European Commission

Draft Renewal Assessment Report prepared according to the Commission Regulation (EU) N° 1107/2009

Microbial Pest Control Agent (MPCA) Bacillus thuringiensis subsp. *kurstaki* SA-12

Volume 3 B.8 (MPCA)

Fate and behavior in the environment

Rapporteur Member State: Denmark

Co- Rapporteur Member State: The Netherlands

Version history

When	What
2008	DAR
2011	Addendum to the DAR
2019	Initial RAR

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B.8 Fate and behavior in the environment

INTRODUCTION

Bacillus thuringiensis subsp. *kurstaki* SA-12 (in the following abbreviated as Btk SA-12) was one of the existing active substances covered by the Regulation (EC) No 2229/2004 on the implementation of the fourth stage of the program of work referred to in Article 8(2) of Council Directive 91/414/EEC. In Annex I to Regulation (EC) No 2229/2004 the Commission designated Denmark as rapporteur Member State to carry out the assessment of Btk SA-12 on the basis of a joint dossier submitted for the Btk strains SA-11, SA-12 and EG 2348. The notifier for Btk SA-11 and SA-12 was Mitsui AgriScience International SA/NV while EG 2348 was notified by Mitsui AgriScience International SA/NV and Intrachem Bio Italia S.p.A. (now CBC (Europe) S.r.l.). In accordance with the provisions of Article 22(1) of Regulation (EC) No 2229/2004, Denmark submitted in January and February 2008 to the EFSA the draft assessment report, including, as required, a recommendation concerning the possible inclusion of Btk SA-12 in Annex I to the Directive. The Commission examined the draft assessment report, the recommendations by the rapporteur Member State and the comments received from other Member States in consultation with experts from a certain number of Member States. The Commission referred on 12 July 2008 a draft review report to the Standing Committee on the Food Chain and Animal Health, for final examination. The draft review report was finalized in the meeting of the Standing Committee on 12 July 2008. Subsequently Regulation (EC) No 1107/2009 repealed and replaced Directive 91/414/EEC and the active substance Btk SA-12, was deemed to be approved under that Regulation and included in the Annex to Regulation (EC) No 540/2011. EFSA delivered its conclusions on *Bacillus thuringiensis* ssp. *kurstaki* (strains ABTS-351, PB-54, SA-11, SA-12, EG2348) on the 16 December 2011 (published 23 February 2012). Based on this new information available, no need to change the conditions of approval of Btk SA-12 was identified. The Commission filed on 13 December 2013 an updated review report for Btk strains SA-11, SA-12 and EG 2348 to the Standing Committee on the Food Chain and Animal Health for examination.

The approval of Btk SA-12 under the Regulation (EC) No 1107/2009 expires 30 April 2019. In accordance with the same Regulation the original notifier Mitsui AgriScience International SA/NV has filed to the Commission an application for the renewal of the approval of the active substance Btk SA-12 on 30 April 2016. In accordance with Regulation (EU) 2016/183 the notifier submitted to the designated RMS Denmark, the co-RMS The Netherlands as well as to EFSA and Commission a dossier for renewal of Btk SA-12 considering the deadline stated in SANTE-2016-10616–rev. 3.

Btk SA-12 is a wild type strain originating from infested insects. Btk acts highly specific against insect species of the order Lepidoptera and is not expected to have any harmful effects on beneficials and other non-target species of other insect orders. The insecticidal activity of Btk is mainly attributed to spore bound insecticidal pro-proteins (*Cry* toxins) which are ingested by the target pests and activated under alkaline conditions in the midgut of the larvae. The first assessment of the strain proved that it does not have any harmful effects on human or animal health or on groundwater or any unacceptable influence on the environment. The overall conclusion from EFSA (2012) confirms that no critical areas of concern are identified within the framework of the use which was supported.

As the manufacturing process of Btk SA-12 has not been changed since original approval, all data submitted for the original approval of the strain are considered fully applicable for the current evaluation.

For the renewal of the Btk strains SA-11, SA-12 and EG 2348 under Regulation (EC) 1107/2009, a separate dossier was submitted for each strain only including data, which have previously not been submitted or evaluated. Nevertheless, there is some information which is applicable to all three Btk strains, e.g. published information for Btk in general obtained during searches for peer reviewed literature according to EFSA Guidance (2011)[1] carried out for relevant sections.

In the following for ease of information, full study summaries/sections taken from the DAR (2008) or its Final Addendum (2011) are included if they are considered relevant for renewal of Btk SA-12. In order to facilitate discrimination between new data and data already evaluated during the first approval process, the headline “New Data” begins the section with data, which have previously not been submitted or evaluated. Data and their evaluations from the original DAR and addenda to the DAR are highlighted by grey background.

1 Guidance of EFSA: 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

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 numerous localities and from most terrestrial habitats. In the natural environment, under favourable conditions, *B. 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 β-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 β-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, *B. thuringiensis* is a poor infectious agent and rarely recycles. While vegetative cells and spores can be produced in insect cadavers, *B. 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.

New Information

Relevant information has been already provided during original approval of Btk strain SA-12 and is still valid. Please refer to the DAR Volume 3, Section B8 and Addendum to the DAR. A literature search covering the last 10 years was performed on subspecies level (Cornelese, 2016b; KMA 8.1/01). Please see below Vol.3 MA, B.8.4. The relevant literature found in the search is evaluated in the chapters below.

Cited references

Report KMA 8.1/01 Cornelese A. (2016b). Literature review on *Bacillus thuringiensis* subsp. *kurstaki* strain SA-12 and metabolites: Fate and behaviour in the environment.

Unpublished report

Evaluation RMS	The literature search was accepted as valid, both regarding inclusion of databases and use of search terms. Please refer to point B.8.4.

Summary of available information on persistence of *Bacillus thuringiensis* in the environment

It is generally agreed that persistence of Bt populations on plant surfaces is low. Half-life times recorded in the literature range from some hours to a maximum of 10 days (e.g. Griego & Spence, 1978; Pedersen et al., 1995; Dent, 1993; Martin, 1994). Factors restricting field persistence are UV-mediated degradation of spores, rain fall and plant growth (dilution effects), lack of nutrients and low humidity. Natural levels of Bt on plant surfaces range between 3 and nearly 1000 CFU/g or cm² (Smith & Couche, 1991; Ignoffo et al., 1974; Hostetter et al., 1975). These studies indicate also that Bt populations quickly decrease to background levels upon treatment.

As seen from the data on persistence presented below, no multiplication occurs on leaves due to sensitivity to solar radiation, foliage exudates and microbial competition. Spore germination and growth was observed in sterile soils, when amended with nutrients, but never in natural bulk soils (Akiba et al., 1986; Saleh et al., 1970; Petras & Casida, 1985). Vegetative cells disappeared rapidly within 1-2 days after inoculation, but cells were able to form spores (Akiba et al., 1986). Germination of spores occurs only if conditions are appropriate, which is only the case after ingestion by insects (Pedersen et al., 1995) or earthworms, or in the rhizosphere of several, but not all plants (Hendriksen & Hansen, 2002). Long term persistence in soil occurs at levels close to background levels (Hendriksen & Hansen, 2002).

In the table below a summary of data on environmental persistence of Btk already provided for Annex I inclusion of Btk SA-12 and of new data is provided.

Table 8.1-01 Half-live of Bt cells, spores and protoxins in soil, water, air

Compound	Experimental approach	Germination/ growth	Half-live time	Factors affecting Bt loss	References
Soil					
Cells	Natural soil (laboratory)	No (only in sterile soil amended with nutrients)	1-2 d*	Nutrient restriction	Akiba et al, 1986[1]
Spores	Natural soil (laboratory)	No (only in sterile soil amended with nutrients)	< 4 d	Competition, nutrient restriction, soil type, pH, predation	Saleh et al., 1970[1]
	Natural soils (field)	No	14 d		Petras & Casida, 1985[1]
	Natural clay loam soil (laboratory)	No	< 63 d		Pruett et al., 1980[1]
	Cabbage field soil (field)	No	120 d		Pedersen et al., 1995[1]
	Sterilized/non-sterilized soil	No	48 h*		Sánchez-Yáñez & Peña-Cabriales, 2000[2]
	Field soil	No	100-200 d		Hansen et al 1996[3]
	Cabbage field	No	>13 years		Hendriksen et al 2013[4]
	Cabbage field	No	>7 years		Hendriksen et al 2002[3]
	Cork oak soil (field)	No	7 years (DT90)		Vettori et al 2003[3]
	Forest field	No	<2 years		Smith and Barry 1998[3]
Protoxin	Free protoxin/toxin	-	0.5-4 h†	Adsorption (depending on soil type), consumption	Stotzky 2000[1]
	^{14}C-labelled protoxins, sterilized, amended soil	-	2.7-5.2 d		West et al., 1984a[1]
	Natural soil	-	3-21 d*		West et al., 1984b[1]
	Natural soil (laboratory)	-	< 7 d		Pruett et al., 1980[1]
	[^{14}C]Cry1Ac Natural soil (laboratory)	-	15 d		Acinelli et al., 2008[4]
	Cry1Ab and Cry1Ac Natural soil (laboratory)	-	9.8 and 12.7 days		Marchetti et al., 2007[4]
	Cry3Bb1 Natural soil (laboratory)	-	40 d (DT90)		Icoz et al., 2007[4]
	Cry1Aa Natural soil (laboratory)	-	< 14 d		Helassa et al., 2011[4]
	Cry1Ac Natural soil (laboratory)	-	1.5 d		Li et al., 2013[4]

Com-pound	Experimental ap-proach	Germination/ growth	Half-live time	Factors affecting Bt loss	References
	Cry1Ab paddy soil (anaerobic)	-	141, 138 and 45.9 d		Wang et al., 2007[4]
Water					
Cells	Sterilized tap/lake water, sewage	No	< 24 h	Nutrient re-striction	Furlaneto et al., 2000[1]
Spores	Sterilized tap/distilled water	No	< 20 d	Nutrient re-striction, sedi-mentation / ad-sorption, con-sumption, (salt concentration)	Menon & Mestral, 1985[1]
	Sterilized seawater	No	~ 20 d		
	Sterilized lake water	No	~ 40 d		
	Forestry, river water	No	≤ 13 d*		
	Field application, river water	No	< 30 d*		Buckner, 1974 cited in Glare & O´Callaghan, 2000[1]
Protoxin	Bti protoxin, laborato-ry microcosms	-	3-5 d*	Sedimentation / adsorption	Larget 1981, cited in Glare & O`Callaghan, 2000[1]
	Bti protoxin river	-	1-4 weeks*		Glare & O´Callaghan, 2000[1]
	Cry1Ab hydrolysis	-	130.8 to 93.7 d		Wang et al., 2007[4]
	Cry 1 Ac artificial 'natural' water	-	12.8 d		Li et al., 2013[4]
Air					
Spores	Field application	-	3.3 h – 2.4 d	Solar radiation	Teschke et al., 2001[1]
Foliage/crops					
Spores	Cabbage	No	16 h	Solar radiation, rain fall, plant growth, leaf tem-perature, vapour pressure	Pedersen et al., 1995[1]
	Soy bean	No	< 24 h		Ignoffo et al., 1974[1]
	California redbud, different commercial formulations	No	0.58-1.85 d		Pinnock et al., 1974[1]
	Field, different crops	No	24-48 h*		Leong et al., 1980[1]
	Depending on formu-lation	Not indicat-ed#	4 -10 d		Dent, 1993[5]
	Maize, beans (green-house)	No	3 days*		Sánchez-Yáñez & Peña-Cabriales, 2000[2]
	Ornamental tree	No	1-3 days		Hostetter et al., 1975[2]
	Potato, tomato, green pepper, and eggplant leafs	No	1 day*		Martin, 1994[2]
	Grapes (spraying of commercial products)	Not indicat-ed#	At time of har-vest:		Bae et al., 2004[2]

Com-pound	Experimental ap-proach	Germination/ growth	Half-live time	Factors affecting Bt loss	References
			10^2 - 10^4 CFU/g		
	Cannot be retrieved	No	16-38 h		Hansen et al.,1996[3]
	Broccoli sellerie	No	1 month <<1 month		Madsen et al., 2011[3] Hendriksen et al., 2011[3]
	Cotton, amaranth, rice	No	120 h		Wang et al.,2014[3]
Protoxin	Pecan tree	-	14.3-24.4 h		Sundaram et al., 1997[1]
	Tomato	-	< 48 h		Walgenbach et al., 1991[1]
	Cotton	-	< 48 h		Wilson et al., 1983 (cited in Walgenbach et al., 1991)[1]
	Field, different crops	-	48-96 h*		Leong et al., 1980[1]

* no half-live times provided, therefore data for complete disappearance are given
† Adsorption of free toxins produced by transgenic plants avoids microbial degradation, insecticidal activity remaining after 234 d
# not explicitly mentioned but data suggest absence of germination and growth
[1] OECD dossier M-IIM, Section 5
[2] OECD dossier M-IIM Section 4
[3] EFSA SCIENTIFIC OPINION on Risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs EFSA Panel on Biological Hazards (BIOHAZ), EFSA Journal 2016;14(7):4524
[4] Current Vol. 3 MA section 6 below

The ecology of *B. thuringiensis* is still poorly understood. It can readily be recovered in spore form from a variety of environments, including soil, plants, and dead insects. For many years, *B. thuringiensis* has been regarded solely as an insect pathogen. The belief being that it is present on leaves, which, when consumed by susceptible insects, causes death with multiplication and subsequent sporulation occurring in the cadaver; the progeny spores and crystals of *B. thuringiensis* being then available for ingestion by further, susceptible lepidopteran larvae. Infected larvae would, however, be expected to become recycled in the soil, and it is unclear how Bt could subsequently recolonize the phylloplane. Spores can reach the lower leaves by rain splash, but no other mechanisms have clearly indicated how *B. thuringiensis* in the soil can colonize plants and so have the opportunity to exert its pathogenic nature. Furthermore, several studies following the fate of *B. thuringiensis* introduced into soil indicate that steady rates of decline are observed. Although it has been found that spores of *B. thuringiensis* can germinate in the rhizosphere of some plants and also in the guts of some soil invertebrates, little multiplication seems to occur. Bizarri et al (2008) investigated in a study colonisation ability of leaves by *B. thuringiensis* subsp. *kurstaki* strain HD-1. 10^6 colony forming units (CFU)/g soil of two prepared stain types were mixed into two replicates of sterile and nonsterile soil. In addition, 2×10^6 CFU/g of each of the latter two organisms were mixed together, and the resulting suspension was inoculated into two replicates of sterile and nonsterile soil. Clover seeds (*Trifolium hybridum* var. Alsike) were sown into the soil. In all experiments where sterilized soil was used, the seeds were surface sterilized. Sterile batch, not inoculated with bacteria, was also seeded to provide a negative control and to ensure the absence of contamination between the batches. The resulting seedlings were grown in a glasshouse at 25°C and 12:12 photoperiod for 5 weeks. Samples of leaves were collected at the fourth and fifth week after inoculation, weighed and stomached. There was no difference in colonisation level between the separate exposure of strain types and the mixture. Spores of *B. thuringiensis* in the soil can be the source of colonization of emergent plants, this has been demonstrated in this article. Whether this involves germination in the soil, on the emerging shoot and/or subsequently as the seedling develops above ground is not known, although it has previously been found that *B. thuringiensis* does not normally occur vegetatively in bulk soil but can do so in the rhizosphere of at least some plants.

General information on persistence in the environment in diverse matrices was provided in a study by Van Cuijk et al (2011). They sampled in urban areas after the use of *B. thuringiensis* subsp. *kurstaki* to suppress or eradicate gypsy moth populations. Soil, surface, water, and vegetation samples were collected from two urban areas (Seattle, WA, and Fairfax County, VA), up to 4 years after spraying, and analysed for the presence of viable *B. thuringiensis* subsp. *kurstaki*. One spray area in Fairfax County, VA, was sampled immediately before (background)

and after *B. thuringiensis* subsp. *kurstaki* spraying and then at 6, 12, 24, and 48 weeks after spraying in 2008 and was largely residential area and included soil, wipe, water, grass, and leaf samples. One application of a commercial formulation of *B. thuringiensis* subsp. *kurstaki*; was applied via helicopter at a rate of 470 liters per km^2. In Seattle, WA, samples were collected in locations that the Washington State Department of Agriculture sprayed between 2004 and 2007. Samples from Seattle included soil, wipe, and water samples. Samples were analysed using DNA methods and analysis for viability by culturing. Urban soils were found to be the most reliable reservoir for viable *B. thuringiensis* subsp. *kurstaki*. Soil results are presented graphically in the **Figures 8.1-02** and **8.1-03** below. A downward trend in the percentage of samples containing detectable DNA and viable cultures was observed with increasing time after spraying; however, viable *B. thuringiensis* subsp. *kurstaki* was still detected 4 years after spraying. Other sample types were less reliable reservoirs of *B. thuringiensis* subsp. *kurstaki* compared to soil and exhibited large variability.

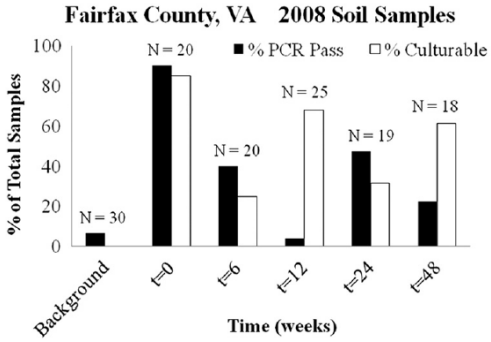

Figure 8.1-01. Soil analysis results as a percentage of total samples passing the *B. thuringiensis* subsp. *kurstaki* PCR screen (■) and *B. thuringiensis* subsp. *kurstaki* culture (□) for Fairfax County, VA, plotted according to weeks after *B. thuringiensis* subsp. *kurstaki* spraying. N, number of sample pools analyzed.

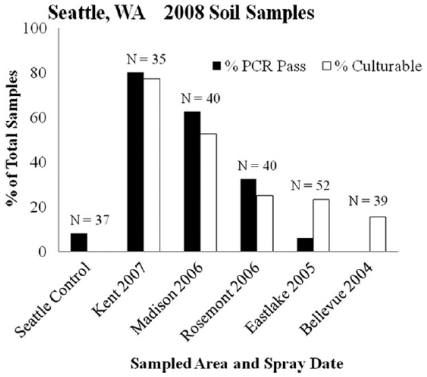

Figure 8.1-02. Soil analysis results as a percentage of total samples passing the *B. thuringiensis* subsp. *kurstaki* PCR screen (■) and *B. thuringiensis* subsp. *kurstaki* culture (□) for Seattle, WA, plotted according to the *B. thuringiensis* subsp. *kurstaki* spray date. N, number of sample pools analyzed.

Cited references

Report KMA 8.1/13 – Bizarri M.F., Bishop A.H. (2008). The Ecology of *Bacillus thuringiensis* on the Phylloplane: Colonization from Soil, Plasmid Transfer, and Interaction with Larvae of *Pieris brassicae*

Published report,

Microb Ecol 56(1):133-139

Abstract: Seedlings of clover (*Triflorium hybridum*) were colonized by *Bacillus thuringiensis* when spores and seeds were co-inoculated into soil. Both a strain isolated in the vegetative form from the phylloplane of clover, 2810-S-4, and a laboratory strain, HD-1, were able to colonize clover to a density of about 1000 CFU/g leaf when seeds were sown in sterile soil and to a density of about 300 CFU/g leaf in nonsterile soil. A strain lacking the characteristic insecticidal crystal proteins produced a similar level of colonization over a 5-week period as the wild type strain, indicating that crystal production was not a mitigating factor during colonization. A small plasmid, pBC16, was transferred between strains of *B. thuringiensis* when donor and recipient strains were sprayed in vegetative form onto leaves of clover and pak choi (*Brassica campestris* var. *chinensis*). The rate of transfer was about 0.1 transconjugants/recipient and was dependent on the plant species. The levels of *B. thuringiensis* that naturally colonized leaves of pak choi produced negligible levels of mortality in third instar larvae of *Pieris brassicae* feeding on the plants. Considerable multiplication occurred in the excreted frass but not in the guts of living insects. Spores in the frass could be a source of recolonization from the soil and be transferred to other plants. These findings illustrate a possible cycle, not dependent on insect pathology, by which *B. thuringiensis* diversifies and maintains itself in nature.

Evaluation RMS	The study is acceptable. The study has demonstrated that spores of *B. thuringiensis* in the soil can be the source of colonization of plants, and thereby shows a possible cycle, not dependent on insect pathology, by which *B. thuringiensis* diversifies and maintains itself in nature.

Report KMA 8.1./14 –Van Cuyk S., Deshpande A., Hollander A., Duval N., Ticknor L., Layshock J., Gallegos-Graves L., and Omberg K.M. (2011). Persistence of *Bacillus thuringiensis* subsp. *kurstaki* in Urban Environments following Spraying.

Published report,

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, 77(22): 7954–7961

Abstract: *Bacillus thuringiensis* subsp. *kurstaki* is applied extensively in North America to control the gypsy moth, *Lymantria dispar*. Since *B. thuringiensis* subsp. *kurstaki* shares many physical and biological properties with *Bacillus anthracis*, it is a reasonable surrogate for biodefense studies. A key question in biodefense is how long a biothreat agent will persist in the environment. There is some information in the literature on the persistence of *Bacillus anthracis* in laboratories and historical testing areas and for *Bacillus thuringiensis* in agricultural settings, but there is no information on the persistence of *Bacillus* spp. in the type of environment that would be encountered in a city or on a military installation. Since it is not feasible to release *B. anthracis* in a developed area, the controlled release of *B. thuringiensis* subsp. *kurstaki* for pest control was used to gain insight into the potential persistence of *Bacillus* spp. in outdoor urban environments. Persistence was evaluated in two locations: Fairfax County, VA, and Seattle, WA. Environmental samples were collected from multiple matrices and evaluated for the presence of viable *B. thuringiensis* subsp. *kurstaki* at times ranging from less than 1 day to 4 years after spraying. Real-time PCR and culture were used for analysis. *B. thuringiensis* subsp. *kurstaki* was found to persist in urban environments for at least 4 years. It was most frequently detected in soils and less frequently detected in wipes, grass, foliage, and water. The collective results indicate that certain species of *Bacillus* may persist for years following their dispersal in urban environments.

Evaluation RMS	The study is acceptable. The collective results indicate that certain species of *Bacillus* may persist for years following their dispersal in urban environments.

B.8.1.1 Soil

No studies on persistence and multiplication in soil of the strains SA-11, SA-12 or EG2348 have been submitted. However, since numerous studies that address this issue have been reported in the scientific literature, these data are regarded as adequate.

Table 8.1-01 is a summary of studies from the scientific literature on the fate of *B. thuringiensis* spores in soil environments. The product does not contain vegetative cells, therefore studies on fate are limited to spores. Four field studies and four laboratory studies are summarized; six of these studies have been performed with *B. thuringiensis* subsp. *kurstaki* strains. It appears from all the studies that no increases in numbers occur as a function of time have been observed, thus no multiplications have been demonstrated. In the field studies where high numbers (log 7.2 cfu/g soil) have been applied a rapid initial decline has been observed, while no initial decline has been observed in the two experiments where a smaller number (log 4.0; log 3.0/cfu/g) were applied. Persistence of numbers between log 2 and log 3 cfu/ g have been observed for up to seven years after the application. In the experiment, which is most similar to the application of Btk SA-12 reported by Pedersen *et al*. (1995) (application of a *B. thuringiensis* subsp. *kurstaki* strain to an agricultural field with cabbage at a level corresponding to the application of one of these *B. thuringiensis* subsp. *kurstaki* strains) the half-life of the spores was estimated to be 120 days, and the persistence in this field has been reported to be more than seven years at a level around log 2.6 cfu/g (Hendriksen & Hansen, 2002). The number of *B. thuringiensis* naturally occurring in soil has been reported to variate between log 2 and log 5 cfu/g (Martin, 1991), so the application of Btk SA-12 to soil is not expected to significantly increase the number of *B. thuringiensis* present in soil.

No studies on the fate of crystallized cry protoxins from Btk SA-12 have been submitted. Studies on the fate of crystallized protoxins in soil from the scientific literature are summarized in **table 8.1-01**. The three studies reported in the DAR are laboratory studies performed with protoxins from either *B. thuringiensis* subsp. *galleriae* or subsp. *aizawai* strains, however these correspond to the protoxins of *B. thuringiensis* subsp. *kurstaki.* It appears from the table that the crystallized pro-toxins disappear with half-lives between 3 days and 50 days; the persistence has been reported to vary between 135 and 1000 days. In a field experiment with Dipel was the pro-toxin documented to be present 840 days after application by an immuno-assay; however the potency of the protoxin in the soil declined to 50% in 10 days. This suggests that only immuno-reactive parts of the protoxin, and not the full pro-toxin, are present in the soil after some time or the persisting pro-toxin cannot be activated as a toxin after ingestion by the insect. In conclusion crystallized protoxins is disappearing from soil, and only small amounts will probably remain in the soil from one growing season to the next. It is most likely that this disappearance is mediated by degradation by microbial proteolytic enzymes.

Numerous publications are investigating the activity and fate of *Bacillus thuringiensis* and *Bacillus thuringiensis* subsp. *kurstaki* in soils. The survival of Bt spores and parasporal crystals in soil in both nature and in the laboratory was examined over an 8-week incubation period by Petras & Casida (1985). The study showed an initial decrease in CFU of 1 log over the first two weeks, which was followed by a steady spore and crystal count for the following 6 weeks.

In a study with a field application of spores of Btk onto a cabbage field, a half-life of 120 days for spores was established after Btk was sprayed directly onto the field soil at a level of 1.2×10^4 CFU/g, which declined to a level of 2.3×10^3 CFU/g after almost one year (Pedersen *et al.*, 1995). A persistent population stabilizing around 6.6×10^2 CFU/g in the same filed was reached during the next three years. Thus persistence at this level was demonstrated for seven years (Hendriksen & Hansen, 2002).

West *et al.* (1985) found that the factor of greatest importance for Bt survival and growth was nutrient availability (which directly relates to the presence of and competition with other soil micro-organisms) as Bt could not grow in soil under most natural soil conditions.

