Renewal
Assessment Report

Bacillus thuringiensis ssp. aizawai strain ABTS-1857

Volume 3 – B.8 Fate and behavior in the environment

Rapporteur Member State: The Netherlands

Co-Rapporteur Member State: Germany

Version history

When	What
September 2018	Initial version of the RAR

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B Summary of the data and information

B.8 Fate and behavior in the environment

Bacillus thuringiensis subsp. *aizawai* strains ABTS-1857, GC-91 were included in Annex I to Directive 91/414/EEC (2008/113/EC) on 1 May 2009 pursuant to Article 24b of the Regulation (EC) No 2229/2004 and has subsequently been deemed to be approved under Regulation (EC) No 1107/2009 in accordance with Commission Implementing Regulation (EU) No 540/2011 as amended by Commission Implementing Regulation (EU) No 541/2011.

European Food Safety Authority (EFSA) revised the draft review report submitted by the European Commission and EFSA's conclusion was published in the peer review (EFSA Journal 2013; 11(1): 3063).

Italy was designated rapporteur Member State and the DAR was issued in 2008. Parts of the original DAR are marked grey.

The plant protection product XenTari® WG is considered representative of uses of the active substance *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 for the purposes of renewal of the approval of the active substance under EC Regulation 1107/2009 according to Regulation EU 283/2013.

Introduction

Bacillus thuringiensis are rod-shaped, gram positive, aerobic bacteria, approximately 1 by 5 microns long. *Bacillus thuringiensis* is a naturally occurring, spore-forming bacteria, which is used as a microbial pest control agent (MPCA). Upon sporulation, *Bacillus thuringiensis* forms crystals of proteinaceous insecticidal δ-endotoxins (also referred to in the literature as crystalline proteins, cry proteins, Cry1Ab protein, Cry1Ab toxins, insecticidal crystal proteins (ICPs), parasporal crystals, parasporal protein-crystal, parasporal crystalline inclusions), which are encoded with *Cry* genes. These crystalline proteins consist predominantly of a single species of polypeptide called protoxins. Once ingested by a susceptible insect (*e.g.* Lepidoptera), the protoxins are solubilised under the alkaline conditions of the insect midgut; the protoxins are proteolytically activated by proteases to become activated Cry toxins. The activated Cry toxins then bind readily to specific receptors on the apical brush border of the midgut microvillae of susceptible insects, leading to cell disruption and consequently death of the insect.

There are several different subspecies (or serovars/serotypes) of *Bacillus thuringiensis*. The fate and behaviour of *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 is assessed. *Bacillus thuringiensis* subsp. *aizawai* is the MPCA component of the product XenTari® WG; a summary of the XenTari® WG GAP is presented in Table B.8-1 below.

Table B.8-1 Summary of critical GAP for XenTari WG

Crop and/or situation	Formulation conc. of MPCA	Application		Application rate per treatment			PHI[1] (days)
		Growth stage & season / Method	Number / Interval between applications (min)	MPCA g/hL min - max	Water L/ha max	MPCA kg/ha max	
Outdoor fruit vegetables (pepper)	54% w/w Approx. $5x10^{13}$ cfu/kg	BBCH 09-BBCH 92 (May-Oct) Start when larvae hatch (L1) Spray	1 – 8 applications, (1 - 3 per generation of pest) 6 – 10 day minimum interval (typically 7 days)	0.027 – 0.054 kg a.s. /hL (0.050 – 0.100 kg f.p./hL)	Typically 500-1000 L/ha	0.270–0.540 kg a.s./ha. (0.500-1.0 kg f.p./ha) Approx. $5x10^{13}$ cfu/ha	0
Indoor fruit vegetables (pepper)	54% w/w Approx. $5x10^{13}$ cfu/kg	BBCH 09-BBCH 92 (Jan-Dec) Start when larvae hatch (L1)	1 – 7 applications, (Typically 1 - 3 per generation of pest) Typically 7 days minimum interval	0.027 – 0.054 kg a.s./hL (0.050-0.100 kg f.p./hL)	400–1000 Typically 1000 L/ha	0.270 – 0.540 kg a.s./ha. (0.500–1.0 kg f.p./ha) Approx. $5x10^{13}$ cfu/ha	0

[1] pre-harvest interval

Definition of the residue

Based on the information provided in the Identity of the Plant Protection Product part, the MPCA present in the formulated product XenTari WG is *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857, which includes non-viable vegetative cells, spores and insecticidal toxins. The product also contains fermentation solids.

The component of concern that will be assessed to address persistence and multiplication in soil is the MPCA *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 only, in its complete form (*i.e.* including non-viable vegetative cells, spores and insecticidal toxins).

The component of concern that will be assessed to address persistence and multiplication in groundwater, surface water and sediment is the MPCA *Bacillus thuringiensis*

subsp. *aizawai* strain ABTS-1857 only, in its complete form (*i.e.* including non-viable vegetative cells, spores and insecticidal toxins).

B.8.1 Persistence and multiplication

B.8.1.1 Soil

The fate and behaviour of *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 in the terrestrial environment was evaluated during the Annex I Inclusion. No additional studies have been performed. The fate and behaviour of *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 in soil is discussed in detail in the draft assessment report (DAR, 2008 and Addendum, 2011) where the references cited from the scientific literature can be found. A brief overview of this information is provided below.

The corresponding part of the DAR is added for reference:

> **B.8.1 Persistence and multiplication**
>
> *Bacillus thuringiensis* occurs naturally and ubiquitously in the environment. It is a common component of the soil micro-flora and has been isolated from most terrestrial habitats. In the natural environment, under favourable conditions, *Bacillus thuringiensis* cells exist in an active vegetative state where growth and colony formation can occur. When conditions for continued growth and survival become unsuitable, sporulation occurs, during which endospores and crystalline inclusions, or proteins are formed and the vegetative cells lyse. The endospores exist in a cryptobiotic state and can be quite durable. The crystalline proteins are the source of delta-endotoxins, which are damaging to highly specific insect species. When insects ingest crystal proteins, alkaline conditions in the gut initiate breakdown of the proteins, releasing the delta-endotoxins. These immediately begin to interfere with internal cell gut structure, leading to a cessation of feeding and eventual starvation. Unlike most insect pathogenic microbes, *Bacillus thuringiensis* is a poor infectious agent and rarely recycles. While vegetative cells and spores can be produced in insect cadavers, *Bacillus thuringiensis* has rarely been recorded causing natural epizootics in insects, leading to speculation that it is essentially a soil micro-organism that possesses incidental insecticidal activity.

It can be concluded that the survival of *Bacillus thuringiensis* in soil is a dynamic process involving sporostasis, germination and sporulation in specific habitats. Survival is influenced by changing conditions regarding soil type, temperature, pH, moisture, photo-degradation, native micro flora, nutrient availability and fertilisation. Therefore, with the exception of some months following application, the application of *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 to soil is not expected to significantly increase the number of *Bacillus thuringiensis* present in soil (background levels of spores reported to range between 2 x 10^2 and

4.9 x 10^4 CFU/g). Vegetative cells of *Bacillus thuringiensis* do not multiply and spores do not germinate in soils where the number of inoculated cells decreases more than 50% before sporulation starts.

The corresponding part of the DAR is added for reference:

B.8.1.1 Soil

Following commercial field applications of formulations containing *Bacillus thuringiensis* endospores and parasporal crystals, some viable spores are expected to survive. These can remain inactive and immobile in soil for several months or even years, during which time a natural breakdown occurs, resulting in gradual spore mortality. However, the vegetative cells and crystal proteins of *Bacillus thuringiensis* are far more rapidly degraded in soil. The actions of indigenous micro-organisms, which compete for nutrients and produce proteolytic enzymes that degrade the protoxin, lead to a rapid loss of potency and insecticidal activity in soil. The photodegradation effects of sunlight also affect the survival and growth of endospores and vegetative cells in the environment. *Bacillus thuringiensis* endospores rarely germinate in soil unless favourable conditions exist in combination such as neutral to alkaline pH, sufficient nutrient availability, favourable soil temperature and moisture content and lack of competition / predation from other soil micro-organisms. *Bacillus thuringiensis* is not therefore adapted to survive as an active member of the soil microbial community and the low potential for spore germination, growth and re-sporulation restricts population growth.

In many of the studies to investigate the behaviour of *Bacillus thuringiensis* in soil, which are reported in the scientific literature, spores and parasporal crystals were initially added to soil at concentrations far greater than is recommended for insect control in the field, in order to measure degradation over time. It is also expected that field applications of *Bacillus thuringiensis* subsp. *aizawai* to foliage will reduce potential soil exposure, resulting in lower soil concentrations, which are well below insecticidal levels. Degradation in soil is also likely to exceed the rate of acquisition from repeated foliar sprays, such that populations of applied *Bacillus thuringiensis* bacteria reaching the soil should decline over time to the fluctuating natural level.

General Comments. Specific studies on the persistence and mobility in soil of *Bacillus thuringiensis ssp. aizawai* strain ABTS-1857 have not been submitted by the notifier. Therefore, the previous statements on *Bacillus thuringiensis ssp. aizawai* strain ABTS-1857 are based on reported characteristics, as found in the scientific literature, under consideration of the envisaged application and relevant properties of *Bacillus thuringiensis ssp. aizawai* strain ABTS-1857.

The new information provided below has been included to address the data gaps identified following the European Food Safety Authority's (EFSA) (EFSA Journal 2013; 11(1): 3063) conclusion on the peer review of the pesticide risk assessment of the active substance *Bacillus thuringiensis* subsp. *aizawai*.

EFSA data gaps

- The scientific literature quoted by the notifier of strain ABTS 1857 in its assessment of the fate and behaviour in the environment are not available in the dossier (relevant for all representative uses evaluated; submission date proposed by the notifier: no date proposed; see section 4).
- Information in relation to potential interferences of *Bacillus thuringiensis* with the analytical systems for the control of the quality of drinking water provided for in Directive 98/83/EC (relevant for all representative uses evaluated; s ubmission date proposed by the notifier: no date proposed; see section 4).
- No information has been provided on the potential transfer of genetic material from *Bacillus thuringiensis aizawai* strains ABTS 1857 and GC-91 to other organisms (relevant for all representative uses evaluated; submission date proposed by the notifier: no date proposed; see section 2 and 4).
- Information to demonstrate that, under the conditions of use, *Bacillus thuringiensis aizawai* strains ABTS 1857 and GC-91 crystalline proteins (δ endotoxins) or their transformation products retaining insecticidal activity will not contaminate groundwater above the regulatory limit of 0.1 µg/L. Further data on the persistenc e, transformation and mobility of δ endotoxins may be needed in order to fulfil this data gap (relevant for all representative uses evaluated; submission date proposed by the notifier: no submission date provided; see section 4).

Summary of new information on persistence and multiplication in soil

The EFSA peer review of the pesticide risk assessment of the active substance *Bacillus thuringiensis* subsp. *aizawai* highlighted that it should be demonstrated that, under the conditions of use, *Bacillus thuringiensis* subsp. *kurstaki* (Btk) crystalline proteins (δ-endotoxins) or any of their transformation products retaining insecticidal activity will not contaminate groundwater above the regulatory limit of 0.1 µg/L. The new information on persistence and multiplication in soil has been included to address this data gap.

With regards physical properties, Calabrese *et al*. (1980) used gel electrophoresis to determine the number and size of the subunits present in the protein crystals from 16 strains of *Bacillus thuringiensis*. The calculated molecular weights (MW) fell into three major categories whose crystals exhibited the following protein banding patterns: type I, high MW only (140,000-160,000 Da); type II, both high MW and medium MW (60,000 and 150,000 Da); and type III, low MW only (40,000-50,000 Da). Faust *et al*. (1974) showed the possible molecular weights of the δ-endotoxin to be 230,000 Da.

To address persistence of the crystalline protein (δ-endotoxin), various half-lives have been found in the literature, ranging from hours and days to months. Haddad *at al*. (2005) found the half-life of viable *Bacillus thuringiensis* spores in maize leaves to be 18.4, 16.5 and 13.6 hours for a half-dose, dose and double-dose of Dipel (containing Btk), respectively. Sundaram *et al*. (1996) measured the effect of two tracer dyes (Erio Acid red (EAR) and Acid Black 48 (AB-48)) on the persistence of *Bacillus thuringiensis* subsp. *kurstaki* δ-endotoxin after spraying two commercial formulations (Foray 48B and Foray 76B). The δ-endotoxin persisted on foliage only for 7 d post-spray when the EAR dye was added to Foray 48B, compared to 10 d when no dye was added. The average DT_{50} of disappearance was 17.4 h for Foray 48B with EAR and 20.9 h when no dye was added. The average DT_{50} was 27.9 h for Foray 76B with EAR and 22.2 h when no dye was added. Persistence was the longest (14 d) when the AB-48 dye was added to the Foray 76B (DT_{50} of 44.9 h). Although these half-lives are measured on foliage and not soil, this information shows that degradation of *Bacillus thuringiensis* is fairly rapid when exposed to sunlight.

West *et al*. (1984; already evaluated in DAR of 2008) found that there was a rapid loss of parasporal crystal insecticidal activity in natural soil, which was attributed to the presence of microbiota. The parasporal crystal was found to retain full insecticidal activity for three days and then decline. The study by West (1984; already evaluated in DAR of 2008) quantified the DT_{50} of the parasporal crystal in untreated soil as 2.7 days. The extent of degradation was significantly reduced by addition of soluble organic supplement (5.2-5.8 days). This study also indicated that the parasporal crystal protoxins are readily degraded by soil microorganisms.

Similar half-lives of *Bacillus thuringiensis* of 2.4-3.1 days and < 3 days have also been determined in field cotton (Ali and Young, 1993) and maize and common bean leaves (Sánchez-Yáñez and Peña-Cabriales, 2000), respectively. As well as finding that *Bacillus thuringiensis* spores had a limited persistence of less than 3 days on maize and common bean leaves,

Sánchez-Yáñez and Peña-Cabriales (2000) also found that *Bacillus thuringiensis* spores were not viable after inoculation in sterilised soil, indicating that the organic matter of the sterilised soil did not favour spore persistence. In the non-sterilised soil, competition and predation from indigenous organisms caused rapid loss of spore viability. This suggests that, at least under these experimental conditions (maize and common bean leaves and the soil tested) *Bacillus thuringiensis* spores were unable to persist.

Bai *et al*. (2007) investigated the impacts of soil water content, pH and temperature on degradation of Cry1Ab protein. Half-life values were measured in the range of 1.8 to 4.0 days. Degradation was found to be affected by soil water content, pH and temperature but effects of soil pH and temperature were relatively greater. Degradation was generally slower under lower soil pH and temperature conditions. Hung *et al*. (2016) determined the half-life of the biopesticide under natural soil conditions to be approximately one week. Feng *et al*. (2011) determined the DT_{50} of Cry1Ab protein released from 34B24 and 1246 x 1482 straw to be 0.97-9.97 d and 0.75-10.89 d, respectively. The results suggested that soil temperature had significant effects on the degradation of Cry1Ab protein, with a higher degradation rate at higher temperature, but soil water content and pH had no obvious effects on the degradation of Cry1Ab protein.

The degradation of the cry protein has been shown to be more rapid in water than soil ($t_{1/2}$ four days in water, nine days in soil) and that presence of the cry protein is fairly uncommon in aquatic environments (Douville *et al*., 2005). In Eastern red cedar, Hostetter *et al*. (1975) found that residual activity of Dipel could still be detected 14 d post-treatment. Wang *et al*. (2007) found rapid degradation of Cry1Ab protein in paddy soils under aerobic conditions, with half-lives ranging from 19.6 to 41.3 days.

The results from the studies by Vettori *et al*. (2003) conducted in Sardinian soils, and Hendriksen and Carstensen (2013) showed that *Bacillus thuringiensis* subsp. *kurstaki* can persist for protracted periods of time in soil. The results from Vettori *et al*. (2003) indicated that *Bacillus thuringiensis* subsp. *kurstaki* and its toxin introduced into soils in sprays can persist for at least 88 months for *Bacillus thuringiensis* subsp. *kurstaki* and at least 28 months for its toxin. The results from Hendriksen and Carstensen (2013) showed that the bacterium could survive at relative low densities in 13 years after spraying. However, it is important to note that the levels of *Bacillus thuringiensis* subsp. *kurstaki* measured were below, or comparable with, typical background levels, thus posing no risk to the environment.

