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ENVIRONMENTAL BIOTECHNOLOGY

Fate of the insecticidal Cry1Ab protein of GM crops in two agricultural soils as revealed by ^{14}C-tracer studies

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Abstract Insecticidal delta-endotoxins of *Bacillus thuringiensis* are among the most abundant recombinant proteins released by genetically modified (GM) crops into agricultural soils worldwide. However, there is still controversy about their degradation and accumulation in soils. In this study, ^{14}C-labelled Cry1Ab protein was applied to soil microcosms at two concentrations (14 and 50 μg g^{-1} soil) to quantify the mineralization of Cry1Ab, its incorporation into the soil microbial biomass, and its persistence in two soils which strongly differed in their texture but not in silt or pH. Furthermore, ELISA was used to quantify Cry1Ab and its potential immunoreactive breakdown products in aqueous soil extracts. In both soils, $^{14}CO_2$-production was initially very high and then declined during a total monitoring period of up to 135 days. A total of 16 to 23 % of the ^{14}C activity was incorporated after 29 to 37 days into the soil microbial biomass, indicating that Cry1Ab protein was utilized by microorganisms as a growth substrate. Adsorption in the clay-rich soil was the most important factor limiting microbial degradation; as indicated by higher degradation rates in the more sandy soil, extremely low concentrations of immunoreactive Cry1Ab molecules in the soils' aqueous extracts and a higher amount of ^{14}C activity bound to the soil with more clay. Ecological risk assessments of Bt-crops should therefore consider that the very low concentrations of extractable Cry1Ab do not reflect the actual elimination of the protein from soils but that, on the other hand, desorbed proteins mineralize quickly due to efficient microbial degradation.

Keywords Protein degradation · Cry proteins · Bt-toxin · Bt-maize · Cry1Ab · Genetically modified plants · Environmental risk assessment · Soil microbial biomass · ^{14}C-labelled compounds · Soil microbiology

Introduction

Genetically modified (GM) crops engineered with insecticidal delta-endotoxins (Cry proteins) naturally produced by *Bacillus thuringiensis* (Bt-crops) provide an alternative to the use of chemical insecticides for controlling insect pests in cotton and maize cultivation (Romeis et al. 2006). Bt-maize expressing the Cry1Ab protein, which confers resistance against the European corn borer (*Ostrinia nubilalis*, Lepidoptera), has already been commercialized for agricultural use in 1996 in the USA. Today, Bt-maize is cultivated on six continents on almost 60 million hectares (James 2014). Among the Bt-maize, varieties expressing Cry1Ab, either as a single event or stacked together with other Cry proteins, are the most prevalent, suggesting that Cry1Ab protein is in fact one of the most abundant recombinant proteins released into agricultural soils worldwide.

In contrast to chemical pesticides, Cry proteins are an organic constituent of the plant itself, and, for most of the currently commercialized Bt-maize, these are typically synthesized in different tissues including leaves, stems and roots; e.g., for Bt-maize MON810, a widely grown genetically modified event also approved for cultivation in the European Community, Cry1Ab was detected in roots at concentrations of up to 4.2 μg per gram fresh weight (Nguyen and Jehle 2007). During plant growth, these Cry proteins enter soils at

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relatively small amounts (Baumgarte and Tebbe 2005; Saxena et al. 2002). The vast amount, however, is released into soil after harvesting, when maize stover (residues including stem and leave material) as well as root stubbles remain on the field (Baumgarte and Tebbe 2005; Hopkins and Gregorich 2005). For a Cry3Bb1-expressing Bt-maize, it was estimated that approx. 820 g are synthesized by roots in 1 ha of a field soil (Nguyen and Jehle 2009). With the lower concentration of Cry1Ab in MON810 (Nguyen and Jehle 2007), the total amount left on agricultural field soil by MON810 could be around 165 g Cry1Ab ha^{-1}, considering only the root biomass (unpublished data).

