Molecular Ecology (2003) **12**, 765–775

Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the field

C. ZWAHLEN,* A. HILBECK,† P. GUGERLI‡ & W. NENTWIG*
*Zoological Institute, University of Bern, Bern, Switzerland; †Geobotanical Institute, Swiss Federal Institute of Technology Zurich (ETH), Zurich, Switzerland; ‡Federal Research Station for Plant Production of Changins (RAC), Nyon, Switzerland

Abstract

Large quantities of *Bacillus thuringiensis* (Bt) corn plant residue are left in the field after harvest, which may have implications for the soil ecosystem. Potential impacts on soil organisms will also depend on the persistence of the Bt toxin in plant residues. Therefore, it is important to know how long the toxin persists in plant residues. In two field studies in the temperate corn-growing region of Switzerland we investigated degradation of the Cry1Ab toxin in transgenic Bt corn leaves during autumn, winter and spring using an enzyme-linked immunosorbent assay (ELISA). In the first field trial, representing a tillage system, no degradation of the Cry1Ab toxin was observed during the first month. During the second month, Cry1Ab toxin concentrations decreased to ≈ 20% of their initial values. During winter, there was no further degradation. When temperatures again increased in spring, the toxin continued to degrade slowly, but could still be detected in June. In the second field trial, representing a no-tillage system, Cry1Ab toxin concentrations decreased without initial delay as for soil-incorporated Bt plants, to 38% of the initial concentration during the first 40 days. They then continued to decrease until the end of the trial after 200 days in June, when 0.3% of the initial amount of Cry1Ab toxin was detected. Our results suggest that extended pre- and post-commercial monitoring are necessary to assess the long-term impact of Bt toxin in transgenic plant residues on soil organisms.

Keywords: *Bacillus thuringiensis*, degradation, ELISA, persistence, soil, transgenic plants

Received 15 April 2002; revision received 5 December 2002; accepted 5 December 2002

Introduction

Following their commercial release in 1996, transgenic crop plants are grown on a large-scale in four countries worldwide. Ninety-nine percent of all transgenic plants are grown in the USA, Argentina, Canada and China (James 2002). In 2001, transgenic crops expressing *Bacillus thuringiensis* (Bt) toxins to control various target pest species were grown on 12 million hectares. The main Bt crop was transgenic corn, which was grown on 5.9 million hectares (James 2002). Most varieties of Bt corn express the Cry1Ab toxin targeting the pest insect, *Ostrinia nubilalis*, the European corn borer. However, some Bt corn varieties may also affect nontarget species, depending on tissue-specific expression levels of the toxin (Hilbeck *et al*. 1998; Losey *et al*. 1999; Hansen Jesse & Obrycki 2000; Wraight *et al*. 2000; but see also Hellmich *et al*. 2001; Sears *et al*. 2001). Also, a new Bt corn variety in the pipeline for commercialization in the USA during 2004, and expressing the Cry3Bb1 toxin against the coleopteran pest *Diabrotica virgifera*, does have high toxin concentration of 62 ± 18 μg/g fresh weight in pollen (Environmental Protection Agency 2002).

When corn is harvested, ≈ 2–2.5 t/ha (silage corn) to 6 t/ha (grain corn) dry matter is left on or in the soil (Zscheischler *et al*. 1984) where it degrades and enters into the soil ecosystem. In principle, there are several ways in which Bt toxins enter the soil: (i) direct input of Bt toxin into soil via root exudates and/or leachates following plant injuries; (ii) Bt protein incorporated in the plant matrix degrades with the plant matrix; and (iii) direct input of Bt protein into soil via leachates from degrading plant material. This results in different exposure scenarios for nontarget soil organisms. For exposure and impact analysis of the incorporated and released Bt toxins in the soil

Correspondence: C. Zwahlen, Department of Entomology, 219 Hodson Hall, University of Minnesota, St Paul, MN, 55108, USA. E-mail: claudia.zwahlen@gmx.net

ecosystem, it is essential to know how long the toxin persists in the plant residues. To our knowledge, only one study to date has quantified the degradation of Cry1Ab toxin in transgenic Bt corn tissue (Sims & Holden 1996). The authors calculated from their bioassay data using *Heliothis virescens*, that 50% of the toxin had degraded 1.6 days after incorporation of the plant material into the soil and 90% had degraded after 15 days. Without any soil contact, they calculated that 50% of the toxin had degraded after 25.6 days and 90% had degraded after 40.7 days. Based on these laboratory data, Sims & Holden (1996) concluded that the Cry1Ab protein in corn plant tissue would be unstable under field conditions and would be likely to degrade rapidly under cultivation practices. As the main growing areas for transgenic crops are currently located in temperate regions with distinct cold seasons, it seems unrealistic that data calculated from laboratory bioassays conducted at constant temperatures of 24–27 °C (Sims & Holden 1996) provide a good estimate of such field conditions. The degradation of Bt toxins depends to a significant extent on microbial activity (Palm *et al*. 1996; Koskella & Stotzky 1997), which is reduced at colder temperatures. Therefore, it must be expected that the degradation in temperate regions differs substantially from that observed in laboratory trials with high constant temperatures.

In most studies that have examined the persistence of Bt toxin from transgenic cotton, the toxin could still be detected when the experiments were terminated after 28–140 days (Palm *et al*. 1994, 1996; Sims & Ream 1997). In addition, Saxena & Stotzky (2001) and Saxena *et al*. (2002) reported that Bt toxin from transgenic corn root exudates and from degrading Bt corn biomass can persist in soil for up to 350 days, the longest time studied. Some studies also report persistence of purified Bt toxins in soil for up to 234 days, i.e. when the trials were terminated (Tapp & Stotzky 1995a; Palm *et al*. 1996; Koskella & Stotzky 1997; Tapp & Stotzky 1998). Bt toxins bind tightly to surface-active particles, such as humic acids and clay minerals (Venkateswerlu & Stotzky 1992; Tapp *et al*. 1994; Tapp & Stotzky 1995a; Crecchio & Stotzky 1998, 2001), where they retain their insecticidal activity (Venkateswerlu & Stotzky 1992; Tapp & Stotzky 1995b) and are protected against microbial degradation (Tapp & Stotzky 1995a; Koskella & Stotzky 1997).

Once the Bt toxin is bound on surface-active particles it is difficult to extract from the soil for quantitative analyses. Sims & Holden (1996) and Sims & Ream (1997) suggest that the degradation of Bt toxin from transgenic plant material within soil be measured using target insect bioassays. Palm *et al*. (1994) proposed an enzyme-linked immunosorbent assay (ELISA) method with various extraction buffers (depending on the soil type) to get a high recovery of Bt toxin. In our first field experiment in 1999/2000, the plant material was cut into large leaf pieces and incorporated into the soil using litter bags with a large mesh size, in simulation of corn plant residues incorporated into the soil in a tillage system. In our second field experiment in 2000/2001, the plant material was left on the surface of the soil, hence, simulating degradation in a no-tillage system. Plant material is incorporated into the soil by soil organisms such as earthworms. Therefore, our second experiment aimed at understanding degradation of Bt toxin in plant material incorporated into the soil by earthworms.