The long-term persistence of Bt viability and parasporal crystal insecticidal potency after incubation in soil was examined by West *et al.* (1984a) and by West *et al.* (1984b). Both studies showed the rate of Bt spors decreased in numbers more rapidly in natural soils as compared to sterilised soils. A quick loss of parasporal insecticidal activity in natural soils occurred between 3 and 21 days after the incubation was initiated. In autoclaved soils, the rate of loss of insecticidal activity was much lower. The authors concluded that Bt parasporal crystals in the soil appear to be susceptible to microbial degradation while the spores of Bt are less susceptible: Thus, the presence of indigenous micro-organisms in natural soil increase the rate of mortality and loss of potency of Bt.

In contrast, Btk, formulated in DiPel 176, was applied in the laboratory to the litter and the fermentation-humus of an acid soil from a coniferous forest in Ontario, Canada (Visser *et al.*, 1994). Application rates were 1.42×10^5 and 1.42×10^8 CFU/g dry weight soil (similar to normal application rate and 1000 x application rate). Over an 8-week period, no significant difference in the numbers occurred, neither in litter nor in the fermentation humus or as a function of the number applied. This indicates long term survival in the litter and the fermentation humus at the microcosmos conditions, exceeding the incubation period of 8-weeks considerably. However, no evidence of growth is presented. The authors refer to another Canadian study where populations of Btk did not decline until 3.5 months after being introduced by aerial spraying to a forest soil in Nova Scotia.

Certainly, the community of micro-organisms present and the physical properties of the test soil (forest soils) differ substantially to an agricultural soil and results reported here are not regarded as being representative for agricultural soils.

The composition of the soil affects the absorption, and consequently the persistence of any Bt inactive protoxin and active toxin proteins in the soil. Several studies have demonstrated the equilibrium adsorption and binding of the purified protoxin and toxins produced by Bt species onto the (predominant) clay minerals, montmorillonite and kaolinite (Venkateswerlu & Stotzky, 1992; Tapp & Stotzky, 1995), and on the clay-, silt-, and sand-size fractions of soil, as well as the adsorption and binding of the toxin from Btk (from DiPel formulation) on humic acids from different soils and on clay humic acid complexes (Crecchio & Stotzky, 1998; Crecchio & Stotzky, 2001).

An important abiotic factor playing a role is solar radiation in the form of ultraviolet and visible light. Survival times of Bt (HD-1 strain) spores and spore-crystal aggregates exposed to natural sunlight between 11 am and 1 pm at 45° latitude and in temperatures of 30 to 38 °C were very short with a half-life of 10 minutes (Griego & Spence, 1978). The greatest effect on survival occurred at a wavelength in the visible range of 400 nm. Exposure of Btk spores to ultraviolet light (280 to 330 nm) for over 60 minutes caused a reduction in survival of spores to 1 % of the initial count (Myasnik *et al.* 2001). Several other studies show fast inactivation of spores due to exposure to UV light.

Sunlight also leads to the inactivation and destruction of Btk (HD-1 strain) purified δ-endotoxin crystals as observed by Pusztal *et al.* (1991). Following a 24 hour irradiation with a spectrum equivalent to the solar spectrum, approximately 35 % of crystal proteins were damaged resulting in total loss of activity. Another study showed half-life values of between 14.3 and 24.4 hours on pecan foliage. As no precipitation occurred during the 96-hour study period, the loss of toxin is primarily attributed to UV degradation (Sundaram *et al.*, 1997).

In consequence the results of these and other studies lead to the conclusion that the survival of *B. thuringiensis* in the soil is a dynamic process involving sporostasis, germination and sporulation in specific habitats and influenced by changing conditions regarding soil type, native micro flora, nutrient availability and fertilization.

Furthermore, these studies demonstrate that after an application of Delfin WG (*Bacillus thuringiensis* subsp. *kurstaki* Strain SA-11) onto grape fields, survival of the endospores of Bt in soil is very likely for a period of a few months during which time a natural breakdown begins and gradually reduces the numbers of spores remain-

ing. Any vegetative cells or crystal proteins however are likely to be far more rapidly degraded. This reduction in numbers will be greatly augmented by the photo degradation effects of sunlight. It is very unlikely that Bt endospores will germinate and grow into vegetative cells, unless encouraging conditions exist, meaning favourable soil pH, soil moisture content, sufficient nutrient availability and lack of competition/predation from other soil micro-organisms.

Thus, the application of *B. thuringiensis* subsp. *kurstaki* SA-11, SA-12 or EG2348 to the soil is not expected to increase the number of *B. thuringiensis* naturally present in soil significantly (reported to variate between 2 x 10^2 and 4.9 x 10^4 CFU/g (Martin, 1991)), except for some months following the application.

New information

Persistence of *B. thuringiensis* spores in soil

Persistence of Foray preparation was evaluated during one-and-a-half-year period in a 195-hectare oak forest on the Krotoszyn Plateau, Poland (Konecka et al, 2014). The study site has not been treated with any bioinsecticide based on *B. thuringiensis* before. Foray 04UL was applied one time at a rate of 2 l per hectare by an airplane. The a.i. is a mixture of spores and crystals with a potency of 21200 IU/mg. Samples of soil had been collected two days before application, and then two days, one month, six months, 12 months and 18 months after spraying. A 1200 cm^3 surface layer of soil (10 cm deep with diameter of 6 cm) was collected each time. Four hornbeam leaves were collected in the same place as the soil samples at each sampling point. Each leaf was gathered from different height of a tree. The sites of sample collection were chosen randomly. Twenty soil and 60 leaves samples were collected each time. Soil in the sampling sites was gleysol, sandy clay loam or sandy cobbly loam with pH 3.7–3.8. Samples were prepared and incubated on *B. cereus* lab agar After a 24-hour incubation at 30°C, white, flat, circular and undulate bacterial colonies were counted and cultured on the sporulation medium for 4 days and then the presence of protein crystals in cultures was evaluated in a light microscope after staining with amido black. The bacterial strains synthesizing non-swollen spores and crystals were identified as *B. thuringiensis*. The average number of *B. thuringiensis* CFU was calculated per 1 g of soil and per 4 cm^2 of leaf surface of each sample. The number of *B. thuringiensis* CFU in the soil of the oak forest units on the Krotoszyn Plateau before biopesticide was applied ranged from 0 to 3.5 × 10^2 CFU/g, no *B. thuringiensis* was found on leaves. RAPD clonal analysis showed that *B. thuringiensis* strains were genetically distinguishable from commercial *B. thuringiensis* subsp. *kurstaki* HD-1 in the product. Two days after application, the number of *B. thuringiensis* in soil increased and ranged from 12.7 × 10^2 to 29.5 × 10^2 CFU/g in soil. On 4 cm^2 surface of leaves there were from 43 to 76.7 CFU of *B. thuringiensis*. Five *B. thuringiensis* strains from each soil sample and five from each leaf were included in the bacterial RAPD typing and all 200 isolates were genetically identical with *B. thuringiensis* HD-1 strain. The differences between the number of bacteria collected before and two days after application were statistically significant for both the soil ($p = 0.003$) and the leaf samples ($p < 0.001$). One month after the bioinsecticide spraying, there was an increase in the number of *B. thuringiensis* in soil (p b 0.001) and a decrease on the leaf surface ($p < 0.001$). The number of *B. thuringiensis* in soil of forest units ranged from 123.5 × 10^2 to 166.5 × 10^2 CFU/g, whereas on leaf surface ranged from 3 to 19 CFU/4 cm^2. The RAPD typing showed that *B. thuringiensis* isolates cultured from all the soil and leaf samples were identical with the *B. thuringiensis* HD-1 strain. Six months after spraying, the number of *B. thuringiensis* in soil was statistically higher than two days after the bioinsecticide application ($p = 0.022$) but lower than a month after the spraying ($p < 0.001$) and ranged from 23.4 × 10^2 to 53 × 10^2 CFU/g in soil and from 0.5 to 3 CFU/4 cm^2 on leaf surface. The RAPD fingerprinting revealed that *B. thuringiensis* isolates cultured from all the soil and leaf samples had the same DNA patterns as the HD-1 strain. Twelve months after application, the number of *B. thuringiensis* was reduced and it was similar to the number before the biopesticide spraying in soil ($p = 0.351$) and on leaf surface ($p = 1$). The number of *B. thuringiensis* in soil ranged from 0 to 10 × 10^2 CFU/g, whereas no *B. thuringiensis* was isolated from the leaves. The RAPD method showed that only on two sampling sites *B. thuringiensis* isolates were genetically identical with the HD-1 strain. The analysis of the samples collected from these two locations 18 months after application revealed that in soil and on leaf surface there were no *B. thuringiensis* isolates clonally related to the HD-1 strain. The results showed that a large number of spores released into the environment to control the insect pest persisted in soil for one year and on leaf surface for half a year. No *B. thuringiensis* was noted on leaf surface one year after spraying and in soil after one and a half years. The survival of *B. thuringiensis* in the environment could result from the germination of *B. thuringiensis* spores in hosts. On the other hand, the study proves that *B. thuringiensis* endospores artificially introduced in a forest environment do not persist infinitely but disappear after a given time.

Long term persistence in a field after treatment after application was described by Hendriksen & Carstensen (2013). In the study a cabbage field was treated in 1993 with 5L spray solution 6.5 x 10^7 CFU/mL *B. thuringiensis* subsp. *kurstaki* DMU67R a rifampicin-resistant strain. In 2001 an area of 1 m^2 was selected within this plot which again was divided in 10×10 cm which were randomly selected for monitoring during the period 2001-

2006. A significant linear decline in DMU67R density was identified. Low values appeared to be associated with water content. In this study a persistence of at least 13 years was demonstrated for *B. thuringiensis* subsp. *kurstaki* DMU67R. Moisture content of the soil seemed to be the most decisive killing factor during the sampling period 2001-2006. Germination and growth has not been directly observed in the field plot but it has been suggested it occurs based on earlier works. The density that is found in the soil during this study is of the same order of magnitude as the density of *B. thuringiensis* often found in soils.

Persistence of endotoxins in soil

In the literature search, several studies have been identified investigating the persistence of Bt cry toxins in soils.

Reference:	KMA 8.1.1/19
Author:	Accinelli C., Koskinen W.C., Becker J.M., Sadowsky M.J. (2008)
Title:	Mineralization of the *Bacillus thuringiensis* Cry1Ac Endotoxin in Soil
Date:	01.09.2008
Doc ID:	Published: Journal of Agricultural and Food Chemistry, 56(3), 1025-1028
Guideline:	Not documented
GLP:	Not documented
Acceptability:	The study is considered to be acceptable.
Material and methods:	
Test material:	Purified ^{14}C labeled CryIAc endotoxin; purified from *E. coli* production batch
Test concentration:	^{14}Clabeled Cry1Ac endotoxin (approximately 2.2×10^{-4} MBq mL-1) was added to attain a final concentration of 10 μg g-1 of soil.
Test system:	2 g soil into 20 mL scintillation vials; sterile soil, non-sterile soil, non-sterile amended soil. Incubation during 20 days in the dark
Temperature:	25 °C
Sampling time points:	Not specified
Method of analysis:	^{14}C LSC
Soil characteristics:	loamy sand, with a pH of 5.8, 86.0% sand, 6.0% clay, and 1.5% organic carbon.

The metabolic potential of microorganisms in soil samples was estimated by measuring mineralization of [^{14}C]glucose. An aqueous solution of ^{14}C-labeled (3.7×10^{-3} MBq mL-1) and unlabeled glucose was applied to soil mixture to obtain a final concentration of 1 μg g-1 glucose. Triplicate 0.3 g (dry weight) soil subsamples from each of the three replicates from the ^{14}C-labeled Cry1Ac and [^{14}C]glucose experiments were combusted. The radioactivity of trapped $^{14}CO_2$ was counted using a liquid scintillation counter.

Results

Mineralization of the ^{14}C-labeled Cry1Ac endotoxin in both unamended and amended nonsterile soil is shown in **Figure 8.1.1-01**. Mineralization did not proceed rapidly until after a 24 h lag period. Once evolution of $^{14}CO_2$ occurred, samples incubated with additional organic material in the form of corn residue mineralized the toxin at a faster rate than those samples without added organic material. Overall, mineralization of the [^{14}C] Cry1Ac toxin was effective, with 63 and 59% converted to $^{14}CO_2$ within samples incubated with and without additional organic material, respectively, over the 20 day period.

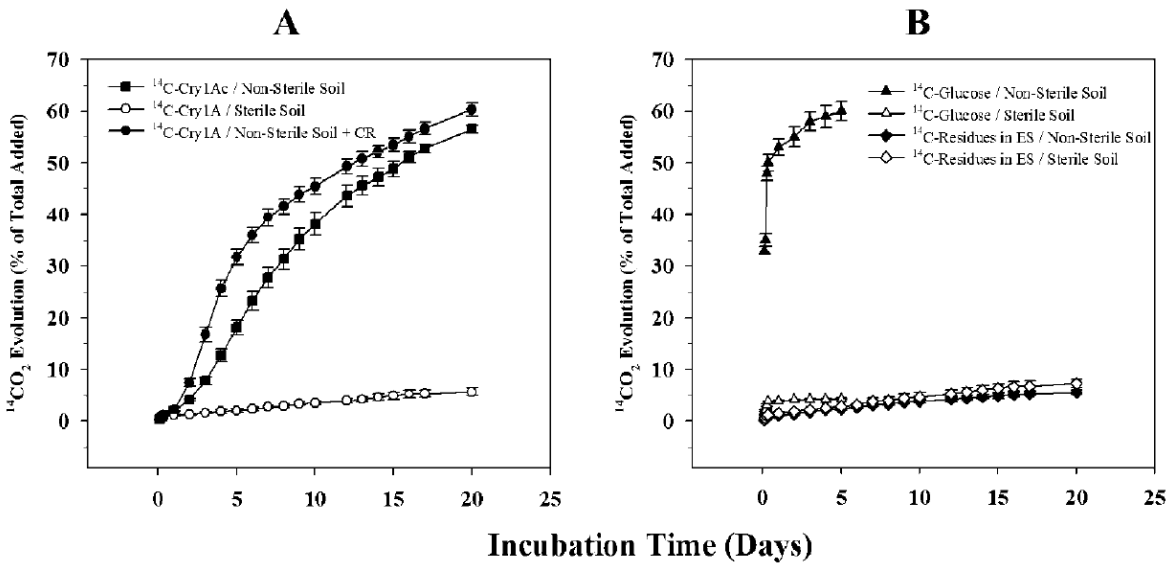

Figure 8.1.1-01. Mineralization of [^{14}C] Cry1Ac toxin (A) and [^{14}C] glucose (B) in nonsterile and sterile soil with or without addition of corn residues (CR). The efficiency of ^{14}C incorporation in the endotoxin was estimated by measuring mineralization of radiolabelled residues remaining in the elution solution (ES) from nonsterile and sterile soil.

After the experiment had run its course, triplicate soil samples were oxidized to determine the amount of radioactivity remaining in the soil as either biomass or sorbed to the soil particles. As shown in **Table 8.1.1-01**, the majority of the radioactivity remained within the soil when applied to sterilized soils. In contrast, less than half this amount remained as bound residue in nonsterile, natural soil.

Table 8.1.1-01. Proportion of the Total Radioactivity of Applied [^{14}C] Cry1Ac and [^{14}C] Glucose Mineralized as $^{14}CO_2$ and Remaining ^{14}C Residues in Nonsterile and Sterile Soils[a]

soil status	treatment	% mineralized	% remaining in soil
nonsterile	[^{14}C]Cry1Ac	58.8 ± 1.4	45.8 ± 2.1
	[^{14}C]Cry1Ac + CR	63.0 ± 1.4	41.8 ± 2.0
	[^{14}C]glucose	58.1 ± 1.2	44.6 ± 2.5
sterilized	[^{14}C]Cry1Ac	6.0 ± 0.8	81.4 ± 5.7
	[^{14}C]glucose	4.4 ± 0.2	75.1 ± 5.0

[a] Experiments conducted with the [^{14}C] Cry1Ac toxin included samples amended with 4.5% corn residue (CR). Numbers represent the mean of triplicate samples (± standard errors).

From the glucose experiment performed to illustrate the metabolic potential of the soil a highly active microbial community within the soil is indicated. Results from the present study clearly demonstrate that the tested Bt toxin was actively metabolized by the soil microbial community at a fairly fast rate within the first 5 days of incubation. Approximately 50% of the toxin was mineralized after 12 and 15 days in soil amended or non-amended with organic matter, respectively.

Similar results were obtained by Marchetti et al (2007).

Reference:	KMA 8.1.1/20
Author:	Marchetti E., Accinelli C., Talamé V., Epifani R. (2007)
Title:	Persistence of Cry toxins and *cry* genes from genetically modified plants in two agricultural soils
Date:	20.02.2007
Doc ID:	Published: Agronomy for Sustainable Development, 27(3), pp. 231-236
Guideline:	Not documented

GLP:	Not documented
Acceptability:	The study is considered to be acceptable.
Material and methods:	
Test material:	Purified Cry 1 Ac and Cry 1 Ab endotoxin; powder
Test concentration:	1 μg g-1 of soil, air dried basis
Test system:	20 g soil into 250 mL screw capped Erlenmeyer flask. Moisture: -33 kPa Incubation 35 days in the dark
Temperature:	25 °C
Sampling time points:	7,14,21,28,35 days
Method of analysis:	Soil extraction plus ELISA and insect bioassay. Average recovery efficiencies of soil extraction/ELISA were 82 and 67% in the sandy and in the clay loam soil, respectively

Soil characteristics:

Soil	Particle size			pH[a]	Organic C	Culturable Bacteria[b]	Bt spores[c]
	Sand	Silt	Clay				
		%		1:2.5	%	Log CFUs[d] g^{-1} soil	
Sandy	87.1	4.9	8.0	7.9	0.9	6.8	Not detected
Clay Loam	33.8	41.1	25.1	6.3	6.6	8.8	1.2

[a] Soil pH measured in 1:2.5 (w/v) soil/deionized water mixture

[b] Size of culturable bacteria estimated according to the method in Acinelli et al 2006)

[c] Number of *Bacillus thuringiensis* spores estimated according to the method proposed by Travers et al (1987)

[d] Colony-forming units

The persistence results obtained following the soil extraction/ ELISA approach are shown in **Figure 8.1.1-02**. The two soils showed a high potential to degrade the Cry1Ab and Cry1Ac toxins. At the end of the 35-day incubation period, the remaining extractable fractions of Cry1Ab and Cry1Ac toxins from the sandy soil were 7 and 10% of the extractable initial amount, respectively.

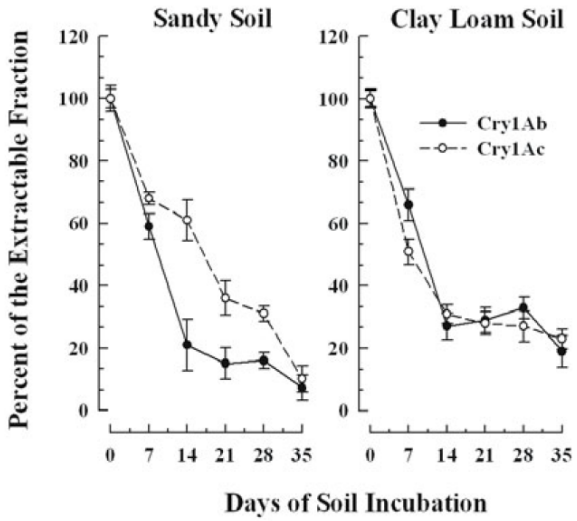

Figure 8.1.1-02 Extractable fractions of Cry1Ab and Cry1Ac toxins in the sandy and clay loam soils during the 35-day incubation period. Bars represent standard deviations of the means.

The half-lives of Cry1Ab and Cry1Ac toxins in the sandy soil were calculated assuming the first-order decay model. The regression coefficients of the log-transformed first-order model for the two soils were >0.89. The estimated half-lives of the extractable Cry1Ab and Cry1Ac toxins were 9.8 and 12.7 days, respectively. Contrary to the sandy soil, the extractable fractions of the two Cry toxins from the clay loam soil showed a rapid decline

during the first 2 weeks, followed by a slower decrease over the remaining incubation time (**Figure 8.1.1-02**). At the end of the incubation period, the extractable fractions of the Cry1Ab and Cry1Ac toxins were 19 and 23% of the initial recovered amount, respectively. The persistence data did not adequately fit the first-order or other decay models.

In another study the degradation in soil of Cry3Bb1 was investigated that is present in Bt corn.

Reference:	KMA 8.1.1/21
Author:	Icoz, I, Stotzky, G. (2008)
Title:	Cry3Bb1 protein from *Bacillus thuringiensis* in root exudates and biomass of transgenic corn does not persist in soil
Abstract:	The Cry3Bb1 protein, insecticidal to the corn rootworm complex (*Diabrotica* spp.), of *Bacillus thuringiensis* (Bt) subsp. *kumamotoensis* was released in root exudates of transgenic Bt corn (event MON863) in sterile hydroponic culture (7.5 ± 1.12 ng/ml after 28 days of growth) and in nonsterile soil throughout growth of the plants (2.2 ± 0.62 ng/g after 63 days of growth). Kitchawan soil, which contains predominantly kaolinite (K) but not montmorillonite (M), was amended to 3 or 6% (vol./vol.) with K (3K and 6K soils) or M (3M and 6M soils) and with 1, 3, 5, or 10% (wt./wt.) of ground biomass of Bt corn expressing the Cry3Bb1 protein and incubated at 25 ± 2°C at the –33-kPa water tension for 60 days. Soils were analyzed for the presence of the protein every 7 to 10 days with a western blot assay (ImmunoStrip) and verified by ELISA. Persistence of the protein varied with the type and amount of clay mineral and the pH of the soils and increased as the concentration of K was increased but decreased as the concentration of M was increased. Persistence decreased when the pH of the K-amended soils was increased from ca. 5 to ca. 7 with $CaCO_3$: the protein was not detected after 14 and 21 days in the pH-adjusted 3K and 6K soils, respectively, whereas it was detected after 40 days in the 3K and 6K soils not adjusted to pH 7. The protein was detected for only 21 days in the 3M soil and for 14 days in the 6M soil, which were not adjusted in pH. These results indicate that the Cry3Bb1 protein does not persist or accumulate in soil and is degraded rapidly.
Doc ID:	Published: Transgenic Res 17(4):609–620
Guideline:	Not documented
GLP:	Not documented
Acceptability:	The study is considered to be acceptable.
Material and methods:	
Test material:	Cry 3 Bb1 endotoxin from Bt corn 1.6 ± 0.20 µg/g of dry biomass
Test concentration:	1, 3, 5, or 10% biomass. Initial conc. in soil 1.8 ng/g 7.8, 40.4, and 90.4 ng/g (estimated by ELISA)
Test system:	50 g of soil/jar. Moisture: -33 kPa Incubation 60 days in the dark
Temperature:	25°C
Sampling time points:	Every 7 days
Method of analysis:	Soil extraction; western blot (ImmunoStrip test) plus ELISA
Soil characteristics:	Kitchawan soil

Property	Soil–clay mixture				
	C	3K	6K	3M	6M
pH	4.9	4.8	4.9	5.4	5.8
Organic matter (%)	2.99	3.04	3.05	2.92	2.81
Total nitrogen (%)	0.13	0.13	0.13	0.13	0.13
CEC (cmol/kg)[a]	11.9	12.4	11.2	15.1	20.5
Sand (%)	56.8	58.2	54.8	57.8	52.2
Silt (%)	33.8	31.2	33.6	30.6	35.8
Clay (%)	9.4	10.6	11.6	11.6	12.0
Dominant clay minerals[b]	K, i	K, i	K, i	K, M, i	K, M, i

[a] CEC: Cation-exchange capacity

[b] K: kaolinite, M: montmorillonite, i: illite (minor component)

Persistence of Cry3Bb1 protein from Bt corn biomass in soil was analysed. Cry3Bb1 protein was added to soil via biomass of Bt corn (mixture of roots, stems, and leaves) which was dried, grounded and incorporated at a concentration of 1, 3, 5, or 10% (wt./wt.) into Kitchawan soil (50 g soil/jar), in which kaolinite is the predominant clay mineral, The soil is tested either unamend (C) or amended to 3 or 6% (vol./vol.) with montmorillonite (M; 3M, and 6M soils) or kaolinite (K; 3K and 6K soils). The concentration of Cry3Bb1 protein in the biomass was estimated to be 1.6 ± 0.20 µg/g of dry biomass by ELISA. The biomass-amended soil–clay mixtures were incubated at 25 ± 2°C for 60 days at their -33 kPa water tension. Every 7 – 10 days, soils in individual jars were mixed with a glass rod, and two replicates of 0.5 g of soil each were analysed for the presence of the Cry3Bb1 protein by western blot. All positive results in the western blot assay were analysed for the presence of the Cry3Bb1 protein by ELISA.

No significant differences in the amount of the protein in soil amended with 1% of biomass (1.8 ng/g of soil initially) during 21 days of incubation, but no protein was detected after 30 days. In soil amended with 3, 5 or 20% biomass the initial concentration was 7.8 ± 0.45, 40.4 ± .56, and 90.4 ± 6.08 ng/g of soil, respectively, and it decreased significantly to 0.7 ± 0.67, 1.8 ± 0.47 and 2.1 ± 1.01 ng/g of soil, respectively, in 40 days; no protein was detected after 50 days (**Figure 8.1.1-03).** Persistence of Cry3Bb1 protein in soil varied with the type of clay mineral present. In soil un-amended with clay and amended with kaolin Cry3Bb1 persisted for ca. 40 days, in soil amended with montmorillonite this was just ca. 21 days. In studies with a combination of Cry3Bb1 protein via biomass and the addition of clay minerals, it is shown that clay minerals increase the dissipation of protein, which is probably related to changes in pH (**Figure 8.1.1-04**).