Concentrations of Cry1Ab found in soil from fields cultivated with Bt-maize for one or more subsequent years are typically extremely low, often non-detectable, or close to the detection threshold, which is, depending on the extraction and detection protocol, in the range of 0.1 to 10 ng g^{-1} of soil. These low concentrations suggest a high instability of Cry1Ab in soils (Baumgarte and Tebbe 2005; Gruber et al. 2012; Margarit et al. 2008). However, several laboratory studies demonstrated that Cry1Ab proteins tend to be adsorbed in soil by the surface-active constituents, including organic material and clay (Sander et al. 2010, 2012; Tapp et al. 1994; Wang et al. 2008). Furthermore, laboratory incubations suggested that the adsorbed Cry1Ab protein is not as easily degradable as the water-dissolved form (Koskella and Stotzky 1997). It can thus be suspected that the fate of Cry1Ab depends on interactions between the soil constituents involved in adsorption and the activity of indigenous soil microorganisms with the capacity to degrade this protein. However, since the adsorbed Cry1Ab is not readily extractable from soil samples (Baumgarte and Tebbe 2005; Shan et al. 2005; Wang et al. 2006) and, thus, has not yet accurately been quantified, the elimination of Cry1Ab from agricultural fields is still not fully understood (Fu et al. 2012).

The objective of this study was to characterize the fate of the Cry1Ab protein in two agricultural soils with contrasting amounts of clay, but high similarities in other parameters, including pH. ^{14}C-labelled Cry1Ab protein was supplied to microcosms to trace it in the background of natural soil proteins and indigenous microbial activities and to quantify its mineralization, its adsorption and the incorporation of its breakdown products into the soil microbial biomass. The contrasting clay contents of the soils, which were accompanied by differences in the amount of soil organic carbon, biomass carbon and cation exchange capacities, allowed an evaluation of the effect of adsorption for limiting the microbial degradation of Cry1Ab. The results of this study demonstrate that dissolved Cry1Ab is highly unstable and utilized by indigenous soil microorganisms for growth but that the degradation of Cry1Ab is limited by its desorption from soil particles, with the clay contents being of paramount importance.

Materials and methods

Origin, sampling and characteristics of soils

Two soils, originating from maize fields of the "Oderbruch" region in Brandenburg, Germany, with no previous cultivation of Bt-maize or other genetically modified crops, were selected for this study and collected with permission of their field owners. One soil is a fluvial loamy sand from Mallnow, and the other is a colluvial sandy clay from Altlangsow. The soils were taken in November, 6 weeks after harvesting, and immediately transported to the laboratory, where they were sieved (mesh size 2 mm, Retsch, Haan, Germany) and stored at 4 °C in the dark for a maximum of 4 months. Soils were analysed for the particle size fractions by sieving and sedimentation according to ISO (ISO 2009). The total soil water holding capacity (WHC) was determined gravimetrically, with water-saturated soil allowed to drain over 6 h in a filter funnel (Fierer and Schimel 2002). Cation exchange capacity was determined with thiourea as described elsewhere (Dohrmann 2006). Total organic carbon was determined by dry combustion using an element analyser (TruSpec CN, Leco, St. Joseph, MI, USA). Soil microbial biomass was quantified with the chloroform fumigation-incubation method (CFI) (Jenkinson and Powlson 1976) using calculations described elsewhere (Vance et al. 1987). The pH values were determined in 20 g of soil suspended in 50 ml of 0.01 M $CaCl_2$ solution. Soil characteristics are summarized in Table 1. It should be noted that the two soils mainly differed in their total amount of clay, which was, however, accompanied by a 12-fold higher cation exchange capacity, a 3.5-fold higher total organic carbon, and a 56 % higher microbial biomass. Before incubation studies with ^{14}C-Cry1Ab, soils were kept for 48 h at room temperature.

Origin and preparation of the ^{14}C-labelled Cry1Ab-protein

The ^{14}C-labelled Cry1Ab protein (^{14}C-Cry1Ab) was produced by an *Escherichia coli* strain, genetically modified to