The objectives of our studies were to: (i) investigate the degradation of the Bt toxin in corn plant material under field conditions from harvest to the next planting season; (ii) evaluate whether the applied methodologies are suitable for monitoring of Bt toxin degradation within transgenic plant residuals in agriculture; and (iii) determine realistic soil temperature ranges for investigating the degradation of the toxin in transgenic corn in temperate regions. This type of information is essential for exposure assessment of nontarget decomposing organisms for risk analyses of transgenic Bt plants.

Materials and Methods

Plants

Two corn hybrids were used in the experiment. One was genetically modified corn from Syngenta (N4640Bt, transformation event Bt11) (referred to as Bt+) containing a truncated, synthetic version of a gene from *Bacillus thuringiensis* ssp. *kurstaki* coding for the expression of the insecticidal *B. thuringiensis* δ-endotoxin Cry1Ab. The other hybrid was the near isoline of N4640Bt, which was not genetically modified (N4640) (referred to as Bt–/control). The plants were cultivated in plastic pots in a growth chamber at an average temperature of 23.3 °C (20 °C for 8 h in dark and 25 °C for 16 h in light). For the first field experiment in 1999/2000, leaves of plants, shortly before and after pollen shed, were cut into ≈ 8 cm pieces. For the second experiment in 2000/2001, leaves of plants were cut 3 weeks after pollen shed into ≈ 2 × 2 cm² pieces. For both experiments, the material was dried at 40 °C for 72 h.

First field trial 1999/2000

Sixty litter bags made of curtain material (20 × 20 cm, 5 mm mesh size) per treatment (Bt+/Bt–) were filled with 2 g (≈ 25–35 leaf pieces) of dried plant material and buried vertically at a depth of 0–20 cm (determined by the size of the litter bags) in the soil of a corn field near Bern, Switzerland, in mid-October 1999. This was done immediately after harvest of the silage corn and ploughing and sowing of winter barley. Two litter bags, i.e. one per treatment (Bt+/Bt–), were buried at 10 locations spread evenly

along one field row ≈ 9 m in length (distance between each location was 1 m). This was repeated six times, i.e. six field rows resulting in a total of 60 locations within a field area of 9×5 m^2. The soil was a Cambisol loam (50% sand, 33% silt, 17% clay, pH 5.7). About 15%, 500 million hectares, respectively, of the potential arable farmland worldwide are Cambisols (Scheffer & Schachtschabel 1989). In monthly intervals from mid-November to mid-June, the litter bags from one location (Bt+/Bt–) were removed for analysis and brought back to the laboratory. The material was frozen at –20 °C until it was analysed by ELISA and used in bioassays.

Second field trial 2000/2001

A second field experiment was carried out one year later in the same area near Bern, Switzerland. The objectives of this experiment were to record degradation of the Bt toxin in corn plant residues left on top of the soil and also to investigate whether the Bt toxin has an impact on the life history parameters of enclosed earthworms, *Lumbricus terrestris* (Annelida: Lumbricidae). Here, we report the data collected on the degradation of the Cry1Ab toxin in the corn plant residues. The data obtained on the life history parameters of the earthworms will be published in Zwahlen *et al*. (in press).

The experimental field was ≈ 300 m from the field where the first experiment was conducted. The soils were very similar, consisting of the same Cambisol as described above. In this field, cob corn had been grown that was harvested in late autumn. The experiment was initiated at the beginning of December 2000, shortly after ploughing and sowing of oats.

Tubular soil cages (height 80 cm, 2.6 cm diameter) (Fig. 1) were made of the same material as the litter bags in the first experiments, except that the mesh size (1 mm) was smaller to prevent the escape of the earthworms. The soil cages were filled with 453 g of soil collected from the field where the experiment was carried out and placed in 60 cm deep column-shaped bore holes, which had been made using a boring rod (3 cm diameter). Nine soil cages were spread evenly along one field row 8 m in length (distance between each location was 1 m). This was repeated 15 times, i.e. 15 field rows (distance between each row was also 1 m). The last row consisted of five soil cages. This resulted in a total of 140 bore holes within a field area of 8×15 m^2. Corn plant material (either Bt+ or Bt–) was placed on top of the soil surface inside the soil cages and the earthworms (one individual/cage) were added. The soil cages were subsequently closed using a piece of string (Fig. 1).

The entire amount of corn leaf material needed to feed the earthworms for the whole trial was added at the beginning of the experiment. Between 0.29 and 1.45 g of leaf material was added per cage depending on the assigned experimentation duration, i.e. 0.65 g (40 days), 1.3 g (80 days), 1.95 g (120 days), 2.6 g (160 days) or 3.25 g (200 days) was provided initially.

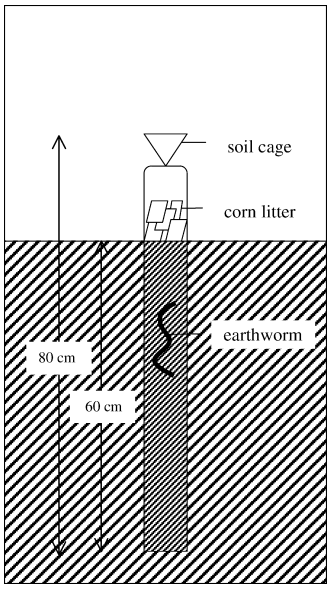

Fig. 1 Soil cages in the second field experiment 2000/2001. Cages are filled with soil (dark grey), and corn litter, either (Bt+) or (Bt–), was put on the surface of the soil. Cages were placed into 60 cm deep column-shaped bore holes.

Most of the corn plant material remained on the surface of the soil in the cages after the end of the experiment. Therefore, these residues were used in the analyses. As *L. terrestris* mainly deposit their cast at the surface of the soil, cast had to be removed from the remaining plant material using deionized H_2O, plant material was then frozen at –20 °C until it was analysed by ELISA and used in bioassays.

Temperature and rainfall measurement

Temperature was measured at a soil depth of 10 cm for the first field experiment and at 5 cm above the surface and at a soil depth of 10 cm for the second experiment at a gauging station in Bern, Switzerland (data provided by MeteoSchweiz, Zurich, Switzerland), ≈ 7 km away from the field site. Rainfall was also measured at the same station.