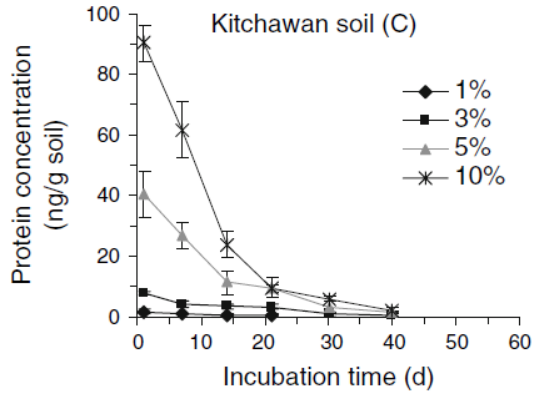

Figure 8.1.1-03 Persistence of Cry3Bb1 protein, as determined by ELISA, in Kitchawan soil (C) amended with 1, 3, 5, or 10% (wt. /wt.) of ground biomass of Bt corn. The data are expressed as the means ± the standard errors of the means

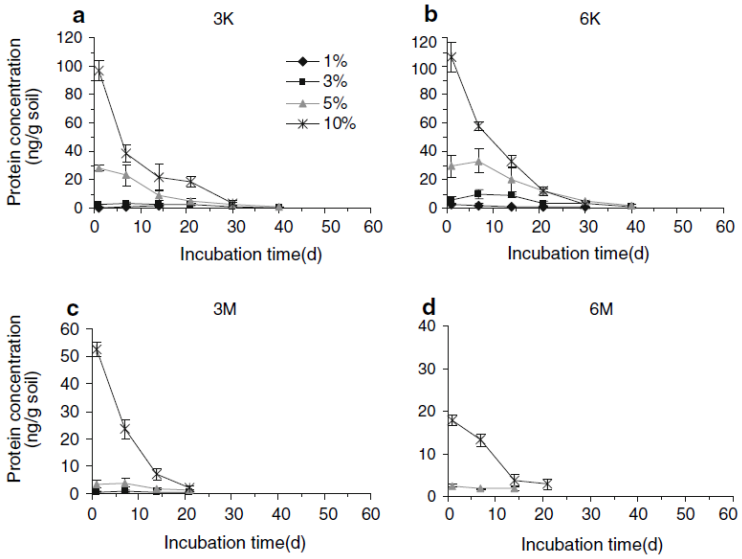

Figure 8.1.1-04 Persistence of Cry3Bb1 protein, as determined by ELISA, in Kitchawan soil amended to 3 or 6% (vol. /vol.) with kaolinite (3K and 6K soils) or amended to 3 or 6% (vol. /vol.) with montmorillonite (3M and 6M soils) and with 1, 3, 5, or 10% (wt./wt.) of ground biomass

Part of the study was also to determine whether the Cry3Bb1 protein is released in root exudates of Bt corn. Two hybrids of corn (*Zea mays* L.), were grown in sterile hydroponic culture and in nonsterile soil. Seedlings were transferred aseptically to sterile test tubes containing sterile Hoagland's solution, or to nonsterile soil in pots. Plants were grown at 25 ± 2°C under a 16-h light and 8-h dark cycle. After approximately 28 days, the Hoagland's solution was analysed for the Cry3Bb1 protein. The amount of protein in root exudates was analysed by SDS-page and Lowry assay. Plants in soil were harvested at the flowering stage (after approximately 63 days) for biomass, and rhizosphere soil (RS). The conclusion of the study indicates that the Cry3Bb1 protein was released in root exudates of transgenic Bt corn and was present in rhizosphere soil throughout growth of the plants. However, the Cry3Bb1 protein was detected in rhizosphere soil un-amended with Bt corn biomass (i.e., only released in root exudates) for only 14 days. The Cry3Bb1 protein was also detected in nonsterile rhizosphere soil of Bt corn after 63 days of growth; the concentration was 2.2 ± 0.62 ng/g of soil.

To gain insight in the persistence of Cry1Aa Bt toxin that can be released from genetically modified crop Helassa et al., (2011) performed a study to follow the fate of Cry1Aa Bt toxin in contrasting soils. Persistence can be influenced by biotic and abiotic factors, and the effect of soil pH and soil microbial activity was investigated. The information can be used to describe the persistence of Cry1Aa Bt toxin in general.

Reference:	KMA 8.1.1/22
Author:	Helassa, N., M'Charek, A., Quiquampoix, H., Noinville, S., Dejardin, P., Frutos, R., Staunton, S. (2011)
Title:	Effects of physicochemical interactions and microbial activity on the persistence of Cry1Aa Bt (*Bacillus thuringiensis*) toxin in soil
Abstract:	Genetically modified crops, that produce Cry insecticidal crystal proteins (Cry) from *Bacillus thuringiensis* (Bt), release these toxins into soils through root exudates and upon decomposition of residues. The fate of these toxins in soil has not yet been clearly elucidated. Persistence can be influenced by biotic (degradation by microorganisms) and abiotic factors (physicochemical interactions with soil components, especially adsorption). The aim of this study was to follow the fate of Cry1Aa Bt toxin in contrasting soils subjected to different treatments to enhance or inhibit microbial activity, in order to establish the importance of biotic and abiotic processes for the fate of Bt toxin. The toxin was efficiently extracted from each soil using an alkaline buffer containing a protein, bovine serum albumin, and a nonionic surfactant, Tween 20. The marked decline of extractable toxin after incubation of weeks to months was soil dependent. The decrease of extractable toxin with incubation time was not related to microbial degradation

	but mainly to physicochemical interactions with the surfaces that may decrease immunochemical detectability or enhance protein fixation. Hydrophobic interactions may play an important role in determining the interaction of the toxin with surfaces.
Doc ID:	Published: Soil Biology & Biochemistry 43(5), 1089-1097
Guideline:	Not documented
GLP:	Not documented
Acceptability:	The study is considered to be acceptable.
Material and methods:	
Test material:	Purified Cry 1 Aa endotoxin
Test concentration:	550 to 1100 ng/g of soil
Test system:	20 g soil into 250 mL screw capped erlenmeyr flask. Moisture: -25 kPa Incubation 35 days in the dark
Temperature:	25°C
Sampling time points:	Not specified, up to 120 days
Method of analysis:	Soil extraction plus ELISA commercial Kit.
Soil characteristics:	Some properties of the soils studied.

Soil	Depth (cm)	[a]Clay (g kg^{-1})	[a]Silt (g kg^{-1})	[a]Sand (g kg^{-1})	[b]C_{org} (g kg^{-1})	pH (water)	[c]CEC (mmol$_c$ kg^{-1})	Location	USDA classification
A	0–30	357	412	203	55.7 (68.2)	7.2	322	Banbury (UK)	Clay loam
B	30–50	345	397	207	17.6 (26.0)	7.8	235	Banbury (UK)	Clay loam
R	0–25	157	160	683	13.5 (15.6)	6.2	79	Marcoule (France)	Sandy loam
T	30–60	73	72	855	2.2 (2.4)	7.5	–	Marcoule (France)	Sandy

[a] sedimentation and gravimetric analysis

[b] Anne method for < 2 mm, (< 200 µm fraction elemental analysis)

[c] Cobaltihexamine method

In **Figure 8.1.1-05** the results of persistence of Cry1Aa Bt toxin in soil with time for the four soils are reported. The effect of increasing or decreasing pH was tested in this study but it had little effect on the persistence. Also with enhancement of microbial activity by adding small amounts of carbon, as glucose or amino acids (or both) did not show a marked difference in the trends of persistence. Also inhibition of microbial activity did not have any effect. No marked differences of sterilization, autoclaving, or ɤ-irradiation were observed for any of the 4 soils.

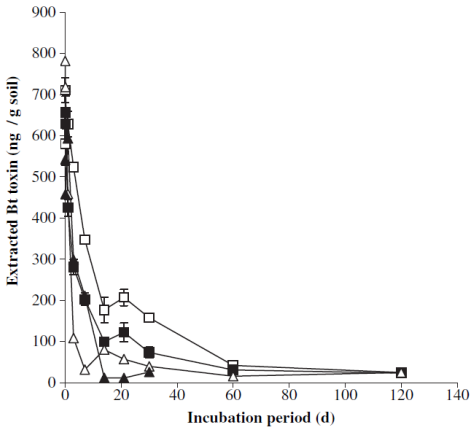

Figure 8.1.1-05 Detection of Cry1Aa toxin as a function of incubation period, determined by ELISA after extraction, in four contrasting soils: clayey soils e soil A (□); soil B (■); and sandy soils e soil R (△); soil T (▲), open symbols for soils richer in organic matter than the closed for the same texture class. The data are expressed as the mean of triplicates ± one standard error.

For each soil, a rapid decline of the amount of extractable toxin during the first 14 days was observed with a decrease of 86 ± 7.5% of the initial value, followed by a slower decrease.

Li et al., (2013) tested the degradation of Cry1Ac in soil, sediment and water (also see Section M-MA 7.1.2, KMA 7.1.2/02) and the effect of sterilisation. Below the results of the soil test are given. Soil was collected from an area with no history of transgenic crop cultivation, sieved and air dried at room temperature. Soil was spiked with crude Bt proteins extracted from transgenic cotton seeds.

Reference:	KMA 8.1.1/23
Author:	Li, Y-L., Du, J., Fang, Z-X., You, J. (2013)
Title:	Dissipation of Insecticidal Cry1Ac Protein and Its Toxicity to Nontarget Aquatic Organisms.
Abstact:	The widespread cultivation of *Bacillus thuringiensis* crops has raised public concerns on their risk to non-target organisms. Persistence of Cry1Ac protein in soil, sediment and water and its toxicity to non-target aquatic organisms were determined. The dissipation of Cry1Ac toxin was well described using first order kinetics, with the half-lives (DT_{50}) ranging from 0.8 to 3.2, 2.1 to 7.6 and 11.0 to 15.8 d in soil, sediment and water, respectively. Microbial degradation played a key role in the dissipation of Cry1Ac toxin and high temperature accelerated the processes. Cry1Ac toxin was more toxic to the midge *Chironomus dilutus* than the amphipod *Hyalella azteca*, with the median lethal concentration (LC_{50}) of *C. dilutus* being 155 ng/g dry weight and 201 ng/mL in 10-d sediment and 4-d water bioassays, respectively. While Cry1Ac toxin showed toxicity to the midges, risk of Bt proteins to aquatic nontarget organisms was limited because their environmentally relevant concentrations were much lower than the LC_{50}s.
Doc ID:	Published: Journal of Agricultural and Food Chemistry, 61(46), 10864-10871
Guideline:	Not documented
GLP:	Not documented
Acceptability:	The study is considered to be acceptable.
Material and methods:	
Test material:	Cotton extracted Cry1Ac endotoxin
Test concentration:	176 ng/g dry soil
Test system:	0.4 g soil, sterilised (121°C for 30 min.) and non-sterilised Moisture: 20% WHC Incubation at 4, 24, and 34°C
Temperature:	three temperatures of 4, 24, and 34°C
Sampling time points:	0, 1, 3, 7, 15, 30, 45, and 60 d
Method of analysis:	Soil extraction plus ELISA, extraction efficiency was 46.5 ± 3.4%. RSD 7.3%; method detection limit of 0.8 ng/g dry weight

Soil characteristics:

pH	**Total OC (g/kg)**	**Total N (g/kg)**	**Total P (g/kg)**
7.47 ± 0.01	11.5 ± 0.15	1.0 ± 0.10	0.94 ± 0.02

After being spiked and throughout the experiments to compensate for the water evaporation, Milli-Q water was added daily to the soil samples to ensure that the moisture contents were 20%. Two sterilization conditions (sterilized and not sterilized) were used to assess the impact of microbial degradation on the dissipation of Cry1Ac protein. At sampling, three replicates were terminated, and concentrations of Cry1Ac protein in the substrates were measured. The Cry1Ac protein was extracted from the soil and the supernatant after centrifugation was decanted and analysed using ELISA. The dissipation of CrylAc protein was fitted with the first order kinetic model in the present study though the measured data as presented in **Table 8.1.1-02**. The kinetic equations and parameters, including coefficients of determination (R^2), *p* values, k, DT_{50}, and DT_{90}, are presented in **Table 8.1.1-03.** The variations in DT_{50} values were the results of different geochemical characteristics of the soils and various experimental conditions.

Table 8.1.1-02. Residues of Cry1Ac Protein in Soil at Different Time, Temperature and Sterilization Conditions

Time (d)	Sterilisation condition	Concentration [ng/g dw][a]		
		4°C	24°C	34°C
0	Not sterilised	176 ± 4.5	176 ± 4.5	176 ± 4.5
1		83 ± 1.9	75 ± 2.7	58 ± 3.9
3		77 ± 4.1	55 ± 1.8	42 ± 3.9
7		30 ± 1.4	27 ± 2.0	16 ± 0.9
15		12 ± 1.6	10 ± 0.5	7 ± 0.6
30		7 ± 0.6	3 ± 0.2	1 ± 0.3
45		3 ± 0.6	2 ± 0.2	1 ± 0.2
60		1 ± 0.2	1 ± 0.2	0 ± 0.3
0	Sterilised	176 ± 4.5	176 ± 4.5	176 ± 4.5
1		109 ± 4.8	98 ± 2.9	90 ± 1.3
3		85 ± 3.4	80 ± 1.2	72 ± 1.3
7		41 ± 1.7	35 ± 1.7	28 ± 2.2
15		18 ± 1.2	16 ± 0.6	14 ± 0.5
30		10 ± 0.5	9 ± 0.6	5 ± 0.3
45		4 ± 0.2	3 ± 0.3	2 ± 0.2
60		2 ± 0.3	1 ± 0.1	1 ± 0.2

[a] Concentrations of Cry1Ac protein were shown as means ± standard deviation of three replicates.

Table 8.1.1-03: First Order Kinetic Equations, the rate constant (k) and the times when concentration of Cry1Ac protein reduced to the half (DT_{50}) and 90% (DT_{90}) of its initial concentration

Matrix	treatment	r^2	*p* value	k [d^{-1}]	DT_{50} [d]	DT_{90} [d]
soil	4°C, NS	0.926	< 0.0001	0.293	2.4	7.9
	4°C, S	0.966	< 0.0001	0.216	3.2	10.7
	24°C, NS	0.934	< 0.0001	0.475	1.5	4.8
	24°C, S	0.952	< 0.0001	0.251	2.8	9.2
	34°C, NS	0.946	< 0.0001	0.830	0.8	2.8
	34°C, S	0.949	< 0.0001	0.312	2.2	7.4

The dissipation curve of Cry 1Ac in soil, but also in water and sediment, at 34°C are presented in **Figure 8.1.1-06**.

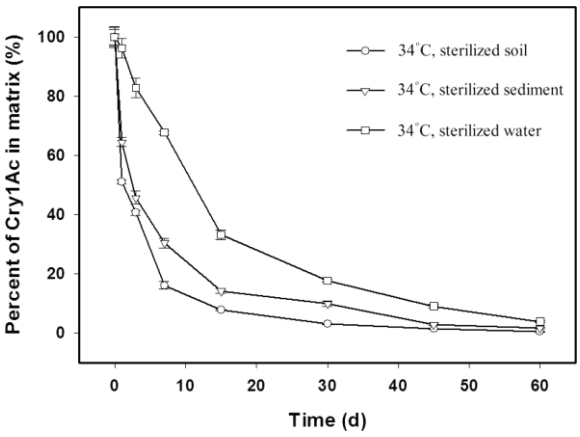

Figure 8.1.1-06 Dissipation curves of Cry1Ac protein in the sterilized soil, sediment and water at 34°C. soil (○),sediment (▽), and water (□), respectively. The symbols are the means of three replicates and the error bars represent the standard deviations.

In conclusion, dissipation of Cry1Ac protein in soil could be well described by the first order kinetic equations. Microbial degradation contributed significantly to the dissipation. High temperature accelerated the dissipation process. Sterilisation led to a slightly higher DT_{50}, however fast transformation was seen under all conditions. The influence of temperature on DT_{50} was larger than that of sterilisation.

Wang et al., (2007) investigated the degradation of Cry1Ab protein under both aerobic and flooded conditions in paddy soils. Five soils were used which have not been exposed to transgenic crops. Soils were air dried mixed and sieved.

Reference:	KMA 8.1.1/24
Author:	Wang, H., Ye, Q., Gan, J., Wu, L. (2007)
Title:	Biodegradation of Cry1Ab protein from Bt transgenic rice in aerobic and flooded paddy soils
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 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 16.9 to 41.3 d. 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 d. These results suggest that the toxin protein, when introduced into a paddy field upon harvest, will undergo rapid removal after the field is drained and exposed to aerobic conditions.
Doc ID:	Published: Journal of agriculture and food chemistry, 55(5):1900-1904
Guideline:	Not documented
GLP:	Not documented
Acceptability:	The study is considered to be acceptable.
Material and methods:	
Test material:	Purified Cry1Ab protein
Test concentration:	560 ng/g dry soil
Test system:	50 g soil in 250 mL flasks loosely covered Moisture: 60% MWHC

	Incubation in the dark Anaerobic incubation using soils S1 to S3, flooded to a water depth of 2 cm
Temperature:	25°C for aerobic, 28°C for anaerobic
Sampling time points:	0, 3, 5, 10, 15, 20 ,30, 60, 90, 120 and 150 d for aerobic, up to 60 days for anaerobic
Method of analysis:	Extraction and analyses by ELISA.
Soil characteristics:	

soil no.	type	pH (H_2O)	OM (g kg^{-1})	CEC (cmol kg^{-1})	clay (%)	silt (%)	sand (%)	total N (%)
S_1	paddy field on quaternary red soil	4.16	8.40	6.62	39.0	41.1	19.9	0.34
S_2	paddy field on red sandstone soil	4.55	6.53	4.53	17.2	7.4	75.4	0.28
S_3	fluvio-marine yellow loamy soil	7.02	30.50	10.83	8.0	71.2	20.8	2.90
S_4	coastal saline soil	8.84	9.50	10.17	24.3	71.1	4.6	1.80
S_5	paddy field on pale muddy soil	5.81	25.11	8.15	49.3	11.4	39.3	2.70

[a] Note: OM, organic matter; CEC, cation exchange capacity.

Purified Cry1Ab protein was obtained from straw of transgenic rice by extraction and purification of crude extracts. The persistence of Cry1Ab in soil was determined in aerobic and flooded soil. An initial concentration of 560 ng/g air dried soil was prepared. The water content of spiked soil samples was brought to 60% of WHC and soils were incubated at 25°C in the dark. At 0, 3, 5, 10, 15, 20, 30, 60, 90, 120 and 150 d of incubation a subsample was removed for analysis. Soils were extracted and the concentration Cry1Ab determined by ELISA. For 3 soils an anaerobic incubation experiment was performed, soils were prepared using the same procedure and the soils were flooded with distilled water to the depth of 2 cm.

In the five soils tested under aerobic conditions dissipation generally followed the first order decay model and half-lives varied from 19.6 to 41.3 days. After 150 days of incubation the Cry1Ab protein was still detected in all of the 5 soils (**Figure 8.1.1-07**). Degradation in paddy soils under flooded conditions was slow and followed a 10-d lag period. In 2 of the 3 soils 80% of the initially added Cry1Ab remained in the soil after 60-d incubation. In soil 3 however, the level decreased rapidly from about 95% on day 10 to 40% on day 60 (**Figure 8.1.1-08).** First order half-lives under anaerobic conditions were estimated to be 141 days for soil 1, 138 days for soil 2 and 45.9 days for soil 3 (**Table 8.1.1-04)**. Analysis of the aqueous part did not show any detectable level of protein. This suggests it was tightly adsorbed top soils which may have protected the protein from degradation and thus extended its persistence.

Table 8.1.1-04 First order regression analysis of the degradation of Cry1Ab protein in paddy soils under aerobic and anaerobic conditions

soil no.	rate constant k (d^{-1})	half-life $t_{1/2}$ (d)	correlation coefficient (r)	p
		Aerobic		
S_1	0.0262	26.5	0.99	<0.005
S_2	0.0168	41.3	0.96	<0.005
S_3	0.0180	38.5	0.96	<0.005
S_4	0.0353	19.6	0.97	<0.005
S_5	0.0293	23.7	0.97	<0.005
		Anaerobic		
S_1	0.0049	141.4	0.99	<0.005
S_2	0.005	138.6	0.90	<0.05
S_3	0.0174	45.9	0.94	<0.005

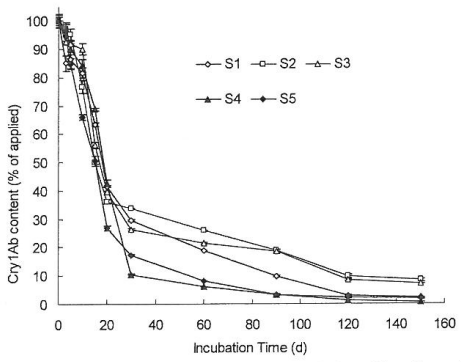

Figure 8.1.1-07 Degradation of purified Cry1Ab protein in paddy soils under unsaturated, aerobic conditions

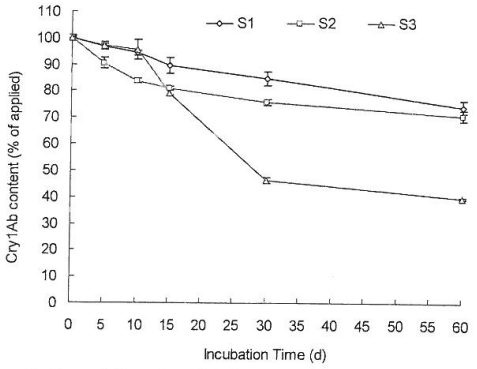

Figure 8.1.1-08 Degradation of purified Cry1Ab protein in paddy soils under flooded, anaerobic conditions.

In a field monitoring experiment Xue et al., (2014) investigated the Cry3Bb1 protein concentration in rhizosphere soils of Bt corn during the growing season at seven sites in New York State (NY), USA. The hypothesis was that 1) the extraction efficiency of the soil Cry3Bb1 protein decreased with higher soil clay content and lower soil pH, while the protein concentration in soils increased with higher soil clay content and lower soil pH; 2) the Cry3Bb1 protein concentration in soils decreased with higher annual temperature, total precipitation and total snow; 3) the Cry3Bb1 protein concentration in soils differed among sites, plant gene constructs, hybrids and their interactions. For one site they characterized the seasonal dynamics of Cry3Bb1 protein in soils and its decomposition in different types of Bt corn residues (cobs, leaves plus stalks, and roots) in litterbags over two years. A commercial ELISA kit was used to determine the Cry3Bb1 protein concentration in sampled soils and Bt corn residues after extraction. By adding a known quantity of standard purified Cry3Bb1 protein (0, 0.1, 0.2,1, 4, 10 or 20 ng g^{-1} soil) into soils collected from the plots planted with non Bt corn, the extraction efficiency of Cry3Bb1 protein from sampled soils was determined. After being adjusted by the extraction efficiency, the Cry3Bb1 protein concentration in rhizosphere soils. The Cry3Bb1 protein concentration at seven sites ranged from 0.25 (± 0.11) ng g^{-1} soil (± SE) in Scipio, NY to 12.12 (± 4.23) ng g^{-1} in Avon, NY. The Cry3Bb1 protein concentration in soils was positively correlated with the soil clay content ($p = 0.003$), but negatively correlated with the total precipitation ($p = 0.005$). The seasonal dynamics of the Cry3Bb1 protein concentration in soils show that the concentration in the mid-season was 10.05 (± 6.77) ng g^{-1} soil. However, the Cry3Bb1 protein was not detected in any of the soil samples collected at planting and its concentration was low at pre-harvest, only 0.10 ng g^{-1} on average. Field degradation of Cry3Bb1 protein in Bt corn residues was assessed by a field litterbag study in Aurora, NY. For residues used to prepare litterbags, various plant parts had significantly different Cry3Bb1 protein concentrations ($p < 0.001$). Leaves had a significantly higher Cry3Bb1 protein concentration [124.2 (±3 6.7) ng g^{-1} tissue] than the other plant parts. The Cry3Bb1 protein concentrations in stalks and cobs were 20.9 (± 5.2) and 19.8 (± 8.5) ng g^{-1} tissue, respectively. In roots, the Cry3Bb1 protein concentration was quite low, only 0.1 ng g^{-1} tissue on average. After 3.5 months, the Cry3Bb1 protein had decomposed nearly completely in residues.

Chen et al., (2011) considered the persistence of Cry1Ac and CpTI (a small polypeptide belonging to the Bowman-Birk type of double-headed serine protease inhibitors) and responses of soil microbial properties and soil enzyme activities involved in nutrient cycling, in a long-term study with transgenic cotton in a potting experiment. Three pairs of cottons, Bt (transgenic Bt cotton ZM30; non-transgenic Bt cotton with its isoline ZM16), CpTI+ (transgenic Bt + CpTI cotton ZM41; non-transgenic Bt + CpTI cotton with its isoline ZM23), and CpTI++ (transgenic Bt + CpTI cotton sGK321; non-transgenic Bt + CpTI cotton with its isoline Shiyuan321) were used. 3 cotton plants per pot were kept. At harvest of the first two years of cultivation, the above-ground plant residues were cut and removed from the pots, but the soil was kept. The soil remains in the pots were fertilized the same as 2004 for additional cultivation of the same cotton variety in the following years. After four years of consecutive cotton cultivations, non-rhizosphere soil samples (~ 150 g) were taken and mixed well after the cotton was harvested; parts of soil samples were stored at –20°C for the determination of Cry1Ac and CpTI proteins. Cry1Ac and CpTI proteins in the supernatant of soil extracts were determined by ELISA. Soil microbial biomass carbon (MBC) was estimated from 15.0 g of soil using the fumigation-extraction technique. Activities of dehydrogenase (DHA), FDA hydrolysis, and catalase as well as Urease, acid phosphomonoesterase, phosphordiesterase, arylsulfatase, and β-glucosidase were detected as described by Tabatabai (1994). Nitratereductase activity was measured by the colorimetric method (Kandeler, 1996). The determination of protease activity was adapted from Ladd & Butler (1972). The amount of Cry1Ac protein in soil under Bt cotton was 12.01 ng/g dry soil and the amount in the two transgenic Bt + CpTI cottons 7.86 and 6.75 ng/g dry soil. The amount of CpTI proteins in soil under the two transgenic Bt + CpTI cottons was 30.65 and 43.60 ng/g dry soil.

