema	LD50>2020 mg /kg b.w.	IIM 5.5.1/01 Holbert (1991a)
Subcutaneous **mouse**	CGA-237218 technical FL 900815	3.8 x 10^6 CFU/animal	No mortalities extremely irritating	LD50>3.8 x 10^6 CFU/animal	IIM 5.5.1/02: Holbert (1991b)
Subcutaneous **mouse**	CGA-237218 technical FL 900816	2.66 x 10^6 CFU/animal	No mortalities slightly irritating	LD50>2.66 x 10^6 CFU/animal	IIM 5.5.1/03: Holbert (1991c)
Subcutaneous **mouse**	CGA-237218 technical FL 900814	1.08 x 10^6 CFU/animal	No mortalities non irritating	LD50>1.08 x 10^6 CFU/animal	IIM 5.5.1/04: Holbert (1991d)
Eye irritation **rabbit**	CGA-237218 technical FL 891267	0.1 g 2.9 x 10^7 CFU per animal	Non irritating	NOAEL is 100 mg	IIM 5.5.1/05 Liggett, 1992
Genotoxicity **In vitro** **Salm. typh.**	Microbial gene mutation	CGA 237218 technical 10% in DMSO	19.5 – 5000 µg/plate	Non genotoxic under the conditions tested	IIM 5.3.5/01 Hertner (1992)
Genotoxicity **In vitro** **Salm. typh.**	Microbial gene mutation	Bt H1, Bt H14 supernatants	0.5 –50 µL 10-fold concentrated supernatant	Non genotoxic under the conditions tested	IIM 5.3.5/02 Carlberg et al. (1995)
Clastogenicity **In vitro** **Human lymphocytes**	Chromosomal aberration	*Bacillus* thuringiensis Serotype 1 or Serotype 3	20% (v/v) of supernatant	Bt 1: Clastogenic at cytotoxic concentrations Bt 3: Not clastogenic under the conditions tested	IIM 5.5.2/03 Meretoja et al. (1977)

Study type	Test item	Dose level	Findings	Conclusions	Report
90 days **oral** **rat**	Bta CGA-237218 technical	10^8 CFU per animal per day for 13 weeks	No adverse effects	NOAEL 10^8 CFU per animal per day	IIM 5.3.7.1/01 Edwards (1993)

Acute oral application

Administration of an acute high dose of *Bacillus thuringiensis kurstaki* by the oral route induced no adverse effects in rats and mice. *Bacillus thuringiensis* passes readily through the gastrointestinal tract and was detected only in the faeces with counts rapidly declining. *Bacillus thuringiensis* remained confined to the gastrointestinal tract and was not systemically distributed and, thus, not detected in the organs.

Upon oral administration of *Bacillus thuringiensis* no toxicity or pathogenicity was observed and there was no infectivity.

Acute inhalative application

Following inhalative exposure to rats no mortalities were noted in rats at high exposure levels. Upon intratracheal instillation in rats 2 of 36 treated animals died at a dose level of 3.76×10^8 CFU per kg b.w.

Acute systemic application

Upon intraperitoneal administration in mice no signs of toxicity or infectivity and no mortalities occurred at a dose level of 10^6 CFU per animal. Mortalities at frequencies of 10% and 82% occurred at dose levels of 10^7 and 10^8 CFU/animal, respectively. All mortalities occurred within two days post-treatment.

No clinical signs of toxicity and no mortalities were noted in a study upon intravenous administration of 7.6×10^7 CFU CGA-237218 per rat. Infectivity of *Bacillus thuringiensis aizawai*, i.e. invasion and multiplication of micro-organisms, could not be demonstrated. Clearance from internal organs was rapid. Only the spleen had significant numbers of the microbe by day 14.

Other acute toxicity endpoints

No mortalities or signs of systemic toxicity were observed upon dermal application of 2020 mg *Bacillus thuringiensis aizawai* /kg b.w. to New Zealand White rabbits for 24 h.

Upon intradermal injection of three different batches of CGA-237218 technical in mice no mortalities or systemic effects were observed. Local effects were observed from extremely irritating to non-irritating.

In a primary eye irritation study, instillation of 0.1 mg CGA-237218 technical FL 891267 (2.9 x 10^7 CFU *Bacillus thuringiensis aizawai* per animal) in the rabbit eye caused no conjunctival irritation or other ocular effects.

Genotoxicity

Suspensions of *Bacillus thuringiensis aizawai* were tested for mutagenic activity in the Ames Salmonella assay. No mutagenic activity was detected in several tester strains with or without metabolic activation by rat liver microsomal fractions.

While cytotoxic concentrations of supernatants from an exotoxin-producing strain (*Bacillus thuringiensis* serotype 1) caused significantly increased chromosomal aberrations in human lymphocytes, no significant clastogenic effect was observed with supernatants from *Bacillus thuringiensis* serotype 3 (ssp. *kurstaki*), which does not produce exotoxins. Since Bt ssp. *aizawai* is very similar to Bt ssp. *kurstaki* and also does not produce exotoxins, it is assumed that Bta will also have no clastogenic effect.

Short term or chronic application

Following thirteen weeks administration of CGA-237218 technical by oral gavage to rats no treatment-related effects were seen on clinical signs, bodyweight gain, ophthalmoscopy, clinical pathology or macroscopic pathology. High counts of *Bacillus thuringiensis* were detected in the caecum but complete clearance was apparent at the end of the 4-week recovery period. The study gave no indication of direct toxicity, infectivity or pathogenicity of *Bacillus thuringiensis aizawai* in the rat upon 13-weeks repeated oral administration.

Overall conclusion

No toxicity or infectivity was noted in experimental studies upon oral, dermal or inhalative exposure even at high dose levels. Upon administration of extremely high dose levels by invasive routes (intranasal, intracerebral or intraperitoneal) mortality occurred in laboratory animals. However, lower doses applied by these routes caused no adverse effects.

Exposure Assessment

Operators and workers

Since no adverse effects were obtained in any study on toxicity, pathogenicity or infectiveness and no target organ exists, no dose-effect response (LOAEL) can be determined. Neither the UK Predictive Operator Exposure Model (POEM) nor the German

BBA model is suitable for calculating a risk assessment for operators based on a non-existing dose-effect relation.

EFSA concludes that the reference values are not necessary for the microorganism and no exposure estimates are required. EFSA however pointed out that due to the data gap for analysis of the potential toxins (e.g. enterotoxins, beta-exotoxins and cytolytic protein) produced after application, the risk assessment cannot be concluded for re-entry workers. In addition, the assessment for operators and bystanders cannot be concluded in view of the data gap in relation to the potential production of enterotoxin during manufacture.

Environmental fate and behaviour

Turex 50 WG with the active substance/organism *Bacillus thuringiensis aizawai* CG-91 (500 g/kg, $3x10^{13}$ colony forming units (cfu)/kg) has been applied for registration in Norway. The area of use is against *Lepidoptera* larvae (caterpillars) in different agricultural crops outdoor and indoor. The application rate is 1-6 applications with 500-1000 g a.s./ha (50-100 g a.s./daa). The highest application rate is to be used in fruit.

This bacterial strain produces crystalline proteins (δ endotoxins) at the time of sporulation. They are exogenous metabolites of *Bacillus thuringiensis* with insecticide activity. These proteins are multi-component proteins that are disaggregated to single active components (Cry toxins) under favourable conditions. The production of this kind of protein is the common characteristic of all *Bacillus thuringiensis* species. However, the actual proteins may vary between species and among different strains. The variations usually result in proteins selective to different kinds of insects (EFSA-conclusion, 2013).

The following fate and behaviour assessment is a short summary of information found in chapter 3.1.5 in part A of the Registration Report for the central zone (Germany 2011) and the DAR (Italy, 2007). More information can also be found in a Norwegian assessment of Vectobac 12 AS (*Bacillus thuringiensis israelensis*) from 2001.

Soil

Degradation

Bacillus thuringiensis aizawai (*Bta*) and all other members of the species of *Bacillus thuringiensis* are naturally present in our environment. Therefore, their application in pest control means only a fluctuation of the bacterium population in the biotope of the pest insect.

A natural breakdown of the endospores of *Bta* in soil begins after application onto the fields and gradually reduces the numbers of spores remaining. It appears that *Bt* spores can remain viable in soils of pH above a certain threshold for long periods under conditions which

do not stimulate germination. In a study with a field application of a suspension of spores of *Btk* onto a cabbage field, a half-life of 120 days for colony forming units (CFU) was established after the suspension was sprayed directly onto the field soil. Any vegetative cells or crystal proteins are likely to be far more rapidly degraded. In a study described in the DAR a quick loss of parasporal insecticidal activity in natural soils occurred between 3 and 21 days after incubation began. Predicted loss of insecticidal activity was 77.3 % within 100 days. In another study it was shown that the addition of nutrients to the soil resulted in a reduction of labelled CO_2 evolution and an increase in the insecticidal activity half-life from 2.7 to 5.2 days. The added nutrients may have acted as an alternative substrate for the native micro-organism population decomposing the parasporal crystals or may have stimulated an entirely different microbial fraction which depressed the activity of the crystal decomposing fraction.

The reduction in numbers of *Bta* will be greatly augmented by the photo degradation effects of sunlight. It is very unlikely that *Bta* endospores will germinate and grow into vegetative cells, unless appropriate conditions exist; meaning favourable soil pH, soil moisture content, sufficient nutrient availability and lack of competition/predation from other soil micro-organisms. The survival of *Bta* in the soil is a dynamic process involving sporostasis, germination and sporulation in specific habitats and will be influenced by changing conditions regarding soil type, native micro flora, nutrient availability and fertilization.

Photolysis

In a study described in the DAR *Btk* spores and crystals were almost completely inactivated following 12 hours exposure to UV light. In a study it was observed that sunlight leads to the inactivation and destruction of *Btk* (HD-1 and HD-73 strains) purified δ-endotoxin crystals. Following a 24 hour irradiation to light with a spectrum equivalent to the solar spectrum, approximately 35 % of crystal proteins were damaged resulting in total loss of activity. It has also been found that average half-life values from deposits on artificial samplers, pecan foliage nutlets and budlets from the mid-canopy were 24.4, 17.9, 14.3 and 16.5 hours, respectively. As no precipitation occurred during the 96-hour study period, the loss of toxin is primarily attributed to UV degradation.

Mobility

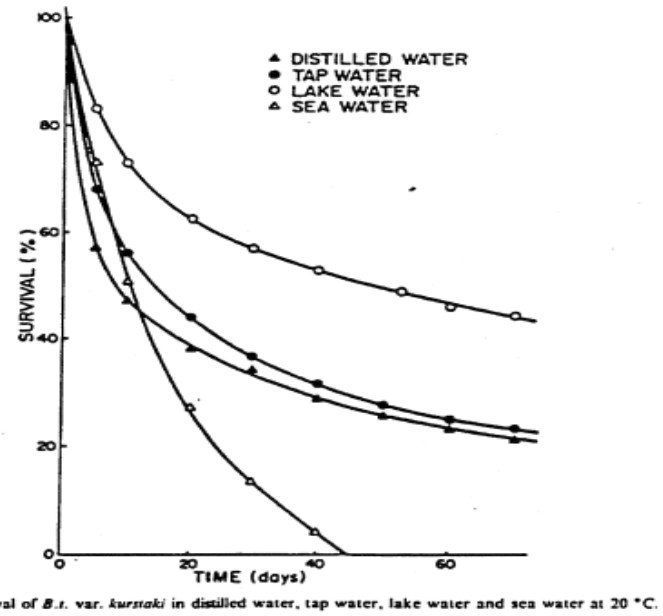
According to the DAR, several studies demonstrated the equilibrium adsorption and binding of the purified protoxin and toxins produced by *Bt* species onto the (predominant) clay minerals, humic acids etc. in soil. Adsorption appeared to be temperature independent and decreased with increasing soil pH, and toxins adsorbed more readily than protoxins. Insecticidal activity was strongly retained indicating that once bound, the protoxins and toxins became inaccessible for consumption by other soil micro-organisms. Bound toxin from *Bt* species had a higher toxicity (i.e., had lower LC50 values) than free toxin, possibly as a result of the toxin being concentrated on the clays.