Table 1 Characteristics of the two agricultural soils used in this study

Soil type (texture)	Loamy sand	Sandy clay
Clay (<2 μm) (%)	5±2	36±8
Silt (2–63 μm) (%)	10±2	13±3
Sand (63–2000 μm) (%)	85±3	51±10
Water holding capacity (g 100 g^{-1} soil)	20±2	39±3
Cation exchange capacity ($mmol_c kg^{-1}$ soil)	19.1±12.2	235.0±45.8
Total carbon (g 100 g^{-1} soil)	0.69±0.10	2.20±0.45
Biomass carbon (μg g^{-1} soil)	110±13	172±37
pH value ($CaCl_2$)	5.0±0.4	5.3±0.2

synthesize the Cry1Ab protein with identity to the protein used in Bt-maize MON810. The *E. coli* was cultivated in batch fermentations, supplying ^{14}C-labelled carbon sources. The production and purification of ^{14}C-Cry1Ab has previously been described (Valldor et al. 2012). For the experimental analyses, two ^{14}C-Cry1Ab batches were utilized. Batch 1 was characterized by a specific activity of 598 Bq mg^{-1} protein and a protein purity of 85 % (*w/w*) Cry1Ab, and batch 2 by 168 Bq mg^{-1} protein and a purity of 90 % (*w/w*). The batch solutions were kept in aliquots in CAPS (3-(cyclohexylamino)-1-propane sulfonic acid) buffer (50 mM CAPS, pH 10.5, 0.25 % *v/v* mercaptoethanol) at −70 °C. Before adding the solutions to the soil microcosms, the pH of the solutions was adjusted to 8.0 by phosphate-buffered saline (PBS buffer; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4).

Soil incubation studies

Each soil microcosm consisted of a 50-ml Erlenmeyer flask filled with 20 g of air-dried soil, amended with one of the two above-mentioned ^{14}Cry1Ab-batch solutions, so that each soil received approximately 10,000 dpm (disintegrations per minute), corresponding to 167 Bq, resulting in initial concentrations of 14 μg Cry1Ab g^{-1} (batch 1) and 50 μg Cry1Ab g^{-1} soil (batch 2), respectively. The amounts added to each soil microcosm represented the amount which would be produced on MON810 maize fields on approx. 170 and 600 cm^2, respectively. The Cry1Ab solutions were carefully added dropwise to soil to reach a 65 % saturation of the total WHC. Each of these treatments was set up in four independent replicate microcosms. In addition, for dpm-background subtractions, the same numbers of soil microcosms were run without the addition of ^{14}C-Cry1Ab.

The microcosms were linked to an airflow system to obtain CO_2-free, humid air. For this purpose, ambient air was passed through a tube of 40 cm length filled with soda lime (granule size 1–2.5 mm; Merck, Darmstadt, Germany) and then passed through two washing bottles; the first filled with 150 ml 2 M NaOH solution, and the second containing the same amount of distilled water. The flow rate was 6–7 ml s^{-1} at a temperature of 20 °C. The microcosm apparatus has been described in more detail elsewhere (Martens 1985). During incubations, the microcosms were kept in the dark. After passage through the soil containing flasks, the airflow of each passed a test tube filled with 5 ml 2 M NaOH solution for absorption of $^{14}CO_2$. The airflow was led into the button of each tube to allow a 15-cm long passage of air bubbles through the NaOH solution to ensure the complete absorption of $^{14}CO_2$. At the beginning of the incubation with ^{14}C-Cry1Ab, these CO_2 traps were daily replaced by fresh 2 M NaOH. Later on, the frequency was reduced, so that, after 3 weeks, the solution was replaced on a weekly basis. The loss of water from the soils and the weights of the soil-containing flasks were frequently determined during the incubation period of up to 135 days, and the weight was readjusted with distilled water to maintain the 65 % saturation of the total WHC.

Determination of the soil microbial biomass and its ^{14}C-content

The quantification of the soil microbial biomass using CFI, as described above, also, as demonstrated in other studies (Okumura et al. 1999; Santruckova et al. 2005), allowed the concomitant measurement of the ^{14}C-signals originating from ^{14}C-Cry1Ab, which had been incorporated into the biomass fraction. For each soil sample, 5 g taken from the soil microcosms were fumigated with 20 ml of chloroform for 24 h in an evacuated desiccator. This fumigated soil and a corresponding non-fumigated soil sample (5 g) were transferred in 100-ml Erlenmeyer flasks, and the WHC was adjusted to 65 % saturation. The Erlenmeyer flasks contained 20-ml beakers filled with 5 ml 2 M NaOH for absorption of $^{14}CO_2$. The soils were incubated for 10 days at 22 °C in the dark. The total CO_2, including $^{14}CO_2$, which evolved during this incubation due to mineralization of the chloroform-killed microbial biomass, was trapped in the NaOH solution. Controls with non-fumigated soils were used for background subtraction of $^{14}CO_2$ production.