The results of these studies show that the persistence of the microbial pest control agent (MPCA) *Bacillus thuringiensis* and its crystalline protein (δ-endotoxin) is largely a function of the study conditions (*i.e.* soil type, temperature, pH, moisture, photo-degradation, native microflora etc.). As such, under the majority of conditions *Bacillus thuringiensis* and its crystalline protein (δ-endotoxin) is degraded relatively quickly, however as demonstrated by Vettori *et al*. (2003) and Hendriksen and Carstensen (2013) it is not impossible for *Bacillus thuringiensis* subsp. *kurstaki* and its crystalline protein (δ-endotoxin) to persist in soils for extended periods of time, although the levels of *Bacillus thuringiensis* subsp. *kurstaki* measured did not exceed typical background levels. Since *Bacillus thuringiensis* subsp. *aizawai* and its crystalline protein (δ-endotoxin) occur naturally and ubiquitously in the environment, it is expected the levels reach background concentrations and densities within weeks after application. Over time (typically in the range of approximately 1-14 days) levels of *Bacillus thuringiensis* subsp. *aizawai* and will decline to numbers of bacteria found in the soil naturally.

The following studies are adequate for the purpose of addressing the data requirements.

New information

Report:	KMA 7.1.1/01, West, A.W., Burges, H.D., White, R.J., and Wyborn, C.H. (1984)
Title:	Persistence of *Bacillus thuringiensis* parasporal crystal insecticidal activity in soil
Document No:	Journal of Invertebrate Pathology (1984), 44: pp.128-133
Guidelines:	No
GLP:	No
Present in DAR (2008)	Yes

Executive summary

Spores and parasporal crystals of a *Bacillus thuringiensis* var. *aizawai* (H-serotype 7), strain HD137, streptomycin-resistant mutant were added to acidic (pH) natural and autoclaved soil and incubated at -0.10 MPa, 25°C. Populations of *B. thuringiensis* in both soil treatments showed exponential rates of mortality which were represented by linear regression, the loss of viability being greater in natural than autoclaved soil. In natural soil, parasporal crystal insecticidal activity was lost at a complex, non-exponential rate. The initial, rapid decrease of activity gradually slowed, and the level of activity stabilised at 10% of the original inoculums level after 250 days incubation, until the end of sampling at > 2 years. In autoclaved soil, no significant loss of parasporal crystal insecticidal activity was detected over the same period,

suggesting that soil microorganisms were responsible for the loss of crystal insecticidal activity in the natural, non-sterilised soil.

I MATERIALS AND METHODS

Parasporal crystal persistence in soil was determined independently by measurement of insecticidal activity, and compared with spore viability and *G. mellonella*-based potency estimates of previous studies. The role of indigenous soil microorganisms in parasporal crystal persistence in soil was indirectly assessed.

A Materials

The soil was an arable, fallow clay loam that had been used for cereal production in the previous season. Soil was composite-sampled in the depth 2-15 cm, and the sample was air-dried to >- 1.57 MPa (permanent wilting point), sieved to ≤ 2 mm particle size, and stored in darkness at 2°C. The pH of natural soil was 5.0 and of autoclaved soil was 5.1. The total organic carbon content was 2.3% by weight, and the carbon:nitrogen ratio was 15:4.

The isolate of *B. thuringiensis* was a streptomycin-resistant mutant, var. *aizawai*, H-serotype 7, strain HD137. This isolate was active against larvae of *Peris brassicae,* therefore a parasporal crystal assay was used based on this insect, and the resistance to streptomycin conferred use of the antibiotic-resistance-selective-medium technique for extraction of *Bacillus thuringiensis* from soil. The media used to estimate both aerobic heterotrophic soil bacteria and inoculated *Bacillus thuringiensis* were based on peptone-yeast extract agar. The soil bacteria medium contained 100 µg nystatin/mL and was incubated for 10 days at 25°C. The *B. thuringiensis* medium contained 50 µg streptomycin and 20 µg nystatin/mL, and was incubated for 3 days at 30°C.

B Inoculation and Extraction

Air-dried portions of natural and autoclaved soil (10 g), held in 29 mL vials plugged with cotton wool, were aseptically wetted with sterile distilled water to give a moisture availability of -0.10 MPa. The water contained a *B. thuringiensis* inoculum of 10^9 spores and crystals/g soil on an oven dry basis. Controls containing no inoculum were also included. The soil was incubated in darkness at 25 ± 1°C and moisture availability was maintained constant. To extract bacteria, duplicate vials of soil containing *B. thuringiensis* and triplicate vials of soil without *B. thuringiensis* were blended for 5 min in 50 m 0.1% peptone water. Suspensions of soil were immediately diluted for bioassay and for total population estimated by pour-plating. Fol-

lowing plating the suspensions were pasteurised and replated to estimate the *B. thuringiensis* spore population as a fraction of the total *B. thuringiensis* (nonpasteurised) population.

C Bioassay

Parasporal crystal amounts in soil were estimated by a bioassay based in the feeding of *P. brassicae* larvae (Burgerjon, 1962). *P. brassicae* larvae were visually selected for even size one day past the third molt. Groups of eight larvae were transferred to 9 cm Petri dishes, each containing a moistened filter paper and a freshly cut square of cabbage leaf (36 cm²). The upper surface of each leaf had previously been coated with 72 µL of 0.1% peptone water containing 0.02% Triton X-100 and 3% polyethylene glycol, with or without a suspension of soil and *B. thuringiensis,* and allowed to dry. Five replicate dishes were used per bacterial concentration, and seven concentrations per treatment. All larvae were left to feed at 20°C for either 24 h or until 80-90% of control leaf squares (no bacteria added) had been consumed. The uneaten leaf area was then measured on a leaf scanner and integrator.

D Statistics

The uneaten leaf was logit-tranformed and plotted against log concentration; the linear response was compared using PARLIN. This calculated an overall reciprocal potency ratio between a bacterial standard used in each experiment and the *Bacillus thuringiensis* extracted from soil. Loss of parasporal crystal insecticidal activity with incubation time in soil was represented by linear regression analysis on log-transformed potency and log time.

II RESULTS AND DISCUSSION

Both *Bacillus thuringiensis* and indigenous soil bacteria in natural soil, and also *Bacillus thuringiensis* in autoclaved soil, declined exponentially. The mortality rate of the indigenous soil bacteria was very low (0.0008) and the predicted regression analysis mortality by 1000 days incubation indicated a reduction of viable organisms of only 84.15%. In comparison, the rate of mortality in natural soil (0.0044) was 5.5 times greater, so that the predicted mortality after 1000 days was 99.996%. The mortality rate in autoclaved soil (0.0010) was considerably less than in natural soil, and similar to that of the indigenous soil bacteria. The predicted reduction in viable spores after 1000 days incubation was 90%. No spore germination was detected by comparison of natural and pasteurised samples.

In natural soil the parasporal crystal retained full insecticidal activity, detected by bioassay, for only 3 days and then declined. Persistence of crystal in natural soil therefore required

analysis by segmented linear regression, utilising log transformations of both the reciprocal potency ratios and the period of incubation, meaning that the rate of loss was not exponential. The predicted loss of insecticidal activity was 77.30% within 100 days and 91.55% within 1000 days incubation. In contrast, in autoclaved soil no significant loss of parasporal crystal insecticidal activity was detected throughout the 842 days of incubation. Soil without *B. thuringiensis* did not show insecticidal activity.

III CONCLUSIONS

The rate of *B. thuringiensis* spore mortality was greater in natural than in autoclaved soil. As no germination or growth of spores was detected in either natural or autoclaved soil, it was assumed that no additional parasporal crystal was produced in soil in the period of incubation. The parasporal crystal rapidly disappeared (loss of insecticidal activity) in natural soil, but remained stable (no loss of activity) in autoclaved soil. As the soil pH was acidic in both soil treatments, this disparity in activity strongly suggests that the loss of crystal in natural soil was due to degradation mediated by the indigenous soil microorganisms. While the parasporal crystal appears susceptible to microbial attack in soil, the spore appeared more stable and resistant.

Comments RMS: Persistence of *B. thuringiensis* in soil is dependent on microbial competition. West et al. (1984) showed that the rate of *B. thuringiensis* spore mortality was greater in natural soils than in sterilised. The abundance of *B. thuringiensis* rapidly diminishes in unsterilized soils but may increase in sterilized soils. The study can be used for risk assessment and is acceptable.

Report:	KMA 7.1.1/02, Bai Y-Y., Jiang, M-X. and Cheng, J-A. (2007)
Title:	Impacts of environmental factors on degradation of Cry1Ab insecticidal protein in leaf-blade powders of transgenic *Bt* Rice
Document No:	Agricultural Sciences in China (2007), 6(2): pp.167-174
Guidelines:	No
GLP:	No
Present in DAR (2008)	No

Executive summary

The impacts of soil water content, pH, and temperature on the degradation of CrylAb protein expressed in the leaves of *Bacillus thuringiensis* rice KMD2 were studied in the laboratory. Three types of paddy soils were used, *i.e.* blue clayey paddy soil, pale paddy soil on quaternary red soil, and marine-fluvigenic yellow loamy paddy soil. Ground powders of KMD2 leaf

blades were mixed with each type of soil, and degradation dynamics of CrylAb were measured using enzyme-linked immunosorbent assay (ELISA). The degradation rate of CrylAb was high at the early experimental stage, but slowed down steadily at middle and later stages, which could be described by exponential equations, with the half-life period of degradation determined as 1.8-4.0 d. The soil water content, pH, and temperature could affect the degradation of Cry1Ab, but the effects of soil pH and temperature were relatively greater. In general, Cry1Ab degradations were slower under lower soil pH and temperature conditions, especially for marine-fluvigenic yellow loamy paddy soil.

I MATERIALS AND METHODS

Degradation dynamics of Cry1Ab toxins obtained from leaf-blade powders of Kemingdao (KMD) were inspected in three paddy soils under different water contents, soil pH and temperatures using enzyme-linked immunosorbent assays (ELISA).

A Materials

Rice plants

The transgenic *Bacillus thuringiensis* line Kemingdao 2 (KMD2) was derived from a Chinese commercial japonica rice variety Xiushui 11 (XS 1 l**),** transformed by *Agrobacterium* infection. KMD2 was homozygous and contained a synthetic *crylAb* gene derived from *B. thuringiensis* under the control of a maize *ubiquitin* promoter. They were reported to be highly resistant to eight lepidopterous pests of rice under laboratory and field conditions. Thirty-day-old plants were transplanted in a greenhouse. The KMD2 leaf blades were collected at mid-tillering stage when Cry1Abs were highly expressed. The leaf blades were chopped into 8-cm long pieces and immediately submerged in 75% alcohol for sterilization for approximately 1 min, and then placed on dry ice before storage at -80°C. Frozen material was lyophilized, ground using a mortar and pestle with liquid nitrogen, and passed through a standard sieve of 2 mm diameter to achieve a homogeneous fine powder for use in the study. ELISA analysis of the lyophilized transgenic rice powder indicated that the level of active Cry1Ab protein was 4.86 µg/g.

Soil preparation

Three types of experimental soils were collected at the depth of 0-15 cm in rice paddies of Hangzhou, Jinhua, and Jiaxing cities of Zhejiang Province, China. The soils were categorised as marine-fluvigenic yellow loamy paddy soil, pale paddy soil on quaternary red clay, and blue clayey paddy soil. The fresh soils were dried in air under shade, passed through a 2 mm

sieve, homogenised and stored in continuous darkness at 25 ± 1°C until use. Each soil was analysed to determine major physic-chemical properties.

B Study Design

Plant tissue systems were maintained in climatically controlled chambers with relative humidity 85-90%. For each type of soil, 2.5 g soil (dry weight) and 0.5 g of transgenic rice tissue (lyophilised powder) were placed into each of 135 glass tubes (1.5 cm diameter, 12 cm long). The tubes were shaken to thoroughly mix the contents. Each tube was covered using a cap with a hole (1-2 mm diameter) at the centre to facilitate air exchange. The pre-incubation concentration of active Cry1Ab protein in each tube was estimated to be 2.43 µg/g. For each environmental factor (water content, pH, temperature), there were three treatments, with each having three replicates for each type of soil.

The moisture contents were maintained at 50, 70 and 100% of water-holding capacity (WHC) for the three treatments by adding distilled water to the dry soil samples. At the 50% soil WHC, the estimated pre-incubation concentration of active Cry1Ab protein was 690, 640, and 650 ng per fresh gram of marine-fluvigenic yellow loamy paddy soil, pale paddy soil on quaternary red soil, and blue clayey paddy soil, respectively. All the tubes were covered with plastics lids having small holes and incubated at 25°C in darkness for 35 d. The moisture was maintained constant by adding distilled water at regular intervals throughout the incubation period. At the intervals of 7 d, three tubes (three replicates) in each treatment were taken out to analyse CrylAb contents. All the results were obtained as the average of the three replicates and were expressed on 50% WHC (water holding capacity) basis.

The moisture content of the soil samples was adjusted first (as above), then soil pH was adjusted to acidic (pH < 6.5), neutral (6.5 < pH < 7.5), and alkaline (pH > 7.5) values; the soil pH was increased by the addition of lime or decreased by the addition of dilute sulfuric acid, followed by incubation at 25 ± 1°C for 7 d. Then, all types of moist soils were dried in oven at 35-45°C and stored at 25 ± 1°C before use. A dry subsample of the soils was used for measurements of soil pH values using a combination electrode in 1:2.5 soil-to-KCl solution ratio using a pH meter of Delta320. The results indicated that acidic, neutral, and alkaline pH values were 6.2, 7.1, and 7.7 in the marine-fluvigenic yellow loamy paddy soil; 5.8, 6.7 and 8.1 in pale paddy soil on quaternary red soil; and 5.0, 6.5 and 8.2 in blue clayey paddy soil, respectively.

Three temperatures, 15, 25, and 35°C were used for assessing the effects of temperature on degradation of *Bacillus thuringiensis* toxin.

C ELISA Analysis

The Cry1Ab concentration in soil samples was determined using a commercially available ELISA kit. Before the assay was performed, the frozen soil samples (0.5 g) were thawed, weighed, added to milk extraction buffer (PBS/0.55% Tween-20/0.4% non-fat dry milk) and mixed. The samples were homogenised using a wheaton overhead stirrer then centrifuged. The supernatant was analysed using the quantitative Cry1Ab ELISA kit. The results were reported as ng/g soil weight in 50% WHC (water-holding capacity).

D Degradation Model

The pattern of CrylAb degradation in paddy soils was described by Bai *et al.* (2004) as follows: exponential equations of first-order reaction kinetics: $Y = C_0 e^{-kt}$, where Y is the residue concentration of CrylAb (ng g^{-1}); C, is the initial concentration of CrylAb (ng/g); k is the degradation rate constant; and t is the time (d). The values of half-life for degradation ($t_{0.5}$) (d) were calculated from the formula: $t_{0.5} = 0.693\ k^{-1}$.

II RESULTS AND DISCUSSION

The results of the study suggested that there was a rapid decline in Cry1Ab concentration up to day 7, followed by a more gradual rate of decline, and only trace Cry1Ab were retained in tissue-soil mixtures during the experimental period of 7-35 days.

In 50, 70 and 100% WHC, the values of half-life for degradation of CrylAb ($t_{0.5}$) were 2.4, 2.5 and 2.5 d in the marine-fluvigenic yellow loamy paddy soil; 2.2, 2.1 and 1.8 d in pale paddy soil on quaternary red soil; and 2.4, 2.5 and 2.5 d in blue clayey paddy soil, respectively.

At acidic, neutral, and alkaline pH, the half-life for degradation of CrylAb ($t_{0.5}$) was 4.0, 2.5 and 2.7 d in the marine-fluvigenic yellow loamy paddy soil; 2.2, 1.8 and 2.0 d in pale paddy soil on quaternary red soil; and 2.4, 2.2 and 2.3 d in blue clayey paddy soil, respectively.

Soil temperature showed a clear effect on the degradation of Cry1Ab. At 15, 25 and 35°C, the half-life for degradation of CrylAb ($t_{0.5}$) was 3.0, 2.4 and 2.5 d in the marine-fluvigenic yellow loamy paddy soil; 2.5, 2.2 and 2.2 d in pale paddy soil on quaternary red soil; and 2.9, 2.4 and 2.4 d in blue clayey paddy soil, respectively.

III CONCLUSIONS

The degradation dynamics of CrylAb in leaf-blade powder of transgenic *Bacillus thuringiensis* rice KMD2 in three types of paddy soils could be described by exponential equations under different soil water contents, soil pH, and soil temperature. All these factors could affect degradation of CrylAb, but comparatively soil pH and temperature had greater impacts, with close relationship being found to soil type. The degradations of CrylAb were slower in acidic (pH < 6.5) than in neutral (6.5 < pH <7.5) or alkaline (6.5 < pH) soil, and slower at lower (15°C) than higher temperature (25°C and 35°C), especially in the marine-fluvigenic yellow loamy paddy soil.