Several studies have indicated that transport of *Bt* through the soil by leaching is not likely to occur. In a sandy clay loam in Denmark the movement of *Btk* in soils was investigated,

showing that after spraying of commercial products containing Bt, 77 % of recovered *Bt* remained in the 0 to 2 cm topsoil layer after 1 year. In experiments in Japan it has been found that under artificially and naturally irrigated conditions, there was no translocation of sprayed *Bt* into the soil down to a depth of 10 cm.

Surface water

Under natural conditions, residues of *Bta* in water are not considered to be able to persist for very long periods due to a combination of natural physical and chemical degradation factors such as solar radiation and predation from resident bacteriophages, protozoans and other lower animal forms. It may be stated that *Bta* GC-91 is inactivated under natural conditions, including water. Figure 5.1 describes the results of a study which investigated survival of *Btk* in water at 20 °C (DAR).



Survival of *B.t. var. kurstaki* in distilled water, tap water, lake water and sea water at 20 °C.

Figure 1: Survival of *Bacillus thuringiensis kurstaki* in water at 20 °C.

Groundwater

Various experiments examining the movement of *Bt* in soils following spraying of commercial products containing *Bt* showed little or no movement neither in laboratory columns nor in the field under natural irrigation conditions. Additionally, adsorption and binding of protoxins and toxins from Btk have been demonstrated to occur readily, rapidly and strongly onto the clay fraction and clay humic acid complexes of soils while desorption occurs far less readily. See paragraph on mobility above and the paragraph on ground water in chapter B.8.1.2 in the DAR. It is thus concluded that no threat of contamination of groundwater exists following applications of Agree 50 WP according to GAP. It has not been possible to estimate a PECgw. (The product Agree 50 WP contains the same active ingredient as Turex 50 WG for which the application has been made in Norway.)

Air

Evaporation and volatility of bacteria is not expected to be a factor to consider in assessing the fate in air. It has been noted that, following field application onto foliage, *Bt* disappears from plant surfaces at rather rapid rates. This can be primarily due to environmental effects such as degradation and breakdown and wash-off by rainfall. A rapid degradation of *Bta* in air is assumed since inactivation by solar radiation is a very important factor causing loss of activity and degradation of bacteria spores and δ-endotoxin crystals in the field environment. Spray drift, however, can occur following an application of *Bta* which may lead to temporary concentrations in the atmosphere which are capable of drifting with wind currents before the spores and crystals in finer spray droplets settle out. However, rapid degradation of *Bta* in air or in these droplets mainly due to inactivation by solar radiation is assumed and confirmed by literature reports.

In the DAR different examples of "air half-lives" have been reported and some of these are summarized briefly here. It has been shown that Btk applied onto cabbage plants had a half-life of 16 hours (r^2 = 0.94) on the leaf during the first 0 to 7 days after application. *Bt* was toxic to target insects for less than 48 hours following application onto tomato plants in the field and the half-life of *Bt* Berliner spores following application onto soybean leaves was determined to be less than 24 hours. Four different *Bt* insecticidal formulations sprayed onto the leaves of the Western redbud *Cercis occidentalis* at three different sites in California resulted in early persistence half-lives that ranged from 0.58 to 1.85 days, depending on location. A study in Canada where aerial application of *Btk* was used, an overall half-life of 2.4 days was observed over a 9-day monitoring period.

Exposure

PECsoil

In order to perform a risk assessment for non-target organisms, the actual concentration of Agree 50 WP upon six applications is calculated as here the highest exposure is expected according to the intended uses. The calculation bases on a maximum application rate of 1 kg Agree 50 WP/ha, assuming as a worst case that no degradation occurs between applications. For the risk assessment the resultant load of Agree 50 WP will be related to the top 5 cm of soil to achieve the highest theoretical soil concentration.

Assumptions

Accumulated dose rate, considering 6 applications: 6 kg Agree 50 WP/ha (= 3 kg *Bta*/ha = 1.8×10^{14} CFU/ha). This application rate is equal to 6x50 g a.s./daa (total 300 g a.s./daa per season) which is the highest proposed application rate in Norway.

Incorporation into the top 5 cm layer (= 50 L soil/m^2). Soil density of 1.5 g/cm^3 (= 75 kg soil/ m^2). Plant interception was not considered for the PEC calculation, as this is the worst case and covers all uses.

According to the PEC calculation the expected initial concentration is 8.0 mg Agree 50 WP /kg dry weight soil (4 mg *Bta*/kg dry weight soil). In terms of CFU, this is equivalent to 2.4 × 10^8 CFU/kg dry weight soil.

PECsurfacewater

Aquatic organisms may be exposed to Agree 50 WP and *Bta* GC-91 through spray drift. Exposure of aquatic organisms from this route was estimated by calculating Predicted Environmental Concentration in surface water (PEC_{SW}).

For 6 applications in flowers and vegetables (drift value of 6.41% for plant heights > 50 cm) assuming worst case conditions of no degradation of Bta GC-91 between the spraying resulting in an accumulated application rate of 6 kg Agree 50 WP/ha (300 g a.s./daa per season), the initial concentration of Agree 50 WP and Bta GC-91 in 30 cm depth in surface waters is as follows:

Table 2: PEC_{SW} values for Agree 50 WP

Test substance	PEC_{sw}
Agree 50 WP	128.07 µg/L
Bta GC-91	64.04 µg/L or 3.84 × 10^6 CFU/L

Ecotoxicology

The text below is taken from the part A of the Registration Report for the central zone (Germany 2011). The application rate used in the risk assessments by Germany is the same as the Norwegian GAP for most crops (100 g Turex 50 WG/daa). In fruits, the Norwegian GAP is twice as high (200 g Turex 50 WG/daa). Looking at the risk assessments, however, the risks are above the triggers except for some Lepidoptera species off-crop.

Effects on Birds

Acute risk assessment

The TER_A values exceed the Annex VI trigger value of 10, indicating that Agree 50 WP poses no risk to birds following application according to the proposed use patterns.

Table 3. Screening assessment for birds following GAP directed application of Agree 50 WP.

Indicator species	Crop	Test item	Toxicity LD_{50}	Application rate[a)]	MAF[b]	Short cut value[c)]	DDD	TER (10)
Small omnivorous bird	Vegetables	Bta GC-91	> 3333 mg/kg b.w.	0.5 kg/ha	1.9	158.8	150.86	> 22.1
Small insectivoreous birds	Orchards and ornamentals/nursery			0.5 kg/ha	1.9	46.8	44.46	> 75.0

[a)] Refers to Bta GC-91 (corresponding to 1 kg Agree 50 WP/ha)

[b)] MAF according to 6 successive applications at intervals of 7 days provided in EFSA Guidance document 2009[2]

[c)] Short cut value based on the 90th percentile of residues provided in EFSA Guidance document 2009[2]

Long-term risk assessment

As the acute TER value indicates no risk to birds and no adverse effects were observed in short-term toxicity studies, no long-term effects are to be expected upon field application of Agree 50 WP according to GAP.

Effects on Terrestrial Vertebrates Other Than Birds

Acute risk assessment

The TER_A values exceed the Annex VI trigger value of 10, indicating that Agree 50 WP poses no risk to mammals following application according to the proposed use patterns.

Table 4. Screening assessment for mammals following application of Agree 50 WP.

Indicator species	Crop	Test item	Toxicity LD_{50}	Application rate[1)]	MAF[2)]	Short cut value[3)]	DDD	TER (10)
Small herbivorous mammals	Vegetables/ ornamentals and nursery[4)]	CGA-237218 technical	> 5050 mg/kg b.w.	0.5 kg/ha	1.9	136.4	129.58	> 39.0

[1)] Refers to Bta GC-91 (corresponding to 1 kg Agree 50 WP/ha)

[2)] MAF according to 6 successive applications provided in EFSA Guidance document 2009[2]

[3)] Short cut value based on the 90th percentile of residues provided in EFSA Guidance document 2009[2]

[4)] This scenario includes: orchards, grassland and vineyards.

Long-term risk assessment

No data on the short- or long-term toxicity of Bta GC-91 or Agree 50 WP are presented here. Due to the absence of toxicity in the acute study and the highly specific mode of action of Bta GC-91, no adverse effects in mammals are to be expected upon prolonged exposure to Agree 50 WP.

Effects on Aquatic Organisms

For the risk-assessment the maximum (initial) PEC_{SW} was compared with the acute ecotoxicological endpoints of aquatic organisms. The toxicity exposure ratios (TER) are given only for worst case scenarios, for which the relevant trigger value is passed. Only drift entries were considered in the PEC_{SW} calculation since this is the only suitable exposure pathway for the use of Agree 50 WP.

Table 5. TER values for Agree 50 WP/Bta GC-91 with exposure via spray drift

Compound	**Organism**	**Endpoint**	**Exposure (PEC_{SW})**	**TER (trigger)**
CGA-237218[a]	Fish	LC_{50} > 2.0 × 10^{10} CFU/L	3.84 × 10^{6} CFU/L	> 5208 (100)
Agree 50 WG[b)]	Daphnids (acute)	LC_{50} > 100 mg/L	128.07 µg/L	> 781 (100)
CGA-237218[a]	Daphnids (chronic)	NOEC >1.57 × 10^{8} CFU/L	3.84 × 10^{6} CFU/L	> 40.88 (10)
CGA-237218[a)]	Algae	3.6 × 10^{9} CFU/L	3.84 × 10^{6} CFU/L	> 938 (10)

a) Synonym for *Bta* GC-91 technical material contained in Agree 50 WP

b) Agree 50 WG contains the same active ingredients and similar co-formulants as Agree 50 WP

The TER values exceed the trigger values indicating that Agree 50 WP poses no risk to aquatic organisms following application according to the proposed use patterns.

Effects on Bees and Other Arthropod species

Bees

Studies assessing the effect of the active ingredient *Bta* GC-91 as well as of the formulation CGD 97220 (= Agree 50 WP) were evaluated as part of the EU review of *Bta* GC-91. The acute risk to honey bees from use Agree 50 WP was assessed using the maximum application rate and the LD_{50} value to calculate hazard quotients. These are considerably less than the trigger value of 50, indicating that bees are not at risk upon field application of Agree 50 WP.

Table 6. Risk to bees from exposure to Agree 50 WP

Compound referred to	Application rate	LD_{50}	Hazard quotient
Bta GC-91	500 g Bta GC-91/ha	10 day oral: 91 µg Bta GC-91/bee	5.5
		48 h oral: > 98.5 µg Bta GC-91/bee	< 5.1

Arthropods other than bees

Effects on non-target arthropods of Agree 50 WP were not evaluated as part of the EU review of *Bta* GC-91. Studies on the toxicity of the formulated Product Turex 50 WG, containing the same content of *Bta* GC-91 as Agree 50 WP, were conducted. In-field and off-field HQ values were calculated for the proposed use patterns and are considered adequate. The obtained HQ values for both scenarios indicate no unacceptable risk for non-target arthropods upon field application of Agree 50 WP.

Table 7. In-field and off-field HQs for non-target arthropods

Species	**LR_{50} (g/ha)**	**In-field foliar**		**Off-field foliar**			**Trigger value**
		PER (g/ha)	**HQ**	**PER (g/ha)**	**Correction factor**	**HQ**	
Typhlodromus pyri	>4500	3200	< 0.71	19.2	10	< 0.004	2
Aphidius rhopalosiphi	>4500		< 0.71			< 0.004	2

PER: predicted environmental rate depending on application rate and drift

HQ: Hazard Quotient

Correction factor: extrapolation from testing just 2 representative species

Lepidoptera species in off-crop habitats

The risk for non-target *Lepidoptera* species in off-crop habitats was assessed using data from open peer reviewed literature. Following the results the HQ values for 4 out of 5 species (*Vanessa cardui*, *Manduca sexta*, *Pieris rapae* and *Heliothis virescencs*) are below the

trigger of 2, although the worst case was assumed. Hence, no negative side effects are expected following field application of Agree 50 WP according to GAP. Assuming the same conditions the HQ value for *Lymantria dispar* slightly exceeds the trigger of 2. However, due to the fast inactivation of Bta no unacceptable risk is expected upon field application of Agree 50 WP.