No Cry1Ac or CpTI protein was found in soil under the non-transgenic isolines. The present study showed that there was the persistence of CpTI protein in non-rhizosphere soils after four years of consecutive cultivation of two transgenic Bt + CpTI cottons.

Microbial biomass carbon, activities of catalase, and FDA hydrolysis in soil were inhibited under CpTI + and Bt cottons compared with their non-transgenic controls. Activities of nitrate reductase, acid phosphomonoesterase, arylsulfatase, and *β*-glucosidase in soil were inhibited under Bt cotton compared with its non-transgenic isoline; activities of nitrate reductase, protease, acid phosphomonoesterase, and arylsulfatase in soil were inhibited under CpTI++ cotton compared with its non-transgenic isoline; and activity of *β*-glucosidase in soil was inhibited under CpTI+ cotton compared with its non-transgenic isoline. No significant difference was found in DHA activity and there were no significant differences in urease and phosphodiesterase activities between transgenic cottons and their non-transgenic controls. Further analysis found that, in contrast to *CpTI*, most soil microbial properties and enzyme activities had a negative correlation with Cry1Ac and Cry1Ac has negative effects on soil enzyme activities.

RMS evaluation	Relevant information has already been provided during original approval of *B. thuringiensis* subsp. *kurstaki* SA-12 and is still valid. No studies on persistence and multiplication in soil of Btk strain SA-12 or its δ-endotoxins have been submitted. However, since numerous studies which address this issue have been reported in the scientific literature, these data are regarded as adequate. *Bacillus thuringiensis* occurs naturally and ubiquitously in the environment. It is a common component of the soil micro-biota and has been isolated from most terrestrial habitats. Available information indicates that *Bacillus thuringiensis* spores may persist from days to years in soil under natural field conditions (**Table 8.1-01**). Numerous publications show that the following factors may have an effect on the survival of *B. thuringiensis* subsp. *kurstaki* in soil: Temperature, pH, moisture, soil type, presence of indigenous micro-organisms and photo-degradation. The low potential for spore germination, growth and re-sporulation in soils minimises multiplication. The crystalline proteins are the source of δ-endotoxins, which are damaging to highly specific insect species. When these insects ingest crystal proteins, alkaline conditions in the gut initiate breakdown of the proteins, releasing the δ-endotoxins. In soil, various half-lives of the crystalline protein (δ-endotoxin) have been found in the literature, ranging from hours and days to months (**Table 8.1-01**) Persistence can be influenced by biotic (degradation by microorganisms) and abiotic factors (physicochemical interactions with soil components such as adsorption and pH of the soils). Overall the results indicate that the endotoxins do not persist or accumulate in soil and are degraded rapidly. **Soil**: Cry1Ac - DT_{50} 15, 12.7, 1.5 days. Cry1Ab - DT_{50} 9.8 days Cry1Aa - DT_{50} < 14 days Cry3Bb1 - DT_{50} 12, 6.3 days
Endpoint: Soil:	<u>Btk SA-12</u> *Bacillus thuringiensis* occurs naturally and ubiquitously in the environment. It is a common component of the soil micro-biota and has been isolated from most terrestrial habitat. Available information indicates that *Bacillus thuringiensis* spores may persist from days to years in soil under natural field conditions. The low potential for spore germination, growth and re-sporulation in soils minimises multiplication. <u>Endotoxins</u> Persistence can be influenced by biotic and abiotic factors. Overall the results indicate that the endotoxins do not persist or accumulate in soil and are degraded rapidly (DT_{50} < 2 weeks).

Cited references

Report KMA 8.1.1/17 –Konecka E., Baranek J., Bielińska I, Tadeja A., Kaznowski A. (2014). Persistence of the spores of *B. thuringiensis* subsp. *kurstaki* from Foray bioinsecticide in gleysol soil and on leaves

Published report,

Science of the Total Environment 472, 296–301

Abstract: The aim of this study was to determine how long the spores of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 from Foray bioinsecticide persist in soil and on leaf surface after application of the bioinsecticide in an oak forest. Foray 04UL was sprayed over a 195-hectare oak forest on the Krotoszyn Plateau in Poland. *B. thuringiensis* was isolated from soil samples and tree leaves taken from randomly chosen sites. *B. thuringiensis* subsp. *kurstaki* HD-1 in the samples was identified upon clonal analysis of the cultured isolates by using the RAPD method. One month after Foray spraying, the number of *B. thuringiensis* increased in soil and decreased on leaf surface comparing to the number estimated two days after the application. The reduction in the number of *B. thuringiensis* was noted six months after the pesticide application and the number was decreasing during the following months. No *B. thuringiensis* was noted on leaf surface one year after Foray spraying and in soil after one and a half years. The study showed that *B. thuringiensis* spores from biopesticide can survive in the forest environment; however, relatively short persistence time does not pose environmental risk.

Evaluation RMS	The study is acceptable and showed that *B. thuringiensis* spores from biopesticide can survive in the forest environment for a relatively short persistence time. The reduction in the number of *B. thuringiensis* was noted six months after the pesticide application and the number was decreasing during the following months. No *B. thuringiensis* was noted on leaf surface one year after product spraying and in soil after one and a half years.

Report KMA 8.1.1/18 – Hendriksen N.B., Carstensen J. (2013) Long term survival of *Bacillus thuringiensis* subsp. *kurstaki* in a field trial

Published report,

Can.J.Microbiol. 59(1), 34-38

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 here was carried out from 2001 to 2007 in a single square meter within the plot using a systematic randomized sampling approach. The bacterium survived at relative low densities in these 13 years after spraying. Statistical analyses revealed that the overall density decreased approximately 40% during years 8 to 13 after 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 the soil temperature exceeds 10°C.

Evaluation RMS	The study is acceptable and showed that strains indistinguishable by RAPD analysis can survive for at least 13 years in soil.

Report KMA 8.1.1/19 – Accinelli, C., Koskinen, W.C., Becker, J.M., Sadowsky, M.J. (2008). Mineralization of the *Bacillus thuringiensis* Cry1Ac Endotoxin in Soil

Published report,

J. Agric. Food Chem. 56(3):1025–1028

Evaluation RMS	The study is acceptable and demonstrates that the Cry1Ac endotoxin was actively metabolized by the soil microbial community at a fairly fast rate within the first 5 days of incubation. Approximately 50% of the toxin was mineralized after 12 and 15 days in soil amended or non-amended with organic matter, respectively.

Report KMA 8.1.1/20 – Marchetti, E., Acinelli, C., Talamé, V., Epifani, R. (2007). Persistence of Cry toxins and *cry* genes from genetically modified plants in two agricultural soils

Published report,

Agron. Sustain. Dev., 27(3):231–236

Evaluation RMS	Although the study is based on Cry toxins from genetically modified plants it is acceptable to study half-lives of Cry toxins originating from Bt. The half-lives of Cry1Ab and Cry1Ac toxins in the sandy soil were calculated assuming the first-order decay model. The estimated half-lives of the extractable Cry1Ab and Cry1Ac toxins were 9.8 and 12.7 days, respectively. Contrary to the sandy soil, the extractable fractions of the two Cry toxins from the clay loam soil showed a rapid decline during the first 2 weeks, followed by a slower decrease over the remaining incubation time. At the end of the incubation period, the extractable fractions of the Cry1Ab and Cry1Ac toxins were 19 and 23% of the initial recovered amount, respectively. The persistence data did not adequately fit the first-order or other decay models.

Report KMA 8.1.1/21 – Icoz, I., Stotzky, G. (2008). Cry3Bb1 protein from *Bacillus thuringiensis* in root exudates and biomass of transgenic corn does not persist in soil

Published report,

Transgenic Res 17(4):609–620

Evaluation RMS	Although the study is based on Cry toxins from genetically modified plants it is acceptable to study half-lives of Cry toxins originating from Bt. Persistence decreased when the pH of the K-amended soils was increased from ca. 5 to ca. 7: the protein was not detected after 14 and 21 days in the pH-adjusted 3K and 6K soils, respectively, whereas it was detected after 40 days in the 3K and 6K soils not adjusted to pH 7. The protein was detected for only 21 days in the 3M soil and for 14 days in the 6M soil, which were not adjusted in pH. The results indicate that the Cry3Bb1 protein does not persist or accumulate in soil and is degraded rapidly.

Report KMA 8.1.1/22 – Helassa, N., M'Charek, A., Quiquampoix, H., Noinville, S., Dejardin, P., Frutos, R., Staunton, S. (2011). Effects of physicochemical interactions and microbial activity on the persistence of Cry1Aa Bt (*Bacillus thuringiensis*) toxin in soil

Published report,

Soil Biology & Biochemistry 43(5):1089-1097

Evaluation RMS	The study is acceptable and showed that the marked decline of extractable toxin after incubation of weeks to months was soil dependent. The decrease of extractable toxin with incubation time was not related to microbial degradation but mainly to physicochemical interactions with the surfaces that may decrease immunochemical detectability or enhance protein fixation. Hydrophobic interactions may play an important role in determining the interaction of the toxin with surfaces.

Report KMA 8.1.1/23 – Li, Y-L., Du, J., Fang, Z-X., You, J. (2013). Dissipation of Insecticidal Cry1Ac Protein and Its Toxicity to Nontarget Aquatic Organisms

Published report,

Journal of Agriculture and Food Chemistry, 61(46), 10864-10871

Evaluation RMS	The study is acceptable. The dissipation of Cry1Ac toxin was well described using first order kinetics, with the half-lives (DT50) ranging from 0.8 to 3.2, 2.1 to 7.6 and 11.0 to 15.8 d in soil, sediment and water, respectively. Microbial degradation played a key role in the dissipation of Cry1Ac toxin and high temperature accelerated the processes.

Report KMA 8.1.1/24 – Wang, H., Ye, Q., Gan J., Wu, L. (2007). Biodegradation of Cry1Ab protein from Bt transgenic rice in aerobic and flooded paddy soils

Published report,

Journal of Agriculture and Food Chemistry, 55(5):1900-1904

Evaluation RMS	Although the study is based on Cry toxins from genetically modified plants it is acceptable to study half-lives of Cry toxins originating from Bt. Rapid degradation of Cry1Ab protein occurred in paddy soils under aerobic conditions, with half-lives ranging from 16.9 to 41.3 d. 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 d. These results suggest that the toxin protein, when introduced into a paddy field upon harvest, will undergo rapid removal after the field is drained and exposed to aerobic conditions.

Report KMA 8.1.1/25 – Xue, K., Diaz, B.R., Thies, J.E. (2014). Stability of Cry3Bb1 protein in soils and its degradation in transgenic corn residues

Published report,

Soil Biology & Biochemistry 76:119-126

Abstract: As the land area planted with transgenic crops expressing insecticidal proteins from *Bacillus thuringiensis* (Bt) continues to increase, and more farmers adopt reduced and no-till practices, the quantity of residues remaining in the fields that may contain Bt proteins is likely to increase considerably. We evaluated the potential of the Cry3Bb1 protein, active against the corn rootworm, to persist in soils with various pH and clay contents at seven sites in NY, USA under different climate conditions, where 15 Bt corn varieties were grown. The within seasonal dynamics of the Cry3Bb1 protein in soils and its decomposition in Bt corn residues were assessed in detail over two years at one site in Aurora, NY, USA. Results showed that soils with higher clay contents had lower Cry3Bb1 extraction efficiencies, but greater protein persistence; while pH was not correlated with any of them. The protein persistence in soils was also inversely correlated with the precipitation. In Aurora, NY, USA, the Cry3Bb1 protein was detectable in rhizosphere soils at anthesis, but not at planting and was barely detectable at pre-harvest. The Cry3Bb1 protein varied in its concentration among different types of transgenic corn residues, lowest in roots, and decomposed nearly completely after 3.5 months. Overall, soil clay content and precipitation were important for the stability or detection of the Cry3Bb1 protein in tested soils. The rapid decomposition of the Cry3Bb1 protein in corn residues and its short persistence in soils indicate that the exposure level of Cry3Bb1 protein to soil-dwelling organisms is likely to be low and transitory. However, the low concentration of Cry3Bb1 protein in roots may hasten the evolution of pest resistance and cause economic losses.

Evaluation RMS	Although the study is based on Cry toxins from genetically modified plants it is acceptable to study persistence of Cry toxins originating from Bt. Soil clay content and precipitation were important for the stability or detection of the Cry3Bb1 protein in tested soils. The rapid decomposition of the Cry3Bb1 protein in corn residues and its short persistence in soils indicate that the exposure level of Cry3Bb1 protein to soil-dwelling organisms is likely to be low and transitory.

Report KMA 8.1.1/26 – Chen, Z.H., Chen, L.J., Zhang, Y.L., Wu, Z.J. (2011). Microbial properties, enzyme activities and the persistence of exogenous proteins in soil under consecutive cultivation of transgenic cottons (*Gossypium hirsutum* L.)

Published report,

Plant Soil Environ., 57(2):67–74

Abstract: One *Bacillus thuringiensis* (*Bt*) and two stacked *Bt* and cowpea trypsin inhibitor (*Bt* + *CpTI*) cottons and their non-transgenic isolines were consecutively cultivated to investigate the soil persistence of *Cry1Ac* and *CpTI* proteins and their effects on microbial properties and enzyme activities involving C, N, P, and S cycling in soil. Results showed that there were persistence of *Cry1Ac* and *CpTI* proteins in soil under 4-year consecutive cultivation of transgenic cottons. *Cry1Ac* proteins varied from 6.75 ng/g to 12.01 ng/g and *CpTI* proteins varied from 30.65 to 43.60 ng/g. However, neither of these two proteins was detected in soil under non-transgenic cottons. Soil microbial biomass carbon, microbial activities, and soil enzyme activities (except urease and phosphodiesterase) significantly decreased in soil under transgenic cottons. Correlation analysis showed that most of microbial properties and enzyme activities in soil had a negative relationship with *Cry1Ac* content, while most of them had a positive relationship with *CpTI* content. Our data indicate that consecutive cultivation by genetically modified cottons with *Bt* and *CpTI* genes can result in persistence of *Cry1Ac* and *CpTI* proteins and negatively affect soil microbial and biochemical properties.

Evaluation RMS	The study is based on *Cry1Ac* and *CpTI* proteins from genetically modified plants and seems to be of minor importance for the risk assessment of bacterial Btk endotoxins in the environment.

B.8.1.2 Water

No studies on persistence and multiplication in water of SA-11, SA-12 or EG2348 have been submitted. However, since several studies that address this issue have been reported in the scientific literature, these data are regarded as adequate.

The persistence of Btk in water in the field was studied by Menon & de Mestral (1985) in Nova Scotia, Canada. Btk was applied as a commercial formulated product (Btk content 0.34%) once per year for two years over selected forestry areas at rates between 4.7 & 9.4 L/ha. Sampling of water from rivers at various distances directly downstream from the spray area and from water in the vicinity took place during and after each spray period. For the first spray period, 22 to 63 colonies of Btk/mL water were recovered during the actual 4-day spray campaign from sampling areas down river of the spray area. For up to 13 days after application, up to 88 colonies/mL water were recovered. No Btk was detected in shellfish directly downriver from the spray area either during spraying or for up to 13 days after spraying. For the second spray period, Btk colonies were recovered from two different sites at up to 8 and 12 days following the spray period at amounts of up to 70 and 175 colonies/mL water, respectively. The transient nature of the recovery pattern of Btk may be attributed to the diluting and removal effect of the flowing water bodies, and the disappearance of Btk from water samples can lead to the conclusion that spores were either adsorbed to clay minerals, destroyed by soil micro-flora and solar radiation or simply remained in the upper soil layers (more likely a combination of all).

Btk was found to be far more persistent in fresh water than in sea water. Sea water is generally considered bactericidal to non-marine bacteria, whereas lake water contains a higher concentration of available nutrients favourable to Btk survival. A continuous and rapid decrease in Btk survival in sea water resulted in approximately 10 % survival after 30 days. By contrast, Btk survival in lake water, although initially declining quickly during the first 20 days, declined more slowly over the remainder of the sampling period to approximately 50 % (Pramer et al., 1963, cited in Menon & deMestral, 1985).

Although Btk was seen in the studies to survive to some extent in water, viability in the natural aquatic environment is influenced by many biological, chemical and physical factors. Predation by bacteriophage, protozoans and other lower animal forms undoubtedly plays a role in controlling the bacteriological population in the aquatic environment.

Btk is not regarded as an autochthonous inhabitant of aquatic environments and does not find optimal conditions for growth e.g. waters are poor in organic C. Therefore, proliferation is not likely to occur. Bacterial cells and especially spores may survive, but will be subject to natural competition in the diverse micro biota of natural waters. Survival of the applied strains SA-11, SA-12 or EG2348 is not expected to cause any environmental or health impact.

Surface water

Under natural conditions, residues of Btk in water are not considered to be able to persist for very long periods due to a combination of natural physical and chemical degradation factors such as solar radiation and predation from resident bacteriophages, protozoans and other lower animal forms.

New information

With reference to Vol. 3MA, section B.2, point B.2.1.2 Btk is not regarded to be a common inhabitant of surface waters. Water is not the natural habitat of *B. thuringiensis*. Reaching aquatic environments e.g. through spray drift during application in agriculture, *B. thuringiensis* comes across unfavourable conditions (e.g. lack of nutrients, UV exposure, temperature) leading to a rapid decline of the population size. Thus, proliferation of this bacterial species in natural water bodies is not expected to occur, and population size will decline upon hostile environmental conditions.

In addition, the literature search covering the past 10 years (Cornelese, 2016b) did not result in any publications on the fate and behaviour of Btk in surface water. The result of the search did provide information on the fate and behaviour of δ-endotoxins of Bt, the Cry proteins. These results are evaluated below.

Endotoxins

Information on hydrolysis of Cry1Ab protein is part of the study by Wang et al (2007). This study is also presented above (KMA 7.1.1/08) for the part on soil degradation.

Reference:	KMA 8.1.2/03
Author:	Wang, H., Ye, Q., Gan, J., Wu, L. (2007)
Title:	Biodegradation of Cry1Ab protein from Bt transgenic rice in aerobic and flooded paddy soils
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 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 16.9 to 41.3 d. 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 d. These results suggest that the toxin protein, when introduced into a paddy field upon harvest, will undergo rapid removal after the field is drained and exposed to aerobic conditions.
Doc ID:	Published: Journal of agriculture and food chemistry, 55(5):1900-1904
Guideline:	Not documented
GLP:	Not documented
Acceptability:	The study is considered to be acceptable.
Material and methods:	
Test material:	Purified Cry1Ab protein
Test concentration:	300, 500, 1000, 2000 and 4000 ng/mL 500 ng/mL for pH range
Test system:	Phosphate buffer at pH 4, 5, 7 and 8. pH 7 for concentration range Incubation in the dark
Temperature:	25°C
Sampling time points:	0, 3, 5, 10, 15, 20, 30, and 60 d
Method of analysis:	ELISA

Purified Cry1Ab protein was obtained from straw of transgenic rice by extraction and purification of crude extracts. Hydrolysis of purified Cry1Ab protein was determined in aqueous solution with different pH, 4.0, 5.0, 7.0, and 8.0 at a final concentration of protein in the solutions of 500 ng/mL. Test solutions were incubated at 25°C in the dark. Hydrolysis as function of the protein concentration, 300, 500, 1000, 2000 and 4000 ng/mL, was also determined at pH 7. In **Figure 8.1.2-01** and **Table 8.1.2-01** the results of the hydrolysis of Cry1Ab protein at different initial concentration are presented. Hydrolysis could be well described by first order kinetics and half-lives ranging from 130.8 to 93.7 days were estimated. The disappearance of Cry1Ab protein in buffer

solutions with different pH also followed the first order decay model (**Figure 8.1.2-02**, **Table 8.1.2-02**). The half-lives estimated increased from 82.5 to 210 days as the solution pH increased from pH 4 to pH 8.

Table 8.1.2-01 first order regression analysis of the hydrolysis of Cry1Ab protein as function of initial

concn (ng/mL)	rate constant $k(d^{-1})$	half-life $t_{1/2}$ (d)	correlation coefficient (r)	p
300	0.0053	130.8	0.98	0.001
500	0.0054	128.4	0.98	0.001
1000	0.0057	121.6	0.98	0.001
2000	0.0060	115.5	0.97	0.001
4000	0.0074	93.7	0.96	0.03

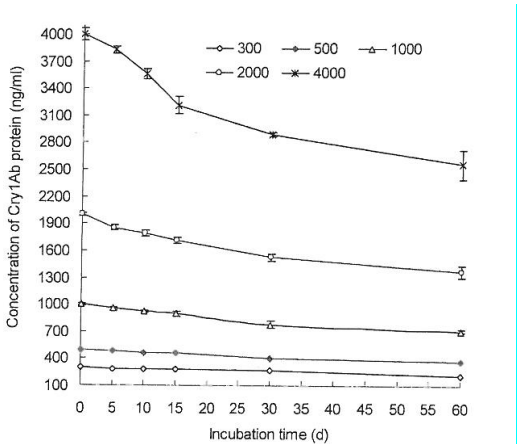

Figure 8.1.2-01 Hydrolysis of purified Cry1Ab protein in aqueous solutions (pH 7) at different initial concentration

Table 8.1.2-02 regression analysis and half-life of the hydrolysis of Cry1Ab protein in aqueous solution at different pH

pH	rate constant $k\ (d^{-1})$	half-life $t_{1/2}$ (d)	correlation coefficient (r)	p
4.0	0.0084	82.5	0.99	0.000
5.0	0.0069	100.4	0.93	0.007
6.0	0.0062	111.8	0.99	0.000
7.0	0.0054	128.4	0.98	0.001
8.0	0.0033	210.0	0.95	0.004

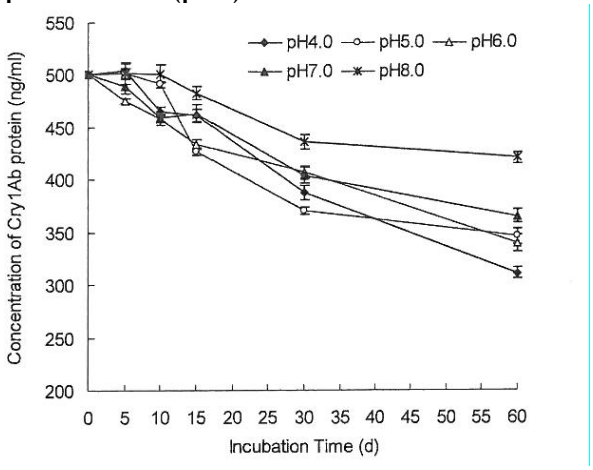

Figure 8.1.2-02 Hydrolysis of purified Cry1Ab protein in aqueous solutions at different pH

Evaluation RMS	Although the study is based on Cry toxins from genetically modified plants it is acceptable to study half-lives of Cry toxins originating from Bt. Rapid degradation of Cry1Ab protein occurred in paddy soils under aerobic conditions, with half-lives ranging from 16.9 to 41.3 d. 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 d. These results suggest that the toxin protein, when introduced into a paddy field upon harvest, will undergo rapid removal after the field is drained and exposed to aerobic conditions.

Li et al., (2013) tested the degradation of Cry1Ac in soil, sediment and water (also see M-MA 8.1.1 above) and the effect of sterilisation. Below the results of the water and sediment test are given. Sediment was collected from a drinking water reservoir in an area with no history of transgenic crop cultivation, sieved and stored at 4°C. Both sediment and water was spiked with crude Bt proteins extracted from transgenic cotton seeds.

Reference:	KMA 8.1.2/04
Author:	Li, Y-L., Du, J., Fang, Z-X., You, J. (2013)
Title:	Dissipation of Insecticidal Cry1Ac Protein and Its Toxicity to Non-target Aquatic Organisms.
Abstact:	The widespread cultivation of *Bacillus thuringiensis* crops has raised public concerns on their risk to nontarget organisms. Persistence of Cry1Ac protein in soil, sediment and water and its toxicity to nontarget aquatic organisms were determined. The dissipation of Cry1Ac toxin was well described using first order kinetics, with the half-lives (DT_{50}) ranging from 0.8 to 3.2, 2.1 to 7.6 and 11.0 to 15.8 d in soil, sediment and water, respectively. Microbial degradation played a key role in the dissipation of Cry1Ac toxin and high temperature accelerated the processes. Cry1Ac toxin was more toxic to the midge *Chironomus dilutus* than the amphipod *Hyalella azteca*, with the median lethal concentration (LC_{50}) of *C. dilutus* being 155 ng/g dry weight and 201 ng/mL in 10-d sediment and 4-d water bioassays, respectively. While Cry1Ac toxin showed toxicity to the midges, risk of Bt proteins to aquatic nontarget organisms was limited because their environmentally relevant concentrations were much lower than the LC_{50}s.
Doc ID:	Published: Journal of Agricultural and Food Chemistry, 61(46), 10864-10871
Guideline:	Not documented
GLP:	Not documented
Acceptability:	The study is considered to be acceptable.
Material and methods:	
Test material:	Cotton extracted Cry 1 Ac endotoxin
Test concentration:	243 ng/g dry sediment, 250 ng/mL water
Test system:	0.5 g sediment, sterilised (121°C for 30 min.) and non-sterilised and 1.5 mL Milli-Q water pH 7 Moisture: 48% WHC for sediment
Temperature:	three temperatures of 4, 24, and 34°C
Sampling time points:	0, 1, 3, 7, 15, 30, 45, and 60 d
Method of analysis:	Sediment extraction plus ELISA, extraction efficiency was 46.5 ± 3.4%. No extraction was needed for water RSD 7.3%; method detection limit of 0.8 ng/g dry weight
Sediment characteristics:	Total OC (%): 2.75

After being spiked and throughout the experiments to compensate for the water evaporation, Milli-Q water was added daily to compensate for losses. Two sterilization conditions (sterilized and not sterilized) were used to assess the impact of microbial degradation on the dissipation of Cry1Ac protein. At sampling, three replicates were terminated, and concentrations of Cry1Ac protein in the substrates were measured. The Cry1Ac protein was extracted from the sediment and the supernatant after centrifugation was decanted and analysed using ELISA. No extraction was needed for water. The dissipation of CrylAc protein was fitted with the first order kinetic model on the measured data as presented in **Table 8.1.2-03** and **-04**. The kinetic equations and parameters, including coefficients of determination (R^2), p values, k, DT_{50}, and DT_{90}, are presented in T**able 8.1.2-05**.