Quantification of the ^{14}C-radioactivity

The $^{14}CO_2$-activity was determined in a liquid scintillation counter (LS1801, Beckman, Irvine, CA, USA). For the quantification of $^{14}CO_2$ in the 2 M NaOH solution, 2.5 to 5 ml of this solution were mixed with 16 ml of scintillation cocktail (Hionic Fluor, Perkin Elmer, Waltham, MA, USA), especially developed for counting alkaline solutions. All other solutions of this study with ^{14}C were analysed by using Rotiszint eco plus (Carl Roth, Karlsruhe, Germany) as a scintillation cocktail. The measuring times of the ^{14}C-samples were adapted to their activity; samples with an activity of less than 100 dpm were counted for 3 h, those above for 1 h.

Extraction and quantification of Cry1Ab and its degradation products from soil

To quantify the amount of Cry1Ab proteins and their degradation products in the soil aqueous phase, soil samples were extracted with a PBST buffer (PBS buffer with 0.05 % (*v/v*) Tween 20 (Carl Roth); pH 7.4). In previous studies, this or similar buffers have been used to extract Cry1Ab proteins from plant materials and soils and as an estimate for biological availability (e.g. Baumgarte and Tebbe 2005; Daudu et al.

2009; Douville et al. 2005; Gruber et al. 2012; Griffiths et al. 2007).

For each extraction, 8 g of soil (wet weight) were suspended in 24 ml of PBST buffer for 1 h in an orbital shaker (GFL, Burgwedel, Germany) and then centrifuged at 4000×*g* for 20 min. The extraction was conducted at 4 °C in the dark. The supernatant was transferred into a scintillation vial, and the amount of ^{14}C-radioactivity was determined as described above. In addition, Cry1Ab proteins were also quantified in duplicates from these supernatants by ELISA (enzyme-linked immunosorbent assay) using the PathoScreen DAS ELISA-Kit Cry1Ab/Cry1Ac (Agdia, Elkhart, IN, USA).

Quantification of non-extractable ^{14}C residues from soil

After determination of the soil microbial biomass and its ^{14}C content, the centrifuged sediments were dried for 24 h at 105 °C and subsequently ground for 10 min in a mortar mill (Pulverisette 2, Fritsch, Idar-Oberstein, Germany). An aliquot of the ground material was then combusted using the Biological Oxidizer OX 300 (R.J. Harvey Instrument Corp., Hillsdale, NJ, USA). During oxidation, the released $^{14}CO_2$-activity in the gas phase was absorbed in a scintillation cocktail consisting of 31 % (*v*/*v*) ethanolamine, 21 % methanol and 48 % Quickszint 212 (Zinsser). Radioactivity was then quantified as described above using the Beckman liquid scintillation counter LS1801.

Results

Mineralization of ^{14}C-Cry1Ab in soil microcosms

Immediately after addition of the ^{14}C-Cry1Ab protein to the soil microcosms, independent of whether their initial concentration was 14 μg Cry1Ab g^{-1} soil (dry weight) or 50 μg g^{-1}, a strong production of $^{14}CO_2$ was detected (Fig. 1).

Higher relative and absolute amounts of $^{14}CO_2$ were released from soils with the higher initial Cry1Ab concentration. For the lower initial concentration, a total of 7 to 9 % of the activity were recovered as $^{14}CO_2$ after 7 days, while the proportion with the higher concentration was 22 to 28 %. During the incubation, the mineralization rates with both soils declined continuously until the end after 101 (for microcosms amended with 50 μg Cry1Ab g^{-1}) and 135 days (initial concentration of Cry1Ab 14 μg g^{-1}), respectively. It should be noted that, due to the purity of 85 and 90 % of the ^{14}C-Cry1Ab stock solutions, some $^{14}CO_2$ may have originated from impurities, probably including fragments of the Cry1Ab protein.