Table 8. Exposure Hazard Quotients for Lepidopteran species in off-crop habitats according to GAP directed use of Agree 50 WP in orchards (3 × 2.0 kg/ha).

Test species	**LR_{50} [kg Agree 50 WP/ha]**	**Exposure scenario**	**Exposure rate [kg/ha]**	**HQ (< 2)**
Lymantria dispar	0.08	off-crop[a]	0.21	2.63
Vanessa cardui, Manduca sexta, Pieris rapae	0.2			1.05
Heliothis virescencs	0.8			0.26

a) In the off-crop scenario, spray drift 23.96% at 3 m is considered, according to JKI (2006).

(Julius Kühn Institute spray drift data from 27. March 2006, http://www.jki.bund.de/fileadmin/dam_uploads/_AT/abdrift-eckwerte/Abdrifteckwerte_xls.xls)

Effects on Earthworms and Other Soil Macro-organisms

The acute toxicity of the formulation CGD 97220 I (equivalent to Agree 50 WP) to the earthworm *Eisenia foetida* was determined in a laboratory study and evaluated as part of the EU review of Bts CG-91. The presented risk assessment calculating the relation between the expected environmental concentration of Agree 50 WP in soil and the endpoint from an acute study with an equivalent concentrated formulation is considered adequate. The obtained TER value indicates no risk for earthworms upon field application of Agree 50 WP.

Table 9. Acute TER value for earthworms

Compound	**LC_{50}**	**Maximum PEC_S for Agree 50 WP**	**TER_A**	**Limit**
Agree 50 WP	> 1000 mg/kg d.w. soil	8 mg/kg. d.w. soil	> 125	10

Effects on organic matter breakdown

No EU data requirement for MPCP.

Effects on Soil Non-target Micro-organisms

Effects on the soil microflora of CGD 97220 I (equivalent to Agree 50 WP) were evaluated as part of the EU review of Bta CG-91. The formulation did not show any influence on the soil microbial activity at a concentration of 20.0 kg/ha. Due to the assumption of the worst case that no degradation of Agree 50 WP occurs between the treatments and the absence of adverse effects observed in the laboratory study with CGD 97220 I containing the same amount of Bta GC-91 as Agree 50 WP, it can be concluded that GAP directed use of Agree 50 WP poses no risk for the soil microflora.

References

T1_E1: EFSA conclusion. 2013. Conclusion on the peer review of the pesticide risk assessment of the active substance *Bacillus* thuringiensis subsp. aizawai (strains ABTS 1857, GC-91)

T2_E2: Draft Assessment Report. Italy, 2007. *Bacillus* thuringiensis subsp aizawai strain GC-91/Agree 50 WP

E3: Draft Registration Report. Germany, 2011. Part A – Risk management

E4: Draft Registration Report. Germany, 2011. Part B – Detailed summary and risk assessment

E5: Evaluation of Vectobac 12 AS. Norwegian Agricultural Inspection Service, 2001.

Terms of reference as provided by the Norwegian Food Safety Authority

Turex 50 WG is a new product containing the new active substance/organism *Bacillus thuringiensis* ssp. *aizawai* GC-91. The intended use is as an insecticide in a wide range of crops.

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

- The human health risk by using *Bacillus thuringiensis* ssp. *aizawai* GC-91 as a plant protection product. It 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 ecotoxicological effects and risks with regard to the use of Turex 50 WG as a plant protection product.

1 Hazard identification and characterisation of *Bacillus thuringiensis* ssp. *aizawai* strain CG-91

1.1 Previous assessments

1.1.1 Assessments in Norway

Plant protection products with the *Bacillus thuringiensis* strain *aizawai* CG-91 has not previously been assessed in Norway. *Bacillus thuringiensis* ssp. *israelensis* H14 in the product Vectobac 12 AS has previously been evaluated in Norway (2001), but was not approved due to insufficient documentation.

1.1.2 Administrative history in EU

The Draft Assessment report (DAR) on *Bacillus thuringiensis* subspecies *aizawai*, strain GC-91 was prepared by Italy in 2007 (Mattilsynets Vedlegg E2-E4) (DAR Italy, 2007). The EU-review was published in 2008 (European Commission, 2008). *Bacillus thuringiensis* ssp. *aizawai* GC-91 was included in Annex 1 in 2009. This means that it is approved for use in EU as an active ingredient in plant protection products. Later, EFSA performed a peer review of the DAR and published their conclusion in 2013 (Mattilsynets vedlegg E1) (EFSA, 2013). The European Commission later (2013) updated the review report to address issues pointed out by EFSA (rev. 4 of SANCO/1538/08) (European Commission, 2013). The Commission found no need for changing the conditions of approval of *Bacillus thuringiensis* ssp. *aizawai*, strain GC-91. The Commission further noted that when the Member States decide on individual plant protection products containing *Bacillus thuringiensis* ssp. *aizawai*, strain GC-91 they shall take into account this review report in accordance with the provisions of Regulation (EC) No 1107/2009, and in particular the provisions of Article 4(1), (2) and (3) of that Regulation and uniform principles laid down in Regulation (EC) No 546/2011.

1.1.3 Northern Zone Registration Report

A registration report (RR) for the product Agree 50 WP with the active ingredient *Bacillus thuringiensis* ssp. *aizawai* GC-91 for use in the Nordic Zone was prepared by the Danish Environmental Protection Agency in 2014 (Registration Report Denmark, 2014). (Mattilsynets vedlegg “Bta GC-91 RR 1107”) The product Agree 50 WP contains the same active ingredient as Turex 50 WG for which the application has been made in Norway.

In general, the Danish Environmental Protection Agency agrees with the Commission, that the points made by EFSA do not indicate any unacceptable risks.

The main issues identified by EFSA, the comments made by the EU Commission/SANCO, and the conclusions in the Danish registration report on the different topics is given below, followed by the considerations and conclusions reached by VKM.

1.2 Active substance – identity, analysis and properties

1.2.1 Introduction

1.2.1.1 Identity and analysis

Bacillus thuringiensis is a facultative anaerobic, gram-positive bacterium that forms characteristic protein inclusions adjacent to the endospore. *Bacillus thuringiensis* subspecies have the ability to produce the parasporal crystalline protein inclusions (δ-endotoxin) which are toxic to certain invertebrates, especially larvae belonging to the insect orders Coleoptera, Diptera and Lepidoptera.

"*Bacillus cereus*-like organisms" consist of *Bacillus cereus sensu stricto*, *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus weihenstephanensis*. What distinguish the members of the *Bacillus cereus* group functionally are mainly genes carried on plasmids. The loss of plasmids of both of *Bacillus anthracis* and *Bacillus thuringiensis* make them indistinguishable to *Bacillus cereus* by morphological and biochemical methods. The reverse process is also possible; that a *Bacillus cereus* gaining a *Bacillus thuringiensis* plasmid becomes indistinguishable from *Bacillus thuringiensis* by morphological and biochemical methods (Gonzalez et al., 1982).

The fundamental difference between *Bacillus thuringiensis* and *Bacillus cereus* is the production of the plasmid encoded δ-endotoxin by *Bacillus thuringiensis*. Extensive genomic studies have concluded that there is no taxonomic basis for separate species status (Carlson et al., 1996; Helgason et al., 2000), since they cannot be separated at the chromosomal level. However, although *Bacillus cereus* can receive plasmids and thereby become δ-endotoxin-producing, and *Bacillus thuringiensis* can lose plasmids to become δ-endotoxin-negative, current taxonomy places them as separate species (Damgaard et al., 1996).

Several techniques have been tested as tools for identification and characterization of these micro-organisms down to strain level. The molecular determination of specific *Bacillus* strains has been reported by comparison of results from hybridization experiments, Cry PCR (Polymerase Chain Reaction), and RAPD-analyses (Random Amplified Polymorphic DNA) (Hansen et al., 1998; Valadares De Amorim et al., 2001). The authors claimed that by the use of these methods together, also *Bacillus thuringiensis* ssp. *aizawai* strains can be

distinguished from other *Bacillus thuringiensis* subspecies, as well as from *Bacillus cereus* strains.

The active ingredient in Turex 50 WG is *Bacillus thuringiensis* ssp. *aizawai* strain CG-91, which is not a naturally occurring strain but a product of a conjugation between the parental strains *Bacillus thuringiensis* ssp. *aizawai* strain HD 135-S4 (recipient strain), and *Bacillus thuringiensis* ssp. *kurstaki* strain HD 191-A2 (donor strain). Plasmid conjugation is a process which allows one bacterium to donate genetic material to another (Gonzalez et al., 1982). The parental strains HD-191-A2 and HD-135-4S differ in their flagella serotype as well as in their δ-endotoxin genes. The parental strains HD-191-A2 (flagella serotype *kurstaki*) and HD-135-4S (flagella serotype *aizawai*) were derived from the wild types HD-191 and HD-135.

GC-91 was selected on the basis of increased size of the parasporal crystal, as determined by microscopy. It has been found that strain GC-91 shows an improved insecticidal activity against certain Lepidopterous pest species and has an effectively broadened spectrum of activity (DAR Italy, 2007). No unusual morphological, physiological, pesticidal or resistance characteristics have been observed for GC-91 which differ from the classical description of the species *Bacillus thuringiensis*.

Common methods for detection and enumeration of *Bacillus cereus*-like organisms in food and clinical settings do not distinguish between *Bacillus cereus* and *Bacillus thuringiensis*. The question has therefore been raised if a proportion of *Bacillus cereus*-like organisms present in ready-to-eat food are in fact *Bacillus thuringiensis*, and consequently, that some of the food borne diseases diagnosed as *Bacillus cereus* infections are actually caused by *Bacillus thuringiensis*.

PCR primers have been designed from the sphingomyelinase gene of *Bacillus cereus* cells. These primers are specific for all *Bacillus cereus* group strains and may be used to detect *Bacillus cereus*-type cells in contaminated food samples in combination with selective agar assays (Hsieh et al., 1999). The preferred technique used to monitor *Bacillus cereus*-like organisms in food items is plating on selective agar or on blood agar medium. These techniques do not normally involve the use of microscopic discrimination between *Bacillus thuringiensis* and *Bacillus cereus*.

Several alternative methods have been tested for ability to discriminate between *Bacillus cereus* and *Bacillus thuringiensis*, for instance 16S rRNA and gyrB gene based PCR methods (Chen and Tsen, 2002). However, when a large number of *Bacillus* strains were tested using this method, the results showed that discrimination between *Bacillus cereus* and *Bacillus thuringiensis* was difficult. Thus, the authors concluded that the simplest practical way to distinguish *Bacillus thuringiensis* from *Bacillus cereus* is via assessment of the presence or not of parasporal crystal protein or Cry gene. The authors of this study concluded that except for PCR, gene sequencing and protein analysis of the Cry gene, they were unable to develop a reliable molecular method for the differentiation between *Bacillus cereus* and *Bacillus thuringiensis* strains (Chen and Tsen, 2002).

The presence of enterotoxin genes, such as haemolysin and enterotoxin can be assayed by polymerase chain reaction (PCR) methods, while enterotoxin activities can be determined using a so-called BCET-RPLA assay, haemolytic patterns on sheep blood agar, or cytotoxicity to Chinese hamster ovary (CHO) or Vero cells.

Recent development in the efficiency and cost reduction of complete genome sequencing has opened for new possibilities to identify organisms down to minor differences between strains or even isolates of the same strain (Ivanova et al., 2003; Kamada et al., 2015; Kunst et al., 1997). It seems likely that sequencing may replace most other methods used for identification of micro-organisms and form the basis for a more comprehensive characterization of properties.