Table 8.1.2-03: Residues of Cry1Ac Protein in Sediment at Different Time, Temperature and Sterilization Conditions

Time [d]	Sterilisation condition	Concentration [ng/g dw][a]		
		4°C	24°C	34°C
0	Not sterilised	243 ± 7.6	243 ± 7.6	243 ± 7.6
1		160 ± 7.7	133 ± 8.6	107 ± 5.1
3		118 ± 6.5	95 ± 6.3	83 ± 2.9
7		89 ± 5.3	76 ± 7.5	63 ± 2.6
15		44 ± 5.8	48 ± 5.4	22 ± 1.9
30		28 ± 2.8	31 ± 3.1	14 ± 0.7
45		18 ± 3.4	23 ± 2.3	4 ± 0.5
60		8 ± 2.9	12 ± 3.3	2 ± 0.4
0	Sterilised	243 ± 7.6	243 ± 7.6	243 ± 7.6
1		201 ± 5.7	187 ± 2.4	157 ± 3.6
3		135 ± 7.6	125 ± 6.2	111 ± 1.3
7		104 ± 4.5	94 ± 7.9	74 ± 5.5
15		65 ± 9.2	55 ± 2.4	35 ± 3.9
30		42 ± 9.7	37 ± 3.1	24± 2.1
45		29 ± 4.0	19 ± 0.6	7 ± 2.0
60		19 ± 3.3	11 ± 1.4	4 ± 0.6

[a] Concentrations of Cry1Ac protein were shown as means ± standard deviation of three replicates.

Table 8.1.2-04 Residues of Cry1Ac Protein in Water at Different Time, Temperature and Sterilization Conditions

Time [d]	Sterilisation condition	Concentration [ng/g dw][a]		
		4°C	24°C	34°C
0	Not sterilised	250 ± 8.8	250 ± 8.8	250 ± 8.8
1		233 ± 7.6	227 ± 6.4	228 ± 3.7
3		210 ± 6.9	203 ± 9.3	203 ± 8.5
7		182 ± 3.2	176 ± 2.8	167 ± 6.3
15		108 ± 5.4	95 ± 5.4	83 ± 5.3
30		65 ± 1.9	52 ± 3.5	42 ± 4.7
45		37 ± 3.1	30 ± 5.1	24 ± 4.7
60		25 ± 2.6	17 ± 1.3	12 ± 1.7
0	Sterilised	250 ± 8.8	250 ± 8.8	250 ± 8.8
1		234 ± 8.7	235 ± 4.4	241 ± 8.0
3		210 ± 8.7	199 ± 11.4	207 ± 8.0
7		190 ± 9.4	170 ± 9.0	169 ± 1.5
15		116 ± 11.3	94 ± 8.7	83 ± 4.1
30		69 ± 4.4	50 ± 3.2	44 ± 1.3

Time [d]	Sterilisation condition	Concentration [ng/g dw][a]		
		4°C	24°C	34°C
45		39 ± 0.9	28 ± 1.8	23 ± 1.4
60		19 ± 2.7	14 ± 1.2	10 ± 0.7

[a] Concentrations of Cry1Ac protein were shown as means ± standard deviation of three replicates.

Table 8.1.1-05 First Order Kinetic Equations, The Rate Constant (k) and the Times When Concentration of Cry1Ac Protein Reduced to the Half (DT_{50}) and 90% (DT_{90}) of Its Initial Concentration

Matrix	treatment	r^2	*p* value	k [d^{-1}]	DT_{50} [d]	DT_{90} [d]
sediment	4°C, NS	0.928	<0.0001	0.137	5.1	16.8
	4°C, S	0.926	<0.0001	0.091	7.6	24.3
	24°C, NS	0.839	<0.0001	0.179	3.9	12.9
	24°C, S	0.936	<0.0001	0.116	6.0	19.8
	34°C, NS	0.882	<0.0001	0.324	2.1	7.1
	34°C, S	0.946	<0.0001	0.181	3.8	12.7
water	4°C, NS	0.993	<0.0001	0.046	15.1	50.0
	4°C, S	0.995	<0.0001	0.044	15.8	52.3
	24°C, NS	0.992	<0.0001	0.054	12.8	42.6
	24°C, S	0.994	<0.0001	0.057	12.2	40.4
	34°C, NS	0.994	<0.0001	0.062	11.2	37.1
	34°C, S	0.994	<0.0001	0.063	11.0	36.5

The dissipation curve of Cry 1Ac in soil, but also in water and sediment, at 34°C are presented in **Figure 8.1.1-06** in section M-MA 8.1.1.

In conclusion, dissipation of Cry1Ac protein in sediment and water could be well described by the first order kinetic equations. Microbial degradation contributed significantly to the dissipation in sediment but not in water. High temperature accelerated the dissipation process.

According to Douville et al., (2007) the growing use of transgenic crops and biopesticides has raised concerns about the release of DNA through either pollen or degradation of plant biomass to recipient environments. The release of exogenous DNA by crops constitutes an important source to the aquatic environment. Thus, an investigation of the occurrence, fate, and persistence of the cry1Ab gene in rivers near transgenic corn planted in fields appeared to be relevant for assessing the potential environmental impacts of these bioproducts. The goals of the study were to develop a methodology for extracting DNA from various environmental compartments (soil, sediment, surface water, and biota) and to determine the concentration of the cry1Ab gene levels in both transgenic corn and Btk in adjacent aquatic environments. The persistence of this exogenous gene in sediment and surface water in the laboratory was also evaluated.

In the study total DNA was analysed in surface water and sediment. DNA extraction from environmental samples resulted in poor recovery and disturbance of qPCR analysis. Though it is described in the study that cry1Ab was readily detected in Bt corn with the selected primers using qPCR a relation with the total DNA from environmental samples is not presented. In a separate experiment sediments and surface water were spiked with genomic DNA from Bt corn to evaluate degradation rates. T0 samples were taken and these corresponded to DNA extracted 1 hour after application. The spiked samples were further incubated for 2, 7, 14, 21, 32, and 40 days at 15 1C (15-h light:9-h dark cycle) The recovery of DNA was constant during time though rather low, ranging between 30 and 35% for 40 days. Again, it is not clear from the study what was exactly analysed. Though it seems total DNA was extracted and analysed a result on the content of the cry1Ab gene from Bt corn is stated. This decreased quickly within the first few days, but it was still detected 21 days later. The half-life ($t_{1/2}$) of the cry1Ab gene from Bt and Bt corn was estimated at 1.7 days for both types of sediment.

The study reports that the Cry1Ab gene persisted in surface water for several weeks, however, the data presented are data on total extracted DNA. Though the material and methods state that Cry1Ab gene is analysed with qPCR methods it is not clear from the reported results this was the actual method used. All results state 'extracted DNA' or 'DNA of corn'. Therefore, it seems total DNA has been analysed and the results on persistence are of DNA instead. The study is not very clear in its details and therefore the study does not provide useful information to derive data on the fate and behaviour of Cry1Ab in surface water systems.

Strain & Lydy (2015) monitored the fate and transport of a commonly used Bt protein, Cry1Ab, in a large-scale agricultural field and related this to environmental observations to an aquatic system using laboratory microcosms. All samples were extracted and quantified using ELISA methods. Below the relevant part of the study on the aquatic microcosms is presented.

The fate of the Cry1Ab protein in maize leaves placed into water and sediment was measured across different environmental conditions over time. Variations in sterility, sediment type, temperature, and ultra-violet (UV) light were tested. Senesced leaves from the research field were used to represent possible routes of exposure to aquatic organisms, water was collected from a river and verified to be devoid of Cry1Ab protein. Three sediment types (experimental field, forest, and creek) were collected from the field with 3.8%, 3.1%, and 1.7% organic matter, respectively. Leaf tissue was grinded with PBST buffer and analysed to determine initial Cry1Ab protein concentration with ELISA and to verify the absence in non-Bt leaves. A clear description of the method is not included.

Sediment (5 g) and river water (75 mL) were added to 120 mL glass jars and left to settle overnight. The following day, 10 leaf discs were added to each replicate, with three non-Bt controls to be taken at 1, 14, and 60 days after addition of the leaves. Another test examined the influence of UV radiation on Cry1Ab stability within the microcosms. Samples were held in a 23°C incubator with a 16:8 h (light:dark) light cycle with full-spectrum daylight (5000 K) linear fluorescent light bulbs. The replicates receiving no treatment were wrapped in aluminium foil to prevent light penetration. Water quality parameters including conductivity, dissolved oxygen, pH, and temperature were monitored throughout all experiments. All soil, sediment, and water samples contained no detectable Cry1Ab levels before the experiment initiation. Results from a preliminary test indicated there was no statistical difference in Cry1Ab concentrations between microcosms with sterile or nonsterile matrices. Therefore, matrices were not sterilized. Time was a highly significant factor in all matrices, treatment groups and experiments. A general trend was observed in control treatments, as the Cry1Ab protein rapidly leached or degraded from the leaf tissue with a half-life of approximately 2 h under all experimental conditions, with the proportion in the water peaking within 2 days following the start of the experiment, and then gradually declining to below the reporting limits around two weeks. No differences in Cry1Ab protein concentrations were observed under different light conditions. As Cry1Ab protein concentrations in the water declined, there was a corresponding accumulation in the sediment according to percentage organic matter, with a peak at two weeks after introduction of Bt maize leaves to the microcosm (**Figure 8.1.2-03**). The effect of sediment type on Cry1Ab concentrations in the sediments was significant ($F_{9, 48} = 13.82$, $P < 0.01$), with Cry1Ab concentrations in farm sediment being higher than those in creek and forest sediments.

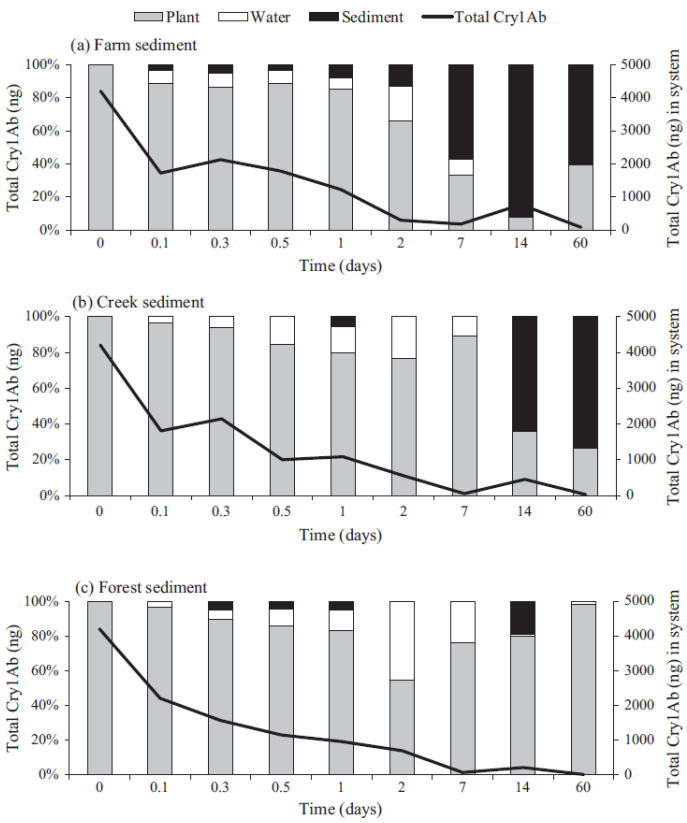

Figure 8.1.2-03 Total degradation of the Cry1Ab protein in microcosms containing (a) farm, (b) creek, or (c) forest sediments. Bar graph shows percentage of Cry1Ab within sediment (■), water (□) and plant material (□). The line represents total Cry1Ab in the system.

The reporting of the materials and methods in the publication is rather limited. There are several separately performed experiments described but the overall line in reporting of results is not clear. Reported results cannot be reproduced based on the information provided. The study is not very clear, and therefore not considered useful for risk assessment. Overall, the results do not differ from other reported results to a great extent, the study should be considered supportive.

Strain et al., (2014) published a method and its validation to analyse Cry1Ab from water matrices. Enzyme-linked immunosorbent assay (ELISA) is commonly used for quantitation of Bt proteins in the environment. The objective of the current study was to optimize the extraction of a Bt protein, Cry1Ab, from three water matrices and validate the ELISA method for specificity, precision, accuracy, stability, and sensitivity. Recovery of the Cry1Ab protein was matrix-dependent and ranged from 40 to 88% in the validated matrices, with an overall method detection limit of 2.1 ng/L. Precision among two plates and within a single plate was confirmed with a coefficient of variation less than 20%. For a more detailed description of the study reference is made to M-MA section 4 point 4.2, KMA 4.2/01.

RMS evaluation	Btk is not regarded to be a common inhabitant of surface waters. Water is not the natural habitat of *B. thuringiensis*. Thus, proliferation of this bacterial species in natural water bodies is not expected to occur, and population size will decline upon hostile environmental conditions. In water, various half-lives of the crystalline protein (δ-endotoxin) have been found in the literature, ranging from days to months (**Table 8.1-01**) Persistence can be influenced by biotic parameters. Overall the results indicate that the endotoxins do not persist or accumulate in water and are degraded rapidly. **Water**: Cry1Ab – DT_{50} 130.8, 93.7 days (hydrolysis) Cry1Ac – DT_{50} 10 – 15 days (biodegradation)
Endpoint: Water:	Btk SA-12 *B. thuringiensis* is not regarded as an autochthonous inhabitant of aquatic environments and does not find optimal conditions for growth. Therefore, prolifera-

	tion is not likely to occur. Endotoxins Persistence can be influenced by biotic and abiotic factors. Overall the results indicate that the endotoxins do not persist or accumulate in water.

Cited references

Report KMA 8.1.2/03 – Wang, H., Ye, Q., Gan, J., Wu, L. (2007). Biodegradation of Cry1Ab protein from Bt transgenic rice in aerobic and flooded paddy soils

Published report,

Journal of Agriculture and Food Chemistry, 55(5):1900-1904

Evaluation RMS	Although the study is based on Cry toxins from genetically modified plants is acceptable to study half-lives of Cry toxins originating from Bt. Rapid degradation of Cry1Ab protein occurred in paddy soils under aerobic conditions, with half-lives ranging from 16.9 to 41.3 d. 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 d. These results suggest that the toxin protein, when introduced into a paddy field upon harvest, will undergo rapid removal after the field is drained and exposed to aerobic conditions.

Report KMA 8.1.2/04 – Li, Y-L., Du, J., Fang, Z-X., You, J. (2013). Dissipation of Insecticidal Cry1Ac Protein and Its Toxicity to Non-target Aquatic Organisms

Published report,

Journal of Agriculture and Food Chemistry, 61(46):10864-10871

Evaluation RMS	The study is acceptable. The dissipation of Cry1Ac toxin was well described using first order kinetics, with the half-lives (DT_{50}) ranging from 0.8 to 3.2, 2.1 to 7.6 and 11.0 to 15.8 d in soil, sediment and water, respectively.

Report KMA 8.1.2/05 – Douville, M. Gagné, F., Blaise, C., André, C. (2007). Occurrence and persistence of *Bacillus thuringiensis* (Bt) and transgenic Bt corn cry1Ab gene from an aquatic environment

Published report,

Ecotoxicology and Environmental Safety, 66(2):195–203

Abstract: Genetically modified corn crops and suspensions of *Bacillus thuringiensis* (Bt) are currently used to control pest infestations of insects of the Lepidoptera family. For this purpose, the cry1Ab gene coding for protein d-endotoxin derived from *B. thuringiensis kurstaki* (Btk), which is highly toxic to these insects, was inserted and expressed in corn. The aims of this study were to examine the occurrence and persistence of the cry1Ab gene from Btk and Bt corn in aquatic environments near fields where Bt corn was cultivated. First, an optimal DNA preparation and extraction methodology was developed to allow for quantitative gene analysis by real-time polymerase chain reaction (qPCR) in various environmental matrices. Second, surface water and sediment were spiked in vitro with genomic DNA from Bt or Bt corn to evaluate the persistence of cry1Ab genes. Third, soil, sediment, and water samples were collected before seeding, 2 weeks after pollen release, and after corn harvesting and mechanical root remixing in soils to assess cry1Ab gene content. DNA was extracted with sufficient purity (i.e., low absorbance at 230 nm and absence of PCR-inhibiting substances) from soil, sediment, and surface water. The cry1Ab gene persisted for more than 21 and 40 days in surface water and sediment, respectively. The removal of bacteria by filtration of surface water samples did not significantly increase the half-life of the transgene, but the levels were fivefold more abundant than those in unfiltered water at the end of the exposure period. In sediments, the cry1Ab gene from Bt corn was still detected after 40 days in clay- and sand-rich sediments. Field surveys revealed that the cry1Ab gene from transgenic corn and from naturally occurring Bt was more abundant in the sediment than in the surface water. The cry1Ab transgene was detected as far away as the Richelieu and St. Lawrence rivers (82 km downstream from the corn cultivation plot), suggesting that there were multiple sources of this gene and/or that it undergoes transport by the water column. Sediment-associated cry1Ab gene from Bt corn tended to decrease with distance from the Bt cornfield. Sediment concentrations of the cry1Ab gene were significantly corre-

lated with those of the cry1Ab gene in surface water (R = 0:83; P = 0:04). The data indicate that DNA from Bt corn and Bt were persistent in aquatic environments and were detected in rivers draining farming areas.

Evaluation RMS	The study is considered acceptable and it was shown that the cry1Ab gene persisted for more than 21 and 40 days in surface water and sediment, respectively.

Report KMA 8.1.2/06 – Strain, K.E., Lydy, M.J. (2015). The fate and transport of the Cry1Ab protein in an agricultural field and laboratory aquatic microcosms

Published report,

Chemosphere, 132:94-100

Abstract: Genetically engineered crops expressing insecticidal crystalline proteins derived from *Bacillus thuringiensis* (Bt), were commercialized almost two decades ago as a means to manage agricultural pests. The Bt proteins are highly specific and only lethal upon ingestion, limiting the scope of toxicity to target insects. However, concern of exposure to non-target organisms and negative public perceptions regarding Bt crops has caused controversy surrounding their use. The objective of this research was to monitor the fate and transport of a Bt protein, Cry1Ab, in a large-scale agricultural field containing maize expressing the Cry1Ab protein and a non-Bt near isoline, and in aquatic microcosms. The highest environmental concentrations of the Cry1Ab protein were found in runoff water and sediment, up to 130 ng L^{-1} and 143 ng g^{-1} dry weight, respectively, with the Cry1Ab protein detected in both Bt and non-Bt maize fields. As surface runoff and residual crop debris can transport Bt proteins to waterways adjacent to agricultural fields, a series of laboratory experiments were conducted to determine the potential fate of the Cry1Ab protein under different conditions. The results showed that sediment type and temperature can influence the degradation of the Cry1Ab protein in an aquatic system and that the Cry1Ab protein can persist for up to two months. Although Cry1Ab protein concentrations measured in the field soil indicate little exposure to terrestrial organisms, the consistent input of Bt-contaminated runoff and crop debris into agricultural waterways is relevant to understanding potential consequences to aquatic species.

Evaluation RMS	The study is considered acceptable and the results showed that sediment type and temperature can influence the degradation of the Cry1Ab protein in an aquatic system and that the Cry1Ab protein can persist for up to two months.

Report KMA 8.1.2/07 – Strain, K.E., Whiting, S.A., Lydy, M.J. (2014). Laboratory and field validation of a Cry1Ab protein quantitation method for water

Published report,

Talanta, 128: 109–116

Abstract: The widespread planting of crops expressing insecticidal proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt) has given rise to concerns regarding potential exposure to non-target species. These proteins are released from the plant throughout the growing season into soil and surface runoff and may enter adjacent waterways as runoff, erosion, aerial deposition of particulates, or plant debris. It is crucial to be able to accurately quantify Bt protein concentrations in the environment to aid in risk analyses and decision making. Enzyme-linked immunosorbent assay (ELISA) is commonly used for quantitation of Bt proteins in the environment; however, there are no published methods detailing and validating the extraction and quantitation of Bt proteins in water. The objective of the current study was to optimize the extraction of a Bt protein, Cry1Ab, from three water matrices and validate the ELISA method for specificity, precision, accuracy, stability, and sensitivity. Recovery of the Cry1Ab protein was matrix-dependent and ranged from 40 to 88% in the validated matrices, with an overall method detection limit of 2.1 ng/L. Precision among two plates and within a single plate was confirmed with a coefficient of variation (CV) less than 20%. The ELISA method was verified in field and laboratory samples, demonstrating the utility of the validated method. The implementation of a validated extraction and quantitation protocol adds consistency and reliability to field-collected data regarding transgenic products.

Evaluation RMS	No remarks

B.8.1.3 Air

No studies on persistence and multiplication in air of *Bacillus thuringiensis* subsp. *kurstaki* strain SA-11, SA-12 or EG2348 have been submitted. However, since a study which addresses this issue has been reported in the scientific literature, no further data is requested.

A rapid degradation of Btk in air is assumed since inactivation by solar radiation is a very important factor causing loss of activity and degradation of bacteria spores and δ-endotoxin crystals in the field environment.

Spray drift can occur following an application of Btk which may lead to temporary concentrations in the atmosphere which are capable of drifting with wind currents before the spores and crystals in finer spray droplets settle out.

Teschke *et al.* (2001) studied the spatial and temporal distribution of Btk following an aerial application program at 4 L formulation/ha (2.1 % Btk concentration) over a wide area in British Columbia, Canada. Average culturable Btk concentrations within the spray zone during the spray period were 739 CFU/m^3 air with an initial (10-hour period from start of spraying) half-life of 3.3 hours. Concentrations decreased rapidly during the first day and gradually over the 9-day monitoring period resulting in an overall half-life of 2.4 days. The authors suggest the contrast between the initial and overall half-life predictions imply that airborne Btk diminishes in two stages, a quick initial stage followed by a slower settling out of aerosol spores and dilution with uncontaminated air.

Indoor concentrations were initially lower than outdoor, but this situation reversed after 5 to 6 hours as outdoor concentrations were diminished by environmental effects such as the action of solar radiation. In areas 125 to 1000 metres away from the spray zone, Btk concentrations to 484 CFU/m^3 air were recorded during the spray period. Anticipated settling time for droplets was less than 5 minutes with target droplet sizes of 110 to 125 µm. However, smaller and larger sized droplets occurred, possibly due to a combination of factors such as the break-up of droplets due to the speed of the plane, and evaporation of water from the aerosols during descent decreasing the droplet sizes. This is supported by evidence that airborne Btk concentrations were higher with lower relative humidity and higher temperatures.

It should be noted that these results report values which are far greater than those that may occur following a land-based broadcast application onto grapes fields. However, they do give an indication of the rapid disappearance of Btk from air.

New information 2016

Emanuel et al., (2012) developed a mutant variant of *Bacillus thuringiensis* subsp. *kurstaki* that eased tracking of applied material. In the study they tested the ability of this barcoded *B. thuringiensis* subsp. *kurstaki* spores to be re-aerosolized. The test was conducted in a 61-m-long ambient breeze tunnel. This tunnel provided a controlled indoor environment that simulated outdoor wind conditions. Vinyl tiles were cleaned with 70% (vol/vol) ethanol-water and placed down the length of the ambient breeze tunnel at 3-m intervals to a distance of 30 m from the contaminated tiles. At the front of the ambient breeze tunnel, a row of tiles enclosed in boxes were carefully seeded with 100 mg of the aerosolized barcoded *B. thuringiensis* subsp. *kurstaki* spores. Five of the seeded tiles were set aside and not subjected to a breeze to serve as the positive-control tiles. Midway down the tunnel, a single dry filter unit (DFU) air sampler was also situated to collect any spores that drifted down the tunnel. To begin the test, fans directed at the seeded tiles were turned on, creating a breeze that was allowed to blow air across the contaminated tiles for a period of 10 min. The velocity of the breeze was measured to be 3.8 m per s by anemometers positioned just above the seeded tiles. The fans were then turned off, and re-aerosolized spores were allowed to settle overnight. In the morning, each tile was taken for analysis.

The outdoor dissemination was performed on a test grid within a field consisting of multiple dry filter unit (DFU) collectors and a single joint biological point detection system was arranged across the field downwind of the release point. The test plan was designed to release 100 g (1.1×10^{13} spores) of dried barcoded *B. thuringiensis* subsp. *kurstaki* spores on the first day and continue to sample the air for an additional 2 days. On the third day after the initial release event, a team of two people used commercial blowers for a 15-min period to simulate air turbulence around a 30-m^2 area near the site of the release. The air samples were monitored for an additional day to detect barcoded spores that might have been re-aerosolized by this event. On the eighth day, a 100-g batch of wild-type (WT *B. thuringiensis* subsp. *kurstaki*; lacking the barcode) spores (approximately 2.6×10^{13} spores) was released in the identical manner, and the air was monitored. The goal of this second release was to demonstrate the selectivity of the detection method. **Figure 8.1.3-01** summarizes the results of the detection for the re-aerosolized spores in the ambient breeze tunnel based on the distance relative to the contaminated tiles. The data show that deposited spores were re-aerosolized and deposited up to 30 m away following simulation of light wind. The average cycle threshold (C_T) values increased with distance from the seeded tiles (**Figure 8.1.3-01** part B) and tracked with the counts of viable spores (**Figure 8.1.3-01** part C). To demonstrate utility of barcoded *B. thuringiensis* subsp. *kurstaki* spores in open-air release studies, outdoor dissemination of both barcoded and WT *B. thuringiensis* subsp. *kurstaki* spores was monitored. The results for the air sampling immediately following each of the release. Immediately following the release, large amounts of viable material producing robust real time (RT)-PCR signals were recovered from several downwind DFUs. The amount of viable material dropped rapidly following each release, with an immediate 3-log drop on the day following the release

and an approximately 1-log decrease per day thereafter. In the field the authors monitored the ability to recover viable spores and generate RT-PCR signals over time following each release by activating each DFU for an 8-h period over subsequent days. While the RT-PCR signals rapidly dropped to undetectable levels, viable material was recovered, though at levels near or below the detection limits for each of the assays.