Comments RMS: The degradation in soil of Bta endotxin Cry1Ab in transgenic rice leaf powder was studied. The results indicate that the Cry1Ab degrade with a half-life of 2.5 days. The influence of pH and temperature was also investigated. Lower temperature showed slower degradation. Acidic soils also showed slower degradation rates than neutral and alkaline soil. The study is acceptable and can be used for risk assessment.

Report:	KMA 7.1.1/03, Sundaram, K.M.S., Sundaram, A. and Sloane, L. (1996)
Title:	Effect of tracer dyes on initial deposits and persistence of *Bacillus thuringiensis* subsp. *kurstaki* toxin after application of two commercial formulations onto spruce trees
Document No:	Journal of Environmental Science and Health (1996), B31(6): pp.1341-1362
Guidelines:	No
GLP:	No
Present in DAR (2008)	No

Executive summary

The effect of two tracer dyes (Erio Acid Red (EAR) and Acid Black 48 (AB-48)) on initial deposits and persistence of *Bacillus thuringiensis* subsp. *kurstaki* toxin (δ-endotoxin) was studied after spraying two commercial formulations, Foray 48B and Foray 76B over potted white spruce (*Picea glauca* (Moench) Voss) seedlings, at a dosage rate of 30 billion international units (BIU) per ha. After spraying, the seedlings were left outdoors under natural conditions for 18 days. Initial deposits and persistence of δ-endotoxin protein in spruce foliage were determined by immunoassay (ELISA) quantification of the toxin. The total protein (inactive plus active) and δ-endotoxin (active protein) concentrations in the two formulations were determined by a gravimetric procedure and by ELISA respectively.

The initial deposit levels of the toxin on foliage were not markedly affected by the addition of either dye, and showed only a narrow range of 1521 to 1625 ng/g foliage (fresh weight) for Foray 48B, and 1789 to 2056 ng/g for Foray 76B. However, the persistence of the toxin was significantly affected by the presence of the dyes. The toxin persisted in foliage only for 7 d post-spray when the EAR dye was added to Foray 48B, compared to 10 d when no dye was added. The average half-life (DT_{50}) of disappearance was 17.4 h for Foray 48 B with EAR and 20.9 h when no dye was added. The average DT_{50} was 27.9 h for Foray 76B with EAR and 22.2 h without the dye. Persistence was the longest (14 d) when the AB-48 dye was added to Foray 76B; the DT_{50} was 44.9 h.

I MATERIALS AND METHODS

The objective of the study was to determine initial deposit and persistence of δ-endotoxin protein following application of two commercial *Bacillus thuringiensis* subsp. *kurstaki* formulations, Foray 48B and Foray 76B, onto potted white spruce. The formulations were sprayed with or without tracer dyes in order to examine the role of dyes and formulation components on photo-activation or photo-inactivation of δ-endotoxin contributing to *Bacillus thuringiensis* subsp. *kurstaki* activity.

A Materials

The two Foray formulations were obtained from Novo Nordisk Bioindustrials Ltd. A portion of the Foray 48B formulation was mixed with Erio Acid Red (EAR, synonymous with Keyacid Red XB400%) dye at 0.1% w/w. A portion of the Foray 76B formulation was mixed with either EAR or Acid Black 48 (AB-48) at 0.1% w/w. This procedure provided a total of five tank mix formulations. The application volume for Foray 48B was 2.0 L/ha, and for Foray 76B was 1.5 L/ha.

Uniform-sized spruce seedlings grown in the greenhouse under the following conditions were selected: temperature, 21 ± 1°C; photo-period, 16 h light, 8 h darkness; relative humidity 70 ± 5%. All plants had abundant foliage. The crown height was 51 ± 5 cm and the crown diameter at the base of the stem was 41 ± 5 cm.

B Study Design

The seedlings were divided equally into five groups (one group per formulation). The seedlings were subdivided into 10 sets of 4, so that 4 seedlings could be used for each of the 10 sampling intervals. The study was conducted outdoors at the Sault Ste. Marie laboratory un-

der the natural conditions of sunlight, wind and rainfall. The experiment was started on 25 July 1995 (pre-spray foliage collection) and spray was applied the next day. Initial deposit was determined at 0.5 h post-spray, and persistence of deposit activity was investigated for a period of 18 days after spray, by collecting foliar samples at different intervals of time. Weather parameters were monitored continuously.

The atomiser (Flak, Micron Agri-Sprayers Canada) was calibrated to deliver drop sizes similar to the small droplets utilised in ULV treatments in operational programs. Kromekote card/glass plate units were used as the artificial samplers for deposit assessment. The volume rates of application were chosen so as to provide 30 BIU/ha for each formulation. The data were grouped into different size categories to facilitate calculation of droplet number and volume distribution percentages, and cumulative number and volume distribution percentages. These data were used to compute the maximum and minimum diameters (D_{max} and D_{min}), the number volume median diameters ($D_{N.5}$ and $D_{V.5}$, respectively), and the number of droplets per cm² (droplet density).

The seedlings were removed from the spray chamber approximately 30 min after application. The seedlings were transported to a secluded outdoor area, where no overhead obstructions or enclosures were present (to allow the seedlings to be exposed to natural sunlight and rainfall conditions). Approximately 0.5 h after spray application, fully developed new growth (branch tip, about 10 cm long) of foliage was clipped from three locations (situated at 120° to one another) of the mid-crown level of each seedling, and the twelve samples collected from the four seedlings were put in a polyethylene bag, mixed by shaking the bag up and down, and divided into three sets of four for triplicate measurements. In addition to 0.5 h sampling, foliage was also sampled at 6 h, 12 h, 1 d, 2 d, 4 d, 7 d, 10 d, 14 d and 18 d after treatment. Two additional seedlings (controls) were used for surface area measurements of spruce needles. Fully developed current-year branch tips, similar to those used above, were clipped from the seedlings and the surface area was measured for fir foliage. From the average area (0.377 cm²), and the mass (5.88 mg) of a needle, the values for 1 g foliage were calculated (*i.e.* 1 g foliage consisted of 170 needles, and the surface area per g foliage was 64 cm².

C ELISA and Gravimetric Analyses

Two standard curves were prepared using a δ-endotoxin standard, one curve for the optical density (OD) values at 690 nm and the other at 450 nm. The plots of $\log_{10}$ δ-endotoxin con-

centration (ng/ml) versus OD values were nearly linear with the coefficient of determination (R^2) values of 0.996 (OD 690 nm) and 0.994 (OD 450 nm). The minimum quantification limit (MQL) was 25 ng/mL when OD's were measured at 690 nm, and 15 ng/ml at 450 nm. Two linear regression equations were prepared (one for each OD) and these were used to determine initial deposits of δ-endotoxin obtained on spruce foliage at 30 min after spray, and also the residual foliar concentrations of the toxin at different intervals of time up to 18 days after treatment.

The total protein content of the two formulations (samples with no dye) was determined by gravimetric analysis. The value was 98 ± 8 g/L for Foray 48B and 78 ± 5 g/L for Foray 76B. Quantification of the δ-endotoxin protein present in the total protein precipitates of the two formulations was done by ELISA. The value was 68 ± 7 g/L for Foray 48B and 58 ± 7 g/L for Foray 76B.

To determine initial deposits on foliage, fully developed branch tips (about 10 cm long) containing current-year needles were collected from the treated seedlings. The needles were sheared using a hair clipper and the stalk discarded. Aliquots of 2.0 g of needles were taken in a centrifuge tube, and 2.0 mL of a sodium carbonate buffer (0.1 M sodium carbonate + 0.02% w/w sodium azide; pH adjusted to 12 by the addition of 0.05 M NaOH) was added. The contents were mixed gently for 60 min, the supernatant was then decanted and the needles were washed with 1.0 mL of buffer. The washings were added to the original extract. The extract was flash-evaporated at 35°C to 0.5 mL. The protein was precipitated by acidifying the concentrated extract with 1:1 glacial acetic acid:water until the pH reached 4.5. The contents were stored for 6 h at 4°C and centrifuged at 2500 rpm to sediment the protein. The supernatant was decanted and the protein pellet was re-dissolved in 0.5 mL of buffer. Aliquots (200 µL) were used to determine the δ-endotoxin concentration by the ELISA method. The OD values at 690 and 450 nm were fitted into the regression equations, and the values were converted into ng/g foliage. In addition to the initial deposits, residual concentrations of δ-endotoxin in foliage collected at different intervals of time after spray were determined in a similar manner.

II RESULTS AND DISCUSSION

Droplet size spectra

Similar droplet size spectra, droplet density and deposit levels were found on the artificial samplers for all five tank mix formulations. The average values showed a narrow range ($D_{N.5}$, 41 to 55 µm; $D_{V.5}$, 51 to 67 µm; droplet density, 70 to 92; volume deposit 928 to 1103 mL/ha).

Initial deposits and persistence in spruce foliage

The initial deposit concentrations of δ-endotoxin on foliage were also similar between formulations, regardless of the addition of a dye tracer or not. For example, Foray 48B gave values between 1521 and 1625 ng/g foliage (fresh weight), and between 1789 and 2056 ng/g foliage for Foray 76B. The persistence data showed noticeable differences between the tank mix formulations. In all cases, the residual concentrations showed a gradual decline with increasing time after spray; although the rate of decline showed appreciable differences between the five sprays.

Foray 48B with and without EAR dye

The δ-endotoxin levels reached the non-quantifiable limits 7 d after spraying with Foray 48B with EAR, whereas they could be measured up to 10 d after spraying when no dye was present. The concentrations obtained during the initial stages of persistence were subjected to regression analysis to determine the best fit. The regression equation was used to calculate the DT_{50}.

The findings indicated that regardless of the type of formulation used (*i.e.* Foray 48B with or without EAR), the DT_{50} value was very similar whether the OD values were measured at 690 nm or 450 nm. For example, the DT_{50} for Foray 48B with EAR was 18.1 h for OD's at 690 nm and 16.6 h for those at 450 nm. Similarly, the DT_{50} for Foray 48B without EAR was 20.6 h at 690 nm and 21.2 h at 450 nm. Nevertheless, there was a significant difference between the two tank mix formulations, because the two DT_{50} values for Foray 48B with EAR were significantly lower than those for Foray 48B without EAR. Therefore, the addition of EAR contributed to a slight decrease in persistence of δ-endotoxin.

Foray 76B with and without EAR and AB-48 dyes

The δ-endotoxin levels could be quantified up to 10 d post-spray with Foray 76B with EAR, and up to 7 d when no dye was present. The half-life of persistence of δ-endotoxin was similar whether the OD values were measured at 690 nm or at 450 nm. The only exception was

the DT_{50} of Foray 76B with EAR, which was 32.1 h at 690 nm but was significantly lower (24.6 h) at 450 nm. The persistence curve showed significant differences between the three tank mix formulations, indicating marked differences in the rate of decline. For example, the two DT_{50} values for Foray 76B with EAR (31.2 and 24.6 h) were significantly higher than those for Foray 76B without dye (21.8 and 22.6 h). Therefore, the addition of EAR contributed to a slight increase in persistence of δ-endotoxin protein. Persistence was longest (14 d) when AB-48 dye was added to Foray 76B, and the DT_{50} was 44.9 h, indicating a marked increase in persistence.

III CONCLUSIONS

The initial deposit levels of the toxin on foliage were not markedly affected by the addition of either dye, and showed only a narrow range of 1521 to 1625 ng/g foliage (fresh weight) for Foray 48B, and 1789 to 2056 ng/g for Foray 76B. However, the persistence of the toxin was significantly affected by the presence of the dyes. The toxin persisted in foliage only for 7 d post-spray when the EAR dye was added to Foray 48B, compared to 10 d when no dye was added. The average half-life (DT_{50}) of disappearance was 17.4 h for Foray 48 B with EAR and 20.9 h when no dye was added. The average DT_{50} was 27.9 h for Foray 76B with EAR and 22.2 h without the dye. Persistence was the longest (14 d) when the AB-48 dye was added to Foray 76B; the DT_{50} was 44.9 h. It was concluded that tracer dyes can contribute significantly to the persistence characteristics of δ-endotoxin deposits on white spruce foliage. However, whether the persistence can be increased or decreased by adding a dye depends on the type of formulation used and the nature of the dye.

Comments RMS: Non GLP peer reviewed scientific literature on the Btk toxins can be used for risk assessment. The half live of of *Bacillus thuringiensis* subsp. *kurstaki* δ-endotoxin is 20.9 hours without addition of dye.

Report:	KMA 7.1.1/04, Haddad, M. de L., Polanczyk, R.A. Alves, S.A. and Garcia, A de O. (2005)
Title:	Field persistence of *Bacillus thuringiensis* on maize leaves (*Zea mays* L.)
Document No:	Brazilian Journal of Microbiology (2005), 36: pp.309-314
Guidelines:	No
GLP:	No
Present in DAR (2008):	No

Executive summary

The relationship between viability loss of *Bacillus thuringiensis* spores on maize leaves and their concentration was investigated, by comparing it with field persistence. The experimental design was split-plot on time, composed by maize plants, in which three concentrations (half, normal and double doses) of a Dipel commercial formulation were applied. In each plot three leaves in the upper part of three plants were randomly selected. Samples of these leaves were collected 3 to 72 hours after treatment, to count the number of viable spores in two foliar dishes with 1 cm in diameter. The field persistence was determined using an exponential model, linearized by a logarithmic transformation of viable spores number in time. Using the log linear method of confidence intervals, there were no significantly differences (P = 0.05) in half-life: 18.2 hours for half-dose, 16.5 hours for normal dose and 13.6 hours for double dose. Assuming a fictitious index of insect consumption equal to one, the effective doses according to concentrations were calculated. It was verified that 77%, 78% and 80.5% of the effective doses (viable spores) remained on the leaf surface after the first day of treatment, respectively.

I MATERIALS AND METHODS

The aim of this study was to investigate the relationship between spore loss viability on maize leaves and the persistence and effective dose in the field.

A Materials

The product used was Dipel® (containing Btk) in the recommended concentration, half recommended concentration, and 2-fold recommended concentration. The number of viable spores in the product determined in the laboratory was 560 million/mL.

B Study Design

The experimental design was split-plot on time in randomized blocks, and was conducted at the "Departamento de Entomologia, Fitopatologia e Zoologia Agrícola" (Escola Superior de Agricultura Luiz de Queiroz, University of São Paulo, Brazil). The climatic conditions during the experimental period, expressed in mean values, were 19.5ºC, 82.2 RH, wind speed of 1.1 m/s, nil rainfall and global radiation 14.62 MJ/m².

The suspensions were applied on 45 maize plants (*Zea mays* L.) between development stages v3 and v4 (11), using a CO_2 sprayer. In each treatment three plants were randomly chosen, and six leaves in the upper part of plants (without shading, and with similar leaf axis and leaf

orientation) were selected. Three leaves were kept in a Biological Oxygen Demand chamber under UV light (25 ± 2ºC and 70 ± 10 RH) and the other three remained in the plant (controls). In each leaf evaluations were done 3h, 8h, 27h, 32h, 51h, 56h and 72h after application of the product, removing 2 disks with 1 cm in diameter from each leaf.

A total of 252 disks were obtained. Each disk was transferred to a glass tube containing 10 mL of distilled water and shaken for one minute. Afterwards, 1 mL was removed and submitted to thermic shock (80ºC and -10ºC, 10 minutes and 3 minutes, respectively) to eliminate contaminants and undesirable microorganisms. Then, 5 μL were dropped in five spots on a plastic Petri dish 10 cm in diameter, containing nutrient medium (0.15% agar, 0.5% yeast and 0.17 M NaCl), for bacterial growth until 48 hours. The number of *Bacillus thuringiensis* colonies in each point was recorded and the numbers of viable spores was determined.

To determine the persistence or half-life (π) two mathematic models (simple linear and segmented linear) were tested.

II RESULTS AND DISCUSSION

The variance analyses of viable spore number on maize leaves showed a significant interaction between DiPel® doses and time of application. The detailing of this interaction, by Tukey Test ($P \leq 0.05$), showed that the number of viable spores, in the double dose, was statistically higher than the other doses, at 3, 8, 27 and 51 hours after application. The number of viable spores at half-dose and dose did not show significant differences until 51 hours after application, but after that the presence of viable spores was only observed in the double dose. The persistence and half-life (π), tested using simple and segmented linear models, presented high significance. Thus the simple linear model was selected. In the double dose a higher number of spores were observed immediately after application; however the velocity of spore fixation in the leaf was lower than in half-dose and dose.