ELISA

Bacillus thuringiensis toxin was quantified by ELISA as described by Gugerli (1979, 1986). Briefly, at each sample date, 10 leaf pieces of (Bt+) and (Bt–) plants were analysed. Thawed leaf pieces were washed with deionized H_2O to remove soil particles, allowed to air dry for 2 h, cut in two and weighed. One part was used for the ELISA, the other was dried at 40 °C for 7 days to determine the dry weight

of the recovered tissue. The test material was put into universal bags (Bioreba AG) and homogenized using a hand homogenizer (Bioreba AG) in 5 mL extraction buffer [10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, 3 mM NaN_3, 2% (v/v) polyvinylpyrrolidone (M_r = 25 000), 0.05% (v/v) Tween®20, pH 7.4] to extract the Bt toxin. In addition, homogenized samples from the second field experiment were put into 14 mL centrifugation tubes (Sarstedt AG) and centrifuged using a Universal 30 RF centrifuge (Hettich AG) at 3857 *g*. for 5 min. The supernatants were used for the analyses (including the calibration curve).

In order to determine the calibration curve, reference samples of purified Cry1Ab toxin were suspended in pooled extracts of the same (Bt–) leaves used for the analyses at concentrations of 1000, 100, 50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1 and 0.01 ng Cry1Ab toxin/mL. All samples were prepared in duplicate. Microtitre immunoassay plates Immunolon® 4 (Dynatech Laboratories Inc.) were analysed using a MRX microplate reader operated with the REVEL software package, Version G3.2. (Dynex Technologies Inc.). Optical density (OD) was measured at 405 nm. Polyclonal coating IgG and alkaline phosphatase-conjugated IgG against the Cry1Ab toxin were produced by Bioreba AG. Diethanolamine and 4-nitrophenylphosphate for the substrate buffer were obtained from Merck.

Detection level. Undiluted samples were used for the ELISA. The threshold values of detectable toxin were defined as optical density (OD) mean + 3 SD of the OD of the (Bt–) leaf samples.

Quantitative analysis. The means of the duplicates were used for the analyses. Data were log-transformed before linear regression was carried out to calculate the concentrations of the Cry1Ab toxin in the samples. Kruskal–Wallis tests were carried out to determine whether the toxin concentrations in transgenic leaves of the different sample dates were significantly different (GRAPHPAD PRISM; GraphPad Software Inc. 2000). Mean concentrations of transgenic leaves of different sample dates were compared using a Dunn's Multiple Comparison Test (GRAPHPAD PRISM).

Herbivore bioassays

To determine the insecticidal activity of the toxin, the remaining sample material was incorporated into meridic diet and fed to the highly susceptible neonate larvae of *Ostrinia nubilalis* Hübner (eggs obtained from French Agricultural Research Inc.) (Koziel *et al*. 1993).

Field experiment 1999/2000. For each treatment and all but two sample dates, 10 larvae were placed into each of 10 vials (5 cm height, 2.2 cm diameter) resulting in 100 larvae per treatment. The vials were closed with Parafilm®. Larvae were kept in a climate-controlled chamber at 25 °C (16 : 8 h light/dark). After 6 days, mortality and the weight of surviving larvae were recorded. For bioassays of the samples at the beginning of the trial and after 2 months, 10 larvae were placed in each of 30 vials and mortality was recorded. Thus, a total of 2600 *O. nubilalis* larvae were examined.

Field experiment 2000/2001. Approximately 50 mg of the remaining sample material was mixed with 250 µL extraction buffer (EnviroLogix) before 300 mg meridic diet was added. For each treatment and sample date 10 larvae were placed into each of 10 vials, resulting in 100 larvae per treatment and a total of 1200 larvae examined. Vials were closed with plastic lids. The larvae were kept in the same climate-controlled chamber as those from the first field experiment. After 5 days, mortality and weight of surviving larvae were recorded.

Data analysis. Mean mortality and average individual weight of the two treatments for each sample date were compared using the Mann–Whitney *U*-test (GRAPHPAD PRISM).

Results

Degradation of Cry1Ab toxin

Field experiment 1999/2000. ELISA analysis indicated that the initial mean (± SD) Cry1Ab toxin concentration in the transgenic (Bt+) leaves was 15.4 ± 4.3 µg/g dry weight (Fig. 2b). No Bt protein was detected in any (Bt–) corn samples at any of the sample dates. We observed a nonlinear degradation curve of the Cry1Ab toxin concentration with a tailing-off, approaching but not reaching zero at the end of the investigation (Fig. 2b). Mean Bt toxin concentrations in leaves were significantly different between the sample dates ($H = 57.98$, $P < 0.0001$). The mean toxin concentration did not decrease significantly during the first month of the trial ($P > 0.05$), but the observed concentrations varied enormously (Fig. 2b). During this month, soil temperature ranged between 14 and 8 °C (Fig. 2a). In December, the Cry1Ab toxin concentration decreased drastically from an initial 15.4 to 3.0 µg toxin/g dry weight, i.e. to 20% of the initial amount, representing the largest degradation step (Fig. 2b). This difference was significant when compared with the initial toxin concentration ($P < 0.05$) but was not significantly different when compared with the mean in November ($P > 0.05$). This was primarily a result of the large variability of the November data (Fig. 2b). Daily mean temperature decreased from 8 to 3 °C during the time from mid-November to mid-December (Fig. 2a). After that, no significant decrease in toxin was observed during

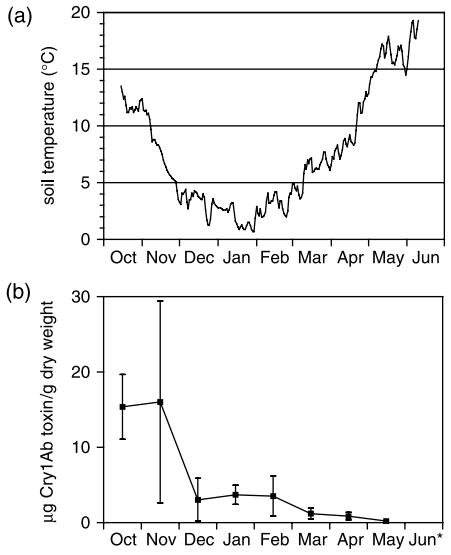

Fig. 2 (a) Average daily soil temperature (°C) at 10 cm depth from mid-October 1999 to mid-June 2000. Mean temperature was 7.6 °C, ranging from a minimum of 0.7 °C in late January to 19.3 °C in mid-June. (b) Mean (± SD) of the Bt toxin concentration (µg Cry1Ab toxin/g dry weight) in transgenic leaf residues. The total number of leaves analysed per treatment from October 1999 to June 2000 was 88. Toxin in June (*) was still detectable, but could not be quantified by ELISA.