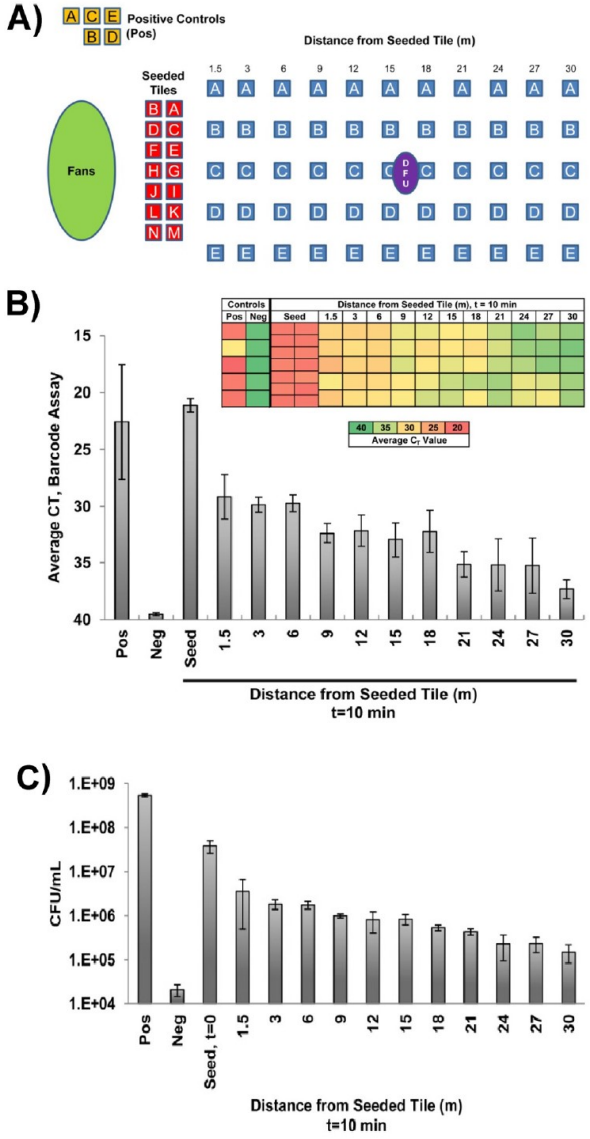

Figure 8.1.3-01 Re-aerolisation in the ambient breeze tunnel tested by PCR. A) experimental design, B) results of re-aerolisation test averages of Ct values, C) enumeration of viable colonies averages

In conclusion, the present study and the data summarized here demonstrated that re-aerosolisation did occur in the ambient breeze tunnel. However, the amount of viable material dropped rapidly following each release. Just the possibility of the process to occur in theory will in practice not change the conclusion that fate and transport via air after application is unlikely to play a role in environmental exposure to *B. thuringiensis* subsp. *kurstaki* spores.

RMS evaluation	Re-aerolisation of applied spores is possible but spores rapidly drop in viability following release to air. Fate and transport via air after application is unlikely to play a role in environmental exposure to *B. thuringiensis* subsp. *kurstaki* spores.
Endpoint: Air:	Re-aerolisation of applied spores is possible but spores rapidly drop in viability following release to air. Fate and transport via air after application is unlikely to play a role in environmental exposure to *B. thuringiensis* subsp. *kurstaki* spores and endotoxins.

Cited references

Report KMA 8.1.3/02 – Emanuel P.A., Buckley P.E., Sutton T.A., Edmonds J.M., Bailey A.M., Rivers B.A., Kim M.H., Ginley W.J., Keiser C.C., Doherty R.W., Kragl F.J., Narayanan F.E., Katoski S.E., Paikoff S., Leppert S.P., Strawbridge J.B.,VanReenen D.R., Biberos S.S., Moore D., Phillips D.W., Mingioni L.R., Melles O., Ondercin D.G., Hirsh B., Bieschke K.M., Harris C.L., Omberg K.M., Rastogi V.K.,Van Cuyk S., and Gibbons H.S. (2012) Detection and tracking of a novel genetically tagged biological simulant in the environment.

Published report,

Applied and Environmental Microbiology, 78(23):8281-8288

Abstract: A variant of *Bacillus thuringiensis* subsp. *kurstaki* containing a single, stable copy of a uniquely amplifiable DNA oligomer integrated into the genome for tracking the fate of biological agents in the environment was developed. The use of genetically tagged spores overcomes the ambiguity of discerning the test material from pre-existing environmental microflora or from previously released background material. In this study, we demonstrate the utility of the genetically "barcoded" simulant in a controlled indoor setting and in an outdoor release. In an ambient breeze tunnel test, spores deposited on tiles were re-aerosolized and detected by real-time PCR at distances of 30 m from the point of deposition. Real-time PCR signals were inversely correlated with distance from the seeded tiles. An outdoor release of powdered spore simulant at Aberdeen Proving Ground, Edgewood, MD, was monitored from a distance by a light detection and ranging (LIDAR) laser. Over a 2-week period, an array of air sampling units collected samples were analyzed for the presence of viable spores and using barcode-specific real-time PCR assays. Barcoded *B. thuringiensis* subsp. *kurstaki* spores were unambiguously identified on the day of the release, and viable material was recovered in a pattern consistent with the cloud track predicted by prevailing winds and by data tracks provided by the LIDAR system. Finally, the real-time PCR assays successfully differentiated barcoded *B. thuringiensis* subsp. *kurstaki* spores from wildtype spores under field conditions.

Evaluation RMS	No remarks

B.8.2 Mobility

No studies on the translocation of spores or protoxins of *B. thuringiensis kurstaki* SA-11, SA-12 or EG2348 to groundwater have been submitted. The scientific literature provides evidence that it is unlikely that the spores or the protoxins/toxin will be translocated to the groundwater. This evidence includes:

- that 77% of the remaining spores was located in the topsoil (0-2 cm) one year after the application of *B. thuringiensis* subsp. *kurstaki* (Pedersen et al, 1995),
- that no translocation of sprayed *B. thuringiensis* to a depth of >10 cm was affected by artificial or natural irrigation (Akiba, 1991).
- that no or only few spores were translocated through 6 cm volcanic ash or alluvium sand in a column artificially irrigated with 450 mm rain (Akiba,1991)
- that no spores were detected in field soils at 10, 20 and 30 cm depth after application (Akiba, 1991),
- that protoxins and toxins have been demonstrated to adsorb and bind rapidly and strongly to clay and clay-humic acid complexes in soils (Venkateswerlu and Stotzky, 1992; Tapp and Stotzky, 1995; Crecchio and Stotzky, 1998; 2001),
- furthermore, most protoxins will disappear from the soil due to enzymatic degradation, notably of the not absorbed protoxins (see section above), which are most likely to be translocated.

New information 2016

Relevant information has already been provided during original approval of *B. thuringiensis* subsp. *kurstaki* SA-12 and is still valid. It can thus be concluded that movement of Bt through the soil by leaching is unlikely to occur. Additionally, adsorption and binding of protoxins and toxins from Btk have been demonstrated to occur readily, rapidly and strongly onto the clay fraction and clay humic acid complexes of soils. Desorption occurs far less readily.

A literature search covering the past 10 years (Cornelese, 2016b) did not result in any publications on the mobility or leaching behaviour of Btk in soil or the environment. The result of the search did provide information on the potential mobility of δ-endotoxins of Bt, the Cry proteins. These results are evaluated below.

Zhou et al (2010) investigated the sorption of purified Bt toxin, produced by genetically engineered *E. coli* onto brown and red soil. Soil properties are reported in **Table 8.2.01** below.

Table 8.2.01 Some properties of soils used

soils	OM g/kg	SSA (m^2/g)	CEC (cmol/kg)	Clay mineral composition
Red soil	28.4	48.5	21.2	Hydromica (20%), 1.4 nm mineral (35%), kaolinite (45%)
Brown soil	67.6	31.7	46.2	Hydromica (45%), vermiculite (35%), kaolinite (20%)

One gram brown soil or red soil were suspended in 100 mL 0.01 mol L^{-1} Tris buffer, respectively, and then sterilized for 20 min. The adsorption isotherms were measured for initial toxin concentrations from 0.25 to 1.0 mg mL^{-1} and adsorbent content of 1.0 mg mL^{-1} at pH 9. The soil-toxin mixtures were shaken at 25±1℃ for 2 h. then the suspension was centrifuged at 20,000 g for 20 min and the absorbance of supernatants was measured at 280 nm. The amount of toxin adsorbed was calculated from the concentration difference. Control experiments were performed with soils in the absence of the toxin. To prepare the toxin solution which contacted with soil, the adsorption was carried out with the initial toxin concentration of 0.8 mg mL^{-1}, the soil concentration of 1 mg mL^{-1}, and the total solution volume of 100 mL. The precipitate (soil-toxin complex) without supernatant was washed with 20 mL 0.01 mol L^{-1} sterilized Tris buffer centrifuged and the concentration of toxin was determined in the supernatant by optical density measurement at 280 nm.

Results: The adsorption isotherms of toxin followed the Langmuir equation (**Figure 8.2-01**, R^2 › 0.9882). The adsorption capacity of brown soil was more than that of red soil. When the toxin concentration was more than 0.8 mg mL^{-1}, adsorption amount increased slowly and then the adsorption tended to gradually come to equilibrium. Therefore, the initial toxin concentration for preparing the adsorption complexes was chosen as 0.8 mg mL^{-1}.

The sorption isotherms are presented in **Figure 8.2-01** below.

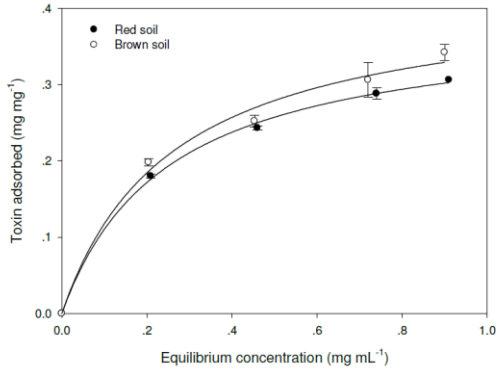

Figure 8.2-01 Adsorption isotherm of toxin on red and brown soil

The study also presented some information on structural changes of the protein after desorption from soil, this information is not considered further as it is considered less relevant for the sorption properties of the toxin. In the study it is not reported which Bt protein was tested.

The adsorption thermodynamics of Cry1Ab in another soil was studied by Zhou et al., (2013). The purified Cry1Ab toxin was prepared from a genetically modified strain *B. thuringiensis* subsp. *kurstaki* HD-1. The molecular weight of the toxin was 66 kDa as determined by polyacrylamide gel electrophoresis (SDS-PAGE). A Chinese latosol was sampled from the 0 - 17 cm layer of a cultivated land. The air-dried samples were homogenized, crushed and passed through a 100-mesh sieve. Some properties of soil are listed in **Table 8.2.02.**

Table 8.2-02 Some soil properties of the tropical soil used

soil	OM (g/kg)	SSA (m^2/g)	CEC (cmol/kg)
Latosol	21.6	35.1	18.9

The adsorption isotherms were measured in the range of toxin concentrations from 4.85×10^{-6} to 1.82×10^{-5} mol/L and soil concentration of 1.0 mg/mL at pH 7.0. The soil-toxin mixtures were shaken at 300 rpm at 5 ± 1 ºC, 25 ± 1 ºC and 45 ± 1 ºC, respectively for 3 h. the suspension was centrifuged and the absorbance of supernatant was measured. As shown in **Figure 8.2-02**, the adsorption isotherms of Cry1Ab toxin followed Langmuir equation ($R^2 > 0.98$), the curves is of the L-type with an initial steep rise in uptake followed by a gradual increase to a more or less flat plateau at 283, 298 and 313 K. The adsorption rose faster at high temperature than at low temperature. The maximum adsorption amount (q_{max}) of the toxin by latosol at three temperatures was 4.50×10^{-6}, 5.99×10^{-6} and 7.55×10^{-6} mol g^{-1}, respectively. The K_{ads} was determined as reported in **Table 8.2-03.**

Table 8.2-03 Langmuir parameters for the adsorption of the Cry1Ab toxin

Temperature (K)	**b (L·mol-1)**	**K**ads	**q**max **(mol·g-1)**	**R^2**
283	2.35×10^5	1.31×10^7	4.5×10^{-6}	0.9970
298	2.34×10^5	1.30×10^7	5.99×10^{-6}	0.9891
313	2.40×105	1.33×10^7	7.55×10^{-6}	0.9987

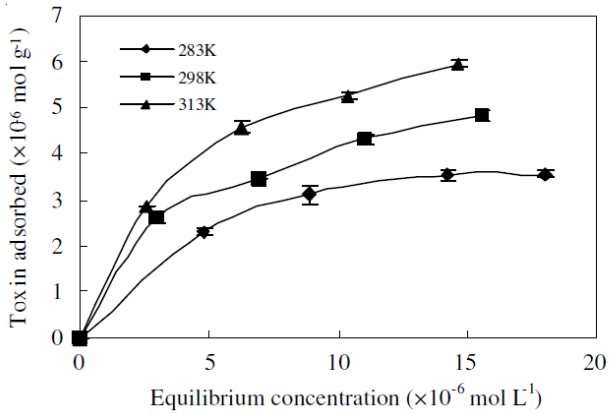

Figure 8.2-02 Adsorption isotherm of Cry1Ab toxin by latosol

From thermodynamic results it is shown that the adsorption of toxin on tropical soil was endothermic, favourable adsorption would occur at higher temperature. Furthermore, results suggested that the adsorption of Cry1Ab toxin in latosol belong to ion exchange processes.

To predict the affinity of Cry proteins for soil, their extractability and persistence in soil a from soil composition a study was conducted by Hung et al (2016) on a number of soils. The aim of this study was to carry out a large-scale screening of the affinity and extractability of contrasting Cry proteins from a large range of soil types. The soils were chosen from the collection of the French Network for the Monitoring of the Quality of Soils (RMQS) and a full analysis of their composition was available. Purified toxin Cry1Ac was derived from a natural strain of *B. thuringiensis* subsp. *kurstaki* HD-73 that produces only Cry1Ac protoxin, purified Cry2A and Cry1C protoxin were derived from genetically modified strains that produce only either the one or the other. Stock solutions of the protein of about 20 g dm^{-3} were stored at 4°C until required in a N-cyclohexyl-3-aminopropanesulphonic acid (CAPS) buffer at pH 10.4 containing 350 mm NaCl to prevent polymerization. Initial protein concentrations for soil suspensions were 3 mg dm^{-3} for Cry1Ac and Cry1C and 40mg dm^{-3} for Cry2A. Forty-one types of soil were selected. Soil samples were air-dried and sieved to <200 µm. Different land-use categories were selected and for each land-use category the soil was chosen so that the full range of clay content, organic matter content and pH were represented. Suspensions were made in triplicate by weighing 0.1 g of each soil into Eppendorf tubes and adding 1ml of solution containing the purified Cry protein to be studied; this was in a background electrolyte of 0.01 m $Ca(NO_3)_2$ solution at the initial concentration indicated above. Suspensions were shaken end-over-end at 25°C for 2 hours. Phases were separated by centrifugation, then 0.8 ml was removed for analysis. The soil was then resuspended in 0.8 ml extraction buffer and shaken vigorously to resuspend. The new

suspensions were shaken again end-over-end at 25°C for 2 hours. Phases were again separated by centrifugation and 0.8 ml supernatant solution removed for analysis. Adsorption and extraction supernatant solutions were diluted as required and the Cry protein quantified with Elisa microplate kits commercially available. The ELISA determinations were calibrated by comparison with the optical density at 290 nm and were found to be linear in the range 1–15 $\mu g \cdot dm^{-3}$ for Cry1Ac and Cry1C and in the range 1–200 $\mu g \cdot dm^{-3}$ for Cry2A. Affinity was assessed from the value of the distribution coefficient, *K*d, measured after 2 hours of incubation. It was calculated by the measured depletion from the initial solution after the addition of soil. The extraction yield was calculated from the ratio of protein desorbed with respect to that initially adsorbed. The influence of land use, soil clay and organic carbon contents, cation exchange capacity and pH as factors on the interaction between protein and soil was statistically evaluated. The ratio of organic carbon to clay contents, Corg:clay, was also included because this reflects the balance between complexed and non-complexed organic matter and has been found to be strongly related to extractable protein for a large number of soil types. A one-way analysis of variance (ANOVA) was used to calculate the significance of each of the variables retained.

Results

The soil properties of the 41 selected soils have a wide range of values as given in **Table 8.2-04**. The range of *K*d values observed and their means are given in **Table 8.2-05**. The range of *K*d values measured is large, 1.6×10^3 to 3.8×10^4, with an average of 1.2×10^4. The large values of *K*d show that the protein has a strong affinity for soil surfaces, which is often observed for proteins on many surfaces.

Table 8.2-04 Maximum and minimum values with the average in brackets of some soil chemical and physical properties

	Clay content / g $\times kg^{-1}$	Corg content / g $\times kg^{-1}$	pH (H_2O) CEC /	CEC / $cmol_c \times kg^{-1}$	C_{org} : clay / %
All soil samples (*n*=41) Range (average)	16–707 (249)	0.6–243 (38)	4.3–8.6 (6.2)	0–39 (11.4)	1–70 (17)
Soils under cereal culture (*n*=16) Range (average)	78–480 (247)	6.9–33 (16.8)	4.6–82 (6.5)	2.3–31.6 (12.9)	3–22 (8)
Soils from (semi-) natural systems (*n*=25) Range (average)	16–707 (250)	0.59–243 (51)	4.3–8.6 (6.1)	0–39 (10.5)	1–70 (23)
Subset of soils studied for all proteins (*n*=19) Range (average)	16–707 (295)	0.59–243 (45)	4.3–8 (6.3)	0–39 (12.6)	1–58 (17)

Table 8.2-05 Minimum and maximum values with the average in brackets of affinity (*K*d / $dm^3 \times kg^{-1}$) for each of the proteins in the soil samples

Protein	Cr1Ac	Cry2A	Cry1C
Full sample set (*n*=41) Range (average)	1630–38 400 (12 100)		
Soils under cereal culture (*n*=16) Range (average)	1630–28 600 (10 100)		
Soils under (semi-) natural land use (*n*=25) Range (average)	2820–38 400 (13 200)		
Soils studied for all proteins (*n*=19) Range (average)	1630–24 400 (11 300)	1560–29 300 (16 100)	837–54 600 (18 300)
Soils under cereal culture (*n*=7) Range (average)	-	1550–26 700 (4700)	5000–54 600 (19 150)
Soils under (semi-) natural land use (*n*=12) Range (average)	-	1560–29 300 (13 700)	837–42 900 (17 700)

There was no significant relation between K_d and soil clay content considered alone although the stepwise correlation identified a significant relation. Helassa et al., (2009) found that the difference in adsorption capacity of two reference clays, montmorillonite and kaolinite, for Cry1Aa, which is similar to Cry1Ac, was in line with their specific surface areas. However, the situation is different for the adsorption of a trace amount of protein orders of magnitude less than the amount required to saturate soil surfaces, which is the case in this study. There was no significant relation between K_d and organic carbon (C_{org}) content, considered alone, although we often observed larger values of K_d for samples with small soil C_{org} contents and smaller values for organic-rich soil. Stepwise analysis identified a significant, inverse relation between K_d and organic carbon content. Simple linear correlation showed no significant relation between affinity and soil pH. In contrast, affinity was significantly inversely related to pH when a stepwise approach was used. A smaller number of soils (19) were used to study the adsorption−desorption properties of the other two Cry proteins, Cry2A and Cry1C. The range and average values of soil clay content, organic matter content, pH and the ratio between C_{org} and clay were similar to those for the full sample set. The range and average value of K_d for Cry2A were the same as for Cry1Ac, and there is no difference in relation to land use. The range of K_d values measured for Cry1C is larger than that for the other proteins, but the average is similar, and again there is no evident effect of land use. As for Cry1Ac, no strong significant relations between affinity of Cry2A or Cry1C and the soil properties were identified by simple regression. The range and average values of extraction yields for each of the proteins from the soil samples were between 43 and 100%, with an average of 74%. The extraction yields of Cry2A were somewhat smaller than those for Cry1Ac, in the range 30 – 100% with an average of 60%.

Conclusion

Cry proteins are all strongly adsorbed by soil and will be effectively immobilized after their release into soil. They differ in their affinity for soil with different textures, organic matter contents and mineralogy. Both affinity and extraction yield vary between soils, and extraction yield is not determined by affinity for all soils and each protein. There is no simple relation between soil properties and either affinity or extraction yield. The amount of Cry protein detected in soil by extraction followed by immune detection is not an absolute measure of the protein present in soil because of differences in extraction yield. The results of our research underline the complexity of the interactions between Cry proteins and soil particles and have important consequences for monitoring the fate of Cry in soil. Reliable quantification of a given Cry protein in soil would require an independent measurement of the extraction yield for each soil type.

Also, Pagel-Wieder et al (2007) studied the influence of soil parameters on the sorption of, in their case, Cry1Ab protein. The aim of this study was not to find the adsorption capacity of the soils from the experimental field site, where Bt corn was cultivated, but rather to characterize the adsorption behaviour of the Cry1Ab protein at con-

centrations typically found at experimental field sites under laboratory conditions. Purified trypsin-activated Cry1Ab protein was microbially produced in a genetically engineered *Escherichia coli.* Na-montmorillonite (M-Na) was commercially purchased and 3 soils were taken from topsoil and subsoil from an experimental field where Bt corn was cultivated. Soil clay fractions were characterised using different methods. To prevent microbial degradation of the Cry1Ab protein, adsorption and desorption experiments were done under sterile conditions. Kinetic tests were carried out at one concentration of Cry1Ab protein to determine the time required for adsorption equilibrium to be attained. An aliquot of 100 ml of a sterile suspension of M-Na (10 mg) and a solution of Cry1Ab protein (40 ng ml^{-1}) were mixed, and deionized water was added to give a final volume of 1 ml. The mixture was incubated in a horizontal shaker at 20±2 °C for 10, 20, 30, and 60 min. At each time interval, the mixture was centrifuged. Concentrations of Cry1Ab protein in the initial and the supernatant equilibrium solution were measured using a commercial enzyme-linked immunosorbent assay (ELISA). The amount of Cry1Ab protein adsorbed or desorbed was calculated from the change in concentrations of protein in the initial and the equilibrium solution. No adsorption of Cry1Ab protein on the walls of the reaction tubes was observed, as determined with control tubes containing only Cry1Ab protein solution without a clay suspension. Dependence of the adsorption of Cry1Ab protein on the concentration of suspended M-Na (pH 7.1) was examined by varying the concentrations of sterile clay from 2.5 to 50 mg of clay ml^{-1}. The Cry1Ab protein solutions (20 or 45 ng ml^{-1}) were mixed with each suspension of M-Na, and deionized water was added to give a final volume of 1 ml. The suspensions were equilibrated in a horizontal shaker at 20±2 °C for 30 min before centrifugation. Concentrations of Cry1Ab protein in solution were determined as described above. Effect of pH on adsorption was investigated by varying pH value of the M-Na Cry1Ab solution in the range of 6 to 7.8. To obtain adsorption isotherms, an aliquot of 100 ml of each sterile clay suspension containing 10 mg of soil clay or M-Na (pH 7.1), was mixed with Cry1Ab protein solutions of concentrations ranging from 0 to 80 $ngml^{-1}$. The pH of the solutions was not adjusted to a defined value in order to measure the adsorption of Cry1Ab on the soil clays at the pH values found at the particular field site (pH values see **Table 8.2-06** for soil properties). Deionized water was added to give a final volume of 1 ml. Concentrations of Cry1Ab protein in the supernatants were measured as described above. Desorption experiments were carried out by re-suspending the pellets obtained after adsorption of Cry1Ab protein in 450 ml of deionized water, $CaCl_2$ and dissolved soil organic matter (50 mg C L^{-1}).

Table 8.2.06 Physical and chemical properties of the soil clays

Clay fraction		pH	Organic carbon (%)	Negative electro-kinetic external surface charge ($mmol_c kg^{-1}$)	Specific external Surface area (m^2 g^{-1})
Soil A	Topsoil	6.2	4.39	20.16	26
	Subsoil	6.7	3.11	16.83	42
Soil B	Topsoil	7.1	2.36	15.52	49
	Subsoil	7.2	0.72	7.74	93
Soil C	Topsoil	6.8	3.15	17.94	27
	Subsoil	7.3	0.40	6.18	81

The results of the equilibrium solution versus time (**Figure 8.2.03** and **-04**) shows Cry1Ab protein rapidly adsorbs to M-Na.

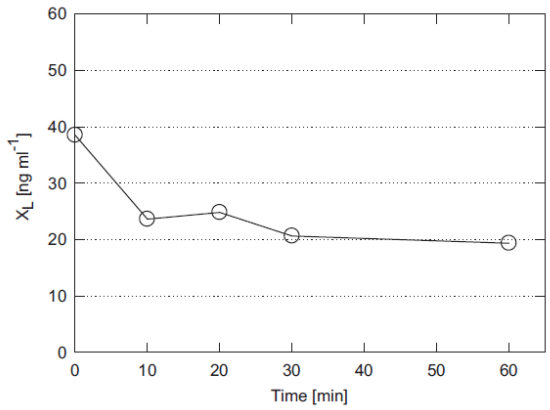

Figure 8.2.03 Effect of time on adsorption of Cry1Ab on Na-montmorillonite, X_L is the concentration Cry1Ab protein in the equilibrium solution.