In maize leaves, the persistence was 18.4, 16.5 and 13.6 hours for half-dose, dose and double dose, respectively. These values did not show significant differences ($P = 0.05$), although the number of viable spores in double dose was superior to other ones after 3, 8, 27 and 51 hours of application.

The effective dose, available dose and percentage of viable spores after the first day is shown in the table below.

Table B.8.1.1-01 Effective dose (L), available dose in the first day (L_1) and percentage of viable spores on maize leaves after the first day of DiPel® application.

DiPel	Effective dose (viable spores) (L)[1]	Available dose (viable spores) (L_1)[2]	Percentage of viable spores in the leaf (L_1/L * 100)
Half dose	6.21	4.79	77
Dose	6.22	4.83	78
Double dose	6.71	5.40	80.5

[1] L = log ® - 0.363+a-log (-b), where r = index of insect consumption
[2] $L_1 = L+\log (1-10^b)$

III CONCLUSIONS

Using the log linear method of confidence intervals, there were no significant differences (P = 0.05) in half-life: 18.2 hours for half-dose, 16.5 hours for normal dose and 13.6 hours for double dose. Assuming a fictitious index of insect consumption equal to one, the effective doses according to concentrations were calculated. It was verified that 77%, 78% and 80.5% of the effective doses (viable spores) remained on the leaf surface after the first day of treatment, respectively.

Comments RMS: Non GLP peer reviewed scientific literature on the Bt spores can be used for risk assessment. Btk spores remained with a 18.2 – 13.6 h on maize leaves depending on the dose.

Report:	KMA 7.1.1/05, West, A.W. (1984)
Title:	Fate of the insecticidal, proteinaceous parasporal crystal of *Bacillus thuringiensis* in soil
Document No:	Soil Biology and Biochemistry, 16(4): 357-360
Guidelines:	No
GLP:	No
Present in DAR (2008)	Yes

Executive summary

Persistence of *Bacillus thuringiensis* parasporal protein-crystal in an arable soil (pH 5.2) incubated at -0.01 MPa at 35°C, was monitored by radioisotope labelling (evolution of $^{14}CO_2$

from soil with or without (±) organic supplement, inoculated with ^{14}C-labelled crystal) and by bioassay (loss of insecticidal activity toward susceptible insect larvae, in soil ± $CaCO_3$ ± organic supplement). The protein-crystal was decomposed by soil microorganisms. The extent of decomposition was significantly reduced ($P < 0.05$) by addition of soluble organic supplement to soil. Insecticidal activity fell rapidly at exponential rates in all soil treatments. The loss rate did not differ significantly between soil and soil + $CaCO_3$ ($t_{0.5}$ = 2.7 days), but was significantly reduced in soil + organic supplement ($t_{0.5}$ = 5.2 days), and in soil + $CaCO_3$ + organic supplement ($t_{0.5}$ = 5.8 days).

I MATERIALS AND METHODS

The study investigated the fate and persistence of the crystal protein under differing soil conditions.

A Materials

Soil

The soil was an arable sandy silt loam previously tilled for cereal cultivation (Pulborough, West Sussex, UK); pH 5.2, clay content 18%, total carbon 1.7%, C:N ratio 14). Fallow soil was composite sampled in winter (3-18 cm depth), air dried, ground and sieved (<2 mm), and stored in darkness at ≥ 1.57 MPa at 2°C. An organic supplement used to increase soil nutrient status comprised 5 mg yeast extract, 1 mg glucose and 0.25 mg K_2HPO_4/g oven-dry soil. Soil pH was raised to pH 7.3 by amendment with 30 mg $CaCO_3$/g oven-dry soil. Soils ± $CaCO_3$ were wetted and stored for 8 days at 2°C before use.

Bacterial Isolate

An asporogenous mutant of *B. thuringiensis* var. *aizawai* H-Serotype 7, strain HD137, induced by temperature (43°C, 2 h) was used for parasporal protein-crystal production. The insecticidal activity of the mutant protein-crystal did not differ from that of the parent strain.

Production and purification of ^{14}C-labelled parasporal protein-crystal

The bacteria were grown overnight at 25°C, by submerged culture in 50 mL of modified Proflo medium, in 250 mL conical flasks on an orbital shaker at 300 rev/min. A 1 mL inoculum was then transferred to each of 5 fluted 250 mL flasks containing 19 ml of fresh modified Proflo medium, incubated at 30°C at 300 rev/min for 9 h (to mid-stationary growth phase) and 4 µCi of high specific activity, uniformly labelled ^{14}C-L-amino acids were then added per flask. The flasks were further incubated for ca. 48 h until protein-crystal production was com-

plete. For crystal purification, cultures were centrifuged at 4000 *g* for 20 min, then re-suspended in 0.1% sodium dodecyl sulphate, and sonicated for 0.5 min, centrifuged twice on continuous gradients of 30-60% w/v sucrose + 0.01% Triton X-100. The lowest band was harvested and washed free of sucrose; this fraction comprised labelled crystal and had a specific activity of 0.17 µCi/mg protein.

B Study Design

Monitoring evolution of labelled CO_2 from soil

Labelled parasporal crystal (measured as 11.6 mg protein by extinction at 260 and 280 m), was diluted with 21.7 mg protein unlabelled crystal (produced as above). One mL of this aqueous crystal suspension (containing 2.56 mg crystal protein, specific activity 0.059 µCi/mg) was added to each of 5 replicate flasks containing soil, 5 replicate flasks containing soil and organic supplement, and to 2 autoclaved control flasks, one containing autoclaved soil and the other containing autoclaved soil and organic supplement. All flasks contained 10 g (oven-dry equivalent) soil at 0.01 MPa. Flasks were incubated at 25°C in a water bath. Labelled CO_2 was flushed from the flasks with 300 mL air (100 mL/min) and trapped in 15 mL of a cocktail of scintillant and trapping fluid. Counts were recorded on a liquid scintillation spectrometer and corrected to disintegrations/min (dpm) using a ^{14}C-hexadecane internal standard. Counting was approximately 58% efficient. Where possible, $^{14}CO_2$ evolved was estimated every 24 h. The 13 and 22 day samples were accumulations of the 10-13 and 19-22 day periods, respectively.

Production and purification of parasporal protein crystal for bioassay

The asporogenous *B. thuringiensis* strain was grown on Nutrient Agar in Roux bottles for 10 days at 30°C. Harvested crystals were centrifuged on 50% and then 60% w/v sucrose cushions, each plus 0.02% TX-100. Pelleted material was centrifuged on a continuous 30-80% w/v glycerol gradient at 1730 g for 10 min, resulting in approximately 4 mg purified crystal protein/mL distilled water.

Monitoring loss of parasporal protein-crystal insecticidal activity by bioassay

Approximately 2 mg crystal protein (in aqueous suspension ± organic supplement ± $CaCO_3$) were added to 5 g (oven-dry equivalent) portions of soil held in 28 mL vials plugged with cotton wool, and maintained at a constant – 0.01 MPa at 25°C in darkness. At each sampling time, two replicate soil vials per treatment were assayed. The bioassay was based on reduction of the feeding rate, caused wholly by parasporal protein-crystal, or larvae of the large white

butterfly, *Pieris brassicae.* Larvae were raised on semi-synthetic diet and 8 larvae, selected visually for uniform size 1 day past third moult, were added to 36 cm² cabbage leaf squares. The upper surfaces of the leaf squares had been previously coated with suspensions of soil containing crystal, control soil without crystal or standard *B. thuringiensis* preparations without soil, and permitted to dry. Protein-crystal in the portions of soil had previously been suspended by bending for 2 min with 50 ml 10 mM phosphate buffer containing 0.02% TX-100 and 3% polyethylene glycol. Seven dilutions of soil suspension were made per vial of soil and 5 leaf square replicates used per dilution. Larvae were left to feed for 24 h at 20 °C; an uneaten leaf was then measured on a leaf scanner and integrator. The response of logit transformed uneaten leaf area against log concentration was compared using parallel line analysis. Reciprocal potency ratios were thus calculated between a *B. thuringiensis* var. *galleria* H-serotype 5 standard and the protein crystal extracted from the soil.

Statistical analysis

The rates of $^{14}CO_2$ evolved from soil and soil + organic supplement for each sample time were compared using ANOVA. Treatments were significantly different at $P = 0.05$ if the uncertainty levels centred on the mean log dpm evolved per sample time did not overlap. Loss of insecticidal activity was analysed by linear regression of log-transformed reciprocal potency ratios against linear time. The average of the ratios of the widths of the 95% fiducial intervals to their respective reciprocal potency ratios was 0.93.

II RESULTS AND DISCUSSION

Labelled parasporal crystal

$^{14}CO_2$ was evolved from each soil treatment (± organic supplement), whilst control soils evolved no $^{14}CO_2$. Evolution of labelled CO_2 varied significantly between sample times and treatments, and there was also a significant sample time by treatment interaction (Table B.8.1.1-02). Soil with organic supplement evolved less $^{14}CO_2$ than unsupplemented soil, although the pattern of $^{14}CO_2$ evolution was similar between the two treatments. $^{14}CO_2$ was produced within 24 h of incubation of protein crystal in soil. After incubation for 23-days an estimated 82% of the original ^{14}C activity remained in soil without organic supplement and 87% in soil with organic supplement.

Table B.8.1.1-02 ANOVA of differences in rates of ^{14}C-CO_2 evolution between replicate flasks, soil treatments and sampling times

Source of variation	Degrees of freedom	Mean square	Variance ratio	Significance
Treatments (± organic supplement)	1	0.698	6.529	$P < 0.05$
Flasks within treatments	8	0.107	9.350	$P < 0.001$
Sample time	14	0.668	58.470	$P < 0.001$
Sample time by treatments	14	0.021	1.847	$P < 0.05$
Error	112	0.011		
Total	149			

Insecticidal activity

Loss of parasporal protein-crystal insecticidal activity occurred at exponential rates in all four treatments. The insecticidal half-life ($t_{0.5}$) in untreated soil was 2.7 days. Addition of $CaCO_3$ alone to soil had no effect. However, in soil with organic supplement the $t_{0.5}$ increased to 5.2 days. Addition of $CaCO_3$ in combination with the organic supplement further reduced the rate of loss of insecticidal activity, with a $t_{0.5}$ of 5.8 days. After 23 days, protein-crystal insecticidal activity had fallen by 99.7% in soil ± $CaCO_3$, 95.3% in soil + organic supplement and 93.7% in soil + $CaCO_3$ + organic supplement.

III CONCLUSIONS

Both the radioisotope and bioassay procedures detected a loss of protein-crystal from the soil. The apparent extent of protein ^{14}C metabolised to $^{14}CO_2$ (13% in 23 days) and loss of insecticidal activity (99.7% in 23 days), differed between the two techniques. In this study, the population did not appear to be affected by an increase in soil pH from acidic to neutral conditions. However, the population decomposing the crystal was affected by the soluble organic supplement, which reduced the amount of crystal carbon metabolised and loss of insecticidal activity. The rate of protein-crystal decomposition remained low the for 36 day incubation period.

The loss rate did not differ significantly between soil and soil + $CaCO_3$ ($t_{0.5}$ = 2.7 days), but was significantly reduced in soil + organic supplement ($t_{0.5}$ = 5.2 days), and in soil + $CaCO_3$ + organic supplement ($t_{0.5}$ = 5.8 days).

The indications from this study is that protoxins are readily degraded by soil microorganisms and that any degradates left are not toxic to insects.

Comments RMS: Non GLP peer reviewed scientific literature on the persistence of *Bacillus thuringiensis* parasporal ^{14}C-labeled protein-crystal in an arable soil can be used for risk assessment. The results show that 99.7% of the activity is removed after 23 days.

Report:	KMA 7.1.1/06, Hung, T.P., Truong, L.V., Binh, N.D., Frutos, R., Quiquampoix, H. and Staunton, S. (2016)
Title:	Fate of insecticidal *Bacillus thuringiensis* Cry protein in soil: differences between purified toxin and biopesticide formulation
Document No:	Pest Management Science (2016), online publication
Guidelines:	No
GLP:	No
Present in DAR (2008):	No

Executive summary

Sunlight accelerated loss of detectable Cry under laboratory conditions, but little effect of shade was observed under field conditions. The half-life of biopesticide proteins in soil under natural conditions was about 1 week.

I MATERIALS AND METHODS

B. thuringiensis biopesticide spray

A commercial spray, Vi-Bt, produced by Hubei Kangxin Agro-Industry Co. Ltd was used according to the supplier's recommendations, by dilution in water. Vi-Bt spray is commonly used in Vietnam. The potency until was given as 16,000 IU mg^{-1}. Vi-Bt is composed of *B. thuringiensis* var. *kurstaki* (isolate HD-1) crystals and spores. The HD-1 strain produces various Cry proteins, including Cry1Ac.

Cry1Ac purified protein

Cry1Ac protein from *B. thuringiensis* strains HD73 was cultivated in shaken Erlenmeyer flasks at 28°C until sporulation (about 48 h). The sterile nutrient solution was composed of 500 µM of $MgSO_4$, 10 µM of $MnSO_4$, 50 µM of $ZnSO_4$, 50µM of $Fe_2(SO_4)_3$, 1 mM of $CaCl_2$, 50 mM of KH_2PO_4, 30 µL L^{-1} of H_2SO_4, 7.5 g L^{-1} of bacteriological peptone and 1% glucose at pH 7.4. The protoxin was solubilised and enzymatically truncated, and the resulting Cry1Ac toxin was purified as has been described in the literature. The protein solution was

stored at 4°C in 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer at pH 10.4 containing 350 mM of NaCl to avoid oligomerisation of the protein. Immediately prior to addition to soil, the storage solution was removed and replaced with a 0.01M $Ca(NO_3)_2$ solution by repeated dilution and concentration in Amicon filter devices.

Soils and study site

The study site for the field experiment and from which soils were sampled for the controlled laboratory experiment was situated in North Vietnam, near Hanoi, in the Plant Protection Research Institute. The climate is subtropical, with most of the annual rainfall of 1700 mm during the rainy season (May-October) and average mean daily temperatures between 16.5°C (January) and 29.5°C (July). The study plot is used for the cultivation of sweet potato (*Ipomoea batatas*). For laboratory studies and soil analysis, triplicate composite soil samples were collected from the top layer of soil (0-5 cm). The soil samples were air dried, sieved (<200 µm), thoroughly mixed and stored until required. The soil was a sandy loam containing 1.1% organic carbon, with a C/N ratio of 13, a cation exchange capacity of 8.2 and a pH of 8.4.

Persistence of the biopesticide Cry proteins under field conditions

The experiment was carried out over one month in winter (December 2013 to January 2014). The temperature ranged from 14 to 17°C at night and from 24 to 26°C in the day. Rainfall was low (5-15 mm in the period) and did not occur soon after spray application. Light intensity was low for Vietnam, with about 70 h of sunshine per month. A sweet potato crop had been planted 21 days prior to spraying. Fertiliser and pesticide treatments were usual for this crop in Vietnam to protect against the lepidopteran pest *Agrius convolvuli*. This plot had not previously received any *Bacillus thuringiensis* treatment, and Cry1Ac was not detectable. Spray was prepared by dilution in water (5 g dm^{-3}), then sprayed at a rate of 0.2 dm^3 m^{-2} to give an application rate of 1.6 x 10^7 IU m^{-2}). Spraying was carried out in the morning, except for one treatment where the crop was sprayed in the afternoon when sun intensity was lower.

Three spray-application and soil-sampling variables were chosen to follow the persistence of Cry1Ac proteins from *Bacillus thuringiensis* spray in the field: (i) soil sampled under leafy canopy; (ii) soil sprayed directly and sampled inter-row; (iii) spray applied directly to inter-row soil in the afternoon of the first day, when light intensity was lower, and soil sampled inter-row. Three replicate rows were spayed. Soil was sampled over various time intervals (between 1 h and 28 days). Composite samples from each of the three positions were taken to obtain about 5 g of soil, which was placed in plastic bags and returned to the laboratory for analysis. Moisture content was determined by oven-drying of a subsample. Seven repetitions

of about 0.2 g of equivalent dry soil were accurately weighed into Eppendorf tubes, and protein was extracted with 1 mL of a solution containing 10 mM of CAPS, 140 mM of NaCl, 1% Tween 20 and 4% bovine serum albumin (BSA) at pH 11. The suspensions were shaken end over end for 30 min, then centrifuged for 30 min at 19000 x *g* to separate the aqueous and solid phases. Supernatant solution was removed and diluted as required, and Cry proteins were assayed using ELISA kits following manufacturer's instructions.

Persistence of biopesticide Cry proteins under controlled laboratory conditions

A commercial *Bacillus thuringiensis* preparation was suspended in distilled water (50 g dm^{-3}). Soil (10 g) was weighed into petri dishes and *Bacillus thuringiensis* suspension was sprayed onto the soils to give a moisture content of 20%; the amount of solution added was determined by weighing the petri dishes. Moisture content was adjusted to 40% by pipetting distilled water onto the soils. The soils were incubated under the required conditions, and weight was checked daily and adjusted for moisture loss as required. The incubation variables were temperature (4 or 25°C) in darkness or direct sunlight. At intervals soil was sampled in three places from each sample to give composite samples of approximately 1 g of soil, from which five replicates of 0.1 g were weighed into Eppendorf tubes, and protein was extracted and assayed as above. All incubations were carried out in triplicate.

Effect of nutrients and soluble soil components on biopesticide Cry proteins under controlled laboratory conditions

Detectable Cry protein from *Bacillus thuringiensis* commercial formulation was monitored in solution for up to 14 days at either 25 or 4°C. The solutions were (i) distilled water; (ii) the nutrient solution used for *Bacillus thuringiensis* culture at three dilutions (1:1, 1:10 or 1:100); or (iii) an aqueous extract of the soil. The soil aqueous extract was obtained by shaking a suspension of soil (1 g: 10 mL) for 30 min end-over-end and then separating phases by centrifugation at 19000 x *g*. At the end of each incubation period, an aliquot of each solution was taken, and Cry1 proteins were assayed by ELISA test.

Persistence of purified Cry1Ac protein in soil under controlled laboratory conditions

Purified Cry1Ac was added to soil by pipetting solution onto soil in Eppendorf tubes. Four treatments, with three repetitions of each, were made. Soil was either wetted directly with Cry solution or pre-wetted with water three days prior to the addition of Cry, to allow the microbial flush to dissipate. Moisture content was adjusted to 40% with distilled water after the addition of the required volume of Cry solution. Soils were incubated at either 25 or 4°C. At the end of the required incubation period, samples were destructively sampled, extraction solution

was added (to give a soil:solution ratio of 1:5), the suspension was shaken and then centrifuged (as above) and the Cry1Ac content was assayed by ELISA detection.

II RESULTS AND DISCUSSION

Persistence of the commercial formulation of Bacillus thuringiensis crystal proteins in field soil under natural conditions

Soil samples collected after field spraying of commercial preparations of HD-1 *Bacillus thuringiensis* formulated biopesticide were assayed. Soil was collected under the leaf canopy, sprayed directly and sampled between rows or sprayed and sampled in the afternoon between rows to give contrasting exposure to sunlight in comparison with the previous treatment. The decrease in detection of *Bacillus thuringiensis* toxins in soils (as given by anti-Cry1 ELISA tests) was similar whatever the sample. There was no coherent effect of canopy protection from sunlight. Detectable Cry tended to be greater for afternoon-sprayed soil for the first week, but his was not observed for each row, and the effect was not significant when the full dataset was considered and compared with either of the other treatments ($P > 0.05$). Similarly, detectable Cry was lower for the morning-sprayed inter-row samples than for the other treatments during the first week after spraying, but taking the full dataset, the effect was not significant ($P>0.05$). The decline in detectable Cry1A followed approximately first-order kinetics. The half-life of detectable protein was about 1 week by visual appraisal and calculated to be 9-10 days by linear regression after log transformation of the data.

Persistence of the commercial formulation of Bacillus thuringiensis crystal proteins in field soil under laboratory conditions

At 25°C in the dark there was a slow gradual decline, with about 70% of the initially detectable protein remaining after 1 week. In some cases an increase in Cry was initially observed before a net decline. In contrast, at 4°C there was a fast initial decrease in detectable toxins, reaching less than 20% of the initial load after only 1 day, followed by a slower decline. Sunlight accelerated the rate of decrease in detectable Cry1A toxins with respect to soil maintained at 25°C in the dark. However, this effect was less than that of low temperature. The time dependence of detectable Cry was significantly different for each of the three treatments ($P<0.05$).

Effect of sunlight on persistence

The results confirmed that sunlight degrades Cry proteins as evidenced by a strong decrease in detectable protein in the laboratory experiment where soil was exposed to sunlight com-

pared with the soil that was kept in the dark at 25°C, for which the decrease was slower. The greater effect of sunlight in the laboratory by comparison with conditions of shade in the field suggests that in the field, differences in sunlight intensity are not sufficient to cause differences in the rate of loss of detectable Cry. The protein content of soils sampled between rows of plants, exposed to more sunlight, relative to the initial content, tended to be lower for the first week, but this was not observed when the soil had been sprayed directly. There was no significant effect for spraying in the afternoon, to ensure a shorter and less intense exposure to sunlight during the first day, than under standard conditions when the spray was applied in the morning. The variability in the field study prevented the sunlight effect from being established. Other effects were stronger and dominated. The larger effect of sunlight in the laboratory conditions may be in part due to the shallow soil layer (5 mm), affording less protection to Cry.

Effect of temperature on persistence in soil

A strong effect of temperature on biopesticide Cry in laboratory conditions was also observed, but it was the reverse of the effect for purified Cry, namely a positive effect of higher temperature on detectability of the toxin. One of the important differences between biopesticide and purified protein is the presence of spores as well as crystal protein in the former. It was thus postulated that that the temperature effect for biopesticide in the laboratory is predominantly biological, with the possibility that spores could produce more protein, thus counteracting the decline that dominates the trend in the field. This hypothesis is strengthened by the fate of the biopesticide in aqueous solutions, designed to test the effect of temperature without the effect of adsorbing surfaces. Detectable biopesticide protein increased with time when incubation conditions favoured microbial development, namely at 25°C rather than 4°C and in solutions containing nutrients, either aqueous soil extract or nutrient solutions, rather than pure water. The temperature effect of soil and nutrient solutions on the increase in Cry concentration could be interpreted as the solubilisation of crystals, but this would not explain the effect of nutrient solution concentration and the absence of an effect in water. Spore germination with protein production occurring at 25°C in the presence of nutrients seems a more likely explanation.

III CONCLUSIONS

Sunlight accelerated loss of detectable Cry under laboratory conditions, but little effect of shade was observed under field conditions. The half-life of biopesticide proteins in soil under

natural conditions was about 1 week. Strong temperature effects were observed, but they differed for biopesticide and purified protein, indicating different rate-limiting steps.

For the biopesticide, the observed decline in detectable protein was due to biological factors, possibly including the germination of *B. thuringiensis* spores, and was favoured by higher temperature. In contrast, for purified proteins, the decline in detectable protein was slower at low temperature, probably because the conformational changes of the soil-adsorbed protein, which cause fixation and hence reduced extraction efficiency, are temperature dependent.

The persistence of Cry proteins in the field results from average conditions of sunlight and temperature. The rate of decline in detectable protein from biopesticide differs from that of purified protein. For purified protein, ongoing fixation of the adsorbed protein, leading to decreasing extractability, dominates the observed time trends. Biopesticide protein trends depend on the additional processes of spore germination and the protective effects of commercial additives.

Comments RMS: Relevant non-GLP peer reviewed literature on the degradation of Btk Cry proteins and the effect of sunlight on the degradation of the proteins. The half-life is about a week. The study is acceptable and reliable and the endpoints can be used for risk assessment for the Cry proteins/toxins.

Report:	KMA 7.1.1/07, Douville, M., Gagné, F., Masson, L., McKay, J. and Blaise, C. (2005)
Title:	Tracking the source of *Bacillus thuringiensis* Cry1Ab endotoxin in the environment
Document No:	Biochemical Systematics and Ecology (2005), 33: pp.219-232
Guidelines:	No
GLP:	No
Present in DAR (2008):	No

Summary

The aim of this study was to quantify levels of Cry1Ab endotoxin and locate its source in the environment. Agricultural soils and surface waters were spiked with crystals (biopesticide-Dipel®) or with pure *Bacillus thuringiensis*-corn endotoxin, Cry1Ab concentrations were then determined with immunoassays. Additionally, surface water, soils and sediments were sampled in an area sprayed with *Bacillus thuringiensis* subsp. *kurstaki* and a site where genetically-modified corn expressing Cry1Ab is grown. Isotopic analysis was performed on the endotoxin from *Bacillus thuringiensis* and *Bacillus thuringiensis*-corn to characterise the propor-

tions of $^{13}C/^{12}C$ and $^{15}N/^{14}N$. The results showed that *Bacillus thuringiensis*-corn endotoxin is degraded more rapidly in water than in soils ($t_{1/2}$: 4 and 9 days, respectively), while crystals appeared to be more resilient, as expected. The isotopic patterns of ^{13}C and ^{15}N in *Bacillus thuringiensis*-corn endotoxin differed markedly from *Bacillus thuringiensis*, making it possible to track the source of Cry1Ab in the environment. Preliminary field surveys indicate that Cry1Ab is fairly uncommon in aquatic environments, being found only at trace concentrations when it is detected.

Comments RMS: Relevant non-GLP peer reviewed literature on Bt endotoxin Cry1Ab. The toxin is degraded with a half-live of 4 days in surface water and 9 days in soil.

Report:	KMA 7.1.1/08, Calabrese, D.M., Nickerson, K.W. and Lane, L.C. (1980)
Title:	A comparison of protein crystal subunit sizes in *Bacillus thuringiensis*
Document No:	Canadian Journal of Microbiology (1980), 26: pp. 1006-1010
Guidelines:	No
GLP:	No
Present in DAR (2008):	No

Summary

Gel electrophoresis was used to determine the number and size of the subunits present in the protein crystals from 16 strains of *Bacillus thuringiensis*. The calculated molecular weights (MW) fell into three major categories whose crystals exhibited the following protein handing patterns: type I, high MW only (140,000-160,000); type II, both high MW and medium MW (60,000 and 150,000); and type III, low MW only (40,000-50,000).

Comments RMS: Relevant non-GLP peer reviewed literature on subunit size of Bt protein crystal of 16 Bt strains. The study only gives an indication of the molecular weight op protein crystals.

Report:	KMA 7.1.1/09, Ali, A. and Young, S.Y. (1993)
Title:	Effects of rate and spray volume of *Bacillus thuringiensis* var. *kurstaki* on activity against *Heliothis virescens* (Lepidoptera: *Noctuidae*) and persistence in cotton terminals
Document No:	Journal of Economic Entomology (1993), 86(3): pp.735-738
Guidelines:	No
GLP:	No
Present in DAR (2008):	No

Abstract

Effects of rate and total spray volume on activity of *Bacillus thuringiensis* var. *kurstaki* against *Heliothis virescens* (F.) in cotton terminals was determined in field cotton. Higher *Bacillus thuringiensis* rates caused higher initial mortality than the lower rates and maintained higher efficacy up to 3 d. Persistence did not differ significantly ($P \leq 0.05$) among rates, and half-lives ranged from 2.4 to 3.1 days. Half-lives for *Bacillus thuringiensis* applied at a rate of 1.12 kg/ha were 3-3.1 days for 46.7, 93.4 and 140 L/ha.

Comments RMS: Non-relevant non-GLP peer reviewed scientific literature on the efficacy of Btk. Half lives vay between 3-3.3 days.

Report:	KMA 7.1.1/10, Faust, R.M., Hallam., G.M. and Travers, R.S. (1974)
Title:	Degradation of the parasporal crystal produced by *Bacillus thuringiensis* var. *kurstaki*
Document No:	Journal of Invertebrate Pathology (1974), 24: pp. 365-373
Guidelines:	No
GLP:	No
Present in DAR (2008):	No

Absstract

Toxicity was found associated mainly with a protein peak of 230,000 MW (molecular weight) although other toxic peaks were found in the tailing. After digestion of parasporal crystals with clarified midgut juice, five peaks causing toxicity and having molecular weights of approximately 235,000, 67,000, 30,200, 5000 and 1000, respectively were identified. Treatment of *Bacillus thuringiensis* δ-endotoxin with α-chymotrypsin gave peaks causing mortality of approximate MW 235,000, 34,000, 5000 and 1000. Trypsin, pronase, carboxypeptidase, and enterokinase digests of the *Bacillus thuringiensis* δ-endotoxin gave toxic components ranging from 235,000 to 30,000 MW. The protein protoxin molecules digested to give small toxic subunits that may be of practical value for structural determinations and for molecular mode of action studies.

Comments RMS: Non-relevant non-GLP peer reviewed scientific literature on the insect gut degradation of Btk parasporal crystals.

Report:	KMA 7.1.1/11, Hostetter, D.L., Ignoffo, C.C. and Kearby, W.H. (1975)

Title:	Persistence of formulations of *Bacillus thuringiensis* spores and crystals on eastern red cedar foliage in Missouri
Document No:	Journal of the Kansas Entomological Society (1975), 8(2): pp.189-193
Guidelines:	No
GLP:	No
Present in DAR (2008):	No

Abstract

The persistence of four formulations of *Bacillus thuringiensis* on Eastern red cedar, a primary ornamental tree in residential areas of the mid-western United States was evaluated. The formulations used were: Thuricide HPC®, Thuricide + carbon, Thuricide + carbon and molasses, and Dipel®. Samples of foliage were taken prior to treatment, immediately after treatment, and at 1, 3, 7, 14, and 28 days post-treatment. The number of viable spores and the remaining insecticidal activity was calculated for these time intervals. Addition of carbon and molasses significantly protected spore viability and insecticidal activity. Residual activity for Dipel and Thuricide + carbon could still be detected up to 14 days post-treatment.

Comments RMS: Relevant non-GLP peer reviewed scientific literature on the influence of formulations on the viability of the spores. For the fate assessment no relevant endpoints can be retrieved.

Report:	KMA 7.1.1/12, Sánchez-Yáñez, J.M. and Peña-Cabriales, J.J. (2000)
Title:	Persistence of *Bacillus thuringiensis* spores on soil and maize and bean leaves
Document No:	Journal Terra Latinoamericana (2000), 18(4): pp.325-331
Guidelines:	No
GLP:	No
Present in DAR (2008):	No

Conclusion

Results showed limited persistence of both Btk spores on maize and common bean leaves, less than 3 d after application due to their sensitivity to solar radiation and drying. In non-sterilised soil, as in sterilised soil, the *Bacillus thuringiensis* spores were not viable after inoculation, indicating that the organic matter of the sterilised soil did not favour spore persistence, while in the non-sterilised soil, competition and depredation from native organisms caused rapid loss of spore viability. This suggests that, at least under these experimental con-

ditions, maize and bean leaves and the soil are not appropriate niches for the persistence of *Bacillus thuringiensis* spores.

Comments RMS: Relevant non-GLP peer reviewed scientific literature on the viability of the spores on maize and bean leaves. On these leaves the spores can only persist few days and in soils after 48 hours the spores were not viable.

Report:	KMA 7.1.1/13, Vettori, C., Paffetti, D., Saxena, D. Stotzky, G. and Giannini, R. (2003)
Title:	Persistence of toxins and cells of *Bacillus thuringiensis* subsp. *kurstaki* introduced in sprays to Sardinia soils
Document No:	Soil Biology & Biochemistry (2003), 35: pp.1635-1642
Guidelines:	No
GLP:	No
Present in DAR (2008):	No

Conclusion

The persistence of *Bacillus thuringiensis* subsp. *kurstaki* and its toxin, released in sprays (FORAY 48B®), in soils of cork oak stands, located in Orotelli, Tempio Pausania and Calangianus (Sardinia) were investigated. In the Calagianus soil, the numbers of *Bacillus thuringiensis* subsp. *kurstaki* remained essentially constant for 28 months (the longest time studied) after spraying, indicating that *Bacillus thuringiensis* subsp. *kurstaki* was able to compete with the indigenous microbial community; the toxin was detected 28 months after spraying by immunological assay, but at a reduced concentration; but the larvicidal activity decreased essentially linearly to 14 months and then decreased markedly between 14 and 28 months. In the Tempio Pausania and Orotelli soils, cells of *Bacillus thuringiensis* subsp. *kurstaki* were detected, whereas the toxin was not detected by immunological and larvicidal assays, 52 and 88 months (the longest times studied) after spraying, respectively. Results indicated that *Bacillus thuringiensis* subsp. *kurstaki* and its toxin introduced into soils in sprays can persist for long periods (at least 88 months for *Bacillus thuringiensis* subsp. *kurstaki* and at least 28 months for its toxin). Persistence of the toxin was probably the results of its binding on surface-active particles in soil, which protected the toxin against biodegradation. It should be noted that the levels of *Bacillus thuringiensis* subsp. *kurstaki* measured are comparable with or below normal background levels.

Comments RMS: Relevant non-GLP peer reviewed scientific literature on the persistence of cells and toxins of Btk. Btk and toxins remained active for a long period (840 days) after application.

Report:	KMA 7.1.1/14, Feng, Y., Ling, L., Fan, H., Lui, Y., Tan., F., Shu, Y. and Wang, J. (2011)
Title:	Effects of temperature, water content and pH on degradation of Cry1Ab protein released from Bt corn straw in soil
Document No:	Soil Biology & Biochemistry (2011), 43: pp. 1600-1606
Guidelines:	No
GLP:	No
Present in DAR (2008):	No

Conclusion

The effects of soil temperature (15°C, 25°C, 35°C), water content (20%, 33%, 50%) and pH (4.5, 7.0, 9.0) on the degradation of Cry1Ab protein released from the straw of *Bacillus thuringiensis*-corn varieties 34B24 and 1246 x 1482 both expressing Cry1Ab protein were investigated. The results showed that Cry1Ab protein released from both 34B24 and 1246 x 1482 straw was degraded in a similar way in all treatments, which demonstrated a rapid decline in the early stage but a slow decline in the middle and late stages. In the late stage (180 days after the experiment commenced) 0.03%-1.51% and 0.02%-0.91% of initial Cry1Ab protein released from 34B24 and 1246 x 1482 straw was detected in soil. In addition, degradation dynamics of Cry1Ab protein under different environmental conditions was well described by the shift-log model. DT_{50} of Cry1Ab protein released from 34B24 and 1246 x 1482 straw was 0.97-9.97 d and 0.75-10.89 d, respectively, and DT_{90} was 4.66-162.45 d and 6.44-57.46 d, respectively. The results suggested that soil temperature had significant effects on the degradation of Cry1Ab protein, with a higher degradation rate at higher temperature, but soil water content and pH had no obvious effects on the degradation of Cry1Ab protein.

Comments RMS: Relevant non-GLP peer reviewed scientific literature on the influence of environmental factors influencing the degradation of Cry1AB Bt corn straw. DT50 were 0.97-9.97 d and temperature had the most impact on the degradation rate.

Report:	KMA 7.1.1/15, Ferreira, L.H.P.L., Suzuki, M.T., Itano, E.N., Ono, M.A. and Arantes, M.N. (2003)
Title:	Ecological aspects of *Bacillus thuringiensis* in an Oxisol
Document No:	Scientia Agricola (2003), 60(1): pp.19-22
Guidelines:	No
GLP:	No
Present in DAR (2008):	No

Abstract

Survival and conjugation ability of *Bacillus thuringiensis* strains were investigated; vegetative cells were evaluated in non-sterile soil. Vegetative cells decreased rapidly in number, and after 48 hours the population was predominantly spores. No plasmid transfer was observed in non-sterile soil, probably because the cells died and the remaining cells sporulated quickly. Soil is not a favourable environment for *Bacillus thuringiensis* multiplication and conjugation. The fate of purified *Bacillus thuringiensis* toxin was analysed by extractable toxin quantification using ELISA. The extractable toxin probably declined due to binding on surface-active particles in the soil.

Comments RMS: Relevant non-GLP peer reviewed scientific literature on the proliferation of vegative cells in soils and the fate of toxin. Within 48 hours the inoculated Btk culture predominately consisted of spores.

Report:	KMA 7.1.1/16, Vilas-Bôas, L.A., Vilas-Bôas, G.F.L.T., Saridakis, H.O., Lemos, M.V.F., Lereclus, D., and Arantes, O.M.N. (2000)
Title:	Survival and conjugation of *Bacillus thuringiensis* in a soil microcosm
Document No:	FEMS microbiology Ecology (2000), 31: pp.255-259
Guidelines:	No
GLP:	No
Present in DAR (2008):	No

Abstract

The survival and conjugation ability of sporogenic and asporogenic *Bacillus thuringiensis* strains were investigated in broth, non-amended sterile clay soil monoculture and in mixed soil culture. The 75 kb pHT73 plasmid carrying an erythromycin resistance determinant and a *cry*1Ac gene was transferred in mating broth and soil microcosm. Survival of strains was as-

sessed in soil monoculture and in mixed soil culture for up to 20 days. Sporogenic strains rapidly formed viable spores which were maintained until the end of the experiment. The asporogenic strains were no longer recovered after 8 days of incubation. This study shows that the environmental impact of asporogenic *Bacillus thuringiensis* strains is lower than that of sporogenic *Bacillus thuringiensis* strains. Thus, use of asporogenic strains may significantly reduce any potential risk (gene transfer, soil and plant contamination) due to the dissemination of *Bacillus thuringiensis*-based biopesticides in the environment.

Comments RMS: Relevant non-GLP peer reviewed scientific literature on the persistence of a sporogenic and asporogenic Bt. The sporogenic strain formed rapidly spores and survived in the test systems.

Report:	KMA 7.1.1/17, Wang, H., Ye, Q., Gan, J. and Wu, L. (2007)
Title:	Biodegradation of Cry1Ab protein from *Bt* transgenic rice in aerobic and flooded paddy soils
Document No:	Journal of Agriculture and Food Chemistry (2007), 55: pp.1900-1904
Guidelines:	No
GLP:	No
Present in DAR (2008):	No

Abstract

Degradation of Cry1Ab protein from *Bt* transgenic rice was examined under both aerobic and flooded conditions in five paddy soils and in aqueous solutions. The hydrolysis rate of Cry1Ab protein in aqueous solutions was correlated inversely with the solution pH in the range of 4.0 to 8.0, and positively with the initial concentration of Cry1Ab protein. Rapid degradation of Cry1Ab protein occurred in paddy soils under aerobic conditions, with half-lives ranging from 19.6 to 41.3 days. The degradation was mostly biotic and not related to any specific soil property. Degradation of the Cry1Ab protein was significantly prolonged under flooded conditions compared with aerobic conditions, with half-lives extended to 45.9 to 141 days. These results suggest that the toxin protein, when introduced into a paddy field upon harvest, will probably undergo rapid removal after the field is drained and exposed to aerobic conditions.

Comments RMS: Relevant non-GLP peer reviewed scientific literature on Bt_Cry1Ab endotoxin. Half-lives ranging from 16.9 to 41.3 d for aerobic soils and half-lives extended to 45.9 to 141 d for anaerobic soils.

Report:	KMA 7.1.1/18, Hendriksen, N.B. and Carstensen, J. (2013)
Title:	Long-term survival of *Bacillus thuringiensis* subsp. *kurstaki* in a field trial
Document No:	Canadian Journal of Microbiology (2013) , 59: pp.34-38
Guidelines:	No
GLP:	No
Present in DAR (2008):	No

Abstract

Long-term survival of *Bacillus thuringiensis* subsp. *kurstaki* DMU67R has been investigated in a field trial. An experimental cabbage plot was sprayed with DMU67R in 1993 and was allowed to lie fallow since then. The investigation reported in the study was carried out from 2001 to 2007 in a single square meter within the plot using the systemic randomised sampling approach. The bacterium survived at relatively low densities in these 13 years after spraying. Statistical analyses revealed that the overall density decreased approximately 40% during years 8 to 13 after this application; however, the trend was not uniform and contained periods of both increases and decreases in density of DMU67R, with decreases in density notably related to conditions of low water content in the soil. Long-term survival of DMU67R in this field plot seems to include germination and growth, possibly related to growth in insect hosts, and death or inactivation during dry periods, both phases occurring during May to October where soil temperature exceeds 10°C.

Comments RMS: Relevant non-GLP peer reviewed literature on Btk and long term survival.

B.8.1.2 Water

The fate and behaviour of *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 in water was evaluated during the Annex I Inclusion. No additional studies have been performed and no further data are provided. The fate and behaviour of *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 in water is discussed in detail in the corresponding document of the EU review dossier where the references cited from the scientific literature can be found. A brief overview of this information is provided below.

Although *Bacillus thuringiensis* subsp. *aizawai* has been shown to survive to some extent in water, viability in the natural aquatic environment is influenced by many biological, chemical and physical factors. Predation by bacteriophages, protozoans and other lower animal forms

undoubtedly plays a role in controlling the bacteriological population in the aquatic environment.

Bacillus thuringiensis subsp. *aizawai* is not regarded as an autochthonous inhabitant of aquatic environments and does not find optimal conditions for growth in the aquatic environment (*e.g.* waters are poor in organic carbon content). Therefore, proliferation is not likely to occur. Bacterial cells and especially spores may survive, but will be subject to natural competition in the diverse microbiota of natural waters. Survival of the applied ABTS-351 is not expected to cause any environmental or health impact.

The corresponding part of the DAR is added for reference:

8.1.2 Water

Bacillus thuringiensis may survive to a limited extent in water. However, its survival and viability in the natural aquatic environment are influenced by the complex interaction of a number of biological, chemical and physical factors. Predation by protozoans and lower animal forms undoubtedly plays a significant role in controlling the population of *Bacillus thuringiensis* in the aquatic environment. The effects of solar radiation may destroy *Bacillus thuringiensis* endospores, crystal proteins and vegetative cells in the upper layers of an aquatic system and extremes of water temperature may have a detrimental effect on survival and insecticidal activity. The adsorption of bacterial cells to the sediment layer in the natural aquatic environment is also expected to occur. Spores are unlikely to be capable of germinating and multiplying in sediment and the crystalline proteins become inaccessible to insect larvae.

The *Bacillus thuringiensis* subsp. *aizawai*, XenTari WG formulation is not intended for direct application to water. Although there may be some potential for surface water exposure resulting from spray drift from field applications, spray drift from application to developed foliage is unlikely to be significant. Concentrations of *Bacillus thuringiensis*, which are deposited in surface water bodies, are therefore expected to be extremely diluted and well below insecticidal effect levels.

Worst-case initial surface water PECs for use in the environmental risk assessment have been calculated assuming deposition of residues from spray drift onto a static ditch (0.3 m depth) with worst-case spray drift values for grapevines (late application) at 3 m distance (Rautmann *et al*., 2001)[1]. The spray drift values for grapevines are considered to be most representative in this case, as the formulation will be applied to peppers in the field using air-assisted sprayers, appropriate for a 3-dimensional crop. In accordance with guidelines provided in the EC Guidance Document on Aquatic Ecotoxicology, (SANCO/3268/2001 1st October 2001), PEC_{sw} values were calculated using a 90th percentile drift value of 8.02% (assuming a single application) and a 70th percentile drift value of 6.41% (assuming 6 applications at 7 day intervals).

[1] Rautmann, D., Streloke, M., Winkler, R. (2001) New basic drift values in the authorisation procedure for plant protection products. In Forster, R., Streloke, M. Workshop on Risk Assessment and Risk Mitigation Measures in the Context of the Authorisation of Plant Protection Products (WORMM). Mitt.Biol.Bundesanst.Land-Forstwirtsch. Berlin-Dahlem, Heft 381

In the case of multiple applications, the accumulated concentration in surface water after the final (6^{th}) application was taken as the initial PEC_{sw}. A conservatively estimated dissipation half-life for biological activity of 14 days in water (assumed first order) was used to account for carry-over between successive applications. This is based on the findings of several studies reported in the scientific literature that have investigated the persistence of *Bacillus thuringiensis* subsp. *israelensis* in water. In most studies, larvicidal activity was reported to have disappeared within 1 – 4 weeks and in laboratory microcosm studies larvicidal activity was retained for only 3 – 5 days (Anon., 2005). This value is therefore considered to be generally representative and is a realistic worst-case estimate of the biological degradation rate of *Bacillus thuringiensis* in surface water. The calculated initial PEC_{sw} values are 14.4 ug MPCA (TP)/L (single application, corresponding to about $6{,}6x10^5$ CFU/L) and 34.5 ug MPCA (TP)/L (6 applications, corresponding to about 1.58 $x10^6$ CFU/L).

General Comments. Specific studies on the persistence and multiplication in water of *Bacillus thuringiensis ssp. aizawai* strain ABTS-1857 have not been submitted by the notifier. Therefore, the previous statements on *Bacillus thuringiensis ssp. aizawai* strain ABTS-1857 are based on reported characteristics, as found in the scientific literature, under consideration of the envisaged application and relevant properties of *Bacillus thuringiensis ssp. aizawai* strain ABTS-1857.

Comments RMS: The RMS has the opinion that the literature search was too narrow (see B.8.4) and relevant and reliable data are missed. No information is provided on the persistence of Cry toxins in water.

B.8.1.3 Air

The fate and behaviour of *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 in the atmosphere was evaluated during the Annex I Inclusion. No additional studies have been performed and no further data are provided. The fate and behaviour of *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 in air is discussed in detail in the corresponding document of the EU review dossier where the references cited from the scientific literature can be found. A brief overview of this information is provided below.

Following application of the XenTari® WG formulation as proposed, spray drift can occur which may result in temporary concentrations of the microbial pest control agent in the atmosphere. However, *Bacillus thuringiensis* subsp. *aizawai* is expected to undergo rapid degradation in the atmosphere since inactivation by solar radiation is a very important factor causing loss of activity and degradation of bacterial spores and δ-endotoxin crystals in the field environment. The survival and persistence of *Bacillus thuringiensis* subsp. *aizawai* in air is therefore expected to be very limited.

The corresponding part of the DAR is added for reference:

B.8.1.3 Air

Potential atmospheric exposure of *Bacillus thuringiensis* may occur following commercial field applications. However, rapid atmospheric degradation is expected since inactivation by solar radiation is a key factor causing the degradation and loss of activity of *Bacillus thuringiensis* bacteria spores and crystal proteins. Atmospheric concentrations of *Bacillus thuringiensis* may be transported by spray drift and air currents before the spores and crystals on finer spray droplets settle out. However, as the spatial and temporal distribution of *Bacillus thuringiensis* in the environment is limited by its poor survival and limited ability to sustain infections in populations, atmospheric dispersal is likely to result in reduced inoculum density and subsequent toxicity levels.

General Comments. Specific studies on the persistence and multiplication in air of *Bacillus thuringiensis ssp. aizawai* strain ABTS-1857 have not been submitted by the notifier. Therefore, the previous statements on *Bacillus thuringiensis ssp. aizawai* strain ABTS-1857 are based on reported characteristics, as found in the scientific literature, under consideration of the envisaged application and relevant properties of *Bacillus thuringiensis ssp. aizawai* strain ABTS-1857.

Comments RMS: The RMS has the opinion that the literature search was too narrow (see B.8.4) and relevant and reliable data are missed.

B.8.2 Mobility

The mobility of *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 was evaluated during the Annex I Inclusion and has been described above under Section B.8.1.1. No additional studies have been performed and no further data are provided. The mobility of *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 is discussed in detail in the corresponding document of the draft assessment report (DAR, 2008 and Addendum, 2011) where the references cited from the scientific literature can be found. A brief overview of this information is provided below.

Various experiments examining the movement of *Bacillus thuringiensis* in soils following spraying of commercial products containing *Bacillus thuringiensis* showed little or no movement. Even one year following an application onto a sandy clay loam soil in a cabbage field in Denmark, 77% of recovered *Bacillus thuringiensis* remained in the 0 to 2 cm topsoil layer. In experiments in Japan, it was found that under artificially and naturally irrigated conditions, there was no translocation of sprayed *Bacillus thuringiensis* into the soil down to a depth of 10 cm. Artificial irrigation with 450 mm simulated rainfall in a soil column showed no movement through 6 cm of volcanic ash and only a few bacteria were detected in the flow through water from movement through a 6 cm column of alluvium sand. Under natural rainfall conditions, a reduction of *Bacillus thuringiensis* numbers of 71 to 99% in the top 0 to 1 cm of surface soil occurred in the first week of a 34-day post-application observation period. No dispersion of *Bacillus thuringiensis* was detected in the field soils below 1 cm to investigated depths of 9 to 10 cm, 19 to 20 cm and 29 to 30 cm. It can therefore be concluded that movement of *Bacillus thuringiensis* through the soil by leaching is unlikely to occur. In addition, the adsorption and binding of crystalline proteins (protoxins) and Cry toxins from *Bacillus thuringiensis* has been demonstrated to occur readily, rapidly and strongly onto the clay fraction and clay humic acid complexes of soils, with desorption occurring far less readily.

Comments RMS: The RMS has the opinion that the literature search was too narrow (see B.8.4) and relevant and reliable data are missed, especially concerning the mobility of Cry toxins. Additionally, the notifier cites various experiments examining the movement of Bt in soil, but no references are included. Also regarding the adsorption of the protoxins no references are provided.

B.8.3 Effects of the micro-organism on drinking water analysis

In the admissibility check the RMS indicated that there was no data on effects of micro-organisms on drinking water analysis. The notifier replied that the study KMA 4.1.5/02 can be used for this data point.

Report:	KMA 4.1.5/02 Hart, H. (2016)
Title:	Effect of XenTari and DiPel Technical Powders on Recovery and Enumeration of Generic *E. coli* in Drinking Water
Document No.	Research Project Number GNV-160008
Guidelines:	Not stated
GLP:	No

Principle of Method:

Sample preparation – Samples of XenTari and DiPel Technical Powders were separately prepared by adding 0.5 g of the test material to 100 mL of sterile deionized water. This resulted in a *B. thuringiensis* concentration of approximately 2.5 x 10^8 CFU/mL in the stock solution.

Serial dilutions were performed on the stock solution to result in test portions with B. *thuringiensis* concentrations of 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 (ca. 250) and 10^1 (ca. 25) CFU/mL.

Sample portions were then inoculated with an *E. coli* suspension (25-50 CFU/mL in sterile water) by aseptically transferring 1,000 µL to each 99 mL product suspension. This inoculation technique resulted in a cell density of ca. 34 CFU/100mL. Three portions of sterile deionized water (99 mL) were likewise inoculated with the E. coli suspension to serve as inoculums recovery controls. The samples were then analysed via membrane filtration using Sartorius Biosart 100 Monitors (47 mm, 0.45 µm). Petri dishes were inverted and incubated at 36 ± 2°C for 21 ± 3h. Typical colonies (dark-blue to violet) were counted, and results were expressed as CFU/100mL.

Results:

Table B.8.3-01 *E. coli* Recovery from DiPel Technical Powder Suspension

DiPel Concentration	*E. Coli* Recovery		
	Log_{10} CFU/mL		
CFU/mL	Average	St. Dev	N
Control	1.54	0.01	3
10^1	1.49	0.13	3
10^2	1.52	0.06	3
$*10^3$	1.55	0.10	3
$**10^4$	1.48	0.03	3
$**10^5$	1.37	0.16	3
$**10^6$	1.50	0.07	3
$**10^7$	1.66	0.09	3

* E. coli colony size slightly restricted
** E. coli colony size restricted (very small or pinpoint in size)

Table B.8.3-02 *E. coli* Recovery from XenTari Technical Powder Suspension

XenTari Concentration	*E. Coli* Recovery		
	Log_{10} CFU/mL		
CFU/mL	Average	St. Dev	N
Control	1.54	0.01	3
10^1	1.56	0.02	3
10^2	1.56	0.08	3
$*10^3$	1.59	0.05	3
$**10^4$	1.38	0.07	3
$**10^5$	0.16	0.28	3
$**10^6$	0.48	0.00	3
$**10^7$	1.13	0.15	3

* E. coli colony size slightly restricted
** E. coli colony size restricted (very small or pinpoint in size)

Conclusion:

Method ISO 9308-1:2014 was considered to be reliable for the enumeration of *E. coli* in water when DiPel and XenTari were present in the solution up to concentrations of 10^3 CFU/mL. Reliability was questionable at DiPel and XenTari concentrations exceeding 10^3 CFU/mL (i.e., $10^4 – 10^7$ CFU/mL of *B. thuringiensis*).

Comments RMS: This study shows that Bt from Dipel and XenTari at higher concentrations (10^4-10^7 CFU/mL) results in non-reliable *E.coli* counts and thereby might affect drinking water analysis.

According to Council Directive 98/83/EC for drinking water the following microbiological parameters must be subject to check monitoring:

- *Escherichia coli*

- Enterococci

And conditionally:

- *Clostridium perfringens* – necessary only if the water originates from or is influenced by surface water;
- *Pseudomonas aeruginosa* and colony count at 22°C and at 37°C – necessary only in the case of water offered for sale in bottles or containers.

No data on the potential interference with the detection of the other organisms is available.

B.8.4 References relied on

Literature search for Bta was conducted (Duffy, 2016) according to the EFSA document; Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009. EFSA Journal 2011; 9(2) : 2092 for public domain literature on *Bacillus thuringiensis aizawai* (Bta).

The review was made in order to identify scientific peer-reviewed open literature on the active substance *Bacillus thuringiensis aizawai* (Bta) which may affect the assessment on human health and/or the environment.

The relevance criteria and databases and search terms are given in the following tables.

Table B.8.3-01 Relevance criteria for fate and behaviour

Data requirement(s) indicated by the correspondent OECD data point number(s)	**Criteria for relevance**
Fate and behaviour in the environment (OECD code: IIA 7)	1. Any information that could affect the environmental parameters, degradation profile or endpoints used in the risk assessment 2. Any environmental monitoring information relevant to the usage pattern. 3. Well defined test material (including its purity and impurity profile). 4. Well described test methodology – appropriate guideline referenced

Table B.8.3-02 Reporting of the search process for scientific peer-reviewed open literature in bibliographic databases

Data requirement (s) captured in the search	**Details of the searches**				
Bacillus thuringiensis aizawai ABTS-1857 (Bta) Covers all data requirements	**BIOSIS**	**Toxcenter**	**Medline**	**CAplus**	**CABA**
	Justification for choosing the source: BIOSIS Previews® is the largest and most comprehensive life science database in the world. Amongst others subject coverage includes Agriculture, Biochemistry, Biophysics, Botany, Environmental Biology, Physiology, Toxicology. Sources include periodicals, journals, conference proceedings, reviews, reports, patents, and short communications. Nearly 6,000 life source journals, 1,500 international meetings as well as review articles, books, and monographs are reviewed for inclusion. Bibliographic information, indexing terms, abstracts, and CAS Registry Numbers are all searchable.	Toxicology Center covers the pharmacological, biochemical, physiological, and toxicological effects of drugs and other chemicals. TOXCENTER is composed of the following subfiles: BIOSIS (1969 to date), CAplus (1907 to date), IPA (1970 to date), and MEDLINE (1953 to date). Sources include abstracts, books and book chapters, bulletins, conference proceedings, journal articles, letters, meetings, monographs, notes, papers, patents, presentations, research and project summaries, reviews, technical reports, theses, translations, unpublished material, web reprints. Records contain bibliographic data, abstracts, indexing terms, chemical names, and CAS Registry Numbers	MEDLINE is the U.S. National Library of Medicine® (NLM) premier bibliographic database that contains more than 23 million references to journal articles in life sciences with a concentration on biomedicine. A distinctive feature of MEDLINE is that the records are indexed with NLM Medical Subject Headings (MeSH®). MEDLINE is the online counterpart to MEDLARS® (MEDical Literature Analysis and Retrieval System) that originated in 1964.	Chemical Abstracts Plus covers worldwide literature from all areas of chemistry, biochemistry, chemical engineering, and related sciences. Since October 1994 all articles from more than 1,600 key chemical journals are added including citations for documents not covered by CA. Coverage includes applied, macromolecular, organic, physical, inorganic, and analytical chemistry. Current sources include over 8,000 journals, patents and patent families from 38 national patent offices and 2 international patent organizations, technical reports, books, conference proceedings, dissertations, product reviews, bibliographic items, book reviews, and meeting abstracts. Electronic-only journals and Web preprints are also covered.	The CAB Abstracts database covers worldwide literature from all areas of agriculture and related sciences including biotechnology, forestry, and veterinary medicine. Sources for CABA include journals, books, reports, published theses, conference proceedings, and patents. Bibliographic information, indexing terms, abstracts, and CAS Registry Numbers are searchable.
	Date of the search: August 2016				
	Date span of the search: 1 January 2005 – 23 August 2016 (BTa)				
	Date of the latest database update included in the search: At 02/2016 24.8 million records were available, the database is updated weekly.	**Date of the latest database update included in the search:** At 01/2016 12.2 million records were available, the database is updated weekly.	**Date of the latest database update included in the search:** At 01/2016 25.3 million records were available, updated daily.	**Date of the latest database update included in the search:** At 01/2016 8 million records were available, updated weekly	**Date of the latest database update included in the search:** At 01/2016 8 million records were available, updated weekly

	Search strategies used for this data requirement						
	Terms searched:						
	"*Bacillus thuringiensis* AND aizawai" – this search term was applied to each of the search terms listed below by scientific area using the 'AND' operator (for example "Bacillus thuringiensis AND aizawai AND growth").						
	Biological Properties:						
	growth	spore coat	hydrophobicity	adherence	food poisoning		
	Human Toxicology:						
	?toxi?	?toxi? and mammal	human	worker	clinical	occup?	adverse
	sensitis?	sensitiz?	allergen	hypersens?	infect?	infect? AND mammal?	pathogen?
	pathogen? AND mammal		immunocomp	genotox?			
	Residues:						
	residu?	food					
	Environmental Fate:						
	distribution	soil	soil and europ?	water	water and europ?	air	
	Ecotoxicology:						
	non-target	fish or lepomis? or oncorhynchus or salmonidae or pimephales or cyprinid? or minnow or carp or zebraf? or goldfish? daphni? or asellus or chironom? or cloeon? or hyalella or 'aquatic inverte-brate'				alga? or selenastrum? or scenedesmus? or chlorella? or skeletonema? or navicula? or anabaena?	
	(nontarget? and plant) and (lemna? or chara? or elodea? or myriophyl-lum? or glyceria? or duckweed? or pondweed)			arthro? or lacewing? or hoverfly? or ladybird# or syrphid? or phytosei-ulus? or aphidius? or typhlodromus?			ecotox?
	adverse	pathogen?	enviro?	?toxi? and bird? or avian or mallard? or quail or bobwhite or repro? or oral or chronic			
	honeybee# or bumblebee# or apis or bom-bus? or bee###		honeybee and contact or oral or larvae or feed?	earthworm? or eisenia? or lumbricus or allobophora? or dendrobaena? or aporrectodea? or dendro-drilus? or hypoaspis? or collembola? or springtail or terrestrial			
	staphylinid? or coccinel? or pardosa? or orius? or bembidion? or hymenopt? or chrysopid?				bird? or avian or mallard? or quail or bobwhite or repro? or oral or chronic		
	Efficacy:						
	n/a						
	Resistance:						
	n/a						
	Total number of summary records retrieved: 1771						
						Total number of summary records retrieved after removing duplicates	**N = 519**

In total, 519 summary records were retrieved from bibliographic databases and were screened by expert reviewers and grouped into two categories according to their likely relevance after rapid assessment of titles and, when available, abstracts:

1. Obviously not relevant: 485 summary records.

These summary records (titles and/or abstracts) did not contain specific information relevant to the criteria specified in Table 1.

2. Not excluded after rapid assessment: 34 summary records were classified as potentially relevant and thus were assessed in detail, a full assessment of the full-text documents.

3. Following assessment 26 of the full text documents were excluded from the dossier.

4. Following assessment 8 of the full text documents were included in the dossier.

The results are presented in detail below in Tables B.8.3-03

Table B.8.3-03 Results of the study selection process, for each data requirement or group of data requirements searched

Data requirement(s) captured in the search (as indicated in Table 2)	**N**
Total number of *summary* records retrieved after *all* searches of peer-reviewed literature (excluding duplicates)	519
Number of *summary records* excluded from the search results after rapid assessment for relevance	485
Total number of *full-text documents* assessed in detail	34
Number of *studies* excluded from further consideration after detailed assessment for relevance	26
Number of *studies* included for relevance after detailed assessment	8

For fate and behavior no relevant and reliable literature was obtained.

Comments RMS: The environmental literature search is conducted according the EFSA guidelines however the search terms (*Bacillus thuringiensis* AND *aizawai*, distribution, soil, soil and europ?, water, water and europ?, air) are narrow and relevant articles might be missed. Search terms like fate, proliferation, mobility, aquat?, lake, river, pond, field, rhizosphere should be included. No metabolites search terms were used and other Bt strains might have relevant data on metabolites and persistence in soil, water/sediment and air as well.

The NL is also RMS of another Bta dossier for which approximately 30 reliable and relevant studies were retrieved from 4 different databases. Therefore the RMS concluded that this literature search is not acceptable since the search is too narrow.

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protec-tion claimed Y/N	Justification if data protection is claimed	Owner
IIM 7.1/01	Anonymous	2005	The Environmental Fate and Behaviour of *Bacillus thuringiensis* Expert Review for EU Dossier JSC International Report-no. VBC/03/016 GLP: no Published: no **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	VBC
IIM 7.1.1/01	Visser, S., Addison, J.A., Holmes, S.B.	1994	EFFECTS OF DIPEL 176, A BACILLUS THURINGIENSIS SUBSP. KURSTAKI (B.T.K.) FORMULATION, ON THE SOIL MICROFLORA AND THE FATE OF B.T.K. IN AN ACID FOREST SOIL: A LABORATORY STUDY - Canadian Journal for Forestry Research, Volume 24, 1994, pp. 462-471 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protec-tion claimed Y/N	Justification if data protection is claimed	Owner
IIM 7.1.1/02	West, A.W., Burges, H.D., Dixon, T.J., Wyborn, C.H.	1985	SURVIVAL OF BACILLUS THURINGIENSIS AND BACILLUS CEREUS SPORE INOCULA IN SOIL: EFFECTS OF PH, MOISTURE, NUTRIENT AVAILABILITY AND INDICENOUS MICROORGANISMS - Soil Biology & Biochemistry, Volume 17, No. 5, 1985, pp. 657-665 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.1/03	Petras, S.F., Casida Jr., L.E.	1985	SURVIVAL OF BACILLUS THURINGIENSIS SPORES IN SOIL - Applied and Environmental Microbiology, Volume 50, December 1985, pp. 1496-1500 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.1/04	Saleh, S.M., Harris, R.F., Allen, O.N.	1970	FATE OF BACILLUS THURINGIENSIS IN SOIL: EFFECT OF SOIL PH AND ORGANIC AMENDMENT - Canadian Journal of Microbiology, Volume 16, 1970, pp. 677-680 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protec-tion claimed Y/N	Justification if data protection is claimed	Owner
IIM 7.1.1/05	Akiba, Y.	1986a	MICROBIAL ECOLOGY OF BACILLUS THURINGIENSIS VI. GERMINATION OF BACILLUS THURINGIENSIS SPORES IN THE SOIL - Japanese Journal of Applied Entomology and Zoology, Volume 21 (1), 1986, pp. 76-80 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.1/06	Pedersen, J.C., Darmgaard, P.H., Eilenberg, J., Hansen, B.M.	1995	DISPERSAL OF BACILLUS THURINGIENSIS VAR. KURSTAKI IN AN EXPERIMENTAL CABBAGE FIELD - Canadian Journal of Microbiology, Volume 41, 1995, pp. 118-125 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.1/07 KMA 7.1.1/01	West, A.W., Burges, H.D., White, R.J., Wyborn, C.H.	1984	PERSISTENCE OF BACILLUS THURINGIENSIS PARASPORAL CRYSTAL INSECTICIDAL ACTIVITY IN SOIL - Journal of Invertebrate Pathology, Volume 44, September 1984, pp. 128-133 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protec-tion claimed Y/N	Justification if data protection is claimed	Owner
IIM 7.1.1/08	West, A.W., Burges, H.D., Wyborn, C.H.	1984	EFFECT OF INCUBATION IN NATURAL AND AUTOCLAVED SOIL UPON POTENCY AND VIABILITY OF BACILLUS THURINGIENSIS - Journal of Invertebrate Pathology, Volume 44, September 1984, pp. 121-127 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.1/09 KMA 7.1.1/05	West, A.W.	1984	FATE OF THE INSECTICIDAL, PROTEINACEOUS PARASPORAL CRYSTAL OF BACILLUS THURINGIENSIS IN SOIL - Soil Biology & Biochemistry, Volume 16, No. 4, 1984, pp. 357-360 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.1/10	Pruett, C.J.H., Burges, H.D., Wyborn, C.H.	1980	EFFECT OF EXPOSURE TO SOIL ON POTENCY AND SPORE VIABILITY OF BACILLUS THURINGIENSIS - Journal of Invertebrate Pathology, Volume 35, March 1980, pp. 168-174 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protec-tion claimed Y/N	Justification if data protection is claimed	Owner
IIM 7.1.1/11	Venkateswer lu, G., Stotzky, G.	1992	BINDING OF THE PROTOXIN AND TOXIN PROTEINS OF BACILLUS THURINGIENSIS SUBSP. KURSTAKI ON CLAY MINERALS - Current Microbiology, Volume 25, 1992, pp. 225-233 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.1/12	Tapp, H., Stotzky, G.	1995	INSECTICIDAL ACTIVITY OF THE TOXINS FROM BACILLUS THURINGIENSIS SUBSPECIES KURSTAKI AND TENEBRIONIS ADSORBED AND BOUND ON PURE AND SOIL CLAYS - Applied and Environmental Microbiology, Volume 61, No. 5, May 1995, pp. 1768-1790 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.1/13	Crecchio, C., Stotzky, G.	1998	INSECTICIDAL ACTIVITY AND BIODEGRADATION OF THE TOXIN FROM BACILLUS THURINGIENSIS SUBSP. KURSTAKI BOUND TO HUMIC ACIDS FROM SOIL - Soil Biology & Biochemistry, Volume 30, No. 4, April 1998, pp. 463-470 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protec-tion claimed Y/N	Justification if data protection is claimed	Owner
IIM 7.1.1/14	Crecchio, C., Stotzky, G.	2001	BIODEGRADATION AND INSECTICIDAL ACTIVITY OF THE TOXIN FROM BACILLUS THURINGIENSIS SUBSP. KURSTAKI BOUND ON COMPLEXES OF MONTMORILLONITE-HUMIC ACIDS-A1 HYDROXYPOLYMERS - Soil Biology & Biochemistry, Volume 33, No. 4-5, 01 April 2001, pp. 573-581 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.1/15	Akiba, Y.	1991	ASSESSMENT OF RAINWATER-MEDIATED DISPERSION OF FIELD-SPRAYED BACILLUS THURINGIENSIS IN THE SOIL - Japanese Journal of Applied Entomology and Zoology, Volume 26 (4), 1991, pp. 447-483 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-

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IIM 7.1.1/16	Griego, V.M., Spence, K.D.	1978	INACTIVATION OF BACILLUS THURINGIENSIS SPORES BY ULTRAVIOLET AND VISIBLE LIGHT - Applied and Environmental Microbiology, Volume 35, No. 5, May 1978, pp. 906-910 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.1/17	Myasnik, M., Manasherob, R., Ben-Dov, E., Zaritsky, A., Margalith, Y., Barak. Z.	2001	COMPARATIVE SENSITIVITY TO UV-B RADIATION OF TWO BACILLUS THURINGIENSIS SUBSPECIES AND OTHER BACILLUS SP. - Current Microbiology, Volume 43, March 2001, pp. 140-143 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-

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IIM 7.1.1/18	Teschke, K., Chow, Y., Bartlett, K., Ross, A., van Netten, C.	2001	SPATIAL AND TEMPORAL DISTRIBUTION OF AIRBORNE BACILLUS THURINGIENSIS VAR. KURSTAKI DURING AN AERIAL SPRAY PROGRAM FOR GYPSY MOTH ERADICATION - Environmental Health Perspectives, Volume 109, No. 1, January 2001, pp. 47-54 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.1/19	Pinnock, D.E., Milstead, J.E., Kirby, M.E., Nelson, B.J.	1977	STABILITY OF ENTOMOPATHOGENIC BACTERIA - Environmental Stability of Microbial Insecticides, 1977 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.1/20	Leong, K.L.H., Cano, R.J., Kubinski, A.M.	1980	FACTORS AFFECTING BACILLUS THURINGIENSIS TOTAL FIELD PERSISTENCE - Environmental Entomology, Volume 9, 1980, pp. 593-599 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-

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IIM 7.1.1/21	Pusztal, M., Fast, P., Gringorten, L., Kaplan, H., Lessard, T., Carey, P.R.	1991	THE MECHANISM OF SUNLIGHT-MEDIATED INACTIVATION OF BACILLUS THURINGIENSIS CRYSTALS - Biochemical Journal, Volume 273, Part 1, January 1991, pp. 43-47 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.1/22	Sundaram, K.M.S., Sundaram, A., Huddleston, E., Nott, R., Sloane, L., Ross, J., Ledson, M.	1997	DEPOSITION, DISTRIBUTION, PERSISTENCE AND FATE OF BACILLUS THURINGIENSIS VARIETY KURSTAKI (BTK) IN PECAN ORCHARDS FOLLOWING AERIAL AND GROUND APPLICATIONS TO CONTROL PECAN NUT CASEBEARER LARVAE - Journal of Environmental Science and Health, Part B, Volume 32 (5), 1997, pp. 741-788 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-

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KMA 7.1.1/02	Bai Y-Y., Jiang, M-X. and Cheng, J-A.	2007	IMPACTS OF ENVIRONMENTAL FACTORS ON DEGRADATION OF CRY1AB INSECTICIDAL PROTEIN IN LEAF-BLADE POWDERS OF TRANSGENIC *BT* RICE - Agricultural Sciences in China 2007, 6(2):167-174 Report-no. not applicable GLP: no Published: yes	N	N	-	-
KMA 7.1.1/03	Sundaram, K.M.S., Sundaram, A. and Sloane, L.	1996	EFFECT OF TRACER DYES ON INITIAL DEPOSITS AND PERSISTENCE OF *BACILLUS THURINGIENSIS* SUBSP. *KURSTAKI* TOXIN AFTER APPLICATION OF TWO COMMERCIAL FORMULATIONS ONTO SPRUCE TREES - Journal of Environmental Science and Health (1996), B31(6):1341-1362 Report-no. not applicable GLP: no Published: yes	N	N	-	-
KMA 7.1.1/04	Haddad, M. de L., Polanczyk, R.A. Alves, S.A. and Garcia, A de O.	2005	FIELD PERSISTENCE OF *BACILLUS THURINGIENSIS* ON MAIZE LEAVES (*ZEA MAYS* L.) - Brazilian Journal of Microbiology (2005), 36:309-314 Report-no. not applicable GLP: no Published: yes	N	N	-	-

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KMA 7.1.1/06	Hung, T.P., Truong, L.V., Binh, N.D., Frutos, R., Quiquampoix, H. and Staunton, S.	2016	FATE OF INSECTICIDAL *BACILLUS THURINGIENSIS* CRY PROTEIN IN SOIL: DIFFERENCES BETWEEN PURIFIED TOXIN AND BIOPESTICIDE FORMULATION - Pest Management Science (2016) Report-no. not applicable GLP: no Published: yes (online)	N	N	-	-
KMA 7.1.1/07	Douville, M., Gagné, F., Masson, L., McKay, J. and Blaise, C.	2005	TRACKING THE SOURCE OF BACILLUS THURINGIENSIS CRY1AB ENDOTOXIN IN THE ENVIRONMENT - Biochemical Systematics and Ecology 33 (2005) 219-232 Report-no. not applicable GLP: no Published: yes	N	N	-	-
KMA 7.1.1/08	Calabrese, D.M., Nickerson, K.W. and Lane, L.C.	1980	A COMPARISON OF PROTEIN CRYSTAL SUBUNIT SIZES IN *BACILLUS THURINGIENSIS* - Canadian Journal of Microbiology (1980), volume 26 Report-no. not applicable GLP: no Published: yes	N	N	-	-

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KMA 7.1.1/09	Ali, A. and Young, S.Y.	1993	EFFECTS OF RATE AND SPRAY VOLUME OF BACILLUS THURINGIENSIS VAR. KURSTAKI ON ACTIVITY AGAINST *HELIOTHIS VIRESCENS* (LEPIDOPTERA: NOCTUIDAE) AND PERSISTENCE IN COTTON TERMINALS - Journal of Economic Entomology 86(3): 735-738 (1993) Report-no. not applicable GLP: no Published: yes	N	N	-	-
KMA 7.1.1/10	Faust, R.M., Hallam., G.M. and Travers, R.S.	1974	DEGRADATION OF THE PARASPORAL CRYSTAL PRODUCED BY *BACILLUS THURINGIENSIS* VAR. *KURSTAKI* - Journal of Invertebrate Pathology 24, 365-373 (1974) Report-no. not applicable GLP: no Published: yes	N	N	-	-
KMA 7.1.1/11	Hostetter, D.L., Ignoffo, C.C. and Kearby, W.H.	1975	PERSISTENCE OF FORMULATIONS OF *BACILLUS THURINGIENSIS* SPORES AND CRYSTALS ON EASTERN RED CEDAR FOLIAGE IN MISSOURI - Journal of the Kansas Entomological Society (1975), 8(2) Report-no. not applicable GLP: no Published: yes	N	N	-	-

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KMA 7.1.1/12	Sánchez-Yáñez, J.M. and Peña-Cabriales, J.J.	2000	PERSISTENCE OF *BACILLUS THURINGIENSIS* SPORES ON SOIL AND MAIZE AND BEAN LEAVES - Journal Terra Latinoamericana (2000), 18(4) Report-no. not applicable GLP: no Published: yes	N	N	-	-
KMA 7.1.1/13	Vettori, C., Paffetti, D., Saxena, D. Stotzky, G. and Giannini, R.	2003	PERSISTENCE OF TOXINS AND CELLS OF *BACILLUS THURINGIENSIS* SUBSP. *KURSTAKI* INTRODUCED IN SPRAYS TO SARDINIA SOILS - Soil Biology & Biochemistry 35 (2003) 1635-1642 Report-no. not applicable GLP: no Published: yes	N	N	-	-
KMA 7.1.1/14	Feng, Y., Ling, L., Fan, H., Lui, Y., Tan., F., Shu, Y. and Wang, J.	2011	EFFECTS OF TEMPERATURE, WATER CONTENT AND PH ON DEGRADATION OF CRY1AB PROTEIN RELEASED - Soil Biology & Biochemistry 43 (2011) 1600-1606 Report-no. not applicable GLP: no Published: yes	N	N	-	-

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KMA 7.1.1/15	Ferreira, L.H.P.L., Suzuki, M.T., Itano, E.N., Ono, M.A. and Arantes, M.N.	2003	ECOLOGICAL ASPECTS OF *BACILLUS THURINGIENSIS* IN AN OXISOL - Scientia Agricola (2003), 60(1): pp.19-22 Report-no. not applicable GLP: no Published: yes	N	N	-	-
KMA 7.1.1/16	Vilas-Bôas, L.A., Vilas-Bôas, G.F.L.T., Saridakis, H.O., Lemos, M.V.F., Lereclus, D., and Arantes, O.M.N.	2000	SURVIVAL AND CONJUGATION OF *BACILLUS THURINGIENSIS* IN A SOIL MICROCOSM - FEMS microbiology Ecology (2000), 31: pp.255-259 Report-no. not applicable GLP: no Published: yes	N	N	-	-
KMA 7.1.1/17	Wang, H., Ye, Q., Gan, J. and Wu, L.	2007	BIODEGRADATION OF CRY1AB PROTEIN FROM *BT* TRANSGENIC RICE IN AEROBIC AND FLOODED PADDY SOILS - Journal of Agriculture and Food Chemistry (2007), 55: pp.1900-1904 Report-no. not applicable GLP: no Published: yes	N	N	-	-

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KMA 7.1.1/18	Hendriksen, N.B. and Carstensen, J.	2013	LONG-TERM SURVIVAL OF *BACILLUS THURINGIENSIS* SUBSP. *KURSTAKI* IN A FIELD TRAIL - Canadian Journal of Microbiology (2013) , 59: pp.34-38 Report-no. not applicable GLP: no Published: yes	N	N	-	-
IIM 7.1.2/01	Menon, A.S., de Mestral, J.	1985	SURVIVAL OF *BACILLUS THURINGIENSIS* VAR. *KURSTAKI* IN WATERS - Water, Air, & Soil Pollution, Volume 25, 1985, pp. 265-274 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.2/02	Furlaneto, L., Saridakis, H.O., Arantes, O.M.N.	2000	SURVIVAL AND CONJUGAL TRANSFER BETWEEN BACILLUS THURINGIENSIS STRAINS IN AQUATIC ENVIRONMENT - Brazilian Journal of Microbiology, Volume 31, No. 4, Oct./Dec. 2000, pp. 233-238 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-

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IIM 7.1.3/01	Griego, V.M., Spence, K.D.	1978	INACTIVATION OF BACILLUS THURINGIENSIS SPORES BY ULTRAVIOLET AND VISIBLE LIGHT - Applied and Environmental Microbiology, Volume 35, No. 5, May 1978, pp. 906-910 Report-no. not applicable GLP: no Published: yes **Submitted in: K IIM 7.1.1/16** **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.3/02	Myasnik, M., Manasherob, R., Ben-Dov, E., Zaritsky, A., Margalith, Y., Barak. Z.	2001	COMPARATIVE SENSITIVITY TO UV-B RADIATION OF TWO BACILLUS THURINGIENSIS SUBSPECIES AND OTHER BACILLUS SP. - Current Microbiology, Volume 43, March 2001, pp. 140-143 Report-no. not applicable GLP: no Published: yes **Submitted in: K IIM 7.1.1/17** **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-

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IIM 7.1.3/03	Pusztal, M., Fast, P., Gringorten, L., Kaplan, H., Lessard, T., Carey, P.R.	1991	THE MECHANISM OF SUNLIGHT-MEDIATED INACTIVATION OF BACILLUS THURINGIENSIS CRYSTALS - Biochemical Journal, Volume 273, Part 1, January 1991, pp. 43-47 Report-no. not applicable GLP: no Published: yes **Submitted in: K IIM 7.1.1/21** **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.3/04	Pedersen, J.C., Damgaard, P.H., Eilenberg, J., Hansen, B.M.	1995	DISPERSAL OF BACILLUS THURINGIENSIS VAR. KURSTAKI IN AN EXPERIMENTAL CABBAGE FIELD not applicable Canadian Journal of Microbiology, Volume 41, 1995, pp. 118-125 Report-no. not applicable GLP: no Published: yes **Submitted in: K IIM 7.1.1/06** **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-

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IIM 7.1.3/05	Walgenbach, J.F., Leidy, R.B.; Sheets, T.J.	1991	PERSISTENCE OF INSECTICIDES ON TOMATO FOLIAGE AND IMPLICATIONS FOR CONTROL OF TOMATO FRUITWORM (LEPIDOPTERA: NOCTUIDAE) - Journal of Economic Entomology, Volume 84, No. 3, June 1991, pp. 978-986 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.3/06	Ignoffo, C.M., Hostetter, D.L., Pinnell, R.E.	1974	STABILITY OF BACILLUS THURINGIENSIS AND BACULOVIRUS HELIOTHIS ON SOYBEAN FOLIAGE - Environmental Entomology, Volume 3, No. 4, 1974, pp. 117-119 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.1.3/07	Pinnock, D.E., Brand, R.J., Jackson, K.L., Milstead, J.E.	1974	THE FIELD PERSISTENCE OF BACILLUS THURINGIENSIS SPORES ON CERCIS OCCIDENTALIS LEAVES - Journal of Invertebrate Pathology, Volume 23, May 1974, pp. 341-346 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-

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IIM 7.1.3/08	Teschke, K., Chow, Y., Bartlett, K., Ross, A., van Netten, C.	2001	SPATIAL AND TEMPORAL DISTRIBUTION OF AIRBORNE BACILLUS THURINGIENSIS VAR. KURSTAKI DURING AN AERIAL SPRAY PROGRAM FOR GYPSY MOTH ERADICATION - Environmental Health Perspectives, Volume 109, No. 1, January 2001, pp. 47-54 Report-no. not applicable GLP: no Published: yes **Submitted in: K IIM 7.1.1/18** **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-
IIM 7.2/01	Meadows, M.P., Ellis, D.J., Butt, J., Jarrett, P., Burges, H.D.	1992	DISTRIBUTION, FREQUENCY, AND DIVERSITY OF BACILLUS THURINGIENSIS IN AN ANIMAL FEED MILL - Applied and Environmental Microbiology, Volume 58, No. 4, April 1992, pp. 1344-1350 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-

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IIM 7.2/02	Akiba, Y.	1986b	MICROBIAL ECOLOGY OF BACILLUS THURINGIENSIS. VII. FATE OF BACILLUS THURINGIENSIS IN LARVAE OF THE SILKWORM, BOMBYX MORI, AND THE FALL WEBWORM, HYPHANTRIA CUNEA. - Japanese Journal of Applied Entomology and Zoology, Volume 30 (2), 1986, pp. 99-105 Report-no. not applicable GLP: no Published: yes **PREVIOUSLY EVALUATED. IN DAR (2008)**	N	N	-	-