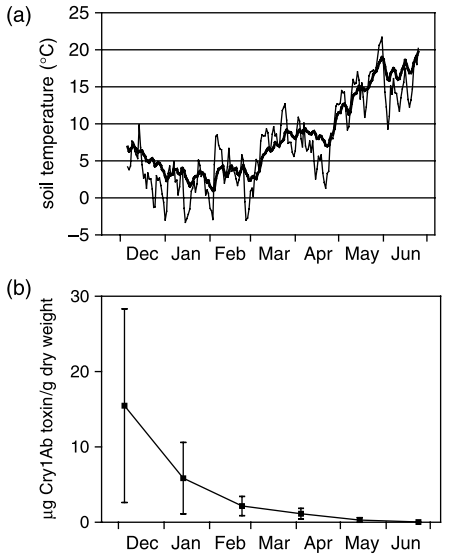

Fig. 3 (a) Average daily soil temperature (°C) at 10 cm depth (thick line) and 5 cm above (thin line) the soil from the beginning of December 2000 to the end of June 2001**.** Mean temperature was 8.2 °C (ranging from a minimum of 1.0 °C in early February to 19.7 °C in late June) in 10 cm depth and 7.3 °C (ranging from a minimum of –3.3 °C in mid-January to 21.7 °C in early June) 5 cm above the soil. (b) Mean (± SD) of the Bt toxin concentration (µg Cry1Ab toxin/g dry weight) in transgenic leaf residues. The total number of leaves analysed per treatment from December 2000 to June 2001 was 60.

winter when average soil temperatures were between 0.7 and 5 °C at 10 cm depth (Fig. 2a). The next decrease of Cry1Ab toxin occurred between mid-February and mid-March when another 12% of the initial amount of toxin degraded, but this decrease was not statistically significant. Soil temperatures ranged between 2 and 7 °C. After that, the toxin concentration declined very slowly. By mid-May, 1.5% of the initial amount of toxin was still present in the remaining corn tissue (Fig. 2b). In May, for 3 of the 10 transgenic leaves, the OD values were below the threshold value. This was the first time that the toxin could not be detected in some leaf samples. However, in June, eight months after incorporation of the plant material, the toxin could still be detected (i.e. above the threshold value) in 7 of the 10 leaves analysed, but there was not enough plant material left to quantify the concentration of the toxin.

Field experiment 2000/2001. Initial mean (± SD) Cry1Ab toxin concentration in the transgenic Bt leaves was again 15.5 ± 12.9 µg/g dry weight (Fig. 3b). Overall mean Bt toxin concentrations were significantly different between the sample dates ($H = 43.07$, $P < 0.0001$). Within the first 40 days from December to mid-January, Cry1Ab toxin concentration decreased to 5.9 ± 4.7 µg/g dry weight, i.e. 38% of the initial amount of toxin (Fig. 3b). Temperatures above ground ranged between –3 and +10 °C (Fig. 3a). At the end of February, 80 days after the beginning of the trial, the Cry1Ab toxin concentration of 2.2 ± 1.3 µg/g dry weight was ≈ 14% of the initial amount. Temperatures increased again from early March onwards and 7% of the initial amount of toxin was still left in early April, i.e. 1.1 ± 0.7 µg Cry1Ab toxin/g dry weight. After 160 days, in mid-May, another 5% of the initial toxin amount had degraded. For the first time since the beginning of the experiment, a statistically significant difference occurred: mean Cry1Ab toxin concentration was significantly lower than the initial concentration ($P < 0.001$) and the concentration after 40 days (sample from January) ($P < 0.001$). At the end of the experiment after 200 days, in late June, 0.05 ± 0.04 µg Cry1Ab toxin/g dry weight was left in the residues. This concentration was significantly lower than the initial concentration ($P < 0.001$), and the concentrations after 40 ($P < 0.001$), 80 ($P < 0.01$) and 120 ($P < 0.05$) days. The OD values of all (Bt+) leaves were above threshold values with the exception of two leaf samples from the last sample date in June

Rain

Mean (± SD) daily rainfall during the first field experiment was lower (2.3 ± 4.5 mm) than that during the second (3.7 ± 6.6 mm) (Table 1). This difference can be explained in

Table 1 Daily mean (± SD) rainfall during time periods between two sample dates

Period	Rain (mm/day)
First field experiment 1999/2000	
mid-Oct–mid-Nov (30 days)	2.9 ± 5.1
mid Nov–mid-Dec (60 days)	1.4 ± 2.6
mid Dec–mid-Jan (90 days)	2.7 ± 4.5
mid Jan–mid-Feb (120 days)	1.2 ± 3.4
mid-Feb–mid-Mar (150 days)	3.3 ± 4.6
mid-Mar–mid-Apr (180 days)	0.9 ± 1.9
mid-Apr–mid-May (210 days)	2.3 ± 4.9
mid-May–mid-Jun (240 days)	3.7 ± 7.1
Mean (mid-Oct–mid-Jun)	2.3 ± 4.5
Second field experiment 2000/2001	
early Dec–mid-Jan (40 days)	3.1 ± 6.8
mid-Jan–late Feb (80 days)	1.7 ± 3.3
late Feb–early Apr (120 days)	7.0 ± 9.0
early Apr–mid-May (160 days)	3.3 ± 5.2
mid-May–late Jun (200 days)	3.9 ± 6.9
Mean (early Dec–late Jun)	3.7 ± 6.6

part by low rainfall during January and from mid-March to mid-April in the first field experiment and high rainfall during March in the second field experiment. Rainfall during the first period of both field trials (30 and 40 days in the first and second field trial, respectively) was very similar, whereas the degradation curves for the Bt toxin in the two field trials were different (see Figs 2b and 3b). This suggests that rainfall was not the main factor causing this large difference.

Herbivore bioassays

Field experiment 1999/2000. Mean mortality of *O. nubilalis* larvae fed (Bt+) leaves was significantly higher in October, November and December than that of larvae fed (Bt–) leaves (Table 2). After that, except for April, no lethal effect was observed when larvae were raised on a Bt toxin-containing diet. However, significant sublethal effects, i.e. reduced larval weight, were observed until February. In March and April, surviving *O. nubilalis* larvae fed on (Bt+) diets weighed significantly more than those fed on (Bt–) diets. In May (Bt+) fed larvae weighed again significantly less than in the (Bt–) control. In June, no significant differences in mortality or larval weight were observed. But very little decaying plant material remained available for the tests at that time.

Field experiment 2000/2001. Mean mortality of *O. nubilalis* larvae fed (Bt+) leaves was significantly higher after 0, 40 and 80 days of degradation than that of larvae fed (Bt–) leaves (Table 3). After that, no significant differences in mortality were observed between the two treatments. Because of the high mortality in the (Bt+) treatment group after 0 and 40 days, larval weight could not be recorded. Although mortality in the (Bt+) treatment group was significantly higher than that in the (Bt–) treatment group after 80 days, there was no significant reduction in weight of surviving larvae fed (Bt+) corn. All surviving larvae seemed to be unaffected and developed similar to those in the control group. No significant differences between the (Bt+) and (Bt–) treatments were observed after 120 and 160 days. After 200 days, at the end of the experiment, larvae raised on the (Bt+) diet again weighed significantly less than those raised on the (Bt–) diet.

Discussion

To our knowledge, these are the first studies investigating the degradation of Bt toxin in transgenic corn leaves under field conditions. Our results only partially confirm previous laboratory studies with transgenic plant material (Palm *et al.* 1994, 1996; Sims & Holden 1996; Sims & Ream 1997). Whereas most laboratory studies reported shorter degradation times than we observed in the field, in general,

Table 2 Mean (± SD) mortality (%) and individual larval weight (mg/individual) of Ostrinia nubilalis reared on (Bt+) leaves or (Bt–) corn leaves from different sample dates (leaf pieces incorporated into meridic diet)

	Mortality (%)				Weight (mg/ind.)			
	Bt+	Bt–	*P*	*U*	Bt+	Bt–	*P*	*U*
October	78.5 ± 27.6	15.0 ± 11.0	< 0.01	2.5	—	—	—	—
November	33.5 ± 22.9	6.4 ± 8.4	< 0.01	13.0	0.48 ± 0.23	1.02 ± 0.23	< 0.01	6.0
December	20.5 ± 6.5	2.2 ± 2.4	< 0.01	0.0	—	—	—	—
January	9.3 ± 9.1	1.3 ± 4.0	> 0.05	25.0	0.43 ± 0.07	0.67 ± 0.19	< 0.01	13.0
February	11.2 ± 7.7	5.2 ± 5.5	> 0.05	32.5	0.41 ± 0.14	0.83 ± 0.20	< 0.01	0.0
March	8.0 ± 7.9	5.4 ± 8.9	> 0.05	40.0	0.98 ± 0.22	0.64 ± 0.09	< 0.01	3.0
April	13.4 ± 6.5	0.0 ± 0.0	< 0.01	5.5	0.56 ± 0.14	0.45 ± 0.09	< 0.05	23.0
May	11.0 ± 8.8	8.9 ± 11.5	> 0.05	44.0	0.69 ± 0.10	0.94 ± 0.27	< 0.05	19.5
June	9.1 ± 10.0	6.8 ± 11.8	> 0.05	38.5	0.41 ± 0.09	0.44 ± 0.13	> 0.05	24.5

Table 3 Mean (± SD) mortality (%) and individual larval weight (mg/individual) of *Ostrinia nubilalis* reared on (Bt+) leaves or (Bt-) corn leaves from samples removed at day 0 (initial material), 40, 80, 120, 160 or 200 (leaf pieces incorporated into meridic diet)

	Mortality (%)				Weight (mg/ind.)			
	Bt+	Bt–	*P*	*U*	Bt+	Bt–	*P*	*U*
0 days*	100.0 ± 0.0	3.6 ± 5.8	< 0.0001	0.0	—	0.25 ± 0.08	—	—
40 days*	92.0 ± 25.3	9.3 ± 11.8	< 0.0001	2.0	—	0.15 ± 0.07	—	
80 days	50.7 ± 44.9	2.0 ± 6.3	< 0.01	16.5	0.29 ± 0.21	0.30 ± 0.12	> 0.05	24.0
120 days	29.9 ± 37.9	12.2 ± 8.3	> 0.05	38.0	0.26 ± 0.13	0.16 ± 0.06	> 0.05	18.5
160 days	53.7 ± 38.4	25.1 ± 23.1	> 0.05	29.5	0.20 ± 0.10	0.28 ± 0.14	> 0.05	24.0
200 days	0.0 ± 0.0	2.1 ± 4.5	> 0.05	36.0	0.15 ± 0.06	0.26 ± 0.12	< 0.01	11.0

*Weight of larvae in the (Bt+) treatments could not be compared with the weight of larvae in the (Bt–) treatment due to the high mortality rates when larvae were raised on the (Bt+) diet.

the toxin could also still be detected at the end of the experiments. In contrast to the results of the laboratory trials, toxin concentrations in the first field experiment, when leaf residues were incorporated into the soil, did not decrease significantly during the first month. This time lag may have been the result of the unprocessed plant material first undergoing a highly variable process of breakdown by soil organisms. This step may be necessary before degradation processes can begin. In most laboratory trials, the plant material was provided in a form in which it was readily utilizable by microorganisms: i.e. the leaf material was ground, sieved and lyophilized (Palm *et al*. 1994; Sims & Holden 1996; Sims & Ream 1997). In the field, raw unprocessed plant material will enter the soil or remain on top of the soil similar to the methods used in our trials. However, in some cases, incorporated plant material may be in a more senescent stage than the corn material used in our experiments. In a environmental assessment report of Bt plants, the US Environmental Protection Agency (2000) stated that the expression level of Bt toxin in Bt11 corn leaves was highest at physiological maturity (3.3 µg/g fresh weight), but decreased rapidly after 84 days. By contrast, Wandeler *et al*. (2002) still quantified high Bt protein concentrations in senescent (brown) Bt11 corn leaves of 19.7 ± 6.6 µg/g dry weight (mean ± SD). These and our data suggest that nontarget soil organisms will encounter Bt corn plant material with greatly varying initial Bt toxin concentrations.

In the second field experiment, when the leaf residues were left on the soil surface, ≈ 62% of the initial Cry1Ab toxin concentration was already degraded after 40 days. In contrast to our first field trial, toxin degradation continued during the winter indicating that degradation of the Cry1Ab toxin in plant residues left on the soil surface differs from that when plant residues are incorporated into the soil. One reason may have been the addition of earthworms to the litter bags. *Lumbricus terrestris* deposit large amounts of their cast at the surface of the soil, thereby providing a favourable micro-environment for a wide range of decomposer organisms, including increased populations of microorganisms compared with the surrounding soil (Topp 1981; Lee 1985). This may lead to a faster breakdown and microbial colonization of the cast-surrounding leaf material (Topp 1981). Also, the Bt toxins may have been inactivated by the sunlight (Koskella & Stotzky 1997) when residues were left on the soil surface.

However, in both experiments after 200 and 240 days the Cry1Ab toxin could still be detected, even when only small amounts of plant material were left. This demonstrates that the Bt toxin is present as long as there is plant material left and that soil organisms feeding on plant residues are likely to be continuously exposed to the Bt toxin, although at low concentrations, particularly, if the field is repeatedly planted with a transgenic Bt crop. Our results indicate a faster initial degradation of the Cry1Ab toxins in plant residues in a no-tillage than in a tillage system during autumn and winter. After that, in both systems, the Bt toxin degrades only slowly and gradually in conjunction with degradation of the plant material. Further studies in other corn-growing regions with different soil types, temperature, rainfall and cultivation practices are necessary to assess whether our data are also representative for other regions.

Our field studies show extended degradation times compared with those observed by Sims & Holden (1996) who estimated from their bioassay data with *Heliothis virescens* that 50% of the Bt toxin from transgenic corn in soil had degraded after 1.6 days and 90% after 15 days. In our first field study, 50% had degraded after ≈ 45 days and 90% after 145 days (Fig. 2b), thus taking ≈ 28 and 10 times longer, respectively. In our second field study, 50% had degraded after ≈ 35 days and 90% after 105 days (Fig. 3b), thus taking ≈ 22 and 7 times longer. Sims & Holden (1996) used lyophilized, ground and sieved plant material (particle size < 425 µm), providing a large surface area for colonization by microorganisms. Furthermore, temperatures between 24 and 27 °C enhanced microbial activity, likely resulting

in a faster degradation of Bt toxin in plant material. Moreover, the degradation data provided were not obtained experimentally but estimated indirectly based on a number of assumptions the validity of which remains to be proven. The authors used the bioassay data to carry out a nonlinear regression analysis by fitting a 3-parameter logistic function and, thereby, obtained an EC_{50} value, i.e. 'effective concentration of test suspension' (Sims & Holden 1996) at which larval growth of *H. virescens* larvae was only half of that in the control. Sims & Holden (1996) assumed that the EC_{50} value is inversely proportional (i.e. linear relationship) to the concentration of biologically active Bt protein in the soil and that degradation follows first-order kinetics. However, the figures shown by Sims & Holden (1996) suggest a nonlinear relationship, indicating that a simple first-order kinetics law may be inadequate to fully describe Bt toxin degradation. Also, data to the degradation of a novel Bt corn (MON 863) variety from Monsanto expressing the Cry3Bb1 toxin also implicate a nonlinear degradation rate of Bt toxin, i.e. do not follow first-order rate kinetics (Environmental Protection Agency 2002).

Based on their laboratory data, Sims & Holden (1996) concluded that the Cry1Ab protein in corn plant tissue will be unstable under field conditions and likely to degrade rapidly under normal cultivation practices. In contrast, we found in our field studies under two different cultivation practices that the Cry1Ab protein in plant tissue was stable and degraded only as the plant material degraded. Plant residues acted as a 'time-release' mechanism whereby Bt protein exposure to the ambient soil environment is a gradual process rather than an instantaneous flush into the environment.

Our data are in relatively good agreement with laboratory results that have reported variable and extended degradation times with purified Bt toxins (Tapp & Stotzky 1998), toxin in exudates from roots and in biomass of transgenic Bt corn (Saxena *et al*. 1999, 2002; Saxena & Stotzky 2000, 2001) or transgenic Bt cotton and purified Bt toxin (Palm *et al*. 1996). Some agreement between our field data was found with experiments using lyophilized and ground transgenic Bt Cry2A cotton powder in polypropylene centrifuge incubation tubes in the field (Sims & Ream 1997). These experiments were also conducted during autumn and winter. Based on their bioassay results with *H. virescens*, Sims & Ream (1997) calculated that ≈ 17.1% of the initial amount of toxin must still be present in the tissue after 120 days. From this, they further calculated that 50% had degraded after 31.7 days. The remaining difference between our data and theirs may be explained by the difference in type of plant material used and the method used to quantify the toxin concentration (as explained above in the comments to Sims & Holden 1996).

Palm *et al*. (1996) reported that in most of the experiments with transgenic Bt cotton (Cry1Ab or Cry1Ac) and purified Bt toxin (Cry1Ac), an initial rapid decline in extractable toxin concentration was observed during the first 14 days, followed by a slower decline until the end of the experiments. At the end of the experiments, Bt toxin from transgenic cotton plants was < 0.1% of the starting concentration in two experiments, and 3, 16 and 35% of the initial amount in the other experiments, measured using ELISA (Palm *et al*. 1996). In another study (Palm *et al*. 1994), the toxins of two different transgenic Bt cotton lines (expressing Cry1Ac or Cry1Ab toxin) were also detectable by ELISA at every time point during a 30-day study. The higher expressing cotton line (containing the Cry1Ab toxin) also showed a rapid decline in extractable toxin concentration during the first 7 days followed by a slow decline until the end of the experiment. However, the shapes of the degradation curves (Palm *et al*. 1994, 1996; Sims & Ream 1997) of toxin in transgenic Bt plants show a two-phase degradation: a rapid decline during the first 10–40 days followed by a slow decline. Usually, a low amount of toxin was still detectable at the end of the trials. The data from our second field experiment showed a similar trend. In contrast, in the first field experiment, the rapid initial decline occurred with a lag time of 30–60 days after the beginning of the experiment and no toxin degraded during winter when mean soil temperatures were < 5 °C (Fig. 2a), probably due to reduced microbial activity in the soil. When temperature increased in early spring (Fig. 2a), the amount of toxin again declined, resulting in a further degradation, then followed by a slow decline but not reaching zero at the end of the experiment. Consequently, the two main differences between the studies discussed above (Palm *et al*. 1994, 1996; Sims & Holden 1996; Sims & Ream 1997) and our first field study are: (i) a lag time before the first rapid decline phase, and (ii) no further degradation during winter until temperatures again increased. However, data from the second field study also showed a two-phase degradation pattern, a rapid initial decline of Cry1Ab toxin followed by a slow decline of toxin resulting in a low amount of Bt toxin persisting for several months, which is in relative good agreement with data of the studies discussed (Palm *et al*. 1994, 1996; Sims & Holden 1996; Sims & Ream 1997).

Head *et al*. (2002) did not detect the Cry1Ac toxin in soil samples taken 3 months after the last planting from fields planted with Bt cotton over several consecutive years. The authors did not separate organic matter from nonorganic matter. Their soil samples may or may not have contained an unspecified amount of Bt cotton material in an unspecified state of degradation. Hence, the plant material content of their soil samples was not quantified. But as most soil-dwelling organisms will preferentially ingest organic material (such as plant residues, detritus, dead animals, other soil organisms) rather than soil particles, no conclusions regarding the exposure of soil organisms feeding on degrading Bt plant material can be drawn from this study.