Clearly and independent of the initial concentration, the mineralization of Cry1Ab was more active in the loamy sand soil compared to the sandy clay. In contrast, the microbial biomass was higher in the sandy clay (Table 1), indicating that this parameter was not the limiting factor for the mineralization of the Cry1Ab protein. Overall, at the end of the soil incubation, the released $^{14}CO_2$ with the lower initial concentration of Cry1Ab (14 μg g^{-1}) added up to 18 % with the loamy sand and 14 % with sandy clay, while the corresponding values for the higher concentration of Cry1Ab (50 μg g^{-1}) were 43 and 35 % mineralized, respectively.

Quantification of ^{14}C from the soil microbial biomass

After 29 (initial concentration 14 μg Cry1Ab g^{-1} soil) and 37 days (50 μg Cry1Ab g^{-1}), soil samples from three replicate microcosms were sacrificed, and soil aliquots were analysed for the amount of ^{14}C activity in the microbial biomass. Then, the amount of total residual ^{14}C activity in soil was determined directly (without extraction) by combustion (see Materials and methods). Combined with the amount of $^{14}CO_2$ produced up to this point and the amount of ^{14}C of the microbial biomass, this allowed the determination of the total recovery of added ^{14}C (see Materials and methods for more details).

Total recovery rates were in the range of 90 to 110 % (Fig. 2). With the lower initial concentration, 82 to 85 % of the ^{14}C activity was still in soil after 29 days (Fig. 2a). A total of 17 and 16 % of this activity were recovered as the microbial biomass for the loamy sand and sandy clay, respectively. This incorporation of ^{14}C into the microbial biomass demonstrates that Cry1Ab was used by microorganisms as a growth substrate. With the higher initial concentration, the amounts still detected in soil after 37 days were 60 % of the total activity from sand and 69 % for clay loam; 23 (loamy sand) and 18 % (sandy clay) of these activities originated from the microbial biomass (Fig. 2b). Even though for both initial concentrations the average amount of ^{14}C recovered from the microbial biomass was lower for the clay than for sand, these differences were not significant, due to the high variability between the replicate microcosms. Thus, the efficiency by which Cry1Ab served as a microbial growth substrate was relatively independent of the soil texture.

Recovery of ^{14}C from buffer extractable and non-extractable soil fractions

In order to estimate the biologically available fraction of the Cry1Ab proteins including their water-soluble breakdown products, aliquots of the soil samples from three replicate microcosms of each soil and the two initial Cry1Ab concentrations, which had been sacrificed after 29 or 37 days of incubation (depending on the initial concentration; see above), were extracted with PBST buffer. Combined with the non-extractable soil fraction and the amount released as CO_2, the total recovery rates of ^{14}C ranged between 94 and 103 % (Table 2).

Fig. 1 Mineralization of ^{14}Cry1Ab in two soils (*grey circles*: loamy sand; *black circles*: sandy clay) under laboratory conditions. Initial concentrations of Cry1Ab were 14 μg g^{-1} soil (dry weight) (**a**) and 50 μg g^{-1} soil (**b**), respectively. The study was conducted with four replicates of each treatment until day 29 (with 14 μg Cry1Ab g^{-1} soil) and day 37 (50 μg Cry1Ab g^{-1} soil) and subsequently with single microcosms for each

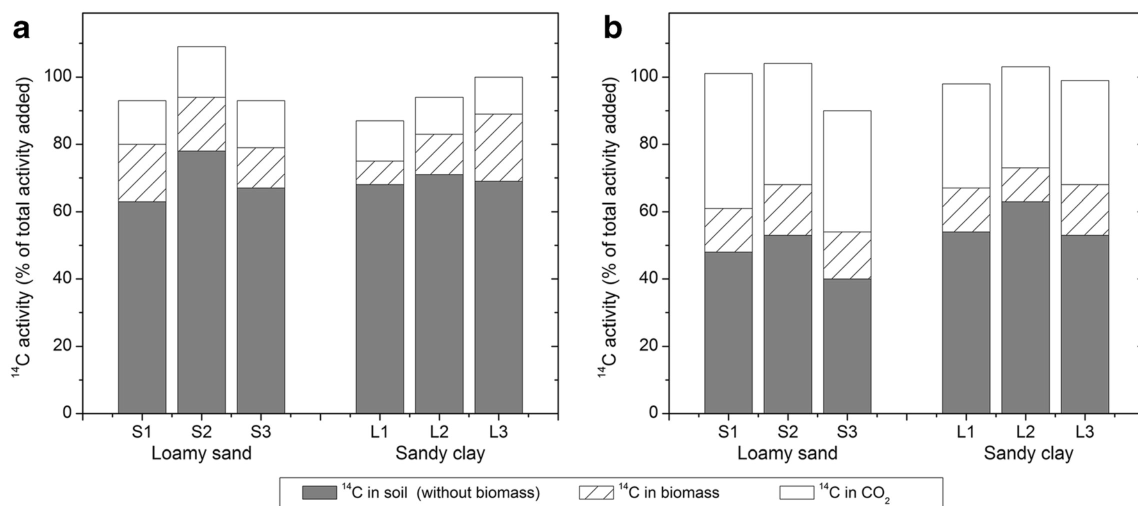

Fig. 2 Recovery of ^{14}C activity from microcosms incubated with initial concentrations of 14 μg ^{14}C-Cry1Ab g^{-1} soil (**a**) and 50 μg g^{-1} (**b**), respectively

Table 2 Partitioning and recovery of ^{14}C activity, after soil amendments with ^{14}C-Cry1Ab in microcosms

Soils in microcosms	$^{14}CO_2$	^{14}C extracted with PBST	^{14}C in the soil fraction, non-extractable with PBST	Total recovery
Initial concentration 14 μg Cry1Ab g^{-1} soil (% recovery of ^{14}C activity, 29 days after its addition to soils)				
Loamy sand	14.0^{a}	8.7^{a}	73.0^{a}	96.3
Sandy clay	11.3^{b}	2.0^{b}	82.5^{b}	95.0
Initial concentration 50 μg Cry1Ab g^{-1} soil				
Loamy sand	37.3^{c}	5.3^{c}	51.0	93.7
Sandy clay	30.5^{d}	1.7^{b}	70.5	103.0

Values with different letters in columns indicate significant differences ($p<0.05$)

Only 1.7 to 8.7 % of the initially applied ^{14}C-Cry1Ab activity was extracted from the soils, and three to four times more were extracted from loamy sand than from the sandy clay. The proportion of the ^{14}C-extracted fraction from the soil matrices compared to the activity remaining in soil appeared to be independent of the initial concentration: 10 to 11 % from loamy sand and only 2.4 % from sandy clay, indicating stronger binding of Cry1Ab to the soil with the higher clay content.

Assuming that the total radioactivity extracted with PBST from the soil samples would still be caused by intact, non-degraded Cry1Ab molecules, the amounts for initially supplied 14 μg g^{-1} soil would correspond to 1.2 μg extracted from 1 g of loamy sand and 0.3 μg from 1 g of sandy clay. Correspondingly for the higher initial concentrations, the amounts extracted from 1 g of sand would be 3.1 μg and from clay 1.0 μg.

Quantification of intact Cry1Ab proteins from PBST-extracted soils

With initially 14 μg Cry1Ab g^{-1} soil applied, the extracted amount of Cry1Ab detected with ELISA only corresponded to 0.8 ng g^{-1} loamy sand, and no Cry1Ab was detected in extracts from the sandy clay (threshold of detection, 0.02 ng g^{-1} soil) (Table 3). With the initially higher amount of Cry1Ab (50 μg Cry1Ab g^{-1} soil), ELISA indicated an average of 5.2 ng intact Cry1Ab g^{-1} loamy sand, and only non-quantifiably traces of Cry1Ab were detected in PBST extractions from the clay loam. Thus, the detected amounts of Cry1Ab proteins only represented less than 0.2 % of the total radioactivity of that originated from extractable ^{14}C-Cry1Ab, demonstrating that the vast majority of the extracted radioactivity originated from non-immunoreactive degradation products of the Cry1Ab protein.