1.2.1.2 Biological properties

Bacillus thuringiensis produce crystalline parasporal inclusions, so-called Cry proteins or δ-endotoxin. Different *Bacillus thuringiensis* strains may produce different Cry proteins with highly specific activity against certain insects. More than 100 different Cry proteins have been characterized. The Cry proteins expressed by the strain GC-91 are Cry1Ac, Cry1C, Cry1D and Cry2A.

Following ingestion of *Bacillus thuringiensis* by the larvae, the crystalline protein inclusions dissolve in the larval midgut, releasing insecticidal crystal (Cry) proteins (δ-endotoxins). Most of the crystal proteins are pro-toxins, and are converted proteolytically into smaller toxic polypeptides under the alkaline conditions in the insect midgut. The activated Cry toxins interact with the midgut epithelium cells of the susceptible insects. After binding to specific midgut receptors, they insert into the apical membrane to create ion channels, or pores, disturbing the osmotic balance, permeability and the regulation of the trans-membrane electric potential. This results in colloid-osmotic lysis of the cells. Spore germination and proliferation of the vegetative cells may result in septicaemia, contributing to enhanced mortality of the insect larvae.

Turex 50 WG is applied at a dose rate of 1.0- 2.0 kg/ha with 3-6 applications, depending on type of crops. The following pests or group of pests against which the product is to be used belong to the following Lepidopteran families (moths and caterpillars): *Geometridae, Plutellidae, Noctuidae, Pieridae, Crambidae, Lymantriidae, Lasiocampidae, Yponomeutidae, Tortricidae,* and *Gelechidae* (Schnepf et al., 1998).

The active ingredient in Turex 50 WP is considered to be gentle against beneficial organisms; including biological control agents (macro and micro-organisms) and pollinating insects. (See chapter 1.8 on ecotoxicology)

Bacillus cereus, *Bacillus thuringiensis* and *Bacillus anthracis* are distinguished principally by their plasmid content. These bacteria have similar 16S and 23S rRNA sequences indicating that they have diverged from a common evolutionary line relatively recently.

Bacillus cereus is known to induce emetic syndrome, caused by small-molecular weight cyclic toxin; cereulide, and diarrheal syndrome, caused by enterotoxins: haemolysin (HBL) and non-haemolytic enterotoxin (NHE). The diarrheal syndrome caused by these enterotoxins is relatively mild and short-lived, while a similar syndrome caused by cytotoxin K (CytK) is rarer and more serious.

Similarly, the pathogenicity of *Bacillus anthracis* is associated with the presence of two plasmids, pXOq and pXO2, the former coding for the anthrax toxin and the latter for capsule formation (Drobniewski, 1993).

The virulence genes of *Bacillus cereus*, on the other hand, are chromosomal (Guttmann and Ellar, 2000; Ivanova et al., 2003). Unlike *Bacillus thuringiensis* strains, *Bacillus cereus* strains and *Bacillus anthracis* strains lack parasporal inclusions. *Bacillus anthracis* is additionally distinguishable from *Bacillus thuringiensis* by its sensitivity to ampicillin, non-mobility and its requirement of thiamine for growth.

The presence of haemolysin and enterotoxin genes can be assayed by polymerase chain reaction (PCR) methods, while enterotoxin activities can be assayed by immunological methods, haemolytic patterns on blood agar, or cytotoxicity to cells *in vitro*.

The exact role of the factors responsible for the diarrhoeal syndrome caused by *Bacillus cereus* is not fully understood. It has for instance been discussed if one or more components are needed for the diarrhoeal syndrome (Agata et al., 1995; Beecher et al., 1995; Granum, 1994; Heinrichs et al., 1993).

In a study by Hsieh et al. (Hsieh et al., 1999), 12 different profiles of enterotoxin were determined for 98 *Bacillus cereus* group strains collected. If any of the three types of enterotoxins was present in the *Bacillus cereus* group cells, the cells were shown to be cytotoxic to the CHO cells. Similar enterotoxin profiles were observed among strains of *Bacillus cereus*, *Bacillus mycoides* and *Bacillus thuringiensis*. Thus, all *Bacillus cereus* group strains may potentially be toxic, and the detection of these cells in foods could therefore be important.

1.2.2 EFSA

"In the area of **identity of the microorganism / biological properties / physical and technical properties and methods of analysis** the main data gaps for the strain GC-91 are: to demonstrate that the level of microbial contamination complies with international standards; validation for the methods of analysis for parasporal protein, beta-exotoxins, contaminating microorganisms, and for identification of the strain; validation of the bio-potency method; batch analysis for enterotoxins; shelf-life of the formulation; effects of light, temperature and humidity on technical characteristics of the plant protection product."

1.2.3 EU Commission/SANCO

"It has been established that for the active substance notified by the main data submitter none of the manufacturing impurities considered are, on the basis of information currently available, of toxicological or environmental concern. However, strict maintenance of environmental conditions and quality control analysis during the manufacturing process shall be assured by the producer, in order to ensure the fulfilment of the limits on microbiological contamination as referred to in the Working Document SANCO/12116/2012."

1.2.4 Northern Zone Registration Report

Not part of the NZRR report.

1.2.5 VKM

Identity and analysis of the active ingredient

The active ingredient is produced from a fermentation product consisting of fermentation solids, spores and insecticidal toxins. The ingredients are described by CFU/g (colony forming units per gram) and standardized for potency (International Units (IU) per mg) using the cabbage looper *Trichoplusia ni* as test organism. The mean of 3-5 different fermentation batches is reported to show a 6.1 x 10^{10} CFU/g and 53,436 IU/mg. The variation between batches was however not reported. It is not clear to what extent each batch of manufactured product is provided with individual analysis data.

The average spore concentration in a Agree 50 WP formulated product (similar to Turex 50 WG) has been reported as 3.05 x 10^{13} spores/kg, with an average content of 37.5 g/kg δ-endotoxin. The exact composition of each preparation batch involves however industrial and commercial secrets for which confidentiality has been demanded.

It has been demonstrated that the ABTS 1857 and GC-91 strains do not produce significant quantities of β-exotoxin and cytolytic proteins in the production process. EFSA also concludes that it has been shown that the strain ABTS 1857 does not produce enterotoxins during the production process, but that a data gap for enterotoxin production exists for strain GC-91. EFSA furthermore states that analysis for content of contaminating microorganisms has not been fully addressed, and that procedures to unequivocally identify the organisms down to strain level are not available. Other sources of information claim however that such methodology is available (Hansen et al., 1998; Hsieh et al., 1999; Valadares De Amorim et al., 2001).

The VKM Panel considers exact knowledge of the content of each production batch to be important for safe use. Because of the close relationship with other toxin-producing bacterial strains, as well as the possibility for gene transfer between different bacterial strains, it is important to verify that each batch of Turex 50 WG consists of strain GC-91 only, and that

the level of enterotoxin in the product is low. Low potency for enterotoxin production should also be documented for each batch. Thus, each production batch should be analysed and labelled with: Number of spores determined as Colony Forming Units per gram (CFU/g); activity (IU/mg) and content (g/kg) of δ-endotoxin; level of enterotoxin produced by the vegetative cells.

1.3 Health risk – mammalian toxicology

1.3.1 Introduction

Available information on the toxic potential of *Bacillus thuringiensis* ssp. *aizawai* CG-91 consists mainly of data for acute toxicity, irritation and sensitization together with studies on the pathogenicity and infectivity of the bacterial products. Most studies have been conducted in rats.

EFSA (EFSA, 2013) concluded on the basis of a 90 day oral study in rats that there is no evidence of toxicity, pathogenicity or infectivity. EFSA stated however that the microorganism may cause sensitization reactions and eye irritation and highlights the potential of food-borne poisoning, related to *Bacillus cereus*-type toxins (enterotoxins). The overall conclusion on toxicity according to EFSA is that no toxicity or infectivity was noted for oral, dermal or inhalation exposure even to exceedingly high dose levels, while for extreme dose levels by invasive routes (intranasal, intracerebral or intraperitoneal) mortality occurred in laboratory animals, while lower doses by these routes caused no adverse effects. Since no adverse effects have been reported for operators and workers, EFSA considers that reference values are not necessary.

Response was obtained in a skin sensitisation study with the formulated strain GC-91. Induction of specific IgE antibodies against *Bacillus thuringiensis* has been found, but no adverse health effects were observed. Since microbes in general are regarded as potential sensitizers, the warning phrase “Microorganisms may have the potential to provoke sensitising reactions” should be used. EFSA has also identified a gap with regard to genotoxicity testing of the relevant toxins, also since it cannot be ruled completely out that production of additional toxins may take place after application. With the exception of case reports on ocular and dermal irritation, no adverse effects have been reported after occupational exposure to *Bacillus thuringiensis* products (Siegel, 2001).

Spores of *Bacillus thuringiensis* ssp. *kurstaki* and *israelensis* have been shown to germinate in the intestine of rats. Heat treatment of the spores, such as cooking, does not affect the ability to germinate. No *in vivo* production of enterotoxins has been detected by application of rat intestinal samples to Vero cell assays, possibly because the numbers of vegetative *Bacillus thuringiensis* cells present in the intestinal samples were too low to produce the amount of enterotoxins exceeding the detection limit of the cell assay. *In vitro* studies have shown that at least 10^6 vegetative cells/ml are needed to produce a response in the cell

assays. Rodents may however not be the best model to reveal human relevant pathogenic traits in bacteria.

Contradictory reports about *in vivo* germination of *Bacillus* spores exist in the literature. The members of the *Bacillus cereus* group are known to produce various virulence factors, such as haemolysins, phospholipases and cytolysin, which could be involved in the destruction of host tissue components and thereby promote invasion of the bacteria. *Bacillus thuringiensis* have been isolated from organs including spleen.

During the production process *Bacillus thuringiensis* strain GC-91 is harvested at the end of the exponential growth phase, and spores are spray dried to a technical powder by removing the culture filtrate subsequent to the fermentation process. The toxin is heat sensitive and shown to be inactivated by boiling. Thus, *Bacillus cereus*-like toxins or other metabolites, released into the fermentation broth, are not likely to occur in the product. Production batches have been examined for microbial and non-microbial impurities, and reportedly, neither microbial impurities nor toxic metabolites have been detected.

It has however been shown that *Bacillus* strains other than *Bacillus cereus* can produce enterotoxins. *Bacillus* diarrheal enterotoxin in 18 hour cultures of different *Bacillus* species has been determined using a Tecra ELISA immunoassay (Damgaard, 1995). The results are shown in Table 1.3.1.1.

Table 1.3.1.1 Titre of *Bacillus* diarrheal enterotoxin of *Bacillus* cultures, determined by Tecra VIA immunoassay kit. Data from (Damgaard, 1995).

Strain / Product	Titre
Bacillus cereus	
F4433/73	1629 (1350-2051)
***Bacillus* thuringiensis**	
HD-I	182 (120-367)
NRRL B-4066	86 (60- 148)
Bactimos	242 (194-321)
DiPel	14 (13-15)
Florbac FC	15 (14-17)
Foray 48B	56 (46-71)
Novodor FC	80 (57- 136)
Turex	21 (18-27)
VecTobac	120 (100-151)
XenTari	23 (18-33)

The table shows the reciprocal value of the highest dilution showing positive response.
95% confidence intervals are shown in parentheses.

The *Bacillus cereus* strain F4433/73 was isolated from what is described as a typical diarrheal, food poisoning outbreak, and has been shown to cause diarrhoea in a monkey feeding assay.

Commercial *Bacillus thuringiensis*-based insecticides were in this study found to produce varying amounts of diarrheal enterotoxin. The highest enterotoxin level was observed in the plant protection product Bactimos, which contains the AM65-52 strain of *Bacillus thuringiensis* ssp. *israelensis*, and the HD-I strain of *Bacillus thuringiensis* ssp. *kurstaki*. The lowest level of enterotoxin was found in DiPel, containing as the active ingredient the ABTS-351 strain of *Bacillus thuringiensis* ssp. *kurstaki*. The titre of toxin was determined in 18 hour cultures, and the level of enterotoxin in the cultures of different commercial *Bacillus thuringiensis* cultures seems to vary between approximately 1/5 and 1/100 of that observed in the *Bacillus cereus* sample. Based on these data it seem reasonable to conclude that *Bacillus thuringiensis* strains, also commercial ones, are capable of producing significant amounts of diarrhoeal enterotoxins. It is also stated in the DAR that strains of *Bacillus thuringiensis* are capable of producing diarrhoeal enterotoxins, at a level one order of magnitude lower compared to what is found in *Bacillus cereus*.