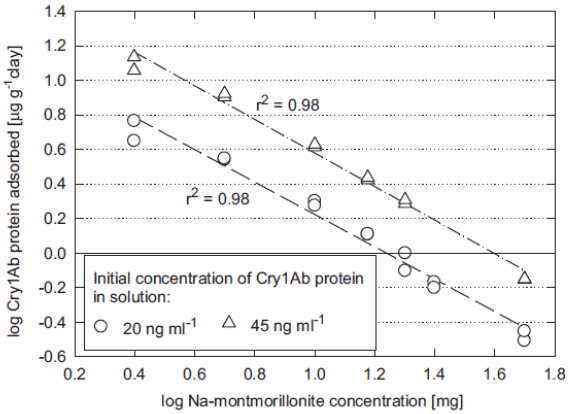

Figure 8.2.04 Effect of the concentration of Na- protein montmorillonite on adsorption of Cry1Ab protein of on Na-montmorillonite.

The amount of Cry1Ab protein adsorbed as a function of the concentration of M-Na is shown in **Figure 8.2.04.** A significant negative correlation between the amount of Cry1Ab protein adsorbed and the amount of M-Na was observed. When the clay concentration was varied and the added Cry1Ab protein concentration was maintained constant at 20 or 45 ng ml^{-1}, the relative adsorption of the Cry1Ab protein decreased as the clay concentration was increased.

Adsorption of the Cry1Ab protein was studied at pH values between 6 and 8.7, which were measured on the field site. The concentration of protons had a significant effect on the adsorption of the protein on M-Na: adsorption was highest at pH 6 and decreased as the pH of the suspensions increased (**Figure 8.2.05**).

The amount of Cry1Ab protein adsorbed on M-Na as a function of the concentration of Cry1Ab protein in the equilibrium solution is shown **Figure 8.2.06**. The adsorption isotherm was described by a linear regression of the data.

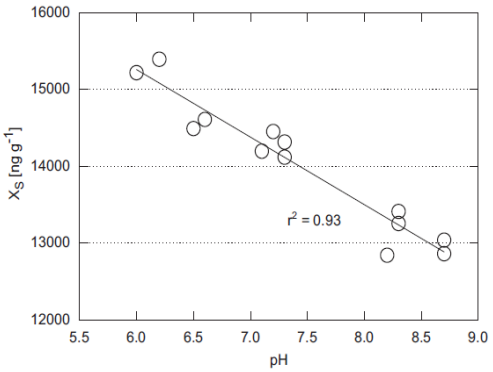

Figure 8.2.05. Effect of pH on adsorption of Cry1Ab protein on Na-montmorillonite. The initial concentration of Cry1Ab protein in solution was 80 ng ml^{-1}. X_S is the concentration of Cry1Ab protein adsorbed on Na-montmorillonite.

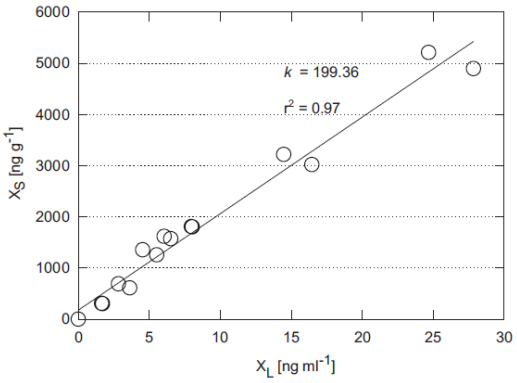

Figure 8.2.06. Adsorption of Cry1Ab protein on Na-montmorillonite. XS is the amount of Cry1Ab protein on Na-montmorillonite. X_L is the concentration of Cry1Ab protein in the equilibrium Solution. Adsorption isotherm was described by using the following formula: $X_S = kX_L$. The slope of the regression gives the parameter *k*, comparable to the distribution of Cry1Ab protein on clay.

Sorption on the clay fractions of the tested soils can also be described mathematically by linear regression. Less than 10% of adsorbed Cry1Ab protein was desorbed by washing with deionized water. Desorption of the Cry1Ab protein from the clay fractions of the subsoils was higher than that from the clay fractions of the top-soils. The desorption efficiency of water was higher than that of $CaCl_2$ and dissolved organic carbon.

Fu et al (2009) also investigated the sorption of Bt toxin on soil and focused on the kinetic and thermodynamic adsorption and investigated the adsorption characteristics of the Bt toxin taking into consideration the equilibrium, kinetic and thermodynamic aspects. Adsorption isotherms of purified toxin to samples of montmorillonite (Mont), kaolinite (Kaol) and silicon dioxide (SiO_2) and goethite (Goet) were derived. This study used Langmuir

and Freundlich isotherm models to establish the relationship between the amount of the Bt toxin adsorbed and its equilibrium concentration in the aqueous solution. The thermodynamics of adsorption were evaluated. The changes in the standard free energy (ΔrG^{θ}_{m}), standard enthalpy (ΔrH^{θ}_{m}), and standard entropy (ΔrS^{θ}_{m}) associated to the adsorption process were calculated.
The adsorption of the Bt toxin by minerals fitted to the Langmuir equation (R^2>0.97, n = 6). The equilibrium data were also fitted to the Freundlich equation for the adsorption of Bt toxin by minerals, and the parameters were shown in **Table 8.2.07**. The Freundlich model was more suitable to the adsorption of the toxin by the minerals than the Langmuir model, as the correlation coefficients R^2 were more than 0.991. The adsorption of Bt toxin belonged to the pseudo-second-order (PSO) model, and the intra-particle diffusion was not the only rate-controlling step. The adsorption of the toxin by the minerals was spontaneous, and the adsorption of the toxin by Mont was endothermic while the adsorption by the other three minerals was exothermic. The degree of freedom of the adsorption increased after the toxin adsorbed by Mont, Kaol, Goet, and decreased after the toxin adsorbed by SiO_2.

Table 8.2.07 Freundlich parameters for the adsorption of the *B. thuringiensis* subsp. *kurstaki* toxin by four minerals.

Mineral	Temperature								
	288 K			298 K			308 K		
	K_F	n	R^2	K_F	n	R^2	K_F	n	R^2
Mont	90.16	3.90	0.997	28.01	2.33	0.998	7.71	1.60	0.999
Kaol	45.70	2.58	0.999	39.86	2.55	0.998	13.02	1.87	0.995
Goet	91.91	4.57	0.999	53.58	3.58	0.998	6.43	1.79	0.991
SiO_2	20.04	2.86	0.997	16.97	3.04	0.999	1.81	1.89	0.999

K_F and n are the Freundlich constants, and R^2 is the correlation coefficient. Mont = Montmorillonite, Kaol = kaolinite, Goet = goethite, and SiO_2 = silicon dioxide.

RMS evaluation	The EFSA peer review of the pesticide risk assessment of the active substance *Bacillus thuringiensis* subsp. *kurstaki* highlighted that it should be demonstrated that, under the conditions of use, *Bacillus thuringiensis* subsp. *kurstaki* 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. It is not possible to address this data gap strict quantitatively. In a study by Huang et al. (2016) The soil properties of the 41 selected soils were studied by assessing affinity from the value of the distribution coefficient, *K*d, measured after 2 hours of incubation. The range of *K*d values measured was large, 1.6×10^3 to 3.8×10^4, with an average of 1.2×10^4. The large values of *K*d show that the protein has a strong affinity for soil surfaces, which is often observed for proteins on many surfaces. Minimum and maximum values with the average in brackets of affinity (*K*d / $dm^3 \times kg^{-1}$) for each of the proteins in the soil samples was: Cry1Ac: K_d 1630 – 38400 Cry2A: K_d 1560 – 29300 Cry1C: K_d 837 – 54600 In conclusion, Cry proteins are all strongly adsorbed by soil and will be effectively immobilized after their release into soil. Therefore, under the conditions of use, it is highly unlikely that *Bacillus thuringiensis* subsp. *kurstaki* crystalline proteins (δ-endotoxins) or any of their transformation products retaining insecticidal activity will contaminate groundwater. They differ in their affinity for soil with different textures, organic matter contents and mineralogy. Both affinity and extraction yield vary between soils, and extraction yield is not determined by affinity for all soils and each protein. There is no simple relation between soil properties and either affinity or extraction yield. Reliable quantification of a given Cry protein in soil would require an independent measurement of the extraction yield for each soil type.
Endpoint: Mobility:	Btk SA-12 Mobility of spores of *B. thuringiensis* subsp. *kurstaki* SA-12 can be considered limited. Endotoxins

	Cry proteins are strongly adsorbed by soil and will be effectively immobilized after their release into soil.

Cited references

Report KMA 8.2/08 – Zhou X., Gao J., Huang Q., Xiong J. (2010) Conformation Studies of the Interaction of the Pest-Resistant toxin from *Bacillus thuringiensis* with Brown and Red Soils

Published report,

International Journal of Chemical Reactor Engineering 8, 1-14

Abstract: The secondary structure of the toxin from *Bacillus thuringiensis* (Bt) is directly correlated with the insecticidal activity and persistence. The conformational changes of Bt toxin before adsorption and after contact with, or adsorbed from, red soil and brown soil were characterized using circular dichroism (CD) and fluorescence spectroscopy. The toxin of Bt contacted with soils for 2 h, the content of α-helix increased 14.1%-19.0%, and β-sheet increased 5.1%-5.8%, but the content of the non-regular coil decreased. There was a minor change bout the content of the β-turns. The fluorescence intensity of contacted toxin decreased and retained 72.8%-93.7% of the intensity of native toxin. The blue-shifts (2.5-3.5 nm) of maximum emission wavelength were observed. The influential trend of desorption was in agreement with the contact course, the influential extent, however, the former was higher than the latter. The content of α -helix of desorbed toxin increased 33.1%-33.8%, and β-sheet increased 17.3%-26.8%. The fluorescence intensity retained 70.4%-91.4% and blue-shifts of 3.5 nm were observed.

Evaluation RMS	In this study the sorption of purified Bt toxin, produced by genetically engineered *E. coli,* onto brown and red soil was investigated. The adsorption isotherms of toxin followed the Langmuir equation. The adsorption capacity of brown soil was more than that of red soil. When the toxin concentration was more than 0.8 mg mL-1, adsorption amount increased slowly and then the adsorption tended to gradually come to equilibrium. Therefore, the initial toxin concentration for preparing the adsorption complexes was chosen as 0.8 mg mL-1. The study is acceptable.

Report KMA 8.2/09 – Zhou X.-Y., Liu H-F., Lu X.-Z., Hao J.-C., Dong Q.-J (2013) Adsorption Thermodynamic Characteristics of Cry1Ab Toxin from *Bacillus thuringiensis* in Chinese Latosol

Published report,

Asian Journal of Chemistry; Vol. 25, No. 13, 7319-7322

Abstract: The adsorption thermodynamics of Cry1Ab toxin of *Bacillus thuringiensis* (Bt) in Chinese latosol was studied. The adsorption isotherms of Cry1 Ab toxin followed Langmuir equation ($R^2 > 0.98$) and the curves belonged to L type. The adsorption increased with the increased temperature and the maximum adsorption amount between 283 and 313 K ranged from 4.50 × 10-6 to 7.55 × 10-6 mol g^{-1}. The results show that the environmental risk of Cry1Ab toxin in Chinese latosol probably enhances if temperature increases. The adsorption of Cry1Ab toxin in Chinese latosol was a spontaneous, endothermic and entropy-increasing process. The separation factor ranged from 0.1865 to 0.4682, indicating that the adsorption of Cry1Ab toxin in Chinese latosol was favourable. The adsorption energy for Cry1Ab toxin ranged from 8 to 16 kJ mol^{-1}, indicating that it was an ion-exchange mechanism.

Evaluation RMS	The adsorption thermodynamics of Cry1Ab was studied. The purified Cry1Ab toxin was prepared from a genetically modified strain *B. thuringiensis* subsp. *kurstaki* HD-1. The adsorption of Cry1Ab toxin in Chinese latosol was a spontaneous, endothermic and entropy-increasing process. The separation factor ranged from 0.1865 to 0.4682, indicating that the adsorption of Cry1Ab toxin in Chinese latosol was favourable. The study is acceptable.

Report KMA 8.2/10 – Hung T.P., Truong V., Binh N.D., Frutos R., Quiquapoix H., Staunton S. (2016) Comparison of the affinity and extraction yield of trace amounts of three Cry proteins from *Bacillus thuringiensis* in contrasting types of soil

Published report,

European Journal of Soil Science, 67(1), 90–98

Abstract: The use of insecticidal proteins known as Cry or Bt, either as biopesticides used in agriculture or as vector control or originating from commercial genetically modified crops (GM), is increasing rapidly. The fate of these proteins in the environment depends strongly on their adsorption on the organo–mineral complexes of soil. Environmental monitoring requires the quantification of the proteins and this entails their chemical extraction from soil. Three Cry proteins, Cry1Ac, Cry1C and Cry2A, present in commercial biopesticide formulations or synthesized by GM plants or both were studied. The adsorption of trace amounts of Cry proteins on over 40 types of soil with contrasting properties was measured in dilute suspension. After a short incubation the extraction yield was measured with a previously tested alkaline solution that contained surfactant and another protein. Each of the proteins had a strong affinity for soil. No soil property was observed to determine either the affinity for soil or the extraction yield. There was no simple relation between the affinity (assessed from the distribution coefficient, *K*d) and the extraction yield, although there was a significant inverse relation ($P<0.05$) for two of the proteins, Cry1Ac and Cry2A. The proteins differ in both their affinity for soil and their extraction yields. We conclude that these insecticidal proteins will be largely immobile in soil, but that routine environmental monitoring can give only semi-quantitative values for protein in soil.

Evaluation RMS	The aim of this study was to carry out a large-scale screening of the affinity and extractability of contrasting Cry proteins from a large range of soil types. The range of *K*d values measured is large, 1.6×10^3 to 3.8×10^4, with an average of 1.2×10^4. The large values of *K*d show that the protein has a strong affinity for soil surfaces, which is often observed for proteins on many surfaces. There was no significant relation between K_d and soil clay content considered alone. The study is acceptable.

Report KMA 8.2/11 –Helassa N., Noinville S., Szponarski W., Quiquampoix H., Staunton S. (2009) Adsorption and desorption of monomeric Bt (*Bacillus thuringiensis*) Cry1Aa toxin on montmorillonite and kaolinite

Published report,

Soil Biology & Biochemistry 41(3), 498–504

Abstract: The use of insecticidal proteins known as Cry or Bt, either as biopesticides used in agriculture or as vector control or originating from commercial genetically modified crops (GM), is increasing rapidly. The fate of these proteins in the environment depends strongly on their adsorption on the organo–mineral complexes of soil. Environmental monitoring requires the quantification of the proteins and this entails their chemical extraction from soil. Three Cry proteins, Cry1Ac, Cry1C and Cry2A, present in commercial biopesticide formulations or synthesized by GM plants or both were studied. The adsorption of trace amounts of Cry proteins on over 40 types of soil with contrasting properties was measured in dilute suspension. After a short incubation the extraction yield was measured with a previously tested alkaline solution that contained surfactant and another protein. Each of the proteins had a strong affinity for soil. No soil property was observed to determine either the affinity for soil or the extraction yield. There was no simple relation between the affinity (assessed from the distribution coefficient, *K*d) and the extraction yield, although there was a significant inverse relation ($P<0.05$) for two of the proteins, Cry1Ac and Cry2A. The proteins differ in both their affinity for soil and their extraction yields. We conclude that these insecticidal proteins will be largely immobile in soil, but that routine environmental monitoring can give only semi-quantitative values for protein in soil.

Evaluation RMS	Three Cry proteins, Cry1Ac, Cry1C and Cry2A, present in commercial biopesticide formulations or synthesized by GM plants or both were studied. The adsorption of trace amounts of Cry proteins on over 40 types of soil with contrasting properties was measured in dilute suspension. No soil property was observed to determine either the affinity for soil or the extraction yield. There was no simple relation between the affinity (assessed from the distribution coefficient, *K*d) and the extraction yield, although there was a significant inverse relation ($P<0.05$) for two of the proteins, Cry1Ac and Cry2A. The proteins differ in both their affinity for soil and their extraction yields. The study is acceptable.

Report KMA 8.2/12 – Pagel-Wieder S., Niemeyer J., Fischer W.R., Gessler F. (2007) Effects of physical and chemical properties of soils on adsorption of the insecticidal protein (Cry1Ab) from Bacillus thuringiensis at Cry1Ab protein concentrations relevant for experimental field sites

Published report,

Soil Biology & Biochemistry 39, 3034–3042

Abstract: The adsorption of the insecticidal Cry1Ab protein of *Bacillus thuringiensis* (Bt) on Na-montmorillonite (M-Na) and soil clay fractions was studied. The aim of this study was not to find the adsorption capacity of the soils from the experimental field site, where Bt corn (MON810) was cultivated, but rather to characterize the adsorption behaviour of the Cry1Ab protein at concentrations typically found at experimental field sites. In kinetic experiments, the Cry1Ab protein adsorbed rapidly (<60 min) on M-Na. As the concentration of M-Na was varied and the added Cry1Ab protein concentration was kept constant (20 and 45 ng ml^{-1}), the adsorption per unit weight of Cry1Ab protein decreased with increasing concentrations of M-Na. Adsorption of Cry1Ab protein on M-Na decreased as the pH value of the suspension increased. All adsorption isotherms could be described mathematically by a linear regression with the parameter k, the distribution coefficient, being the slope of the regression line. Although their mineralogical composition was nearly identical, the soil clay fractions showed different k values. The different k values were correlated with the physical and chemical properties of the soil clay fractions, such as the organic carbon content, the specific external surface area, and the electrokinetic charge of the external surfaces of the clays, as well as with the external surface charge density. An increase in the amount of soil organic matter, as well as an increase in the electrokinetic external surface charge of the soil clays, decreased the distribution coefficient k. An increase of the specific external surface areas of the soil clays resulted in a higher distribution coefficient k. Less than 10% of adsorbed Cry1Ab protein was reversibly adsorbed on the soil clays and, thus, desorbed. The desorption efficiency of distilled water was higher than that of a solution of $CaCl_2$ (2.25 mmol) and of dissolved organic carbon (50 mg C l^{-1}).

Evaluation RMS	The aim of this study was not to find the adsorption capacity of the soils from the experimental field site, where Bt corn was cultivated, but rather to characterize the adsorption behaviour of the Cry1Ab protein at concentrations typically found at experimental field sites under laboratory conditions. The study is acceptable.

Report KMA 8.2/13 – Fu Q., Deng Y., Li H., Liu J., Hu H., Chen S., Sa T. (2009) Equilibrium, kinetic and thermodynamic studies on the adsorption of the toxins of *Bacillus thuringiensis* subsp. *kurstaki* by clay minerals

Published report,

Applied Surface Science 255(8), 4551–4557

Abstract: The persistence of *Bacillus thuringiensis* (Bt) toxins in soil is further enhanced through association with soil particles. Such persistence may improve the effectiveness of controlling target pests, but impose a hazard to non-target organisms in soil ecosystems. In this study, the equilibrium adsorption of the Bt toxin by four clay minerals (montmorillonite, kaolinite, goethite, and silicon dioxide) was investigated, and the kinetic and thermodynamic parameters were calculated. The results showed that Bt toxin could be adsorbed easily by minerals, and the adsorption was much easier at low temperature than at high temperature at the initial concentration varying from 0 to 1000 mg L^{-1}. The adsorption fitted well to both Langmuir and Freundlich isotherm models, but the Freundlich equation was more suitable. The pseudo-second-order (PSO) was the best application model to describe the adsorption kinetic. The adsorption process appeared to be controlled by chemical process, and the intra-particle diffusion was not the only rate-controlling step. The negative standard free energy (ΔrG^{θ}_{m}) values of the adsorption indicated that the adsorption of the Bt toxin by the minerals was spontaneous, and the changes of the standard enthalpy (ΔrH^{θ}_{m}) showed that the adsorption of the Bt toxin by montmorillonite was endothermic while the adsorption by the other three minerals was exothermic.

Evaluation RMS	In this study, the equilibrium adsorption of the Bt toxin by four clay minerals was investigated, and the kinetic and thermodynamic parameters were calculated. The results showed that Bt toxin could be adsorbed easily by minerals, and the adsorption was much easier at low temperature than at high temperature at the initial concentration varying from 0 to 1000 mg L^{-1}. The study is acceptable.

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

Interference with analytical methods for the control of the quality of drinking water

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.

The methods of analysis of microbiological parameters are specified in the Directive and are the following: ISO 9308-1 (*E. coli* and coliforms), ISO 7899-9 (Enterococci), ISO 12780 (*P. aeruginosa*), and ISO 6222 (colony count at 22°C and 37°C). For *C. perfringens* the method described in the Directive 98/83/EC is proposed.

In principle, drinking water monitoring requires a concentration step, usually membrane filtration, as drinking water does not contain high densities of micro-organisms. Afterwards, the concentrated samples are subjected to cultivation procedures on media which are highly selective for the indicator species mentioned above. In most cases the media contain specific substrates which are metabolized by the indicator species resulting in a particular color reaction which is then used as a discrimination criterion. The following media/substrates are used:

−*E. coli* or coliforms: chromogenic agar (tryptone bile salts agar), red or pink color due to glucuronidase activity

−Enterococci: Slanetz-Bartley medium (triphenyltetrazolium chloride is recued to formazan, red to maroon colorization of enterococci colonies) and/or KAA agar (kanamycin, aesculin, azide), black colorization of enterococci colonies due to esculin-hydrolase activity

−*C. perfringens:* membrane *Clostridium perfringens* medium (m-CP); lack of β-D-glucosidase activity (an enzyme involved in cellobiose fermentation), fermentation of sucrose and production of acid phosphatase are used to differentiate presumptive *C. perfringens* colonies from other *Clostridium* spp.; lack of β-D glucosidase activity means that *C. perfringens* does not cleave the chromogen, indoxyl β-D glucoside, in the medium. Furthermore, as the organisms ferment the sucrose in the medium, reducing the pH, bromocresol purple changes from purple to yellow. This results in characteristic opaque yellow *C. perfringens* colonies.

−*P. aeruginosa:* acetamide reaction on cetrimid agar (fluorescing colonies) and growth at 42°C for distinction between *P. aeruginosa* and *P. fluorescence*

Quality assurance for the specific media always includes testing of false positive signals for other indicator species. As such *E. coli* is used as a negative control for *C. perfringens* m-CP medium and for Enterococcus KAA and Slanetz-Bartley medium, and Enterococcus and *Bacillus cereus* are used as negative controls for *E. coli* CCM medium. In these cases, no growth of negative controls is observed. This already indicates that the media are highly specific and that it is very unlikely that another bacterial species will grow on them and/or exhibit the required enzymatic activities for metabolism of the applied substrates used for the chromogenic detection of the indicator species.

B. thuringiensis optimal growth temperatures range between 28-32°C and it was shown that most plant protection product strains of this species were inhibited at 43°C (Hansen et al., 2011). Microbiological methods that are applied for detection of indicator species such as *C. perfringens* and *P. aeruginosa* include incubation of the filter membrane with the concentrated bacteria at 44 and 42°C, respectively. At these temperatures it is unlikely that Btk will grow, and therefore it cannot interfere with the drinking water microbiological monitoring.

Additionally, it has to be noted that the technical material of Btk SA-12 was screened for the presence of the microbial contaminants using the same microbiological identification principles (please refer to Vol. 3 MA, Section B.5). The product was for example checked for the presence of coliforms/*E. coli* using violet red bile agar, similar to the one proposed for drinking water testing, and no bacterial growth was observed. Despite a high density of Btk SA-12 in the test material no false positive signals or any other kind of interference with the applied methods have been observed.

Al-Wasify et al. (2013) examined the sensitivity and specificity of chromogenic media for detection of pathogens in water, examining a total of 140 various water samples, including groundwater, river Nile surface water,

wastewater and marine water for detection of *Staphylococcus aureus, C. perfringens* and *Candida albicans*. The authors determined the limits of detection for culture-based methods and evaluated the specificity of the methods by PCR colony identification techniques. Results showed that chromogenic media were rapid, specific and sensitive for detection of the pathogenic indicator bacteria, with the low limits of detection of 1-20 CFU/100 mL determined in the spiked filter sterilized drinking water samples.

It can therefore be concluded, that Btk SA-12 will not interfere with the microbiological methods used for drinking water monitoring.

Evaluation RMS	The analytical methods for the control of the quality of drinking water are based on highly specific media. It is very unlikely that another bacterial species will grow on them and/or exhibit the required enzymatic activities for metabolism of the applied substrates used for the chromogenic detection of the indicator species. It can be concluded, that Btk SA-12 will not interfere with the microbiological methods used for drinking water monitoring.

Report KMA 8.3/02 – Al-Wasify, R.S., Al-Sayed, A.A., Kamel, M.M. (2013). Sensitivity and specificity of chromogenic media for detection of some pathogens in water Published report,

International Journal of Environment and Sustainability, 2(1): 1-9

Abstract: The main aim of the study was to evaluate the performance of some chromogenic media for detection of some pathogenic microorganisms in water such as; *Staphylococcus aureus, Clostridium perfringens* and *Candida albicans*. Sensitivity, specificity and limit of detection of these media were studied. A total of 140 water samples were examined; groundwater (40), River Nile surface water (70), wastewater (20) and marine water (10). The first purpose of the study was to evaluate the specificity and sensitivity of chromogenic media; HiCrome Aureus Agar Base (HAA, Himedia, India), M-CP Agar Base (MCP, Himedia, India) and HiCrome Candida Differential Agar (HCD, Himedia, India) for detection of *Staphylococcus aureus*, *Clostridium perfringens* and *Candida albicans* in water samples, respectively. The second purpose was to determine the limit of detection for these chromogenic media. The isolated colonies were confirmed using PCR technique. Results showed that these chromogenic media were rapid, specific and sensitive for detection of the previous pathogenic microorganisms in different water samples, also, these chromogenic media showed a low recovery level of the examined microorganisms.

Keywords: Chromogenic media, pathogenic microorganisms, membrane filtration, water.

B.8.4 References relied on

Several literature review reports have been provided according to the guidance of EFSA (Guidance of EFSA: 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). The aim of these reports was to provide a global overview of peer-reviewed literature concerning potential side effects of *B. thuringiensis* subsp. *kurstaki* strain SA-12.