Discussion

Microbial metabolism and nutrient cycling in soils are mainly fuelled by compounds originating from the decomposition of plants, animals and microorganisms. Among these compounds, proteins represent a major group, and consequently, all natural soils harbour microbial communities with a broad capacity to degrade these macromolecules (Kielland et al. 2007; Lipson et al. 1999). The rapid release of $^{14}CO_2$ immediately after the addition of the Cry1Ab solutions to both soils of this study demonstrated that this capacity also applies to the degradation of Cry1Ab, when supplied at microgram quantities per gram of soil. Possibly, some of the immediately released $^{14}CO_2$ originated from impurities of the stock solutions, but the kinetic of the degradation rates showed a homogenous decline rather than abrupt changes, suggesting no strong effect of the contaminants. In fact, in the only other available study with ^{14}C-labelled Cry proteins, similar rates of mineralization were found for a Cry1Ac protein in loamy sand with a comparably low pH (Accinelli et al. 2008). In this study, the detection of ^{14}C from ^{14}C-Cry1Ab incorporated into the soil microbial biomass at amounts of 16 to 23 % already after 29 to 37 days after its addition provides unequivocal evidence that microorganisms utilize this protein as a carbon source, just as would be expected from any natural protein entering the soil.

Table 3 Detection of Cry1Ab proteins extracted with PBST buffer from soil microcosms after 29 days of incubation for the initial concentration of 14 μg Cry1Ab g^{-1}, and after 37 days of incubation for 50 μg g^{-1} soil with ^{14}C-labelled Cry1Ab proteins

Soil microcosms	Cry1Ab in soil (ng g^{-1} soil)	
	Initial concentrations	
	14 μg Cry1Ab g^{-1}	50 μg Cry1Ab g^{-1}
Loamy sand, replicate 1	0.90	3.23
Loamy sand, replicate 2	0.66	6.12
Loamy sand, replicate 3	0.88	6.30
Sandy clay, replicate 1	n.d.	<0.1
Sandy clay, replicate 2	n.d.	n.d.
Sandy clay, replicate 3	n.d.	<0.1

n.d. not detected (threshold of detection 0.02)

After 29 to 37 days of incubation, a significant amount of the ^{14}C activity added to soil with ^{14}C-Cry1Ab was neither mineralized nor incorporated into the microbial biomass but still present in soil. As a result of a lower mineralization activity, the percentage of this fraction was higher for an initial concentration of 14 μg Cry1Ab g^{-1} soil compared to 50 μg g^{-1}, and it was relatively similar for both soils of this study.

However, when the soils were extracted with PBST buffer, the yield of ^{14}C activity, for both initial concentrations, was three to fourfold higher in loamy sand compared to the sandy clay, indicating that binding capacities were much stronger due to a larger organo-mineral surface area, higher cation exchange capacity and total organic carbon, which were all connected to the higher amount of clay. The adsorption of Cry1Ab to clay is especially strong due to patch-controlled electrostatic interactions (Sander et al. 2010). Other buffers with pH of 10 or above (Fu et al. 2012; Helassa et al. 2011), or addition of alkaline artificial gut fluids (Shan et al. 2005), have shown an increased efficiency to extract Cry proteins from the soil matrix. However, the PBST buffer (pH 7.4), which keeps the Cry1Ab protein in a soluble native form, was selected in this study to estimate the concentration of water-dissolved Cry1Ab and its potential metabolites, reflecting the bioavailability, as also done in several other studies (e.g. Daudu et al. 2009; Douville et al. 2005; Feng et al. 2011; Griffiths et al. 2007; Gruber et al. 2011; Margarit et al. 2008).

The comparison of the ^{14}C activity found in the PBST extracts from soils after 29 to 37 days of incubation to the amount of immunoreactive Cry1Ab protein, as detected by ELISA, demonstrated that most ^{14}C activity originated from non-immunoreactive fragments and not from Cry1Ab itself. As for the CO_2 release, some ^{14}C may have also originated from impurities of the ^{14}C-Cry1Ab stock solutions, considering that the solutions had a purity of 85 and 90 %, respectively.

Before the Cry1Ab protein can enter microbial cells, it needs to undergo cleavage by extracellular proteases into smaller peptides or amino acids. Extracellular proteases are common in soil (Quiquampoix and Burns 2007), and the potential of such enzymes to degrade Cry1Ab has previously been demonstrated (Padmaja et al. 2008). The analyses of this study do not allow one to decide whether the breakdown products of Cry1Ab detected in the PBST extracts were intermediates generated by proteolytic activity *before* they were taken up by metabolizing microbial cells, or whether they were Cry1Ab-metabolites, e.g. organic acids, excreted by microbial cells after intracellular metabolism. In any case, the high PBST-extracted amounts of ^{14}C activity in contrast to the low concentrations of Cry1Ab detected by ELISA therein were proof of an irreversible elimination of the recombinant Cry1Ab from soil. In contrast, studies of Cry protein degradation without isotope-labelled compounds, only based on ELISA, were actually not capable of directly distinguishing between dissipation by adsorption and actual degradation (Badea et al. 2010; Baumgarte and Tebbe 2005; Fu et al. 2012; Helassa et al. 2011; Wang et al. 2006).

Given the high apparent conformational stability of the Cry1Ab protein (Madliger et al. 2011), it is likely that the major proportion of the soil-bound ^{14}C activity, not extractable with PBST, was in fact caused by Cry1Ab and not by degradation products. While being adsorbed, the Cry1Ab protein is partially protected against microbial degradation (Chevallier et al. 2003; Koskella and Stotzky 1997). On the other hand, the very low concentrations of Cry1Ab detected in PBST-extracted fraction demonstrate that the compound is quickly degraded in the soil aqueous phase. This instability of the water-dissolved Cry1Ab is also indicated by their higher mineralization rates found in loamy sand compared to the sandy clay, with more adsorption of Cry1Ab in the latter. This high instability of Cry1Ab in soil water was also reported in other studies (Daudu et al. 2009), and it explains why the protein is not, or only hardly, detected in studies on agricultural fields, even after repeated cultivations of Bt-maize over several years (Baumgarte and Tebbe 2005; Gruber et al. 2011). Therefore, a low concentration of a Cry protein, or even a lack of detection, does not necessarily mean that the protein is eliminated and does not persist or accumulate on fields.

This discrepancy between the low amount of Cry1Ab detected in the soil aqueous phase and the non-extractable high amounts in soil clearly indicates that the rate-limiting step for the degradation of Cry1Ab in the soils of this study was desorption of the protein rather than the indigenous microbial activity. Similar conclusions were drawn when the fate of a non-isotope-labelled Cry1Aa was studied in a sandy loam and in a clay loam under varying conditions (Helassa et al. 2011). As time proceeds, increasing amounts of $^{14}CO_2$ produced by the soils will originate from metabolites and microbial products, rather than from the Cry1Ab protein itself. Thus, the $^{14}CO_2$ data cannot directly be used to calculate the period of time that would be required for complete elimination of the biologically active protein, as Cry1Ab degradation will be much faster than the oxidation of its last carbon atom to CO_2. Under field conditions, the mineralization rate of Cry1Ab would probably also be affected by the presence of other proteins released from Bt-crops during their decomposition.

The exposure of non-target organisms to water-dissolved Cry1Ab in agricultural field soil used for cultivation of Bt-maize will be very low, but it may last longer than indicated by a lack or low level of detection due to desorption processes. On the other hand, non-target organisms which come into contact with soil-adsorbed Cry proteins, e.g. by ingestion of clay or organic particles, would possibly be exposed to much higher concentrations of Cry1Ab than suggested by ELISA

detection in aqueous soil extracts. Whether the adsorption process of Cry1Ab by soils actually results in an accumulation of Cry1Ab on agricultural fields depends therefore on the desorption rates rather than on microbial degradation potentials. The desorption rates will be greatly affected by soil properties, agricultural management or other factors, as demonstrated by the importance of solution chemistry for this process (Madliger et al. 2010, 2011). Thus, the implication of these factors may deserve more scientific attention than the actual measurement of water-dissolved Cry1Ab for predicting the long-term persistence of this recombinant protein in soil.

In conclusion, environmental risk assessments of Cry1Ab should primarily consider the potential amounts of Cry1Ab produced and released into field soils and the amount stabilized by adsorption onto soil particles rather than a low level or lack of detection of Cry1Ab in aqueous soil extracts.

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Conflict of interest The authors declare that they have no competing interests.

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