1.3.2 EFSA

In the area of **mammalian toxicology**, the risk assessment cannot be finalised for operators and bystanders and re-entry due to the data the gaps identified in relation to the formation of toxins during manufacture and after application respectively.

1.3.3 EU Commission / SANCO

It has been established that for the active substance notified by the main data submitter none of the manufacturing impurities considered are, on the basis of information currently available, of toxicological or environmental concern. However, strict maintenance of environmental conditions and quality control analysis during the manufacturing process shall be assured by the producer, in order to ensure the fulfilment of the limits on microbiological contamination as referred to in the Working Document SANCO/12116/2012.

1.3.4 Northern Zone Registration Report

1.3.4.1 Operator Exposure

Bacillus thuringiensis acts in a highly specific mode and is not pathogenic to mammals. This has been shown in many tests on toxicity, pathogenicity and infectiveness to vertebrates, all without adverse effects.

No harmful effects have been observed on personnel in research or industrial mass production, over a production period of more than 20 years.

Since no adverse effects were found in any study on toxicity, pathogenicity or infectiveness, calculations on the health risk for operators are considered unnecessary: no target organ exists and no dose-effect response (LOAEL) can be determined.

1.3.4.2 Bystander Exposure

Direct exposure of bystanders or residents, or successive exposure of a resident later the same day or on other days, will only be a fraction of operator exposure during spraying and thus negligible.

Hence, exposure of bystanders or residents is not expected to pose any unacceptable risk.

1.3.4.3 Worker Exposure

Worker exposure is considered to be negligible because dermal exposure is not relevant for *Bacillus thuringiensis* and inhalation exposure is not relevant for cultivation work.

Hence, exposure of workers is not expected to pose any unacceptable risk.

1.3.4.4 Summary and evaluation of health effects

All submitted toxicological studies and supplemental information on *Bacillus thuringiensis* including Agree WP prove that these are non-toxic and non-infectious to mammals and impose no health risk for operators, bystanders or workers. – The preparation is not irritating to the skin and only transiently and mildly irritating to the eye. Classification and labelling regarding skin sensitisation is required. Considering a very conservative approach, the application of Agree 50 WP according to the GAP is considered safe for the operator based on exposure estimates of the German BBA model.

It can be concluded that there are acceptable exposure scenarios for consumers since no AOEL or ADI were set based on the lack of pathogenicity and infectivity in the available data. These conclusions are based on the fact that the active components (spores and crystal proteins (δ-endotoxins)) of *Bacillus thuringiensis* ssp. *azawai* strain GC-91 are not toxic or pathogenic to humans.

In principle the regulation for classification is applicable to chemicals and not to microbials. Therefore classification and labelling regarding sensitisation for Agree 50 WP is not required. *Bacillus thuringiensis* ssp. *azawai* strain GC-91, is to be considered, as any microorganism, a potential sensitizer. Conditions of use shall include risk mitigation measures, like the use of adequate personal protective equipment, where appropriate.

1.3.5 VKM

Bacillus thuringiensis is often considered to be an environmentally friendly and harmless pesticide ingredient with no harmful effects on humans, and has been in use for more than 50 years with few reported incidences of related *Bacillus thuringiensis* infections (Green et al., 1990; Jackson et al., 1995; Noble et al., 1992).

It is however possible that *Bacillus thuringiensis* may have been involved in more cases of human diseases than reported because the distinction between *Bacillus thuringiensis* and *Bacillus cereus* involves specialised techniques not normally used when identifying cases of food poisoning by *Bacillus cereus*-like organisms. The Nordic Committee on Food Analysis states in the guidelines for examination of foods for *Bacillus cereus*: "Since these species (*Bacillus thuringiensis* and *Bacillus cereus*) are very closely related and both may produce enterotoxins, differentiation is not necessary in foods" (Nordic Committee, 1993).

Different strains of *Bacillus thuringiensis* produce enterotoxins, known to cause human diarrhoea, although at lower levels than *Bacillus cereus*. Since the spores are viable and have been shown to survive the acidic conditions in the stomach, they pose the risk of causing gastroenteritis when the right conditions occur. The question has been raised if only diarrhoeal enterotoxin-negative strains should be allowed used as insecticides. Such an approach has been the case for beta-exotoxin producing bacteria, where beta-exotoxin-positive strains have been banned in USA since 1971 due to their potential toxicity in humans (Damgaard, 1995).

The strain GC-91 has been reported to be low in enterotoxin production, and shown to have low infectivity and pathogenicity. It should however be recognized that many of these studies have been carried out in rats, which with regard to infectious sensitivity may differ from that of humans.

The current guidelines for testing of active substances and products of microbiological pesticides for EU registration do not contain experiments that would reveal the amount of diarrhoeal enterotoxin produced (EU, 1994).

VKM has noted the many uncertainties pointed to by EFSA, while at the same time it is argued that different strains of *Bacillus thuringiensis* have been used as insecticides for many years with no clear evidence of any serious health related consequence.

It may be questioned if the tests that the products undergoes before registration are designed to reveal necessary knowledge for a proper evaluation of possible health related effects (EU, 1994). It is the opinion of VKM that there are more quantitative than qualitative differences between *Bacillus cereus* and *Bacillus thuringiensis* with regard to aspects of importance for possible influence on human health. The picture that has been painted of *Bacillus cereus* as pathogenic in strong contradiction to the unproblematic image of *Bacillus thuringiensis* seems not to be supported by available data. It would be advantageous if the data from rodent toxicological testing could be supplemented with comparative toxicological characterization of the different strains of *Bacillus thuringiensis* used as insecticides and *Bacillus cereus*-like strains known to be pathogenic to humans. Preferably also non-rodent species should be used in these studies and the oral route of administration should be used. Methods are now available to provide quantitative data on both the efficiency as insecticide, content of toxic substances such as enterotoxins, strain identity and purity.

Bacillus thuringiensis ssp. *azawai* strain GC-91 should as any microorganism be considered as a potential sensitizer. Thus, appropriate protective equipment should be used.

1.4 Health risk – residues in crops

1.4.1 Introduction

Health and residues

Turex 50 WG is intended to be used against insects in vegetables in greenhouse and field, ornamentals in greenhouse, fruit trees in field and plastic tunnels, berries (incl. strawberry), plant nurseries, urban landscape and forestry. NIBIO has evaluated the efficacy of the product and suggested that the intended use of the product should be specified on the label.

Methods for identification of *Bacillus cereus*-like bacteria in food and clinical settings do not distinguish between *Bacillus cereus* and *Bacillus thuringiensis*. Thus, the presence of *Bacillus thuringiensis* in food and the role of this organism in food poisoning may not be fully understood.

Fresh fruits and vegetables are normally not associated with *Bacillus cereus*-related diarrhoea. However, when used as ingredients, these products may contaminate complex food dishes, such as starchy dishes, in which there are good conditions for growth, especially if the final dishes are improperly cooled after heat treatment (Frederiksen et al., 2006; Rosenquist et al., 2005).

Among 48,901 Danish samples of ready-to-eat food products, 0.5% had counts of *Bacillus cereus*-like bacteria above 10^4 CFU/g (Rosenquist et al., 2005). 31 out of 40 randomly selected *Bacillus cereus*-like strains was classified as *Bacillus thuringiensis* due to crystal production and/or content of cry genes, and could be contaminants or residues of *Bacillus thuringiensis* insecticides. The genetic relationship with commercial *Bacillus thuringiensis* strains was not investigated in this study, but the finding of high counts (>10^4 CFU/g) of *Bacillus cereus*-like organisms in fresh cucumbers and tomatoes suggested to the authors that this could result from spraying with *Bacillus thuringiensis*-based insecticides.

In a later Danish study, 128 *Bacillus cereus*-like strains were isolated from fresh fruits and vegetables sold in Denmark. 39% (50/128) were classified as *Bacillus thuringiensis* on the basis of their content of Cry genes determined by PCR or crystal proteins visualized by microscopy (Frederiksen et al., 2006). 23 of the 50 *Bacillus thuringiensis* strains were of the same subtype as *Bacillus thuringiensis* strains used as commercial bio-insecticides, 14 *Bacillus thuringiensis* ssp. *kurstaki* HD1 and 9 *Bacillus thuringiensis* ssp. *aizawai*. The frequency of enterotoxin genes was higher among the commercial strains than among the other *Bacillus thuringiensis* and *Bacillus cereus*-like strains (Frederiksen et al., 2006).

Isolates from the majority of the Danish tomatoes and cucumbers contained bacterial strains that were indistinguishable from the strain used in the plant protection product DiPel, allowed for use in Denmark. At the time of this study the product Turex was not allowed in Denmark, but it was in The Netherlands, and the majority of the isolates from products produced in The Netherlands were indistinguishable from the *Bacillus thuringiensis* strain in Turex (Frederiksen et al., 2006). Although one cannot completely exclude that the findings are due to natural isolates indistinguishable from commercial strains, the data suggest, perhaps not very surprising, that the findings are actual residues of commercial plant protection products.

The presence of enterotoxin-encoding genes in commercial *Bacillus thuringiensis* strains has also been found in other studies (Gaviria Rivera et al., 2000; Hansen and Hendriksen, 2001; Perani et al., 1998). Several studies have shown that enterotoxin genes in commercial strains are not only present but also expressed *in vitro* (Damgaard, 1995; Jensen et al., 2002; Rosenquist et al., 2005; Tayabali and Seligy, 2000). Since all commercial strains harbour genes for all of the three known enterotoxins, HBL, NHE, and CytK, there seems to be a risk that high levels of these organisms may cause human disease.

In another Danish study, Cry protein inclusion bodies were found in six of the seven strains isolated from food (Damgaard et al., 1996). The serotype of six of the isolated strains was found to be *kurstaki*, which is the most widely used serotype for insect pest control in Denmark. Only one of the seven strains isolated from food in this study did not produce enterotoxin (Damgaard et al., 1996).

Diarrhoeal type of food poisoning has been considered caused by enterotoxins formed by vegetative *Bacillus cereus* in the small intestine (Granum and Lund, 1997). The fact that *Bacillus cereus* spores can survive the conditions of the gastrointestinal tract and adhere to the gut epithelium may be another contributing factor (Andersson et al., 1998; Drobniewski, 1993). *Bacillus thuringiensis* strains have been claimed to be responsible for human infections similar to those caused by strains of *Bacillus cereus* (Damgaard et al., 1997a; Damgaard et al., 1997b; Jackson et al., 1995), while in the DAR it is stated that the medical literature does not show case reports where commercially used *Bacillus thuringiensis* is directly associated with food poisoning (Siegel, 2001).

Bacillus thuringiensis is however capable of producing diarrhoeal enterotoxins at a level about one order of magnitude lower as compared to *Bacillus cereus*. The significance of this difference in enterotoxin produced as a cause of human disease is at the moment not fully understood.

It is stated in the DAR (DAR Italy, 2007) that the *Bacillus thuringiensis* spores or crystal proteins are not toxic to man or domestic animals, and that the persistence of *Bacillus thuringiensis* products above ground on leaves and fruits is low. Half-life of viable spores is about 1 day in sunlight. Applied as a spray, the δ-endotoxins are rapidly degraded, and endospores are rapidly inactivated by UV radiation. Thus, the DAR of 2007 concludes that because of this residue data are not required. “The derivation of reference values was not

considered necessary as *Bacillus thuringiensis aizawai* strains ABTS 1857 and GC-91 was not shown to be pathogenic or infective based on the available data."

Since *Bacillus thuringiensis* has been considered not to be a human pathogen, no restraining time has been imposed upon pre-harvest or post-harvest of agricultural commodities. Cases of *Bacillus cereus* diarrhoeal outbreaks from ingestion of raw contaminated vegetable sprouts (Rosenquist et al., 2005) and from improperly stored cooked green beans (Schnepf et al., 1998) have been described. Levels as low as 10^3-10^4 cells/g products have been considered as relevant contamination by the food industry (Granum, 1994; Rosenquist et al., 2005). Food borne poisoning caused by other *Bacillus* spp. has been linked to more than 10^6 CFU per g. Levels of 10^6–10^7 *Bacillus thuringiensis* spores /g of leaf material are not considered unusual when applied to vegetables for insect control under field conditions (Pedersen et al., 1995).

On the other hand, in the DAR it is stated that *Bacillus cereus* is frequently isolated as contaminant in various foods (Drobniewski, 1993; Erlendur Helgason, 1998), and that the consumption of foods that contain more than 10^5 CFU *Bacillus cereus* per gram may result in food poisoning (Anonymous, 2005; Kramer and Gilbert, 1989).

1.4.2 EFSA

EFSA and others have pointed out the risk of microbial contamination of the product, and that *Bacillus thuringiensis* may produce enterotoxins.

"For **residues** the risk assessment cannot be finalised as the possible formation of enterotoxins cannot be excluded."

1.4.3 EU Commission / SANCO

See previous section

1.4.4 Northern Zone Registration Report

The Danish report concluded that: "there are acceptable exposure scenarios for consumers since no AOEL or ADI were set based on the lack of pathogenicity and infectivity in the available data. These conclusions are based on the fact that the active components (spores and crystal proteins (δ-endotoxins)) of *Bacillus thuringiensis* ssp. *aizawai* strain GC-91, are not toxic or pathogenic to humans."

1.4.5 VKM

Dispute exists with regard to the need for reference values for maximally allowed levels of *Bacillus thuringiensis* residues in crops. On one hand it is argued that since CG-91 were not considered to be pathogenic or infective based on available data, and lack of demonstrated

association between human illness and the long term use of *Bacillus thuringiensis* as insecticide, reference values are not necessary.

On the other hand, cases of food poisoning possibly caused by *Bacillus thuringiensis* have been reported (Green et al., 1990; Jackson et al., 1995; Noble et al., 1992), and some EU member states are of the opinion that certain episodes of human illness may be caused by *Bacillus thuringiensis* contaminated crops. Such an episode has been documented in Germany, and has resulted in a German demand for introducing analysis methodology and reference values for *Bacillus thuringiensis* residues in crops (See Appendix from Mattilsynet: "DE comments on Bt").

It could be argued that authorities responsible for food safety should consider the amount of *Bacillus thuringiensis* insecticide residues left on products after harvest. EFSA has recommended that food processors should ensure that levels of *Bacillus cereus* bacteria between 10^3 and 10^5/g are not reached at the day of consumption (Anonymous, 2005). EFSA also stated in a 2005 opinion on *Bacillus cereus* that food poisoning incidents in some cases could be caused by levels as low as 10^3 CFU/g *Bacillus cereus*. It has been argued that this statement should also in principle apply to residues of commercial *Bacillus thuringiensis* strains, since commercial *Bacillus thuringiensis* stains have been demonstrated to produce enterotoxins, at a level that by EFSA has been described as one order of magnitude lower than that of *Bacillus cereus*. The frequency of enterotoxin genes was also higher among the commercial strains than among the other *Bacillus thuringiensis* and *Bacillus cereus*-like strains (Frederiksen et al., 2006). It has been considered if this could be linked to the fact that the commercial strains have been selected based on effectivity as insecticide.

Bacillus thuringiensis has been discussed in the Standing Committee on the Food Chain and Animal Health (SCFCAH) regarding if and how to consider pesticide residues from this agent. There is at present disagreement among member states about the inclusion of the microorganism on the Annex IV of the Regulation (EC) 396/2005 which contains a list of active plant protection substances for which maximum residue levels (MRLs) are considered not to be required.

EFSA concludes that the active components of commercial *aizawai* strains GC-91 preparations are not toxic or pathogenic to humans, and that the only remaining issue concerning consumer exposure is that *Bacillus thuringiensis* species carry the genetic material that encodes for the *Bacillus cereus* enterotoxin, and that it is not known if this can be expressed, and if so under what conditions. The scenario in this case is if the spores in the product experience conditions that allow germination followed by production of enterotoxin by the vegetative bacterial cells. It seems reasonable that this may occur both in stored food items, as well as in the intestines of people following ingestion of spore-infected produce. The question of whether this may result in human illness would then depend on number of vegetative cells, again depending on conditions and number of spores.

It is the opinion of VKM that based on available data it cannot be ruled out that intake of *Bacillus thuringiensis* spores as residues in food items sprayed with plant protection products may under certain conditions cause intestinal human illness caused by the production of enterotoxins by vegetative *Bacillus thuringiensis* cells. It has been shown that the spores will survive the environment in the stomach, and are able to germinate in the intestine and produce enterotoxins. However, if each batch of product is properly characterised and controlled with respect to enterotoxin production ability and species characterization, this could reduce the concern about food poisoning.

1.5 Health risk – drinking water

1.5.1 EFSA

No information has been provided in relation to **potential interferences of *Bacillus thuringiensis* with the analytical systems for control of the quality of drinking water** provided for in Directive 98/83/EC.

1.5.2 EU Commission / SANCO

Due to the lack of close relationship with the microorganisms listed under Directive 98/83/EC, the risk of interference is considered negligible.

1.5.3 Northern Zone Registration Report

Under natural conditions, residues of *Bacillus thuringiensis* ssp*. aizawai* in water are not considered to be able to persist for very long periods due to a combination of natural physical and chemical degradation factors such as solar radiation and predation from resident bacteriophages, protozoans and other lower animal forms. It may be stated that *Bacillus thuringiensis* ssp*. aizawai* GC-91 is inactivated under natural conditions, including water.

1.5.4 VKM

VKM shares the opinion of the Northern Zone Registration Report that the prescribed use of *Bacillus thuringiensis* as an insecticide is unlikely to pose a threat to human health via drinking water, but care should be taken not to use drinking water sources/private wells in agricultural areas close to where the product is used.

1.6 Transfer of genetic material

1.6.1 Introduction

Transfer of plasmids between *Bacillus thuringiensis* bacteria
Bacillus thuringiensis ssp. *aizawai* strain CG-91 is a trans-conjugant of *Bacillus thuringiensis* ssp. *aizawai* strain HD 135-S4 (recipient strain), with a *Bacillus thuringiensis* ssp. *kurstaki* strain HD 191-A2 (donor strain). The new trans-conjugant strain GC-91 is a product of a natural crossing (conjugation) between the two strains. The genes coding for the δ-endotoxins (cry proteins) are located on plasmids that may be transferred by conjugation. This requires that vegetative cells are present under favourable conditions.

It has been reported a study where Bacillus thuringiensis ssp. *israelensis* were used both as donor and receptor cells, and GFP tagged plasmids were analysed by flow cytometry. Full life cycle of *Bacillus thuringiensis* were demonstrated in the rat gut, and plasmid transfer demonstrated in gnotobiotic rats, as a worst-case model.

1.6.2 EFSA

No information has been provided on **the potential transfer of genetic material** from *Bacillus thuringiensis aizawai* strain GC-91 to other organisms. The original scientific papers quoted in the fate section of the dossier have not been provided, and therefore a data gap has been identified.

1.6.3 EU Commission / SANCO

Potential transfer of genetic material to other organisms is not considered as a process which is likely to increase under the proposed conditions of use.

1.6.4 Northern Zone Registration Report

This issue is not addressed in the NZRR.

1.6.5 VKM

In the soil environment, the conditions for vegetative growth are not favourable and *Bacillus thuringiensis* exists mainly as spores. The presence of a natural flora of microorganisms in the soil further reduces the potential for transfer of plasmids (Vilas-Boas et al., 2000). Thus, the potential for transfer of genetic material in the environment is considered low. Furthermore, the cry-genes found in the GC-91 strain are already present in the gene pool of the *B. thuringiensis* populations in the environment.

The proposed use of *Bacillus thuringiensis* ssp*. aizawai* strain GC91 as a microbial crop protection agent is not likely to cause any adverse effects linked to transfer of genetic

material. It is however the opinion of VKM that the fact that such gene transfer may take place highlights the importance of strict procedures for analysis of the purity, and genotypic and phenotypic properties of the individual batches of product prior to marketing.

1.7 Groundwater and soil contamination

1.7.1 Introduction

Turex 50 WP is applied by spraying on the foliage of the crops to be protected from attack from *Leptoderian* larvae. Some of the spores and crystalline proteins will be deposited on the soil during application and later on they can be washed off from the foliage and end up in the soil beneath. Additional vegetative cells and spores of *Bacillus thuringiensis* ssp*. aizawai* may be released from insect larvae invaded and killed by the bacteria. Assuming that all the applied bacteria and crystalline proteins end up in the soil, and are mixed into the upper 5 cm of the soil profile, the concentration of *Bacillus thuringiensis* ssp*. aizawai* in the soil following 3 applications of 2 kg/ha of Turex 50WP is calculated at 2.4×10^8 CFU/kg dry weight soil. (See background provided by Mattilsynet)

Spores of *Bacillus thuringiensis* rapidly inactivated and δ-endotoxins are rapidly degraded when exposed to UV radiation. Spores and crystals are almost completely inactivated after 12 h UV and all activity lost after 24 h in sunlight. Therefore *Bacillus thuringiensis* is more stable in the soil than on plant foliage.

EFSA has noted that no studies of the fate of the specific strain *Bacillus thuringiensis* ssp*. aizawai* GC-91 have been reported. However, in field studies with other strains of *Bacillus thuringiensis* (ssp. *kurstaki* and *aizawai*) reviewed by NZRMS no multiplication in the soil was demonstrated. Most studies show a rapid initial decline in numbers of spores, but persistence of numbers between log 2 and log 3 CFU/g have been observed for up to seven years after the application. This is within the normal range of naturally occurring *B. thuringiensis* in soil, and significantly lower than the density of *B. cereus* group bacteria (log 5,2/g) reported from a Danish soil.

The mobility of spores of *Bacillus thuringiensis* in soil is low. Field studies reviewed by the NZRMS showed that spores remain in the top layer of the soil for several years and no allocation was found below 10 cm.

Also the horizontal dispersion of *Bacillus thuringiensis* spores in soil appears to be low. DeLucca et al. (DeLucca et al., 1981) showed that *Bacillus thuringiensis* does not move in the soil, as two serotypes sprayed in close proximity did not become cross-contaminated. A field study in a wetland area in Switzerland where *Bacillus thuringiensis* ssp. *israeliensis* had been applied annually for 22 years showed that the spores were not displaced to other locations outside the treated area (Guidi et al., 2011).

The toxic potency of the cry-proteins has been found to decreases rapidly in soil. This is caused by microbial degradation and adsorption and binding to clay and clay-humic complexes in the soil (NZRMS, 2014).

1.7.2 EFSA

A data gap was identified for a **groundwater exposure assessment of the crystalline proteins** and transformation products that retain any insecticidal activity, and therefore the assessment of potential groundwater contamination cannot be finalised.

1.7.3 EU Commission / SANCO

Potential contamination by δ-endotoxins is considered unlikely since the crystalline proteins are rapidly degraded by the actions of indigenous microorganisms and photo-degradation. Regarding the microorganism itself, *Bacillus thuringiensis* ssp. *aizawai* occurs naturally ubiquitously in soil.

1.7.4 Northern Zone Registration Report

Groundwater

The scientific literature provides evidence that it is unlikely that neither the spores nor the protoxins/toxins will be translocated to groundwater. No threat of contamination of groundwater exists following applications of Agree 50 WP according to GAP.

Soil

Bacillus thuringiensis ssp. *aizawai* occurs naturally ubiquitously in soil. The application of *Bacillus thuringiensis* ssp. *aizawai* GC-91 to soil is not expected to increase the density of neither *Bacillus thuringiensis* nor *Bacillus cereus* group bacteria in Nordic soils significantly.

Crystallized protoxins disappear from soil, and only small amounts will probably remain in the soil from one growing season to the next. It is most likely that this disappearance is affected by degradation by microbial proteolytic enzymes. It is therefore unlikely that the cry-toxins will persist in soils in significant potent concentrations for times exceeding a year.

1.7.5 VKM

VKM supports the conclusions of NZRMS that:

- It is unlikely that neither the spores nor the protoxins/toxin will be translocated to groundwater

- Application of *B. thuringiensis* ssp. *aizawai* GC-91 to soil is not expected to increase the density of neither *B. thuringiensis* nor *B. cereus* group bacteria in Nordic soils significantly.
- It is unlikely that the cry-toxins will persist in soils in significant potent concentrations for times exceeding a year.

1.8 Ecotoxicology

1.8.1 Introduction

Bacillus thuringiensis constitutes a large family of subspecies found world-wide in different habitats. Several phylogenetically different strains of *B. thuringiensis* have also been isolated from environmental samples in Norway (Ticknor et al., 2001). In the environment, *Bacillus thuringiensis* exist mainly as spores, which can persist in soil for long periods. Germination of spores has been observed in nutrient amended, sterile soil, but seems to be rare under natural conditions. However, occasionally, *Bacillus thuringiensis* spores may be ingested by animals and germinate in the digestive tract as shown eg. in nematodes, earthworms, insects, birds , rats and cows, which act as paratenic hosts for the bacteria (Argolo-Filho and Loguercio, 2013). Such paratenic host routes may be important for the maintenance of *Bacillus thuringiensis*.

Effects on non-target organisms

Effects of application of *Bacillus thuringiensis* ssp*. aizawai* CP-91 as a microbial pest control agent on non-target terrestrial and aquatic organisms have been assessed in the NZRR. The risk assessment procedure is similar to the procedure used for assessment of chemical pesticides. The exposure of various non-target organisms has been calculated using standard exposure scenarios based on the recommended application rates of Agree 50 WP and compared to toxic concentrations found in tests with representative organisms. The toxicity/exposure ratios (TER) or Hazard quotients (HQ) have been compared to the accepted trigger values for estimation of risk of adverse effects. In general, the risk assessment has indicated that no unacceptable risk to non-target organisms is expected upon field application of A *Bacillus thuringiensis* ssp*. aizawai* CP-91 according to GAP. (See section 1.8.4).

1.8.2 EFSA

Data gaps were identified in the **ecotoxicology** section to address the risk to aquatic organisms, earthworms and other soil-dwelling non-target arthropods from exposure to GC-91 crystalline proteins (δ-endotoxins), to further address the risk to bees (strain GC-91)

1.8.3 EU Commission / SANCO

"However, *Bacillus thuringiensis* is a common component of the soil micro-biota and has been isolated from most terrestrial habitats. It is not considered an autochthonous inhabitant of aquatic environments and does not find optimal conditions for growth under these conditions. Because of these reasons and the high host specificity (susceptible species of the order Lepidoptera), the risk to soil and aquatic organisms is considered acceptable;

- The risk assessment performed for bees (*Apis mellifera*) could not be considered finalised. However, the fact of the high host specificity of the present *Bacillus thuringiensis* strain (which excludes *Hymenoptera* as *Apis spp*), the absence of a systemic mode of action at crop level, and the available data would indicate a low risk to bees."

1.8.4 Northern Zone Registration Report

Effects on Birds and mammals

"Due to the highly specific mode of action of *Bacillus thuringiensis* ssp*. aizawai* and its low field persistence birds and mammals are not considered to be at risk upon application of Agree 50 WP. This was confirmed by the absence of toxicity upon oral administration in birds and rats and TER values exceeding the Annex IV trigger of 10 for all indicator species considered in screening assessments for these two vertebrate groups, respectively."

"As the acute TER values indicate no risk to birds and mammals and no adverse effects were observed in short-term toxicity studies, no long-term effects are to be expected upon field application of Agree 50 WP according to GAP."

Effect on aquatic species

Aquatic organisms may be exposed to Agree 50 WP through spray drift from the application site into adjacent water bodies. The PEC calculation was performed on the basis of three applications in orchards, as here the highest exposure of aquatic non-target organisms is to be expected. Following the Guidance Document on Aquatic Ecotoxicology (SANCO/3268/2001), the maximum drift rate is 23.96% (considering three applications x 2 kg/ha) at a distance of 3 m to surface waters. As a worst case, no degradation between the applications is assumed. Drift was considered according to Rautmann et al (Rautmann et al., 2001).

The predicted environmental concentration with a 3 m buffer-zone of Agree 50 WP and its active ingredient *Bacillus thuringiensis* ssp*. aizawai* GC-91 in surface waters is 478.72 µg/L (239.36 µg *Bacillus thuringiensis* ssp*. aizawai*/L) corresponding to 1.44×10^7 CFU or 11,968 IU/L. Note that calculated PEC_{SW} is higher than shown in the background provided by the Norwegian Food Safety Authority (See table 6.1). The reason for the difference is that the worst case scenario for the Northern Zone (Orchards) includes a higher drift rate than in the

flowers and vegetables scenario as presented in the background provided by the Norwegian Food Safety Authority.

The TER-calculations for aquatic species using the worst case PEC_{SW} for the Northern Zone is shown in Table 1.8.4.1.

Table 1.8.4.1. TER values for Agree 50 WP/*Bacillus thuringiensis* ssp*. aizawai* with exposure via spray drift from three applications in orchards with a buffer-zone of 3 m.

Compound	Organism	Endpoint	Exposure (PEC_{SW})	TER (trigger)
CGA-237218[a]	Fish	LC_{50} > 2.0 × 10^{10} CFU/L	1.44 × 10^{7} CFU/L	> 1389 (100)
Agree 50 WG[b)]	Daphnids (acute)	LC_{50} > 100 mg/L	478.72 µg/L	> 208 (100)
CGA-237218[a]	Daphnids (chronic)	NOEC 1.57 × 10^{8} CFU/L	1.44 × 10^{7} CFU/L	10.9 (10)
CGA-237218[a)]	Algae	>3.6 × 10^{9} CFU/L	1.44 × 10^{7} CFU/L	> 938 (10)

[a] Synonym for *Bacillus thuringiensis* ssp*. aizawai* GC-91 technical material contained in Agree 50 WP

[b] Agree 50 WG contains the same active ingredients and similar co-formulants as Agree 50 WP

Fish

The acute TER value of fish for Agree 50 WP exceeds the Annex VI trigger value of 100 indicating that no adverse effects are to be expected upon field application at recommended use levels. Due to the absence of toxicity in the semi static studies conducted over a period of 30 and 32 days, respectively, no risk for fish is expected even upon prolonged exposure to Agree 50 WP. Furthermore, prolonged exposure is not likely to occur due to the restricted persistence of *Bacillus thuringiensis* ssp*. aizawai* in water. (N.B. the PECsw for the aquatic risk assessments has been calculated assuming a 3 m buffer zone).

Daphnids

The TERA values calculated with the nominal concentration as well as with the mean measured (actual) concentration are above the Annex VI trigger of 100, indicating a low acute risk to *D. magna* following GAP directed application of Agree 50 WP. Considering the absence of acute toxicity and the NOEC obtained in the 21-day static renewal tests (1.57 × 10^{8} CFU/L) that is more than 10-fold higher than the PECSW (1.44 × 10^{7} CFU/L) no adverse effects on daphnids are to be expected even upon prolonged exposure to Agree 50 WP. Prolonged exposure, however, is not likely to occur due to the restricted persistence of

Bacillus thuringiensis ssp*. aizawai* in water. (N.B. the PECsw for the aquatic risk assessments has been calculated assuming a 3 m buffer zone).

Algae

The long-term TER value of algae for Agree 50 WP strongly exceeds the Annex VI trigger value of 10 suggesting that no negative side effect is expected following field application according to GAP.

Bees

The assessment was based on the maximum application rate (2 kg Agree 50WP/ha) which corresponds to 1000 g *Bacillus thuringiensis* ssp*. aizawai* GC-91/ha). This application rate is higher than was used in background provided by the Norwegian Food Safety Authority (Table 6). The hazard quotients with the higher application rate are shown in Table 1.8.4.2.

Table 1.8.4.2. Risk to bees from exposure to Agree 50 WP

Compound referred to	Application rate	LD_{50}	Hazard quotient
Bta GC-91	1000 g Bta GC-91/ha	10 day oral: 91 µg Bta GC-91/bee	11
		48 h oral: > 98.5 µg Bta GC-91/bee	< 10.2

Application of Agree 50 WP at intended use levels represents no risk for honey bees as the calculated Hazardous Quotient is far below the trigger value of 50.

Effects on Arthropods other than bees

The assessment was based on application of 2 kg Agree 50WP/ha in orchards. This gives different environmental rates (PER) for in-field and off-field foliar exposure than shown in the background provided by the Norwegian Food Safety Authority (table 7.5). The hazard quotients are shown in Table 1.8.4.3.

Table 1.8.4.3. In-field and off-field HQs for non-target arthropods

Species	LR_{50} (g/ha)	In-field foliar		Off-field foliar			Trigger value
		PER (g/ha)	**HQ**	**PER (g/ha)**	**Correction factor**	**HQ**	
Typhlodromus pyri	>4500	4600	< 1.02	110.2	10	< 0.024	2
Aphidius rhopalosiphi	>4500		< 1.02			< 0.024	2

PER: predicted environmental rate depending on application rate and drift
HQ: Hazard Quotient
Correction factor: extrapolation from testing just 2 representative species

Following the result of the non-target arthropod risk assessment the HQ values are far below the trigger value of 2 indicating that no unacceptable risk is to be expected upon field application of Agree 50 WP according to GAP.

Effects on non-target lepidopteran species

The risk for non-target Lepidopteran species in off-crop habitats was assessed using data from open peer reviewed literature. The doses causing 50% mortality of five lepidopteran species were converted from IU/µL to kg Agree 50 WP/ha. The risk assessment was performed for GAP directed use in orchards using the maximum application rate (2.0 kg/ha). Due to 3 applications at intervals of 7 days, a MAF of 2.3 and a drift value of 23.96% were used. The resulting hazard quotients are shown in table xx.

Table 1.8.4.4. Exposure Hazard Quotients for lepidopteran species in off-crop habitats

Test species	LR50 (kg Agree 50 WP/ha)	Exposure scenario	Exposure rate (kg/ha)	HQ	Trigger value
Lymantria dispar	0.4			2.76	2
Vanessa cardui, Manduca sexta, Pieris rapae	1.0	Off-crop	1.1	1.10	2
Heliothis virescencs	4.0			0.28	2

a) in the off-crop scenario, spray drift of 23.96% at 3 m is considered, according to JKI (2006)

Following the results the HQ values for 4 out of 5 species (*Vanessa cardui*, *Manduca sexta*, *Pieris rapae* and *Heliothis virescencs*) are below the trigger of 2, although the worst case was assumed. Hence, no negative side effects are expected following field application according to GAP.

Assuming the same conditions the HQ value for *Lymantria dispar* slightly exceeds the trigger of two. However, due to the fast inactivation of *Bacillus thuringiensis* ssp*. aizawai* spores and the behaviour of *L. dispar* to feed in crowns of broadleaf trees, where a lower exposure rate is expected, no unacceptable risk is expected upon field application of Agree 50 WP. Furthermore, *L. dispar* is one of the pest insects to be controlled by Agree 50 WG in forestry.

The intended uses of the product are for the control of lepidopteran larvae on a number of different crops, ornamentals and in forestry in northern Europe. The pest intended to be controlled include a number of significant lepidopteran pests which are commonly occurring in northern Europe, the only exception for this is *Malacosoma neustria* (to be controlled in orchards and forestry), which is a rare species, at least in Denmark, mostly occurring as male immigrants.

Effects on Earthworms

The acute TER value of earthworms for Agree 50 WP exceeds the Annex VI trigger value of 10 indicating that no adverse effects are to be expected upon field application at recommended use levels.

Based on the mode of action, knowledge from other *Bacillus* thuringiensis-strains and studies on the toxins for earthworm no unacceptable risk is expected. In addition in the Draft report of the OECD/Kemi/EC workshop on Microbial Pesticides: Assessment and Management of Risk, 2013 it is recommended: "Earthworm study is not required unless the microbial is not naturally occurring in the soil".

1.8.5 VKM

The highly specific mode of action of the toxins produced by *Bacillus thuringiensis* ssp. *aizawai* suggests that non-target organisms other than lepidopteran species are not likely to be affected. This is supported by the low toxicity of Agree 50 WP found in tests with various terrestrial and aquatic organisms. Based on the TER calculations reported in the NZRR, it can be concluded that the intended use of TUREX 50 WP is not expected to cause adverse effects on birds, mammals, aquatic organisms (providing a buffer-zone of 3 m), bees, arthropods other than bees, and earthworms. Non-target lepidopteran species which are susceptible to *Bacillus thuringiensis* ssp*. aizawai* are not likely to be significantly affected in off-crop areas.

Since *Bacillus thuringiensis* ssp*. aizawai* is a microbial agent with potential for survival, multiplication and dispersion in the environment also long-term ecological effects of release as an agricultural pest control should be considered. *Bacillus thuringiensis* is known to have a worldwide distribution. Several phylogenetically different strains of *B. thuringiensis* have been isolated from environmental samples in Norway (Ticknor et al., 2001), Sweden (Landén et al.) and Denmark (Damgaard et al., 1997b; Hansen et al., 1998). Application of *Bacillus thuringiensis* ssp*. aizawai* as a plant protection agent will cause a local and temporal increase of the population of *Bacillus thuringiensis*, mainly in the soil where spores will be deposited. Due to the limited mobility of spores, they are likely to remain in the upper soil layers (See section 1.8.1).

Due to the specific mode of action of *Bacillus thuringiensis* ssp*. aizawai*, long-term effects could potentially affect populations of *Lepidopteras*, i.e. moths and butterflies whose larvae are susceptible to the cry proteins and may be invaded by the bacteria. In order for spores of *Bacillus thuringiensis* ssp*. aizawai* remaining in the soil from treatments with Turex WP 50 in previous seasons to reach sites where they can be ingested by lepidopteran larvae, the spores must be transferred from the soil to the leaves on which the larvae feed. Possible routes for such transfer is by rain splash or by endophytic colonization of plants from the rhizosphere (Argolo-Filho and Loguercio, 2013). The density of spores on plant foliage that may result from rain splash is likely to be low and rapidly declining due to UV radiation. The

rizosphere transfer route requires that *Bacillus thuringiensis* ssp*. aizawai* spores germinate and form vegetative cells in the soil. However, spore germination requires environmental conditions that rarely occur in natural soil, and the presence of vegetative cells in the rhizophere is likely to be sporadic. The exposure of lepidopteran larvae to *Bacillus thuringiensis* ssp*. aizawai* transferred from soil to plant foliage is therefore not likely to be high enough to affect the populations of lepidopteran species.

VKM concludes that the use of Turex 50 WP according to GAP will not pose an unacceptable risk to the environment.

1.9 Antimicrobial resistance

1.9.1 Introduction

Of 40 *Bacillus cereus*-like organisms (31 of which is *Bacillus thuringiensis*) 36 show penicillin resistance, caused by the intrinsic production of beta-lactamase by *Bacillus cereus*-like organisms. All strains were however sensitive to the other seven antimicrobials tested (Chloramphenicol, Ciprofloxacin, Gentamicin, Streptomycin, Tetracycline, Erythromycin, Vancomycin). Two strains classified as *Bacillus cereus* and two strains classified as *Bacillus thuringiensis* were sensitive to penicillin as well (Rosenquist et al., 2005).

1.9.2 EFSA

Bacillus thuringiensis ssp*. aizawai* strain GC-91 shows high sensitivity to Chloramphenicol, Erythromycin, Streptomycin and Tetracycline and resistance to Penicillin.

1.9.3 EU Commission / SANCO

Bacillus thuringiensis ssp. *aizawai*, Strain GC-91-has been tested for sensitivity to a range of antibiotics.

1.9.4 VKM

Antimicrobial resistance and *Bacillus* thuringiensis ssp. *aizawai*, strain GC-91.

Several studies have shown that *Bacillus thuringiensis* as a species may be resistant against some classes of antimicrobial agents, including clinical relevant antimicrobial agents used in veterinary and human medicines. According to the study performed by Luna and co-workers (Luna et al., 2007), *Bacillus thuringiensis* may be resistant against β-lactam antibiotics like amoxicillin, ampicillin, ceftriaxone, penicillin and oxacillin. This study was also confirmed by Bautista (Bautista et al., 2013), who showed that the isolated strains of *Bacillus thuringiensis* were resistant to β-lactams (amoxicillin and ampicillin). Another study showed that *Bacillus*

thuringiensis isolated from dump soil samples are resistant against amoxicillin and neomycin (Sarker et al., 2010).

Resistance against antimicrobial agents in bacteria may be intrinsic (naturally) or acquired (mutation or acquisition of a resistance gene). Acquired resistance may be transferred to other bacteria, intra- and interspecies and both to a-pathogenic and pathogenic bacteria, including to the bacteria in the environment.

According to the Commission staff working document (European Commission, 2013), *Bacillus thuringiensis* ssp. *aizawai*, strain GC-91 has been tested for sensitivity for a range of antibiotics. However, the information regarding susceptibility of this strain has not been provided.

In order to elucidate the issues related to possible transfer of antibiotic resistance the following information is needed:

- Antimicrobial susceptibility testing (MIC-values) of *Bacillus thuringiensis* ssp. *aizawai*, strain GC-91 against different antimicrobial agents.
- Clarification of the intrinsic and acquired resistance properties.

In the case of acquired resistance:

- The linkage between the resistance gene(s) with transposable elements
- The localization of the resistance gene(s) on chromosome or plasmid.

2 Uncertainty

The uncertainties discussed in this assessment can be summarized as follows:

- The *Bacillus* genus contains a large number of bacterial strains, some of which have acquired genes for pathogenic toxins. Although available techniques to characterize and discriminate between the different strains exist today, data from the use of such methods are almost non-existing.
- Published data suggest that *Bacillus thuringiensis* strains may produce pathogenic enterotoxins, but data is not available to assess the possible role of this.
- Data on the toxicological impact are mainly based on rat studies, and it is uncertain to what extent these data are representative for humans. Data from other species and experimental systems are needed for a more accurate assessment.
- The assessment of environmental fate and behaviour of the specific strain of *Bacillus thuringiensis* used in Turex 50 WP is based on studies and data on various or unspecified strains of *Bacillus thuringiensis*. The uncertainty introduced by this is, however, considered to be insignificant since the strain *Bta* GC-91 diverges from other strains mainly with respect to the genes coding for crystalloproteins, which are not likely to influence the persistence and germination of spores in soil or other environmental compartments.

3 Conclusions

Identity and analysis of the active ingredient

- Because of the close relationship with toxin-producing bacterial strains, and the possibility for gene transfer between bacterial strains, each manufactured product batch should be analysed and documented for relevant parameters including number of spores determined as Colony Forming Units per gram (CFU/g); activity (IU/mg) and content (g/kg) of δ-endotoxin; level of enterotoxin produced by the vegetative cells.

Health risk – mammalian toxicology

- It is the opinion of VKM that there are more quantitative than qualitative differences between different strains of *Bacillus cereus* and *Bacillus thuringiensis* with regard to some of the aspects of importance for possible effect on human health, especially the formation of enterotoxins. The general consideration of *Bacillus cereus* as being pathogenic, and *Bacillus thuringiensis* being unproblematic, seems not to be supported by available data. Also non-rodent species should be considered as test organisms, and existing data on the *Bacillus* strain used should be supplemented with toxicological characterization with now available methods to form a better basis for assessing possible risk to human health from its use as insecticide.

Health risk – residues in crops

- It is the opinion of VKM that it cannot be ruled out that intake of *Bacillus thuringiensis* spores as residues in food items sprayed with plant protection products, or vegetative cells from improperly stored food may under certain conditions cause intestinal human illness resulting from the production of enterotoxins by vegetative *Bacillus thuringiensis* cells. It is recommended to generate data on this using the conditions of use in Norway (Nordic countries)

Health risk – drinking water

- VKM considers that the prescribed use of *Bacillus thuringiensis* as an insecticide is unlikely to pose a threat to human health via drinking water.

Transfer of genetic material

- It is the opinion of VKM that the potential for harmful effects caused by transfer of genetic material in the environment is low. The fact that such gene transfer may take place highlights however the importance of strict procedures for analysis and control of purity, genotypic and phenotypic properties of the active ingredients.

Groundwater and soil contamination

- VKM find it unlikely that the spores or the protoxins/toxins will translocate to groundwater, and that the use of Turex 50 WG will result in increased density of *Bacillus thuringiensis* in Nordic soils.

Ecotoxicology

- VKM concludes that the use of Turex 50 WG according to GAP will not pose an unacceptable risk to the environment.

Antimicrobial resistance

There is a need for more data regarding this topic, including but not limited to:

- Antimicrobial susceptibility testing (MIC-values) of *Bacillus thuringiensis* ssp. *aizawai*, strain GC-91 against different antimicrobial agents.
- Clarification of the intrinsic and acquired resistance properties.

4 Data gaps reported by EFSA

EFSA (2013) (*Bacillus thuringiensis* ssp. *aizawai* strain GC-91):

- Demonstrate that the level of microbial contamination complies with international standards;
- Validation of methods of analysis for parasporal protein, beta-exotoxins, contaminating microorganisms, and for identification of the strain;
- Validation of the bio-potency method;
- Batch analysis for enterotoxins;
- Shelf-life of the formulation;
- Effects of light, temperature and humidity on technical characteristics of the plant protection product.
- Interferences of *Bacillus thuringiensis* with the analytical systems for control of the quality of drinking water provided for in Directive 98/83/EC.
- Potential transfer of genetic material from *Bacillus thuringiensis aizawai* strain GC-91 to other organisms.
- Groundwater exposure assessment of the crystalline proteins and transformation products that retain any insecticidal activity
- Risk to aquatic organisms, earthworms and other soil-dwelling non-target arthropods from exposure to GC-91 crystalline proteins (δ endotoxins)
- Risk to bees

Appendix I

Reports from the Norwegian Food Safety Authorities. These reports may be obtained from Mattilsynet, Seksjon nasjonale godkjenninger, Regionkontoret for Oslo, Akershus og Østfold (Ås).

- Bestilling
- Notat – Turex 50 WP
- E1_T1: EFSA-conclusion (ABTS 1857, GC-91)
- E2_T2: *Bt aizawai* GC-91 DAR (560 sider; inkludert mye data/artikler)
- E3: dRR Part A_Risk Management (Registration Report, Central Zone/Germany, CG-91, 3.2 CONCLUSION)
- E4: dRR Part B_Detailed summary (Fate and behaviour in the environment,
- E5: Vectobac 12 AS_2001
- BELA_Reporting_Form_Käsespätzle_and_Salanova_Lettuce
- DE comments on B. t. - Expert Opinions and Sample Documentation Forms
- Expert_Opinion_CVUA_Stuttgart_A12059790-25-ST_of_13_August_2012
- Expert_Opinion_CVUA_Stuttgart_A12063015-25-HC_of_20_August_2012
- Sample_Documentation_Form_Käsespätzle_Leftover
- Sample_Documentation_Form_Lettuce
- Sample_Documentation_Form_Salanova_Lettuce_1
- Sample_Documentation_Form_Salanova_Lettuce_2
- Bta GC-91 RR 1107 Core Part B Section 3 Agree 50 WP Mitsue AgriScience Int
- Bta GC-91 RR 1107 Core Part B Section 5 Agree 50 WP Mitsue AgriScience Int
- Bta GC-91 RR 1107 Core Part B Section 6 Agree 50 WP Mitsue AgriScience Int
- Bta GC-91 RR 1107 Part A Agree 50 WP Mitsue AgriScience Int DK 2014-09-24

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