Overview of literature reports provided according to the guidance of EFSA

Data point	Author	Year	Title	Section of RMS evaluation
KMA 2.7/12 & 3.5/06	Süß, J.	2016	Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12: Biological properties	Vol. 3MA, B.2.10
KMA 6.1.1/07	Seehase, S.	2016	Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12: Toxicology	Vol. 3MA, B.6.3
KMA 7.1/01	Cornelese, A.	2016a	Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12 and metabolites: Residues in or on treated products, food and feed	Vol. 3MA, B.7.4
KMA 8.1/10	Cornelese, A.	2016b	Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12: Fate and behaviour in the environment	Vol. 3MA, B.8.3
KMA 9/01	Schöbinger, U.	2016	Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12: Effects on non-target organisms	Vol. 3MA, B.9.8

Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12: "Fate and behaviour in the environment" (Cornelese, A., 2016b)

RMS comments on the literature search: "Literature review on *B. thuringiensis* subsp. kurstaki strain SA-12: Fate and behaviour in the environment" (Cornelese, A., 2016b); submitted in Data point KMA 8.01	The review was made in order to identify scientific peer-reviewed open literature on the active substance *Bacillus thuringiensis* subsp. *kurstaki* SA-12 and metabolites which may affect the assessment on human health, animal health and/or the environment. The search strategy was based on a multi-concept approach. **Relevance criteria** • Property investigated was relevant for data requirements of Regulation (EC) 1107/2009 • Subject relevant for environmental properties and occurrence of *Bacillus thuringiensis* subsp. *kurstaki* and metabolites • Subject relevant for population dynamics of *Bacillus thuringiensis* subsp. *kurstaki*? • Test species/system relevant to the environmental fate assessment • Location of studies and geo-climatic conditions of field studies are relevant • Method of application / exposure relevant for environmental fate assessment • Assessment / evaluation of mechanistic aspects or e.g. synergisms • Is it possible to correlate the observations to the agronomic use • Conclusions given in the abstract are robust and clear **Database searched** A search was conducted using the DIMDI database provided by the German Institute of Medical Documentation and comprised of searches in MED-

	LINE, BIOSIS, CAB and SCISEARCH databases **Search methods** Search strategy aimed to find all recent (from 2006 onwards) references that are of relevance regarding fate and behaviour of the concerned microorganism or its close relatives. The following keywords were used in the searches: Active substance *Bacillus thuringiensis* AND *kurstaki* AND fate OR behaviour OR proliferation OR mobility OR dispers? OR persistence OR colonization OR survival OR population dynamics AND soil OR rhizosphere OR field OR phyllosphere, AND water OR aquat? OR aquatic system? OR lake OR river OR pond AND air. Cry? protein OR Cry? toxin AND water OR aquat? OR aquatic system? OR lake OR river OR pond AND proliferat? OR persistence OR survival? OR population AND air. The „?“ is used for the expansion of keywords. Obtained references were first subjected to a *rapid assessment* based on title and the abstract. Summary records that appeared to be relevant passed to a second step in which a detailed assessment of full text documents was conducted. **Results of the study selection process** In total 213 references were retrieved and first subjected to a rapid assessment based on title and the abstract. Summary records that appeared to be relevant passed to a second step in which a detailed assessment of full text documents was conducted. In total 42 references were identified as being potentially relevant. These references were subjected to a full-text assessment. 27 references were finally classified as relevant and supportive and are included in the dossier.
Conclusion	The literature search, regarding fate and behaviour of the concerned microorganism or its close relatives, was accepted as valid, both regarding inclusion of databases and use of search terms. By the searches 27 references were finally considered relevant and reliable and are included in the dossier.

Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KMA 8.1/01	Cornelese, A.	2016b	LITERATURE REVIEW ON BACILLUS THURINGIENSIS SUBSP. KURSTAKI STRAIN SA-12 AND META-BOLITES: FATE AND BEHAVIOUR IN THE ENVIRONMENT Certis USA / CBC (Europe) GAB Consulting GmbH, Heidelberg, Germany Report-no.: S.r.l. 2281384-MA-07-01 GLP/GEP: no Published: no	no	yes	protected	Certis USA	New data for active ingredient, not previously submitted nor evaluated
8.1/02	Griego, V.M., Spence, K.D.	1978	INACTIVATION OF BACILLUS THURINGINSIS SPORES BY ULTRAVIOLET AND VISIABLE LIGHT Applied and Environmental Microbiology, 35(5), 906-910 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
8.1/03	Pedersen, J.C., Darmgaard, P.H., Eilenberg, J., Hansen, B.M.	1995	DISPERSAL OF BACILLUS THURINGINSIS VAR. KURSTAKI IN AN EXPERIMENTAL CABBAGE FIELD Canadian Journal of Microbiology, 41(2), 118-125 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
8.1/04	Dent, D.R.	1993	THE USE OF BACILLUS THURINGIENSIS AS AN INSECTICIDE In: Exploitation of Microorganisms, D.G. Jones (ed.), Chapman & Hall, London Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
8.1/05	Martin, P.A.W., Travers, R.S.	1989	WORLDWIDE ABUNDANCE AND DISTRIBUTION OF BACILLUS THURINGIENSIS ISOLATES Applied and Environmental Microbiology, Oct. 1989, p. 2437-2442 Report-no. GLP: no Published: yes	no	no	not protected	-	DAR 2008

Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
8.1/06	Smith, R.A., Couche, G.A.	1991	THE PHYLLOPLANE AS A SOURCE OF BACILLUS THURINGIENSIS VARIANTS Applied and Environmental Microbiology, 57(1): 331-315 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
8.1/07	Ignoffo, C.M., Hostetter, D.L., Pinnell, R.E.	1974	STABILITY OF BACILLUS THURINGIENSIS AND BACULOVIRUS HELIOTHIS ON SOYBEAN FOLIAGE NOT APPLICABLE ENVIRON ENTOMOL, 3 (4), 117-119 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
8.1/08	Hostetter, D.L., Ignoffo, C.M., Kearby, W.H.	1975	PERSISTENCE OF FORMULATIONS OF BACILLUS THURINGIENSIS SPORES AND CRYSTALS ON EASTERN RED CEDAR FOLIAGE IN MISSOURI not applicable Journal of the Kansas ENTOMOLOGICAL Society, 48, 189-193 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
8.1/09	Akiba, Y.	1986	MICROBIAL ECOLOGY OF BACILLUS THURINGIENSIS VI. GERMINATION OF BACILLUS THURINGIENSIS SPORES IN THE SOIL Appl. Ent. Zool. 21 (1): 76-80 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
8.1/10	Saleh, S.M., Harris, R.F., Allen, O.N.	1970	FATE OF BACILLUS THURINGIENSIS IN SOIL: EFFECT OF SOIL PH AND ORGANIC AMENDMENT Can. J. Microbiol. 16, p. 677-680 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
8.1/11	Petras, S.F., Casida Jr., L.E.	1985	SURVIVAL OF BACILLUS THURINGIENSIS SPORES IN SOIL Appl. Environ. Microbiol., 50(6): 1496-1501 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
8.1/12	Hendriksen N.B., Hansen, B.M.	2002	LONG-TERM SURVIVAL AND GERMINATION OF BACILLUS THURINGINSIS VAR. KURSTAKI IN A FIELD TRIAL Canadian Journal of Microbiology 48(3): 256-261 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.1/13	Bizzarri, M.F., Bishop, A.H.	2008	THE ECOLOGY OF BACILLUS THURINGIENSIS ON THE PHYLLOPLANE: COLONIZATION FROM SOIL, PLASMID TRANSFER, AND INTERACTION WITH LARVAE OF PIERIS BRASSICAE Microb. Ecol. 56(1): 133-139 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.1/14	van Cuyk, S., Deshpande, A., Hollander, A., Duval, N., Ticknor, L., Layshock, J., Gallegos-Graves, L., Omberg, K.M.	2011	PERSISTENCE OF BACILLUS THURINGIENSIS SUBSP. KURSTAKI IN URBAN ENVIRONMENTS FOLLOWING SPRAYING Appl. and Environ. Microbiol., 77(22), 7954 - 7961 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.1.1/01	Pedersen, J.C., Darmgaard, P.H., Eilenberg, J., Hansen, B.M.	1995	DISPERSAL OF BACILLUS THURINGINSIS VAR. KURSTAKI IN AN EXPERIMENTAL CABBAGE FIELD Canadian Journal of Microbiology, Volume 41, 118-125 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.1.1/02	Hendriksen N.B., Hansen, B.M.	2002	LONG-TERM SURVIVAL AND GERMINATION OF BACILLUS THURINGINSIS VAR. KURSTAKI IN A FIELD TRIAL Canadian Journal of Microbiology 48(3): 256-261 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008

Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KMA 8.1.1/ 03	Martin, P.A.W.	1991	DYNAMICS OF BACILLUS THURINGIENSIS TURN-OVER IN SOIL The General Meeting of the American Society for Microbiology, p. 315 (Abst.) Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.1.1/ 04	Petras, S.F., Casida Jr., L.E.	1985	SURVIVAL OF BACILLUS THURINGIENSIS SPORES IN SOIL Appl. Environ. Microbiol., 50(6): 1496-1501 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.1.1/ 05	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(5), 657-665 Report-no.: not applicable GLP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.1.1/ 06	West, A.W., Burges, H.D., White, R.J., Wyborn, C.H.	1984a	PERSISTENCE OF BACILLUS THURINGIENSIS PARASPO-RAL CRYSTAL INSECTICI-DAL ACTIVITY IN SOIL Journal of Invertebrate Pathology, 44(2), 128-133 Report-no. not applicable GLP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.1.1/ 07	West, A.W., Burges, H.D., Wyborn, C.H.	1984b	EFFECT OF INCUBATION IN NATURAL AND AUTOCLAVED SOIL UPON POTENCY AND VIABILITY OF BACILLUS THURINGIENSIS Journal of Invertebrate Pathology, Volume 44 (2), 121-127 Report-no. not applicable GLP: no Published: yes	no	no	not protected	-	DAR 2008

Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KMA 8.1.1/ 08	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, 24(3), 462-471 Report-no.: not applicable GLP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.1.1/ 9	Venkateswerlu, G., Stotzky, G.	1992	BINDING OF THE PROTOXIN AND TOXIN PROTEINS OF BACILLUS THURINGIENSIS SUBSP. KURSTAKI ON CLAY MINERALS Current Microbiology, 25(4), 225-233 Report-no.: not applicable GLP: no Published: Yes	no	no	not protected	-	DAR 2008
KMA 8.1.1/ 10	Tapp, H., Stotzky, G.	1995	INSECTICIDAL ACTIVITY OF THE TOXINS FROM BACILLUS THURINGINSIS SUBSPECIES KURSTAKI AND TENEBRIONIS ADSORBED AND BOUND ON PURE AND SOIL CLAYS Applied and Environmental Microbiology, 61(5), 1768-1790 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.1.1/ 11	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, 30(4), 463-470 Report-no.: not applicable GLP: no Published: yes	no	no	not protected	-	DAR 2008

Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KMA 8.1.1/ 12	Crecchio, C., Stotzky, G.	2001	BIODEGRADATION AND INSECTICIDAL ACTIVITY OF THE TOXIN FROM BACILLUS THURINGIENSIS SUBSP. KURSTAKI BOUND ON COMPLEXES OF MONTMO-RILLONITE-HUMIC ACIDS-A1 HYDROXYPOLYMERS Soil Biology & Biochemistry, 33(4), 573-581 Report-no.: not applicable GLP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.1.1/ 13	Griego, V.M., Spence, K.D.	1978	INACTIVATION OF BACILLUS THURINGIENSIS SPORES BY ULTRAVIOLET AND VISIBLE LIGHT Applied and Environmental Microbiology, 35(5) 906-910 Report-no.: not applicable GLP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.1.1/ 14	Myasnik, M., Manasherob, R., Ben-Dov, E., Zaritsky, A., Margalith, Y., Barak Z.	2001	COMPARATIVE SENSITIVITY TO UV-B RADIATION OF TWO BACILLUS THURINGI-ENSIS SUBSPECIES AND OTHER BACILLUS SP. Current Microbiology, 43(2), 140-143 Report-no.: not applicable GLP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.1.1/ 15	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, 273(1), 43-47 Report-no.: not applicable GLP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.1.1/ 16	Sundaram, K.M.S., Sundaram, A., Huddles-ton, 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, 32 (5), 741-788 Report-no.: not applicable GLP: no Published: yes	no	no	not protected	-	DAR 2008

Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KMA 8.1.1/ 17	Konecka, E. Baranek, J., Bielinska, I., Tadeja, A., Kaznowski, A.	2014	PERSISTENCE OF THE SPORES OF B. THURINGIENSIS SUBSP. KURSTAKI FROM FORAY BIOINSECTI-CIDE IN GLEYSOL SOIL AND ON LEAVES Sci. Total Environ. 472:296-301 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.1.1/ 18	Hendriksen, N.B., Carstensen, J.	2013	LONG-TERM SURVIVAL OF BACILLUS THURINGIENSIS SUBSP. KURSTAKI IN A FIELD TRIAL Can.J.Microbiol. 59(1), 34-38 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.1.1/ 19	Accinelli, C., Koskinen, W.C., Becker, J.M., Sadowsky, M.J.	2008	MINERALIZATION OF THE BACILLUS THURINGIENSIS CRY1AC ENDOTOXIN IN SOIL Journal of Agricultural and Food Chemistry, 56(3), 1025-1028 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.1.1/ 20	Marchetti. E., Accinelli, C., Talamé, V., Epifani, R.	2007	PERSISTENCE OF CRY TOXINS AND CRY GENES FROM GENETICALLY MODIFIED PLANTS IN TWO AGRICULTURAL SOILS Agronomy for Sustainable Development, 27(3), 231-236 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.1.1/ 21	Icoz, I., Stotzky, G.	2008	CRY3BB1 PROTEIN FROM BACILLUS THURINGIENSIS IN ROOT EXUDATES AND BIOMASS OF TRANSGENIC CORN DOES NOT PERSIST IN SOIL Transgenic Research 17(4):609-620 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated

Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KMA 8.1.1/ 22	Helassa, N., M'Charek, A., Qui-quampoix, H., Noinville, S., Dejardin, P., Frutos, R., Staunton, S.	2011	EFFECTS OF PHYSICO-CHEMICAL INTERAC-TIONS AND MICROBIAL ACTIVI-TY ON THE PER-SISTENCE OF CRY1AA BT (BACILLUS THURIN-GIENSIS) TOXIN IN SOIL Soil Biol. Biochem., 43(5), 1089-1097 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not pro-tected	-	New data for active ingredi-ent, not previous-ly sub-mitted nor eval-uated
KMA 8.1.1/ 23	Li, Y.-L., Du, J., Fang, Z.-X., You, J.	2013	DISSIPATION OF INSECTI-CIDAL CRY1AC PROTEIN AND ITS TOXICITY TO NONTARGET AQUATIC ORGANISMS Journal of Agricultural and Food Chemistry, 61(46):10864- 10871 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not pro-tected	-	New data for active ingredi-ent, not previous-ly sub-mitted nor eval-uated
KMA 8.1.1/ 24	Wang, H., Ye, Q., Gan, J., Wu, L.	2007	BIODEGRADATION OF CRY1AB PROTEIN FROM BT TRANSGENIC RICE IN AEROBIC AND FLOODED PADDY SOILS Journal of Agricultural and Food Chemistry, 55(5), 1900-1904 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not pro-tected	-	New data for active ingredi-ent, not previous-ly sub-mitted nor eval-uated
KMA 8.1.1/ 25	Xue, K., Diaz, B.R., Thies, J.E.	2014	STABILITY OF CRY3BB1 PROTEIN IN SOILS AND ITS DEGRADATION IN TRANS-GENIC CORN RESIDUES Soil Biology and Biochemis-try, 76, 119-126 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not pro-tected	-	New data for active ingredi-ent, not previous-ly sub-mitted nor eval-uated
KMA 8.1.1/ 26	Chen, Z.H., Chen, L.J., Zhang, Y.L., Wu, Z.J.	2011	MICROBIAL PROPERTIES, ENZYME ACTIVITIES AND THE PERSISTENCE OF EXOGENOUS PRO-TEINS IN SOIL UNDER CONSECU-TIVE CULTI-VATION OF TRANSGENIC COTTONS (GOSSYPIUM HIRSUTUM L.). Plant Soil Environ, 57(2), 67-74 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not pro-tected	-	New data for active ingredi-ent, not previous-ly sub-mitted nor eval-uated

Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KMA 8.1.2/ 01	Menon, A.S., de Mestral, J.	1985	SURVIVAL OF BACILLUS THURINGIENSIS VAR. KURSTAKI IN WATERS Water, Air, & Soil Pollution, 25(3), 265-274 Report-no. not applicable GLP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.1.2/ 02	Cornelese, A.	2016b	LITERATURE REVIEW ON BACILLUS THURINGIENSIS SUBSP. KURSTAKI STRAIN SA-12 AND META-BOLITES: FATE AND BEHAVIOUR IN THE ENVIRONMENT Certis USA / CBC (Europe) GAB Consulting GmbH, Heidelberg, Germany Report-no.: S.r.l. 2281384-MA-07-01 GLP/GEP: no Published: no	no	yes	protected	Certis USA	New data for active ingredient, not previously submitted nor evaluated
KMA 8.1.2/ 03	Wang, H., Ye, Q., Gan, J., Wu, L.	2007	BIODEGRADATION OF CRY1AB PROTEIN FROM BT TRANSGENIC RICE IN AEROBIC AND FLOODED PADDY SOILS Journal of Agricultural and Food Chemistry, 55(5), 1900-1904 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.1.2/ 04	Li, Y.-L., Du, J., Fang, Z.-X., You, J.	2013	DISSIPATION OF INSECTICIDAL CRY1AC PROTEIN AND ITS TOXICITY TO NONTARGET AQUATIC ORGANISMS Journal of Agricultural and Food Chemistry 61(46), 10864-10871 Report-no.: not applicable GLP/GEP: no Published: no	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.1.2/ 05	Douville, M., Gagné, F., Blaise, C., André., C.	2007	OCCURRENCE AND PERSISTENCE OF BACILLUS THURINGIENSIS (BT) AND TRANSGENIG BT CORN CRY1AB GENE FROM AN AQUATIC ENVIRONMENT Ecotoxicology and Environmental Safety, 66(2): 195-203 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated

Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KMA 8.1.2/ 06	Strain, K.E., Lydy, M.J.	2015	THE FATE AND TRANSPORT OF THE CRY1AB PROTEIN IN AN AGRICUL-TURAL FIELD AND LABO-RATORY AQUATIC MICRO-COSMS Chemosphere, 132:94-100 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.1.2/ 07	Strain, K.E., Whiting, S.A., Lydy, M.J.	2014	LABORATORY AND FIELD VALIDATION OF A CRY1AB PROTEIN QUANTITATION METHOD FOR WATER Talanta, 128:109-116 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.1.3/ 01	Teschke, K., Chow, Y., Bartlett, K., Ross, A., van Netten, C.	2001	SPATIAL AND TEMPORAL DISTRIBUTION OF AIRBORNE BACILLUS THURIN-GIENSIS VAR. KURSTAKI DURING AN AERIAL SPRAY PROGRAM FOR GYPSY MOTH ERADICATION Environmental Health Perspectives, 109, (1), 47-54 Report-no.: not applicable GLP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 8.1.3/02	Emanuel P.A. Buckley P.E., Sutton T.A., Edmonds J.M. Bailey A.M., Rivers B.A., Kim M.H., Ginley W.J., Keiser C.C., Doherty R.W. Kragl F.J., Narayanan F.E., Katoski S.E., Paikoff S., Leppert S.P., Straw-bridge J.B., Van-Reenen D.R., Biberos S.S., Moore D., Phillips D.W., Mingi-oni L.R., Mel-les O., Ondercin D.G., Hirsh B., Bie-schke K.M., Harris C.L., Omberg K.M. Rastogi V.K., Van Cuyk S., Gibbons H.S.	2012	DETECTION AND TRACKING OF A NOVEL GENETICALLY TAGGED BIOLOGICAL SIMULANT IN THE ENVIRONMENT Applied and Environmental Microbiology, 78(23):8281-8288 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.2/01	Pedersen, J.C., Darmgaard, P.H., Eilenberg, J., Hansen, B.M.	1995	DISPERSAL OF BACILLUS THURINGINSIS VAR. KURSTAKI IN AN EXPERIMENTAL CABBAGE FIELD Canadian Journal of Microbiology, Volume 41, 118-125 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.2/02	Akiba, Y.	1991	ASSESSMENT OF RAINWATER-MEDIATED DISPERSION OF FIELD-SPRAYED BACILLUS THURINGIENSIS IN THE SOIL Japanese Journal of Applied Entomology and Zoology, 26 (4), 447-483 Report-no.: not applicable GLP: no Published: yes	no	no	not protected	-	DAR 2008

Data point	Author(s)	Year	Title Owner Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KMA 8.2/03	Venkateswerlu, G., Stotzky, G.	1992	BINDING OF THE PROTOXIN AND TOXIN PROTEINS OF BACILLUS THURINGIENSIS SUBSP. KURSTAKI ON CLAY MINERALS Current Microbiology, 25(4), 225-233 Report-no.: not applicable GLP: no Published: Yes	no	no	not protected	-	DAR 2008
KMA 8.2/04	Tapp, H., Stotzky, G.	1995	INSECTICIDAL ACTIVITY OF THE TOXINS FROM BACILLUS THURINGINSIS SUBSPECIES KURSTAKI AND TENEBRIONIS ADSORBED AND BOUND ON PURE AND SOIL CLAYS Applied and Environmental Microbiology, 61(5), 1768-1790 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.2/05	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, 30(4), 463-470 Report-no. not applicable GLP: no Published: yes	no	no	not protected	-	DAR 2008
KMA 8.2/06	Crecchio, C., Stotzky, G.	2001	BIODEGRADATION AND INSECTICIDAL ACTIVITY OF THE TOXIN FROM BACILLUS THURINGIENSIS SUBSP. KURSTAKI BOUND ON COMPLEXES OF MONTMO-RILLONITE-HUMIC ACIDS-A1 HYDROXYPOLYMERS Soil Biology & Biochemistry, 33(4), 573-581 Report-no.: not applicable GLP: no Published: yes	no	no	not protected	-	DAR 2008

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KMA 8.2/07	Cornelese, A.	2016b	LITERATURE REVIEW ON BACILLUS THURINGIENSIS SUBSP. KURSTAKI STRAIN SA-12 AND META-BOLITES: FATE AND BEHAVIOUR IN THE ENVIRONMENT Certis USA / CBC (Europe) GAB Consulting GmbH, Heidelberg, Germany Report-no.: S.r.l. 2281384-MA-07-01 GLP/GEP: no Published: no	no	yes	protected	Certis USA	New data for active ingredient, not previously submitted nor evaluated
KMA 8.2/08	Zhou, X., Gao, J., Huang, Q., Xiong, J.	2010	CONFIRMATION STUDIES OF THE INTERACTION OF THE PEST-RESISTANT TOXIN FROM BACILLUS THURINGIENSIS WITH BROWN AND RED SOILS International Journal of Chemical Reactor Engineering, 8, 1-14 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.2/09	Zhou, X.-Y., Liu, H.F., Lu, X.-Z., Hao, J.-C., Dong, Q.-J.	2013	ADSORPTION THERMODY-NAMIC CHARACTERISTICS OF CRY1AB TOXIN FROM BACILLUS THURINGIENSIS IN CHINESE LATOSOL Asian J Chemistry, 25(13), 7319-7322 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.2/10	Hung, T.P., Truong, L.V., Binh, N.D., Frutos, R., Quiquapoix, H., Staunton, S.	2016	COMPARISON OF THE AFFINITY AND EXTRACTION YIELD OF TRACE AMOUNTS OF THREE CRY PROTEINS FROM BACIL-LUS THURINGIENSIS IN CONTRASTING TYPES OF SOIL European J Soil Sci, 67(1), 90-98 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated

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KMA 8.2/11	Helassa, N., Quiquampoix, H., Noinville, S., Szponarski, W., Quiquampoix, H., Staunton, S.	2009	ADSORPTION AND DESORPTION OF MONOMERIC BT (BACULLUS THURIN-GIENSIS) CRY1AA TOXIN ON MONTMORILLONITE AND KAOLINITE Soil Biol. Biochem., 41(3), 498-504 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.2/12	Pagel-Wieder S., Niemeyer J., Fischer W.R., Gessler F.	2007	EFFECTS OF PHYSICAL AND CHEMICAL PROPERTIES OF SOILS ON ADSORP-TION OF THE INSECTICI-DAL PROTEIN (CRY1AB) FROM BACILLUS THURIN-GIENSIS AT CRY1AB PRO-TEIN CONCENTRATIONS RELEVANT FOR EXPERIMENTAL FIELD SITES Soil Biol. Biochem., 39, 3034-3042 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.2/13	Fu, Q., Deng, Y., Li, H., Liu, J., Hu, H., Chen, S., Sa, T.	2009	EQUILIBRIUM, KINETIC AND THERMODYNAMIC STUDIES ON THE ADSORP-TION OF THE TOXINS OF BACILLUS THURINGIENSIS SUBSP. KURSTAKI BY CLAY MINERALS Appl Surface Sci, 255(8), 4551-4557 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 8.3/01	Hansen, B.M., Thorsen, L., Nielsen-LeRoux, C., Wilcks, A., Hendriksen, N.B.	2011	NEW EXPERIMENTAL APPROACHES FOR HUMAN RISK ASSESSEMENT OF MICROBIAL PEST CONTROL AGENTS – EXEMPLIFIED BY THE BACTERIUM BACILLUS THURINGIENSIS Danish EPA, Pesticides Research, 136 2011, 1-128 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated

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KMA 8.3/02	Al-Wasify, R.S., Al-Sayed, A.A., Kamel, M.M.	2013	SENSITIVITY AND SPECIFI-CITY OF CHRO-MOGENIG MEDIA FOR DETECTION OF SOME PATHOGENS IN WATER Int. J. Environ.Sustain., 2(1):1-9 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated