Renewal
Assessment Report

Bacillus thuringiensis subsp. aizawai strain GC-91

- Agree 50 WG -

Volume 3 – B.9 Effects on non-target organisms

July 2018

Rapporteur Member State: The Netherlands

Co-Rapporteur Member State: Germany

Version history

When	What
July 2018	Initial RAR

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Introduction

Bacillus thuringiensis subsp. *aizawai* GC-91 (in the following abbreviated as Bta GC-91) was one of the existing active substances covered by the Regulation (EC) No 2229/2004 on the implementation of the fourth stage of the program of work referred to in Article 8(2) of Council Directive 91/414/EEC. In Annex I to Regulation (EC) No 2229/2004 the Commission designated Italy as rapporteur Member State to carry out the assessment of Bta GC-91 on the basis of a dossier submitted by the notifier Mitsui AgriScience International SA/NV. In accordance with the provisions of Article 22(1) of Regulation (EC) No 2229/2004, Italy submitted in November 2007 to the EFSA the draft assessment report, including, as required, a recommendation concerning the possible inclusion of Bta GC-91 in Annex I to the Directive. The Commission examined the draft assessment report, the recommendations by the rapporteur Member State and the comments received from other Member States in consultation with experts from a certain number of Member States. The Commission referred on 11 July 2008 a draft review report to the Standing Committee on the Food Chain and Animal Health, for final examination. The draft review report was finalised in the meeting of the Standing Committee on 11 July 2008. Subsequently Regulation (EC) No 1107/2009 repealed and replaced Directive 91/414/EEC and the active substance Bta GC-91, was deemed to be approved under that Regulation and included in the Annex to Regulation (EC) No 540/2011. EFSA delivered its conclusions on *Bacillus thuringiensis* ssp. *aizawai* (strains ABTS-1857, GC-91) on the 19 December 2012 (published January 2013). Based on this new information available, no need to change the conditions of approval of Bta GC-91 was identified. The Commission filed on 13 December 2013 an updated review report for Bta GC-91 to the Standing Committee on the Food Chain and Animal Health for examination.

The approval of Bta GC-91 under the Regulation (EC) No 1107/2009 expires 30 April 2019. In accordance with the same Regulation the original notifier Mitsui AgriScience International SA/NV has filed to the Commission an application for the renewal of the approval of the active substance Bta GC-91 on 30 April 2016. In accordance with Regulation (EU) 2016/183 the notifier is submitting to the designated RMS The Netherlands, the co-RMS Germany as well as to EFSA and the Commission a dossier for renewal of Bta GC-91 considering the deadline stated in SANTE-2016-10616–rev. 3. As the manufacturing process of Bta GC-91 has not been changed since original approval, all data submitted for the original approval of the strain are considered fully applicable for the current evaluation. The information from the previous DAR (May 2007) and DAR addendum (February 2013) is included along with new data which resulted during the literature search.

Mode of action

Bta GC-91 is a transconjugant strain originating from a Bta and a Bt subsp. *kurstaki* strain. Bta in general occurs ubiquitous in soils on plants as well as in infested insects. Bta acts highly specific against insect species of the order Lepidoptera and is not expected to have any harmful effects on beneficials and other non-target species of other insect orders. The insecticidal activity of Bta is mainly attributed

to spore bound insecticidal pro-toxins (Cry toxins) which are ingested by the target pests and activated under alkaline conditions in the midgut of the larvae.

Metabolites

Analysis of five batches GC-91 showed that no microbial pathogens of toxicological concern for human and animal health were detected. Quality tests did not reveal the presence of toxigenic pathogens producing nameable amounts of metabolites of toxicological concern for human and animal health. MPCA does not contain any additives.

Representative uses and formulation

Representative uses chosen for renewal of Bta GC-91 cover control of *Lobesia botrana* and *Eupoecillia ambiguella* in grapes (as for original approval) as well as *Cydia pomonella* in pome fruits and *Spodoptera* spp. in turf as field uses, as well as *Tuta absoluta* in tomato in the greenhouse. Both, use by professionals and non-professionals is intended. The maximum intended application rate is 2 kg product/ha with 6 subsequent applications at an interval of 7 days.

It is considered that the Critical GAP of Agree 50 WG chosen for the renewal of the active substance Bta GC-91 covers worst case exposure scenarios for human, non-target organisms and the environment.

According to the information provided in Volume 1, a multitude of products containing *Bacillus thuringiensis* is registered Europe-wide for the control of Lepidopteran larvae in various agricultural and horticultural crops, orchards, and forests.

Agree 50 WG has been approved in the EU for many years. The product of Agree WG (= Turex WG) is similar product Turex WP (please refer to Volume 4). It is considered appropriate to use the data from the studies conducted with Turex WP for the product Agree 50 WG.

Critical GAP of Agree 50 WG for renewal of Bta GC-91

Crop	**F G or I**	**Pest**	**Application**			**Application rate**		
			Method / Kind	**Growth stage of crop**	**Max. number (min. interval between applications)** **a) per use** **b) per crop/ season**	**Kg product / ha** **a) max. rate per appl.** **b) max. total rate per crop/season**	**g as/ha IU/ha** **a) max. rate per appl.** **b) max. total rate per crop/season**	**Water L/ha** **min / max**

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Crop	F G or I	Pest	Application			Application rate		
			Method / Kind	Growth stage of crop	Max. number (min. interval between applications) a) per use b) per crop/ season	Kg product / ha a) max. rate per appl. b) max. total rate per crop/season	g as/ha IU/ha a) max. rate per appl. b) max. total rate per crop/season	Water L/ha min / max
Pome fruits	F	*Cydia pomonella*	Foliar spray	BBCH 53-99 (April-October)	a) 6 (7) b) 6 (7)	a) 2.0 b) 12.0	a) 1000 / 5×10^{10} b) 6000 / 3×10^{11}	1000-1500
Grapes	F	*Lobesia botrana, Eupoecilia ambiguella*	Foliar spray	BBCH 53-99 (April-October)	a) 6 (7) b) 6 (7)	a) 2.0 b) 12.0	a) 1000 / 5×10^{10} b) 6000 / 3×10^{11}	200-1200
Tomato	G	*Tuta absoluta*	Foliar spray	BBCH 12-89 (all seasons)	a) 6 (7) b) 6 (7)	a) 2.0 b) 12.0	a) 1000 / 5×10^{10} b) 6000 / 3×10^{11}	500-1500
Turf, Sports	F	*Spodoptera* spp.	Foliar spray	BBCH 12-89 (all seasons)	a) 6 (7) b) 6 (7)	a) 2.0 b) 12.0	a) 1000 / 5×10^{10} b) 6000 / 3×10^{11}	1000-1500

Biopotency of Agree 50 WG: 25000 IU/mg
Max. CFU content in Agree 50 WG: 3.3×10^{13} CFU/kg

B.9.1 Effects on birds

B.9.1.1 Toxicity to birds

No product study provided. Based on the composition of the formulation it is expected that the active substance data will cover for the product.

Results:

A summary of endpoints is given in the table below.

Table B.9.1.1: Toxic effects / Infectivity / Pathogenicity of active substance to bird

Species	Test duration	Dose range	Results/ Endpoint	Observations	Reference
TOXICITY					
Bobwhite quail *(Colinus virginianus)*	30 days (5 days of treatment)	LC50: >3333 mg/kg >3.53×10^{11}	LC50: >3333 mg/kg >3.53×10^{11}	No signs of mortality.	[redacted] 1990a,

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Species	Test duration	Dose range	Results/ Endpoint	Observations	Reference
		CFU/kg b.w./day	CFU/kg b.w./day		
Mallard duck *(Anas platyrhynchos)*	30 days (5 days of treatment)	3333 mg/kg or approximately 3.53×10^{11} CFU/kg b.w./day	3333 mg/kg or approximately 3.53×10^{11} CFU/kg b.w./day	No signs of mortality.	[redacted] 1990b
INFECTIVENESS					
Not determined.					
PATHO-GENICITY					
Bobwhite quail *(Colinus virginianus)*	30 days	LC50: >3333 mg/kg >3.53×10^{11} CFU/kg b.w./day	LC50: >3333 mg/kg >3.53×10^{11} CFU/kg b.w./day	No signs of pathogenicity.	[redacted] [redacted],1990a,
Mallard duck *(Anas platyrhynchos)*	30 days	3333 mg/kg or approximately 3.53×10^{11} CFU/kg b.w./day	3333 mg/kg or approximately 3.53×10^{11} CFU/kg b.w./day	No signs of pathogenicity.	[redacted] 1990b

B.9.1.2 Infectiveness to birds

Not determined. Considering the mode of action, it is not expected that the product will be infective to birds.

B.9.1.3 Pathogenicity to birds

No signs of pathogenicity were detected in the studies presented.

B.9.1.4 Risk assessment for birds

The previous DAR included a risk assessment according to SANCO document 4145/2000. RMS would like to note that it was agreed in PRAPeR M2 that the guidance document SANCO/4145/2000 was intended for chemical substances and is considered less relevant for plant protection products containing micro-organisms. During PRAPeR M2 it was agreed that, with the lack of appropriate expo-

sure scenario's for micro-organisms, a worst case risk assessment could be performed by comparing the amount of CFU applied, or present in the application liquid to the endpoint of the study.

The RMS included the following risk assessment for birds and mammals.

- **Birds**

The data provided an endpoint of > 3.53 x 10^{11} CFU/kg b.w./day
The density of spores in the WG formulation is 3.3 x10^{13} CFU/kg. The maximum of spores applied is 6.6 x 10^{13} CFU/ha. Considering the minimum water applied for the uses in grapes of 200 L/ha, the maximum concentration in the spray liquid is 3.3 x 10^{11} CFU/L.

The exposure via drinking water is considered relevant. According to the EFSA bird mammal guidance document (EFSA Journal 2009; 7(12):1438), the worst-case for drinking water is a small granivorous bird with a body weight of 15.3 g, with a drinking rate of 7.0 mL/day, equivalent to 0.46 L/kg bw/d. Based on the worst PECsw of 1.22 x 10^{7} CFU/L for the applications in pome fruit, the daily dose is 0.56 x 10^{7} CFU/kg bw/d. This value is below the endpoint for birds and therefore the risk through drinking water is considered acceptable.

- **Mammals**

The acute oral LD_{50} of *Bta* technical was greater than 9.4 x 10^{8} CFU/kg bw (refer to toxicology section). The test substance is not toxic, infective or pathogenic on the basis of the acute oral toxicity study in male and female rats.
The daily water intrake of a small granivorous mammal with a body weight of 21.7 g is 0.24 L/kg bw/d. Considering the PECsw of 1.22 x 10^{7} CFU/L for the applications in pome fruit, the daily dose is 0.29 x 10^{7} CFU/kg bw/d. This value is below the endpoint for mammals and therefore the risk through drinking water is considered acceptable.

B.9.2 Effects on aquatic organisms

B.9.2.1 Effects on fish

The ingredients of the preparation Agree 50 WP formulated as WP are not expected to cause any hazards to aquatic organisms. Therefore, studies on the technical material CGA 237218 (GC-91) are considered applicable and relevant with regard to the evaluation of the formulated product on aquatic organisms. As *Bacillus thuringiensis* spp. *aizawai* is highly specific and only has an effect on very few species of the Lepidoptera order, aquatic organisms are not at risk and studies are not regarded as necessary.

Results:

A summary of endpoints is given in the table below.

Table B.9.2.1: Toxic effects / Infectivity / Pathogenicity of active substance to fish

Test species	Sheepshead minnow (Cyprinodon variegatus)
Toxicity	The LC_{50} value at 32 days was estimated to be > 2.1×10^9 CFU/L (mean measured concentration)
Infectivity / Pathogenicity	No signs of pathogenicity or infectivity.

B.9.2.1.1 Infectiveness to fish

No signs of infectivity.

B.9.2.1.2 Pathogenicity to fish

No signs of pathogenicity.

B.9.2.2 Effects on freshwater invertebrates

B.9.2.2.1 Toxicity to freshwater invertebrates

Plant protection product

Report:	KMP 10.2.2/01 - Dengler, D. (2010)
Title:	Assessment of toxic effects of Agree WG on *Daphnia magna* using the 48 h acute immobilisation test
Document No:	Report No. S10-02545
Guidelines:	OECD Guideline No. 202 (2004)
GLP	Yes
Validity	Yes

Executive summary

In a 48-h acute immobilisation test, *Daphnia magna* were exposed to Agree WG at nominal concentrations of 0 (control) and 100 mg/L under static conditions.

After 24 and 48 hours of exposure, no immobile daphnids were observed in the control and at 100 mg/L.

The 48-h EC_{50} was determined to be above 100 mg/L (nominal) and above 25 mg/L (actual). The NOEC (48 h) was observed at 100 mg/L (nominal) and 25 mg/L (actual).

MATERIAL AND METHODS

Test Item

Designation Agree WG

Active ingredient	*Bacillus thuringiensis aizawai* strain GC91
Characteristics	Light brown granular
Batch no.	1000990
Expiration date	08.06.2011
Purity	8.5×10^{12} cfu/kg (nominal), 1.02×10^{12} cfu/kg (actual)

Test System

Species	*Daphnia magna* Straus, clone V
Source	Rearing stock at the testing facility
Number	Control group: 20, Treated group: 20
Food	Single cell green algae (*Desmodesmus subspicatus*)

Test Conditions

Temperature	From 20.8 to 21.1°C
Photoperiod	16 hour photoperiod daily (~ 1250 lux)
Oxygen content	≥ 96% of air saturation
Hardness	10 °dH (178.48 mg/L as $CaCO_3$)
pH	from 7.85 to 8.37

Study Design and Methods

In-life dates	07.07.2010 to 05.08.2010
System	Static
Duration	48 hours
Test vessel	100 mL glass beaker
Concentration	0 (Control) and 100 mg/L 4 replicates per group
Observations	After 24 and 48 hours the immobilised daphnids were counted.

RESULTS AND DISCUSSION

After 24 and 48 hours of exposure, no immobile daphnids were observed in the control and at 100 mg/L.

CONCLUSIONS

The 48-h EC_{50} was determined to be above 100 mg/L (nominal) and above 25 mg/L (actual). The NOEC (48 h) was observed at 100 mg/L (nominal) and 25 mg/L (actual).

(Dengler, 2010)

Comments and conclusion RMS:

The test was performed according to OECD 202. The validity criteria according to this guideline were fulfilled. According to the study authors, the analyzed content of CFU in Agree WG was 1.02 x 10^9 CFU/g instead of 8.5 x 10^9 CFU/g as specified in the Certificate of Analysis. It is further mentioned that at test start a total mean of 0.26 x 10^9 CFU/g was determined in the test sample and considering the analyzed content the target concentration was 25 mg product/L instead of the nominal of 100 mg product/L. During the recovery method, 19% of the nominal number of cfu were counted on plates

while during the method validation 12% of the nominal was counted. It is considered that this is due to the variation of the spores' viability.

The duration of the test was too short to account for any infectivity and pathogenicity. It can be concluded that under the condition of this test the product is not toxic to daphnids. The LC_{50} > 25.5 x 10^6 CFU/L, based on the actual concentrations measured.

Results:

A summary of endpoints is given in the table below.

Table B.9.2.2: Toxic effects / Infectivity / Pathogenicity of plant protection product to freshwater invertebrates

Test species	*Daphnia magna*
Toxicity of plant protection product	LC_{50} > 25.5 x 10^6 CFU/L (actual measured concentration)

B.9.2.2.2 Infectiveness to freshwater invertebrates

Based on the 21 day static renewal test by Collins, M.K. (1993) it is not expected that the product will be infective to daphnids.

B.9.2.2.3 Pathogenicity to freshwater invertebrates

Based on the 21 day static renewal test by Collins, M.K. (1993) it is not expected that the product will be pathogenic to daphnids.

B.9.2.3 Effects on algae growth

Considering the composition of the product and the mode of action, no effects on the algae growth are expected. The test with the active substance is will cover for the product.

Results:

A summary of endpoints is given in the table below.

Table B.9.2.3: Toxic effects / Infectivity / Pathogenicity of active substance to algae

Test species	*Scenedesmus subspicatus*
Toxicity	$E_bC_{50:}$ > 3.6 x 10^9 CFU/L NOE_bC: 3.6 x 10^9 CFU/L
Infectivity / Pathogenicity	No signs of infectivity or pathogenicity found

B.9.2.4 Effects on plants other than algae

Information not provided. Considering the mode of action, the study is not considered necessary.

B.9.2.5 Summary of the studies on aquatic organisms toxicity, infectiveness and pathogenicity

Table 9.2.5: Summary of the studies on toxicity on aquatic organisms

Species	Test duration	Dose range	Results/ Endpoint	Observations	Reference
Active substance					
Rainbow trout (*Onchorhyncus mykiss*)	32 d	3.90 x 10^{10} CFU/L, 3.90 x 10^{9} CFU/L and 1.0 x 10^{9} CFU/L.	LC_{50} > 2.0 x 10^{10} CFU/L (mean measured concentration).	No signs of toxicity, pathogenicity or infectivity. Increased concentration of cfu were measured in the gills after necropsy, but did not contstitute infection	[redacted] (1991a)
Sheepshead minnow (*Cyprinodon variegatus*)	30 d	3.90 x 10^{10} CFU/L, 3.90 x 10^{9} CFU/L and 1.0 x 10^{9} CFU/L.	LC_{50} > 2.1 x 10^{9} CFU/L (mean measured concnetration)	No signs of pathogenicity or infectivity.	[redacted] (1991b)
Daphnia magna	10 d	Not applicable	Not applicable	No endpoint calculated, due to different purpose of study. It concluded that heat labile	Collins, M.K. (1997)

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Species	Test duration	Dose range	Results/ Endpoint	Observations	Reference
				components from the fermentation broth are carreied over into the technical material. These components elicit the toxicity of GC-91 observed for Daphnids.	
Daphnia magna	21 d	1.17 x 10^{10}, 3.51 x 10^{9}, 1.05 x 10^{9}, 3.16 x 10^{8}, 9.50 x 10^{7} CFU/L	21-day EC_{50} (survival): 3.24 x 10^{8} CFU/L. NOEC(length, offspring and survival): 1.57 x 10^{8} CFU/L	Signs of infectivity were not observed when appying the normal test procedures.	Christensen, K.P. (1991c)
Daphnia magna	21 d	8.5 x 10^{6}, 2.8 x 10^{7}, 9.5 x 10^{7}, 3.15 x 10^{8} and 1.05 x 10^{9} CFU/L	21-day EC50 > 6.2 x 10^{8} CFU/L NOEC ≥ 6.2 x 10^{8} CFU/L	No effects on pathogenicity or infectivity observed	Collins, M.K. (1993)
Palaemonetes vulgaris	30 d	1.58 x 10^{10} CFU/g	21-d NOEC 1.9 x 10^{9} CFU/g (diet exposure)	No signs of treatment related pathogenicity or infectivity were observed.	Christensen, K.P. (1991d)
Scenedesmus subspicatus	72 h	4.2 X 10^{7} 14.1 X 10^{7} 46.5 X 10^{7} 125.5 X 10^{7} 356 X 10^{7} CFU/L	$E_bC_{50:}$ > 3.6 x 10^{9} CFU/L NOE_bC: 3.6 x 10^{9} CFU/L	No signs of infectivity or pathogenicity found	Grade, R. (1993)

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Species	Test duration	Dose range	Results/ Endpoint	Observations	Reference
Product					
Daphnia magna	48 h	1.02 x 10^8 CFU/L	> 25.5 x 10^6 CFU/L (actual measured concentration)	No signs of infectivity or pathogenicity found	Dengler, 2010

B.9.2.6 Risk assessment for aquatic organisms

The maximum PECsw was calculated for applications in pome fruits and grapes and amounts 1.22 x 10^7 CFU/L.

The trigger TER values were validated with data for chemicals. Therefore, RMS will use instead the concept of margin of safety

Species	PECsw CFU/L	Endpoint CFU/L	MoS
Cyprinodon variegatus	1.22 x 10^7	> 2.1 x 10^9	> 172
Daphnia magna	1.22 x 10^7	6.2 x 10^8	51
Daphnia magna	1.22 x 10^7	> 25.5 x 10^6	> 2.1
Palaemonetes vulgaris	1.22 x 10^7	1.9 x 10^9 (diet)	156 (diet)
Scenedesmus subspicatus	1.22 x 10^7	3.6 x 10^9	295

The MoS for exposure via water are above the unit for all organisms.

B.9.3 Effects on bees

B.9.3.1 Toxicity to bees

Plant protection product

Reference: KMP 10.3/01	Kleiner, R. (1992), Testing toxicity to honeybee – *Apis mellifera* L. (laboratory) according to BBA Guideline VI, 23-1 (1991). Unpublished Report No. 92 10 48 068, 11.12.1992
Guideline:	BBA Guideline VI, 23-1 (1991)
GLP:	Yes

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Material and methods:	The study was conducted during the period 07.10.1992 to 17.10.1992, by BioChem GmbH Karlsruhe, Q-7101 Cunnersdorf, Germany. The test material used was CGD 97220 I, content of a.i.: 3×10^{13} CFU/kg; batch no.: P 109001. A total of 30 *Apis mellifera* (10 honey bees per cage) were exposed to CGD 97220 I over a period of 72 hours. The substance CGD 97220 I was tested at a concentration of 0.4% w/v (= 4.0 kg/ha in 1000 L/ha of water) in the respiration test, in the direct contact test (residual effect) and in the indirect contact (direct sprayment) test. In the feeding test the concentration was 1%. To test feeding effects the bees were fed with a defined quantity of a 50% aqueous sugar solution that contained 1% of the test substance. Approx. 0.2 mL (≈ 0.25 g) of the test solution were offered to ten bees per cage for maximum 3 hours. As toxic standard "Wofatox-Konzentrat 50" (Parathion-methyl) was used (0.21 L/ha in 600 L/ha of water = 0.035% v/v). Mortality and abnormal behaviour were observed 24, 48 and 72 hours after application.
Test substance:	CGD 97220 I
Test species:	*Apis mellifera*
Number of test animals:	30 *Apis mellifera* (10 honey bees per cage) were exposed to CGD 97220 I
Treatments:	Direct contact test (residual effect), indirect contact (direct sprayment) test, feeding test
Duration:	3 days
Test conditions:	Not indicated
Deviations from guideline	None
Endpoint:	The mortality in the untreated control was 0%. The test substance caused no mortality of the honeybee in the four laboratory tests (i.e. respiration, direct and indirect contact and feeding test). Consequently the LD_{50} was higher than 197 µg/honeybee. No behavioural anomaly was observed. The reference substance effected total mortality (100%) in all tests.
Observations:	The LD_{50} was higher than 197 µg/honeybee

Results:

A summary of endpoints is given in the table below.

Table 10.3-1 Toxic effects of plant protection product to bees

Test species	*Apis mellifera*
Toxicity of plant protection product	The mortality in the untreated control was 0%. The test substance caused no mortality of the honeybee in the four laboratory tests. Consequently the LD_{50} was higher than 197 µg/honeybee. No behavioural anomaly was observed. The reference substance effected total mortality (100%) in all tests.

Comments: According to the results of this study it can be assumed that the oral LD_{50}/72 h is > 197 µg Agree 50 WP/bee.

Comments RMS: the study was considered acceptable in the original DAR. There was no mortality in the untreated control and 100% mortality in there reference item after 24 h. The test product did not cause mortality in bees at the tested dose.
The tested dose was 4 kg product/ha in 1000 L/ha (i.e. 4 x 10^{-3} kg product/L). Considering the concentration of 500 g a.s./kg P, the dose tested was 2 g a.s./L.
According to the GAP, the maximum spray concentration in pome fruits, grapes and tomato is 0.4% equivalent to 400 g product/HL (i.e. 4 g product/L or 2 g a.s./L). The highest concentration in turf and sports fields is 2 g product/L or 1 g a.s./L. The current test does cover for the maximum spray volumes of the current GAP.
The LD_{50} is > 2 g a.s./L.

Results:
A summary of endpoints is given in the table below.

Table B.9.3.1: Toxic effects / Infectivity / Pathogenicity of plant protection product to bees

Test species	*Apis mellifera*
Toxicity of plant protection product	LD_{50} is > 2 g a.s./L

Report:	Vergé, 2016
Title:	Delfin WG – Acute oral and contact toxicity to the honeybee *Apis mellifera* L. under laboratory conditions.
Document No:	S15-05620
Guidelines:	OECD 213 and 214 (1998) and OPPTS 885.4380
GLP	Yes

Executive Summary

Under the conditions of the study, Delfin WG can be considered not toxic to honey bees. 96-hour oral and contact LD_{50} was determined to be > 82 µg product/bee, and > 100 µg product/bee respectively. At the end of the 19 day test period the oral LD_{50} was > 82 µg product/bee, and at the end of the 15 day test period the contact LD_{50} was > 100 µg product/bee. The oral and contact NOED were > 82 µg product/bee, equivalent to 4.2×10^6 cfu/bee and > 100 µg product/bee equivalent to 5.1×10^6 cfu/bee, respectively.

I. MATERIALS AND METHODS

Test Item

Designation	Delfin WG
Characteristics	Solid, brown
Batch no.	2501595
Active ingredient(s)/Content	*Bacillus thuringiensis* subsp. *kurstaki* at a concentration of 5.1×10^{10} CFU/g
Storage conditions	Dark and dry, ambient
Stability (expiry date)	07.10.2016 (not given by sponsor but assumed to be one year after first receipt)

Test System

Species	Honeybee *(Apis mellifera* L.), derived from a healthy colony
Age	Adult worker bees
Weight	Not provided
Supplier	Mr. Antonio Escriva Moreno, Bee hive exploitation is registered in the Local Government Administration under the official number 176-V-026.
Acclimatisation period	Bees were kept under test conditions until test start.
Diet	50% aqueous sucrose solution

Test Conditions

Housing	Cages not further described
Temperature	30.1 °C - 34.4 °C
Relative humidity	42.4 - 68.4 %

Photoperiod	24 h darkness, except during application and assessments

Study Design and Methods

Conducted at	Trialcamp, Poligon Industrial l'Alter, Avda. Antic Regne de Valencia, 25, S-46290 Alcasser (Valencia), Spain (experimental phase) and Eurofins Agroscience Services EcoChem GmbH / Eurofins Agroscience Services Ecotox GmbH, Eutinger Straße 24, 75223 Niefern-Öschelbronn, Germany
Experimental phase	05.11.2015 to 13.12.2015
Treatment	The oral and contact toxicity tests were carried out as limit test with Delfin WG: 100 µg product/bee (oral and contact test). As reference item "Perfekthion" (a.i. dimethoate, analysed 420.3 g/L) was used in the oral and contact tests. Four doses of the reference item were tested: 0.06, 0.09, 0.14, and 0.21 µg a.i./bee (nominal rates in oral toxicity test) and 0.08, 0.12, 0.18, and 0.27 µg a.i./bee (contact toxicity test). In the oral toxicity test the bees of the control group received a pure 50% (W/v) aqueous sucrose solution. In the contact toxicity test the bees of the control group were treated with deionised water. At each dose and treatment of the oral and contact toxicity tests respectively five replicate groups of 10 bees were tested.
Observations	Assessment of mortality in both oral and toxicity tests was carried out after 4, 24 and 48 and 96 hours after test start. Thereafter mortality was recorded every 24 hours up to 19 days for oral toxicity test and 15 days for contact toxicity test. The reference item group observation period was 48 hours.
Statistics	Statistical calculations were made by using the statistical program ToxRat Professional 3.2.1.

II. RESULTS AND DISCUSSION

In the control group of the oral toxicity test (untreated 50 % (w/v) aqueous sucrose solution), 2.0 % mortality was recorded after 96 hours. A mortality of 20.0 % was recorded 19 days after application. The test was therefore terminated at day 19.

In the control group of the contact toxicity test (deionised water), 2.0 % mortality was recorded after 96 hours. A mortality of 20.0 % was recorded 15 days after application. The test was therefore terminated at day 15.

The 24-hour oral and contact LD_{50} values for the reference item were 0.12 and 0.18 µg dimethoate/bee, respectively. Consequently, validity criteria for both control and reference item mortality were met and the test was considered valid.

In the oral toxicity test, 24.0 % mortality (corrected mortality: 5.0 %) was observed at the target dose of 100 µg product/bee (actual uptake: 82 µg product/bee) 19 days after start of feeding. This was not statistically significantly different compared to the control; the NOED (No Observed Effects Dose) is therefore greater than 82 µg product/bee. One affected bee was recorded 14 and 19 DAA, respectively.

In the contact toxicity test, 8.0 % mortality (corrected mortality: -15.0 %) was recorded at the dose of 100 µg product/bee at the end of the 15 day test period. This was not statistically significantly different compared to the control; the NOED is therefore greater than 100 µg product/bee. No behavioral abnormalities were recorded during the 15 days test period.

III. CONCLUSIONS.

At the end of the 19 day test period the actual oral LD_{50} value for Delfin WG is > 82 µg product/bee, equivalent to 4.2×10^6 CFU/bee.

At the end of the 15 day test period, the contact LD_{50} was determined to be >100 µg product/bee, equivalent to > 5.1×10^6 CFU/bee.

The oral and contact NOED were 82 µg product/bee, equivalent to 4.2×10^6 cfu/bee and 100 µg product/bee equivalent to 5.1×10^6 cfu/bee, respectively.

Comment by RMS: The study was evaluated and considered acceptable by RMS. The endpoints as per study report are considered acceptable for the risk assessment. The test duration was longer than the standard 96 h required for the tests with chemicals however much lower than the 30 days recommended by OPPTS guidelines for studies with microorganisms. However, according to the OECD 67 shorter duration studies of 14 days can be acceptable if the control mortality rate of lower or equal than 10% can be achieved. This was the case in the current test. Infectivity and pathogenicity were not tested.

Considering a consumption of 20 ul/bee in the oral study and a topical application of 2 ul/bee in the contact study, the endpoints can be translated to > 41 kg/L and > 5 kg/L in the oral and contact study, respectively. However, as the studies were conducted with Btk strain SA-11 and considering that it is unknown whether the Btk strain used in this study contains the same plasmids as the one used for the production of the transconjugant strain Bta GC-91, the data can only be used in a weight-of-evidence. The RMS is of opinion that the most appropriate studies for the current risk assessment are going to be the ones conducted with the transconjugant GC-91.

B.9.3.2 Infectiveness to bees

Study duration was too short to investigate infectiveness.

B.9.3.3 Pathogenicity to bees

Study duration was too short to investigate pathogenicity.

B.9.3.4 Summary and risk assessment for bees

For the current risk assessment four studies are acceptable, Mommaerts V. et al. (2009), Parrish, J.R. & Yeager, B. (1994), Vergé (2016) and Kleiner, R. (1992) .

Species	Test type	Micro-organism tested	Endpoints and observations	Reference
Bumble bee *(Bombus terrestris)*	Oral and contact Exposure (11 weeks)	*Bta* (Xentari)	100% mortality orally via sugar water at 1.5 x 10^4 IU/mL 0% mortality orally via sugar water at 1.5 x 10^3 IU/mL 100% reduction in reproduction orally via sugar water at 1.5 x 10^4 IU/mL 0% reduction in reproduction orally via sugar water at 1.5 x 10^3 IU/mL 31% reduction in reproduction orally via pollen at 1.5 x 10^4 IU/mL. No effects via dermal exposure.	Mommaerts, V., Jans, K., Smagghe, G. (2009)
Bumble bee *(Bombus terrestris)*	Oral and contact Exposure (11	*Btk* (Dipel)	3% mortality via contact at 1.6 x10^4 IU/mL	Mommaerts, V., Jans, K., Smagghe, G. (2009)

	weeks)		5% mortality via sugar water at 1.6 x10^4 IU/mL 0% mortality via treated pollen at 1.6 x10^4 IU/mL No effects on reproduction at 1.6 x10^4 IU/mL	
Honey bee *(Apis mellif-era)*	Oral and contact (19 days)	*Btk* (Delfin WG)	96 h and 19-day LC_{50} > 41 kg/L 96 h and 15-day LC_{50} > 5 kg/L	Vergé, 2016
Honey bee *(Apis mellif-era)*	Oral (14 days)	*Bta* Technical grade material CGA 237218 (GC 91), 9.9% delta endotoxin	5-day LC_{50}: 3656 ppm (3652 mg/L, 897 µg/bee) 10-day LC_{50}: 170 ppm (169.8 mg/L, 91 µg/bee) NOEC: 50 ppm Delta-endotoxin (9.9%): 5-day LC_{50}: 88.8 µg/bee 10-dayLC_{50}: 9.0 µg/bee	Parrish, J.R. & Yeager, B. (1994)
Honey bee *(Apis mellif-era)*	Oral, respira-tory, direct and indirect contact (72 h)	CGD 97220 I (Agree 50 WP)	LD50 is > 2 g a.s./L. No behavioural anomaly was observed.	Kleiner, R. (1992)

The applicant claims for pome fruits, grapes and tomato a maximum intended concentration of Agree WG in the spraying liquid of 0.4 % which corresponds to 4 g product/L (i.e. 2 g a.s./L). Considering the concentration of 25000 IU/mg product, this translates to 1 x 10^8 IU/L or 1 x 10^5 IU/mL. For the exposure in turf , a maximum product rate of 2 kg/ha is applied in a minimum of 1000 L/ha. Considering the concentration of 25000 IU/mg product, this translates to 5 x 10^7 IU/L or 5 x 10^4 IU/mL.

These field application rates are higher than the concentrations of the *Bta* containing product at which the toxicity was measured in the test with *Bombus terrestris* (bumble bee) by Mommaerts V. et al. (2009). No toxicity was found for the *Btk* containing product (highest test dose 1.6 x10^4 IU/mL).

The applicant had the following argumentation in order to refine the possible risk to bumblebees:

- *the endpoint is not strain specific (as Bta GC-91 is a transconjugant between Btk and Bta also the endpoint for Dipel could be used) It has to be noted also that both, the exposure level and also the exposure time in the study of Mommaerts et al (2009) represent unrealistic worst case conditions*

- *the concentration in the spraying liquid is the highest possible field exposure only occurring during spraying. Usually dilution factors are applied e.g. a factor of 5 is used for calculation of drinking water exposure for birds and mammals for sources such as puddles or axes of leaves. If such a factor is considered the exposure would decrease to 2 x 10^4 IU/mL already.*
- *the high concentration will not be maintained for long but the Bt spores and associated cry toxins are considered to disappear quickly under field conditions (recorded half-life times of spores and protoxins are in the range of some hours to days)*
- *the authors have been aware about the fact that the chosen exposure scenario represents unrealistic worst case conditions and concluded that in general Bt based products can be considered safe for bumble bees.*

As mentioned above, the Btk-based product Dipel did not show any side effects at the MFRC et al. As Bta GC-91 is a transconjugant strain between a Btk and a Bta, this result should be taken into consideration also.

The RMS agrees that the exposure in the study by Mommaerts V. et al. (2009) represents a worst-case scenario. Regarding the transconjugant, the RMS is of opinion that it is unknown whether the *Bta* and *Btk* strains used in this study contain the same plasmids as the two strains used for the production of the transconjugant strain *Bta* GC-91. As a result, the conclusion from this study is that there can be effects on bumblebees however, it is uncertain what the real effect of the transconjugant will be.

The authors did conclude that in general, the *Bt* strains are safe to bumblebees, but in some cases there were detrimental effects that depend on strain and route of exposure. The authors state that routine testing of lethal and sublethal effects is recommended to ascertain a safe combined use of Bt products and bumblebees in modern agriculture.

No effect on the honey bees were observed in the acute test with the *Btk* at concentrations higher than the current field application rates.

In the case of *Bta* GC-91, the 5 day LC_{50} is higher than the maximum intended field rate for all the crops. The 10 day LC_{50}, however is below the maximum intended field rate for all the crops. Taking these results into account it cannot be excluded that the transconjugant is chronically toxic to bees. The applicant argues that the spores and associated Cry proteins cannot be maintained in the field for a long time with recorded half-lives of hours to days. The RMS requests the applicant to provide data to support this claim.

In the study by Kleiner, R. (1992), the tested dose of 2 g a.s./L covers for the applications as per GAP. The applicant claims maximum spray concentration of 400 g product/ HL which is equivalent to 2 kg a.s./L for the uses in pome fruits, grape and tomato. For the uses in sport and turf the maximum application rate is 1 kg a.s./L. Considering these and that the LD_{50} was higher than the maximum tested dose of 2 g a.s./L, the product containing the transconjugant Bta GC-91 is not acutely toxic to honeybees.

To conclude, the additional data is required in order to assess if the potential risk to bumblebees seen in the study by Mommaerts et al. (2009) is relevant for the current transconjugant and if it is relevant in

the field situations. Furthermore, additional data on the half-lives of the Cry proteins are required in order to exclude the chronic toxicity to bees. Information on infectivity and pathogenicity to honey bees and bumble bees must be submitted. The RMS is of opinion that if no further information is provided a restriction sentence is necessary prohibiting the application of the product when the crop is flowering.

B.9.4 Effects on arthropods other than bees

B.9.4.1 Toxicity to arthropods other than bees

PLANT PROTECTION PRODUCT

Reference: KIIM 10.4/01	Warmers, C. (2005a): TUREX 50 WP: Acute toxicity to the aphid parasitoid. *Aphidius rhopalosiphi* De Stefani Perez (Hymenoptera, Braconidae) in the laboratory (limit test). Unpublished Report No. 20051317/01-NLAp
Guideline:	ESCORT I Guidance Document (Barrett et al., 1994), ESCORT II Guidance Document (Candolfi et al., 2001) IOBC (Mead-Briggs et al., 2000)
GLP:	Yes
Material and methods:	The study was conducted during the period 19.09.2005 to 04.10.2005, by GAB Biotechnologie GmbH & IFU Umweltanalytik GmbH, Niefern-Öschelbronn, Germany. The test material used was TUREX 50 WP (Lot no.: 0091-67-2, content of active ingredient: 3.8% insecticidal toxins (50% insecticidal toxins, spores and fermentation solids), *Bacillus thuringiensis aizawai* (strain GC91) mean potency 25000 International Units/mg –11 BIU/lb (24.3 BIU/kg) (analyzed) was diluted in water and applied with a spray application volume of 200 L/water/ha to glass plates at a rate equivalent to 4.5 kg product/ha. Ten wasps of *Aphidius rhopalosiphi* (5 female and 5 males) were placed into each exposure unit (n = 4 per treatment). Deionised water was applied as control (200 L/ha) and Perfekthion (nominal content of dimethoate: 400.0 g/L) was applied at 0.3 mL product in 200 L water/ha as a reference item treatment. Assessments of direct treatment effects were made after 1 h, 2 h, 24 h and 48 h. To assess any impact on the fecundity of surviving individuals, 17 females from the control group and all unaffected females (up to a maximum of 17) from the 4.5 kg product/ha treatment group were taken after 48 h and confined individually over aphid-infested barley plants (untreated) for a further 24 h period. The number of parasitized aphids that developed was recorded 13 days after parasitation period started.
Test substance:	TUREX 50 WP (Lot no.: 0091-67-2)
Test species:	*Aphidius rhopalosiphi*

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Number of test animals:	Ten wasps of *Aphidius rhopalosiphi* (5 female and 5 males) were placed into each exposure unit (n = 4 per treatment).
Treatments:	TUREX 50 WP was diluted in water and applied with a spray application volume of 200 L/water/ha to glass plates at a rate equivalent to 4.5 kg product/ha.
Duration:	Assessments of direct treatment effects were made after 1 h, 2 h, 24 h and 48 h. To assess any impact on the fecundity of surviving individuals, 17 females from the control group and all unaffected females (up to a maximum of 17) from the 4.5 kg product/ha treatment group were taken after 48 h and confined individually over aphid-infested barley plants (untreated) for a further 24 h period. The number of parasitized aphids that developed was recorded 13 days after parasitation period started.
Test conditions:	Not indicated
Deviations from guideline	1. The counting of the parasitized aphids to evaluate the reproduction rate per female was done 13 days instead of 12 days after start of the fertility test. 2: In the 2nd replicate of the control group fertility cages more than one female was introduced and only one female was caught 24h later.
Endpoint:	After 48 h the mean mortality was 7.50% in the 4.5 kg product/ha treatment group of TUREX 50 WP (Table 1). In the control group a mean mortality of 0.00% was observed. The corrected mortality of *Aphidius rhopalosiphi* after exposure to TUREX 50 WP was calculated to 7.50% in the 4.5 kg product/ha treatment group. The mortality of the reference item was 100%. Mortality was not statistically significantly increased compared to the control in the 4.5 kg/ha TUREX 50 WP treatment. The mean number of mummies per female developed after 13 days in the control group was 11.14. For the treatment group of TUREX 50 WP the mean number of mummies per female was 5.00 and the reduction in reproduction rate was calculated as 55.12%. The reproduction in the TUREX 50 WP treatment was statistically significantly reduced compared to the control.

Table 10.4-1 - Mortality and reproduction rate of *Aphidius rhopalosiphi*, after treatment with 4.5 kg/ha TUREX 50 WP

Application	Mortality[1] after 48 h [%]		Fecundity (mummies/female)	
Control	0.00		11.14	
Application rate product [kg /ha]	Mortality[1] after 48 h [%]	Corrected Mortality[2] after 48 h [%]	Mummies/ female	Reduction in reproduction rate[3] [%]
4.5	7.50	7.50	5.00*	55.12
[1] Based on the number of moribund and dead organisms [2] Corrected mortality according to Schneider-Orelli (1947) [3] Calculated according to Abbot (1947) * Statistically significantly different from the control (Mann-Whitney U-Test, one-tailed, $p \leq 0.05$)				
Observations:	Based on these results it is assumed that TUREX 50 WP will have no adverse effects on mortality of *Aphidius rhopalosiphi* while a significant reduction in the reproduction rate was observed under field conditions up to the tested application rate of 4.5 kg/ha .			

Comments RMS: The test was conducted according to the IOBC guidelines. The validity criteria were fulfilled. The study is acceptable and the results can be used in the risk assessment. Infectivity and pathogenicity were not investigated. The information provided under “observations” refer to the field conditions, however please note that the test is a Tier I.

Reference: KIIM 10.4/02	Warmers, C. (2005b): TUREX 50 WP: Toxicity to the predatory mite, *Typhlodromus pyri* Scheuten (Acari, Phytoseiidae) in the laboratory (limit test). Unpublished Report No. 20051317/01-NLTp
Guideline:	ESCORT I Guidance Document (Barrett et al., 1994), ESCORT II Guidance Document (Candolfi et al., 2001) IOBC (Blümel et al., 2000)
GLP:	Yes

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Material and methods:	The study was conducted during the period 16.09.2005 to 03.10.2005, by GAB Biotechnologie GmbH & IFU Umweltanalytik GmbH, Niefern-Öschelbronn, Germany. The test material used was TUREX 50 WP (Lot no.: 0091-67-2, content of active ingredient: 3.8% insecticidal toxins (50% insecticidal toxins, spores and fermentation solids), *Bacillus thuringiensis aizawai* (strain GC91) mean potency 25000 International Units/mg –11 BIU/lb (24.3 BIU/kg) (analyzed) was diluted in water and applied with a spray application volume of 200 L/water/ha to glass plates ate the rate equivalent to 4.5 kg product/ha. Deionised water was applied as control and Perfekthion (nominal content of a.i.: 400.0 g/L dimethoate:) was applied at 12 mL product in 200 L water/ha as a reference item treatment. Twenty protonymphs of *Typhlodromus pyri* were placed with a fine-bristled brush into each replicate unit (5 units per treatment). Direct treatment effects (mortality) were assessed after 3 and 7 days. The fertility test was conducted with the TUREX 50 WP group and the control group. Reproduction assessments were carried out 9, 11, and 14 days after treatment by counting the number of females and eggs/juveniles present in each test unit and determining the cumulative number of eggs per female.
Test substance:	TUREX 50 WP
Test species:	*Typhlodromus pyri*
Number of test animals:	Twenty protonymphs of *Typhlodromus pyri* were placed with a fine-bristled brush into each replicate unit (5 units per treatment).
Treatments:	200 L/water/ha to glass plates ate the rate equivalent to 4.5 kg product/ha
Duration:	Direct treatment effects (mortality) were assessed after 3 and 7 days. The fertility test was conducted with the TUREX 50 WP group and the control group. Reproduction assessments were carried out 9, 11, and 14 days after treatment by counting the number of females and eggs/juveniles present in each test unit and determining the cumulative number of eggs per female
Test conditions:	T = 25 ± 2°C; Hum.: 75 ± 15% ; Photoperiod 16:8 L:D ; light : 2000-5000 lux
Deviations from guideline	None

Endpoint:	The mean 7-day mortality (defined as the number of dead and missing mites) of *Typhlodromus pyri* after exposure to the glass plates treated with TUREX 50 WP was 8.0% (4.5 kg/ha) compared to 1.0% mortality in the control group (Table 2). Thus, for TUREX 50 WP the corrected mortality was calculated as 7.1% (4.5 kg/ha). In the reference group the mean mortality was 88.0% and the corrected mortality was 87.9%. Mortality was not statistically significantly increased compared to the control in the 4.5 kg/ha TUREX 50 WP treatment. After 3 assessments from day 7 onwards the cumulative mean number of eggs per female in the 4.5 kg/ha TUREX 50 WP treatment was 7.9, compared to 5.9 eggs per female in the control group (Table 2). The reproduction in the TUREX 50 WP treatment was not statistically significantly reduced compared to the control.

Table 10.4-2 - Mortality and reproduction rate of *Typhlodromus pyri*, after treatment with 4.5 kg/ha TUREX 50 WP

Application	Mortality[1] after 7 days [%]		Reproduction (eggs/female)	
Control	1.0		5.9	
Application rate product [kg /ha]	Mortality[1] after 7 days [%]	Corrected Mortality[2] after 7 days [%]	Reproduction [eggs/female]	Reduction in reproduction [%]
4.5	8.0	7.1	7.9	-33.9[3]
[1] Mortality based on the number of dead and missing organisms [2] Corrected mortality according to Schneider-Orelli (1947) [3] Negative reduction in reproduction indicates a better reproduction than in the control group				
Observations:	Based on these results it is assumed that TUREX 50 WP will have no adverse effects on *Typhlodromus pyri* under field conditions up to the tested application rate of 4.5 kg/ha.			

Comments RMS: The test was conducted according to the IOBC guidelines. The validity criteria as per guideline were fulfilled. It can be concluded that the product does not have effects on *Typhlodromus pyri.* Infectivity and pathogenicity were not investigated. The information provided under "observations" refer to the field conditions, however please note that the test is a Tier I.

Results:

A summary of endpoints is given in the table below.

Table B.9.4.1: Toxic effects / Infectivity / Pathogenicity of plant protection product to arthropods other than bees

Test species	*Aphidius rhopalosiphi*
Toxicity of plant protection product	Based on these results it is assumed that TU-REX 50 WP will have no adverse effects on mortality of *Aphidius rhopalosiphi* while a significant reduction in the reproduction rate was observed up to the tested application rate of 4.5 kg/ha (i.e. 2.25 kg a.s./ha).
Test species	*Typhlodromus pyri*
Toxicity of plant protection product	Based on these results it is assumed that TU-REX 50 WP will have no adverse effects on *Typhlodromus pyri* up to the tested application rate of 4.5 kg/ha (i.e. 2.25 kg a.s./ha).

B.9.4.2 Infectiveness to arthropods other than bees

Infectivity was not investigated.

B.9.4.3 Pathogenicity to arthropods other than bees

Pathogenicity was not investigated.

B.9.4.4 Summary and risk assessment for non-target arthropod species other than bees

The toxicity of the plant protection product was investigated in the studies with *Aphidius rhopalosiphi* and *Typhlodromus pyri.* In the study with A. *rhopalosiphi* while there were no effects on mortality, significant effects on reproduction were recorded at the application rate of 4.5 kg product/ha. In case of *Typhlodromus pyri,* no effects on mortality and reproduction were recorded at the application rate of 4.5 kg product/ha.

According to the current GAP, the maximum rate per application is 2 kg product/ha with a max total rate per crop/season of 12 kg a.s./ha. According to the information provided in Volume 1, it is not expected that the spores will survive longer than 10 days (depending on the formulation) and 1 month on broccoli and celery leaves. Therefore, for the current uses it is not expected that an accumulation between applications will occur. Considering these, the tested dose of 4.5 kg/ha from the *Aphidius rhopalosiphi* and *Typhlodromus pyri* tests is considered sufficient to cover the applications as per GAP for the field and greenhouse applications.

Regarding the studies with the active substance, the results were expressed in CFU/g feed which does not allow for a direct comparison with the current application rates.

As the mode of action of the product is against Lepidopteran larvae, some effects might be expected on the non-target Lepidopteran species present in field and in the field margins.

B.9.5 Effects on earthworms

B.9.5.1 Toxicity to earthworms

PLANT PROTECTION PRODUCT

Reference: KIIIM 10.5/01	Winkler, J. (1992a): Acute toxicity earthworm test – *Eisenia foetida* according to the OECD Guideline 207. Unpublished Report No.92 10 49 014, 20.08.1992
Guideline:	OECD Guideline No. 207 (1984) Guideline for testing chemicals: Earthworm, acute toxicity test.
GLP:	Yes
Material and methods:	The study was conducted during the period 10.07.1992 to 14.08.1992, by BioChem GmbH Karlsruhe, O-7101 Cunnersdorf, Germany. The test material used was CGD 97220 I (conc. of a.i.: 3×10^{13} CFU/kg; batch no.: P 109001). Adult *Eisenia fetida* (6 months old) were exposed to concentrations of 500 and 1000 mg CGD 97220 I/kg artificial soil after a preliminary test with a dose range from 1 to 1000 mg/kg dry weight of artificial soil. Adaptation of the earthworms 24 hours before beginning of the test in the artificial soil substrate. For controlling the reproducibility of the test system the reference substance 2-Chloracetamide 100% is tested in the concentration range from 18 to 55 mg/kg dry weight of artificial soil in the interval of about six months. Deionised water was used as control substance. The treated and the control groups were set up in 4 replicates each with 10 earthworms. Mortalities of the earthworms in the individual test cages were recorded after 7 and 14 days of exposure time. Living weight of earthworms (mg/10 earthworms) was recorded at the beginning of the test and at test termination. Behaviour of earthworms was recorded at 0, 7 and 14 days after test initiation. The physico-chemical values (water retaining capacity, moisture content and pH) were measured at the start and at the end of the test.
Micro-organism	CGD 97220 I
Test species:	*Eisenia fetida*
Number of test animals:	The treated and the control groups were set up in 4 replicates each with 10 earthworms
Treatments:	Adult *Eisenia fetida* (6 months old) were exposed to concentrations of 500 and 1000 mg CGD 97220 I/kg artificial soil after a preliminary test with a dose range from 1 to 1000 mg/kg dry weight of artificial soil. Adaptation of the earthworms 24 hours before beginning of the test in the artificial soil substrate
Duration:	14 days
Test conditions:	Not indicated
Deviations from guideline	None
Endpoint:	The test substance CGD 97220 I caused no mortality with the concentrations of 500 and 1000 mg/kg d.w. At both concentrations an increase of weight was observed. This was significant at the concentration of 1000 mg/kg d.w.

Observations:	The LC_{50} and the LOEC of the test substance CGD 97220 I could not be determined, because the test substance is non-toxic at the tested concentrations. The NOEC was determined at the concentration of 1000 mg/kg dry weight of artificial soil.

Comments: The NOEC was determined at the concentration of 1000 mg/kg dry weight of artificial soil.

Comment RMS:

The test was conducted according to OECD 207. In terms of testing the microorganisms, the duration of the test is too short in order to account for infectivity and pathogenicity. It can be concluded that under the conditions of this test the test product is not toxic to earthworms.

Results:

A summary of endpoints is given in the table below.

Table B.9.5.1: Toxic effects / Infectivity / Pathogenicity of plant protection product to earthworms

Test species	*Eisenia fetida*
Toxicity of plant protection product	The test substance CGD 97220 I caused no mortality with the concentrations of 500 and 1000 mg/kg d.w. At both concentrations an increase of weight was observed. This was significant at the concentration of 1000 mg/kg d.w. test duration was too short to investigate infectivity and pathogenicity.

B.9.5.2 Infectiveness to earthworms

Based on the information provided above, *Bta* is not expected to be infective to earthworms.

B.9.5.3 Pathogenicity to earthworms

Based on the information provided above, *Bta* is not expected to be pathogenic to earthworms.

B.9.5.4 Summary and risk assessment for earthworms

The acute toxicity to earthworms was investigated in the test by Winkler, J. (1992a). under the conditions of this test there was no mortality to earthworms at concentrations of 500 and 1000 mg/kg d.w. concentrations which are 31 and 62 times, respectively higher than intial PED in soil. Therefore no acute toxicity to earthworms from the application of the current product is expected.

Earthworm immunity has extensively been studied and earthworms have served as an important experimental model for immunologic research. Earthworms have evolved effective innate defence mechanisms for survival in often hostile habitats, and have experienced a long time of co-evolution

with micro-organisms in their environment (Bilej, 2010). Earthworms are capable to cope with soil-borne micro-organisms without being infected or negatively affected due to the long-time evolutionary co-existence (SANCO/12117/2012)[1]. Only few studies indicated pathogenic effects to earthworms, however the observed pathogenic effects of *Bacillus thuringiensis* subsp. *thuringiensis* in a prolonged study with *Lumbricus terrestris* were not attributed to the tested mBCA (Smirnoff & Heimpel, 1961; cited in SANCO/12117/2012). Addison and Holmes (1996, cited in SANCO/12117/2012) observed detrimental impacts of Bt-formulations on earthworms and other non-target soil organisms, but found no effect of unformulated and aqueous Btk at 1000 times the field concentration.

B.9.6 Effects on non-target soil micro-organisms

Plant Protection Product

Reference:	Winkler, J. (1992b), Effects on the activity of soil microflora according to the BBA Guideline VI, 1-1 (1990). Unpublished Report No. 961049013, 01.12.1992
Guideline:	BBA-Guideline Part VI, 1-1, 2nd edition, March 1990
GLP:	None
Material and methods:	The study was conducted during the period 02.09.1992 to 30.09.1992, by BioChem GmbH Karlsruhe, O-7101 Cunnersdorf, Germany. The test material used was CGD 97220 I (content of a.i.: 3 x 10^{13} CFU/kg; batch number: P 109001). The possible effects of CGD 97220 I on the metabolic activity of the microbial biomass were examined by means of the nitrogen turnover and dehydrogenase activity. Prior to the application, the initial content of mineral nitrogen was measured in 3 parallel soil samples. Following 100 g d.m. soil were mixed thoroughly with 0.5% lucerne meal. - Untreated control soil samples were prepared only with lucerne meal. Two soil types were used . CGD 97220 I was incubated in darkness over a period of 28 days at an amount corresponding to 2.0 L/ha and 20.0 L/ha, assuming a penetration depth of 5 cm and a soil gravity of 1.5 g/cm^3. The water content of the soil type was adjusted to 40% of its maximum water holding capacity. The control consisted of a treatment group with deionised water. A reference group was tested at a concentration of 15 L (soil 1: nitrogen turnover; soil 1 + 2: dehydrogenase activity) and 35 L (soil 2: nitrogen turnover) ARETIT/ha (a.i.:Dinoseb-acetate with 492 g/L) to demonstrate the normal sensitivity of the soil microflora against pesticides. Four study groups with 3 replicates for nitrogen turnover and 4 replicates for dehydrogenase activity were performed. Soil samples were taken 3 h, 14 d, 28 d after the application. For the quantitative determination of the mineralized part of nitrogen a photometrical assay using a continuous analysis automaton (ADM 300) was used. To each sampling a measurement of pH-value was carried out for control of the test system. The dehydrogenase activity assay is based on the enzyme reduction of Triphenyltetrazoliumchloride (TTC) to Triphenylformazan (TPF). For the determination of the TPF-content a Shimadzu UV-VIS Recording Spectrophotometer UV-160 was used at 485 nm wave-length.
Test substance	CGD 97220 I
Test species:	Soil microflora

[1] Working Document to the Environmental Safety Evaluation of Microbial Biocontrol Agents, SANCO/12117/2012-rev.0, September 2012, EUROPEAN COMMISSION HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL Directorate E – Safety of the food chain Unit E.3 – Chemicals, contaminants, pesticides.

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Treatments:	Four study groups with 3 replicates for nitrogen turnover and 4 replicates for dehydrogenase activity were performed. Untreated control soil samples were prepared only with lucerne meal. Two soil types were used
Duration:	Soil samples were taken 3 h, 14 d, 28 d after the application
Deviations from guide-line	None
Endpoint:	The content of NO_3, N_{min} and NH_4-N were normal during the experimental time. The test substance CGD 97220 I in both concentrations and both soils showed no negative effects neither to the nitrogen mineralization nor to the nitrification during the experimental time of 28 days. The reference substance ARETIT flüssig exhibited inhibition of the nitrification in both soils during the experimental time depending on the concentration used. The value of pH in both soils showed no distinct detectable variations. During the experiment the dehydrogenase activity was not affected by CGD 97220 I. The reference substance ARETIT flüssig showed a distinct inhibition of the dehydrogenase activity up to 82%.
Observations:	CGD97220 I had no negative influence neither to the nitrogen turnover nor to the dehydrogenase activity in both soils. In conclusion, the test substance CGD 97220 I represents no hazard to soil microflora.

Comments RMS: RMS agrees with the conclusion of the original DAR, namely that the used doses of 2 and 20 L/ha (2 and 20 kg/ha) corresponded to 2.66 and 26.2 mg product/kg soil dw., respectively, or $7.98x10^7$ and $7.98\ x10^8$ CFU/kg soil dw. No negative influence neither to nitrogen turnover nor to dehydrogenase activity could be determined in both soils.

Risk assessment soil micro-organisms

The maximum tested dose of 26.2 mg product/kg soil dw was 1.6 times higher than the PEDsoil of 16 mg product/kg soil dw. The calculated application rate represents a worst-case as it represents an accumulated application rate.
Since there were no effects at the maximum application rate, it can be concluded that there is no risk to soil microflora from the application of the product.

B.9.7 Effects on terrestrial plants

According to the information provided in the previous version of the DAR *Bacillus thuringiensis* spp. *aizawai*, is toxic specifically to insects of the Lepidoptera order and no effects on terrestrial plants from applications of Bta in insecticidal formulations targeted specifically at these insects is expected This is further envisaged considering results from studies on algae. In addition evidence from over fifty phytotoxicity trials performed on twelve different crops in the USA with Javelin Biological Insecticide and SAN 415I SC 353 support the lack of toxicity expected on terrestrial plants following application of Btk containing formulations in the fields. The highest mean toxicity was observed in trials (n = 3) with Bok choy (*Brassica chinensis*) at 4.3%, the next highest mean toxicity was in trials (n = 10) with Sugar beet (*Beta vulgaris*) at 1.7% (Anonymous, year unknown). These results may be extrapolated to Bta due to their family relationship.

B.9.8 Additional studies

No additional studies were submitted.

B.9.9 References relied on

Data point	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
KMP 10.2/01	Dengler, D.	2010	Assessment of toxic effects of Agree WG on Daphnia magna using 48h Acute Immobilisation Test Certis USA LLC, S10-02545 Eurofins Agroscience Services GmbH GLP: yes Published: no	no	yes	New data for existing formulation, not previously submitted nor evaluated	CEU
KMP 10.3/01	Kleiner, R.	1992	Testing toxicity to honeybee - Apis mellifera L. (laboratory) according to BBA Guideline VI, 23-1 (1991) Certis USA LLC, 92 10 48 068 BioChem Agrar, Cunnersdorf, Germany GLP: yes Published: no	no	no	not protected	CEU

KMP 10.4/01	**Warmers, C.**	**2005a**	**TUREX 50 WP: Acute Toxicity to the Aphid Parasitoid, Aphidius rhopalosiphi De Stefani Perez (Hymenoptera, Braconidae) in the Laboratory (Limit Test)** **Certis USA LLC, 20051317/01-NLAp** **GAB Biotechn. GmbH & GAB Analytik GmbH, Niefern-Öschelbronn** **GLP: yes** **Published: no**	**no**	**no**	**not protected**	**CEU**
KMP 10.4/02	**Warmers, C.**	**2005b**	**Turex 50 WP: Toxicity to the Predatory Mite, Typhlodromus pyri Scheuten (Acari, Phytoseiidae) in the Laboratory (Limit Test)** **Certis USA LLC, 20051317/01-NLTp** **GAB Biotechn. GmbH & GAB Analytik GmbH, Niefern-Öschelbronn** **GLP: yes** **Published: no**	**no**	**no**	**not protected**	**CEU**
KMP 10.5/01	**Winkler, J.**	**1992a**	**Acute toxicity earthworm test - Eisenia foetida according to the OECD Guideline 207** **Certis USA LLC, 921049014** **BioChem GmbH, Cunnersdorf, Germany** **GLP: yes** **Published: no**	**no**	**no**	**not protected**	**CEU**

KMP 10.6/01	**Winkler, J.**	**1992b**	**Effects on the activity of soil microflora according to the BBA Guideline VI, 1-1 (1990)** **Certis USA LLC, 921049013** **BioChem GmbH, Cunnersdorf, Germany** **GLP: yes** **Published: no**	**no**	**no**	**not protected**	**CEU**