These findings are somewhat in contrast to the studies of Saxena & Stotzky (2000, 2001) who reported that Bt toxin released from corn plant material can be adsorbed onto surface-active particles in the soil. Once bound, Bt toxins retain their insecticidal activity (Saxena *et al*. 1999; Saxena & Stotzky 2000, 2001). In a field trial, Saxena & Stotzky (2000) showed that the toxin released from root exudates into soil was still detectable and insecticidally active several months after the occurrence of frost and the death of the plants. Differences between the findings of Head *et al*. (2002) and the studies cited above might be due the different crops, toxins and soil types used. It now needs to be established whether Bt toxin is released from the plant material and can be adsorbed onto surface-active particles.

Impact of temperature and rain

The US Environmental Protection Agency readily accepted data which suggested rather short degradation half-lives for Bt toxin from transgenic Bt plant material in soils, and proposed that 'conditions that favour microbial growth, including the presence of metabolizable organic matter, such as crop residues or rhizosphere secretions, and neutral pH, will favour shorter half-lives' (Environmental Protection Agency 2000). Referring to Sims & Holden (1996), they also suggested that 'degradation rates under field conditions may be higher than those shown in bulk soil experiments' (Environmental Protection Agency 2000). Temperature, a major factor driving degradation processes under natural conditions, was not considered by the Environmental Protection Agency. All laboratory trials were conducted at constant temperatures at or above 20 °C. However, in our field trials, after harvest of the corn, daily average soil temperatures declined rapidly (Figs 2a and 3a). In a cold winter, the temperature can drop well below 0 °C, particularly in regions with a continental climate such as the Midwest of the USA. During both field experiments, average soil temperatures at 10 cm depth hardly ever reached 20 °C (the maximum was 19.3 °C in the first field trial and 19.7 °C in the second), and the average soil temperature during the experiments was 7.6, and 8.2 °C, respectively. Although during the second field trial air temperatures at 5 cm above the soil showed greater fluctuation, the average temperature during the entire experiment was lower (7.3 °C) than in the soil. From this, we propose that one improvement for laboratory experiments investigating the degradation of novel proteins in transgenic plants after harvest would be to use average temperatures (or even better fluctuating temperatures) resembling those in the field of the target environment; for temperate regions, such as the low elevation parts of Switzerland, this would range at or below 10 °C.

In contrast to the important influence of temperature on the degradation of the Bt toxin, rain did not seem to have a strong influence. In our experiments, Bt toxin concentrations decreased during periods when the average rainfall was both low and high. However, more research is necessary to further assess the influence of rain on the degradation process.

Herbivore bioassays

Insecticidal bioassays using *O. nubilalis* larvae that were carried out with leaves from the first field trial confirmed the insecticidal activity of the toxin until February. Mortality was significantly higher in the (Bt+) treatment until and including December. After that, mortality overall was low and the differences between the treatments were not statistically significant, except in April.

Clear sublethal effects, i.e. lower larval weight, were detected until February when larvae had been fed transgenic Bt corn. After February, the weight data were inconclusive.

Bioassay data from the second field trial were similar those the first. Up to and including 80 days after initiation of the experiment, there were clear lethal effects on (Bt+) fed *O. nubilalis*. After that, mortality remained higher in the (Bt+) treatments, except for the last sample after 200 days, but they were no longer statistically significant. Owing to high mortality rates at the beginning and 40 days after the beginning of the experiments weight could not be recorded until 80 days after initiation of the experiment. After that, data were again, as in the first-year bioassays, inconclusive. Affected larvae died, although those that were not lethally affected developed similar to those in the control.

In general, our data show that *O. nubilalis* larvae were lethally and/or sublethally affected by the Bt toxin when toxin concentrations in leaf residues were $> 1.2\,\mu g/g$ dry weight. When toxin concentrations were $< 1.2\,\mu g/g$ dry weight, mortality data often only showed a tendency towards higher mortality in the (Bt+) treatment, but in most cases the data were not statistically significant. Weight data did not provide clear results because they could be confounded by mortality or because too little decaying leaf tissue was left for the tests. This means that it was not possible to determine whether the detected Bt toxin was still in its insecticidal state. However, data by Saxena *et al*. (2002) studying the leaching of Cry1Ab toxin from the same transgenic Bt corn line as used in our trials, showed that Bt toxin remained in its insecticidally active form after 350 days. Based on our data, we conclude that for experiments in which Bt toxin in degrading tissue is at detection limits and tissues are also mixed with artificial diet, thus resulting in a very low amount of Bt toxin in the diet, bioassay data do not always provide conclusive results and can only complement, but not substitute for, immunological analyses (ELISA).

Nevertheless, the litter bag and soil cage methods, in combination with quantitative analyses using ELISA, may be

useful for monitoring degradation of the toxin in plant residues in agricultural soils, for both pre- or post-registrational testing of transgenic plants. Further research is needed to determine whether and how the use of litter bags influences the degradation process of Bt toxin.

Extended field and laboratory research is needed to establish the degree to which nontarget soil organisms and their functions can be affected by persistent exposure to Bt toxins. Possible subtle chronic effects cannot be tested satisfactorily in the laboratory but must be monitored over long periods according to EU law. For these reasons, we recommend that, in addition to temporally extended pre-release tests, post-commercial release research and monitoring for long-term ecological implications of transgenic Bt plants on soil ecosystems is essential.

Acknowledgements

We thank R. Howald, J. Simmen, J. Müller (University of Bern, Switzerland), and K. Barmettler (ETH Zurich, Switzerland) for their technical assistance. We are grateful to Prof Dr G. Stotzky (New York University, USA), Prof Dr R. Kretzschmar (ETH Zurich), Dr L. Kuhn-Nentwig, Dr J.-P. Airoldi, H. Wandeler (University of Bern) and M. Meier (ETH Zurich) for helpful discussions and important advice.

References

GraphPad Software Inc. (2000) *GRAPHPAD PRISM 3.02*. GraphPad Software Inc., San Diego.

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 and Biochemistry*, **30**, 463–470.

Crecchio C, Stotzky G (2001) Biodegradation and insecticidal activity of the toxin from *Bacillus thuringiensis* subsp. *kurstaki* bound on complexes of montmorillonite–humic acids–Al hydroxy-polymers. *Soil Biology and Biochemistry*, **33**, 573–581.

Environmental Protection Agency (EPA) (2000) Issues pertaining to the Bt plant pesticides risk and benefit assessments. Environmental assessment: http://www.epa.gov/scipoly/sap/2000/october/brad3_enviroassessment.pdf, IIC17-IIC30.

Environmental Protection Agency (EPA) (2002) A set of scientific issues being considered by the Environmental Protection Agency regarding: Corn Rootworm plant-incorporated protectant non-target insect and insect resistance management issues. FIFRA Scientific Advisory Panel Meeting, Sheraton Crystal City Hotel, Arlington, Virginia, August 27–29, http://www.epa.gov/scipoly/sap, pp. 26–31.

Gugerli P (1979) Le test immuno-enzymatique (ELISA) et son application pour le diagnostic rapide des viroses de la pomme de terre. *Revue Suisse d'Agriculture*, **11**, 253–260.

Gugerli P (1986) Potato viruses. In: *Methods of Enzymatic Analysis* (ed. Bergmeyer HU), Vol. XI, pp. 430–446. VCH-Verlagsgesellschaft, Weinheim, Germany.

Hansen Jesse LC, Obrycki JJ (2000) Field deposition of Bt transgenic corn pollen: lethal effects on the monarch butterfly. *Oecologia*, **125**, 241–248.

Head G, Surber JB, Watson JA, Martin JW, Duan JJ (2002) No detection of Cry1Ac protein in soil after multiple years of transgenic Bt cotton (Bollgard) use. *Environmental Entomology*, **31**, 30–36.

Hellmich RL, Siegfried BD, Sears MK *et al.* (2001) Monarch larvae sensitivity to *Bacillus thuringiensis* purified proteins and pollen. *Proceedings of the National Academy of Sciences of the USA*, **98**, 11925–11930.

Hilbeck A, Baumgartner M, Fried PM, Bigler F (1998) Effects of transgenic *Bacillus thuringiensis* corn-fed prey on mortality and development time of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Environmental Entomology*, **27**, 480–487.

James C (2002) *Global Review of Commercialized Transgenic Crops: 2001, ISAAA Briefs no. 24: Preview*. ISAAA, Ithaca, NY.

Koskella J, Stotzky G (1997) Microbial utilization of free and clay-bound insecticidal toxins from *Bacillus thuringiensis* and their retention of insecticidal activity after incubation with microbes. *Applied and Environmental Microbiology*, **63**, 3561–3568.

Koziel MG, Beland GL, Bowman C *et al.* (1993) Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. *Biotechnology*, **11**, 194–200.

Lee KE (1985) *Earthworms. Their Ecology and Relationships with Soils and Land Use*. Academic Press, Sydney.

Losey JE, Rayor LS, Carter ME (1999) Transgenic pollen harms monarch larvae. *Nature*, **399**, 214.

Palm CJ, Donegan KK, Harris D, Seidler RJ (1994) Quantification in soil of *Bacillus thuringiensis* var. *kurstaki* δ-endotoxin from transgenic plants. *Molecular Ecology*, **3**, 145–151.

Palm CJ, Schaller DL, Donegan KK, Seidler RJ (1996) Persistence in soil of transgenic plant produced *Bacillus thuringiensis* var. *kurstaki* δ-endotoxin. *Canadian Journal of Microbiology*, **42**, 1258–1262.

Saxena D, Flores S, Stotzky G (1999) Insecticidal toxin in root exudates from Bt corn. *Nature*, **402**, 480.

Saxena D, Flores S, Stotzky G (2002) Vertical movement in soil of insecticidal Cry1Ab protein from *Bacillus thuringiensis*. *Soil Biology and Biochemistry*, **34**, 111–120.

Saxena D, Stotzky G (2000) Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic *Bt* corn *in vitro* and *in situ*. *FEMS Microbiology Ecology*, **33**, 35–39.

Saxena D, Stotzky G (2001) *Bt* toxin uptake from soil by plants. *Nature Biotechnology*, **19**, 199.

Scheffer F, Schachtschabel P (1989) *Lehrbuch der Bodenkunde*. Enke, Stuttgart.

Sears MK, Hellmich RL, Stanley-Horn DE *et al.* (2001) Impact of Bt corn pollen on monarch butterfly populations: a risk assessment. *Proceedings of the National Academy of Sciences of the USA*, **98**, 11937–11942.

Sims SR, Holden LR (1996) Insect bioassay for determining soil degradation of *Bacillus thuringiensis* subsp. *kurstaki* CryIA(b) protein in corn tissue. *Environmental Entomology*, **25**, 659–664.

Sims SR, Ream JE (1997) Soil inactivation of the *Bacillus thuringiensis* subsp. *kurstaki* CryIIA insecticidal protein within transgenic cotton tissue: laboratory microcosm and field studies. *Journal of Agricultural and Food Chemistry*, **45**, 1502–1505.

Tapp H, Calamai L, Stotzky G (1994) Adsorption and binding of the insecticidal proteins from *Bacillus thuringiensis* subsp. *kurstaki* and subsp. *tenebrionis* on clay minerals. *Soil Biology and Biochemistry*, **26**, 663–679.

Tapp H, Stotzky G (1995a) Dot blot enzyme-linked immunosorbent assay for monitoring the fate of insecticidal toxins from *Bacillus*

thuringiensis in soil. *Applied and Environmental Microbiology*, **61**, 602–609.

Tapp H, Stotzky G (1995b) Insecticidal activity of the toxins from *Bacillus thuringiensis* subspecies *kurstaki and tenebrionis* adsorbed and bound on pure and soil clays. *Applied and Environmental Microbiology*, **61**, 1786–1790.

Tapp H, Stotzky G (1998) Persistence of the insecticidal toxin from *Bacillus thuringiensis* subsp. *kurstaki* in soil. *Soil Biology and Biochemistry*, **30**, 471–476.

Topp W (1981) *Biologie der Bodenorganismen*. Quelle und Meier, Heidelberg.

Venkateswerlu G, Stotzky G (1992) Binding of the protoxin and toxin proteins of *Bacillus thuringiensis* subsp. *kurstaki* on clay minerals. *Current Microbiology*, **25**, 225–233.

Wandeler H, Bahylova J, Nentwig W (2002) Consumption of two Bt and six non-Bt corn varieties by the woodlouse *Porcellio scaber*. *Basic and Applied Ecology*, **3**, 357–364.

Wraight CL, Zangerl AR, Carroll MJ, Berenbaum MR (2000) Absence of toxicity of *Bacillus thuringiensis* pollen to black swallowtails under field conditions. *Proceedings of the National Academy of Sciences of the USA*, **97**, 7700–7703.

Zscheischler J, Estler MC, Staudacher W *et al*. (1984) *Handbuch Mais. Anbau, Verwertung*. Fütterung-Verlagsunion Agrar, Frankfurt a Main.

Zwahlen C, Hilbeck A, Howald R, Nentwig W (in press) Effects of transgenic Bt corn litter on the earthworm *Lumbricus terrestris*. *Molecular Ecology*.

This study is part of Claudia Zwahlen's doctoral research under the supervision of Angelika Hilbeck and Wolfgang Nentwig. This PhD project focuses on the degradation of transgenic *Bacillus thuringiensis* (Bt) corn in soil. A. Hilbeck is agroecologist specializing in plant and insect ecology and biocontrol. Paul Gugerli is a virologist and plant pathologist with special experience in immunological analyses of viruses and specific proteins. W. Nentwig is Professor in Community Ecology.
