Exposure and Nontarget Effects of Transgenic *Bt* Corn Debris in Streams

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ABSTRACT   Corn (*Zea mays* L.) transformed with a gene from the bacterium *Bacillus thuringiensis* (*Bt*) comprises 49% of all corn in the United States. The input of senesced corn tissue expressing the *Bt* gene may impact stream-inhabiting invertebrates that process plant debris, especially trichopteran species related to the target group of lepidopteran pests. Our goal was to assess risk associated with transgenic corn debris entering streams. First, we show the input of corn tissue after harvest was extended over months in a stream. Second, using laboratory bioassays based on European corn borer [*Ostrinia nubilalis* (Hübner)], we found no bioactivity of Cry1Ab protein in senesced corn tissue after 2 wk of exposure to terrestrial or aquatic environments. Third, we show that *Bt* near-isolines modify growth and survivorship of some species of invertebrates. Of the four nontarget invertebrate species fed *Bt* near-isolines, growth of two closely related trichopterans was not negatively affected, whereas a tipulid crane fly exhibited reduced growth rates, and an isopod exhibited reduced growth and survivorship on the Cry1Ab near-isoline but not on the stacked Cry1Ab + Cry3Bb1 near-isoline. Because of lack of evidence of bioactivity of *Bt* after 2 wk and because of lack of nontarget effects on the stacked near-isoline, we suggest that tissue-mediated differences, and not the presence of the Cry1Ab protein, caused the different responses among the species. Overall, our results provide evidence that adverse effects to aquatic nontarget shredders involve complex interactions arising from plant genetics and environment that cannot be ascribed to the presence of Cry1Ab proteins.

KEY WORDS   *Bacillus thuringiensis*, genetically modified crops, nontarget effects, stream ecology

Genetically engineered corn (*Zea mays* L.) transformed with a gene from the bacterium *Bacillus thuringiensis* (*Bt*) was first introduced in 1996. In 2008, 57% of all U.S. corn was planted in insect resistant *Bt* hybrids (USDA 2008). Beyond a potential reduction in insecticide use, the advantage of *Bt* transgenic crops over conventional insecticides is their high specificity such that potential toxic effects on nontarget insects should be minimal or nonexistent (MacIntosh et al. 1990, Schuler et al. 1999, Betz et al. 2000). Like any insect management technology, however, transgenic crops expressing the *Bt* gene may present a risk to nontarget organisms, particularly beneficial insects that are taxonomically similar to the target pests (Obrycki et al. 2001). If corn leaves enter streams, the presence of the *Bt* protein in plant material may impact invertebrates that commonly exist and consume plant detritus (also called shredders, Klug and Cummins 1979). Because of their close phylogenetic relationship with Lepidoptera (Grimaldi and Engel 2005), shredders in the order Trichoptera (caddisflies) may be affected by *Bt* expressed in corn leaves that targets lepidopteran pests.

Most types of *Bt* proteins expressed in transgenic crops are biologically active on only one order of insects with susceptibility determined by specific receptors in the membranous lining of the midgut epithelial cells (Shelton et al. 2002). Previous studies have evaluated the potential impact of the transgenic plants expressing the *Bt* Cry toxins on nontarget phytophagous and beneficial species. Most of these studies show no significant effect of these toxins on nontarget arthropods (reviewed by Romeis et al. 2006), suggesting only direct impact on targeted pest species which are selectively susceptible to the mode of action of the expressed *Bt* lepidopteran-specific Cry1Ab proteins.

To our knowledge, only one study has examined the potential nontarget effects of the plant incorporated *Bt* δ-endotoxins on macroinvertebrates in the aquatic environment (Rosi-Marshall et al. 2007), although no study has specifically tested whether the endotoxin persists in the aquatic environment in intact leaf tissue (but see Prihoda and Coats 2008). Given the intimate association between the ecology of first order streams and upland agricultural use, the likelihood of transgenic plant tissue being delivered through wind and water action to these streams is high, although has yet to be quantified over broad spatial scales (but see Stone et al. 2005, Rosi-Marshall et al. 2007). Ecologists

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are becoming increasingly aware of the implications of the cross-ecosystem movement of organisms and resources and the subsequent cascading effects the presence of such allochthonous subsidies can have on food web dynamics (Wallace et al. 1997, 1999). Corn, being the greatest in terms of individual biomass of the transgenic crops, may be the most abundant input of crop detritus to those streams draining agricultural fields.

To assess the risk of senesced *Bt* corn to aquatic macroinvertebrates, we (1) measured the input and standing stock of corn leaves in an agricultural stream, (2) determined the duration of Cry1Ab protein bioactivity in senesced corn tissue subjected to terrestrial and stream field conditions, and (3) assessed possible nontarget effects of senesced transgenic corn tissue to selected stream macroinvertebrates. In addition to using a nontransformed near-isoline as control, we used a near-isoline expressing Cry1Ab as well as a near-isoline expressing two stacked traits (Cry1Ab and Cry3Bb1). The stacked near-isoline served as a second Cry1Ab treatment and to screen the selected taxa for potential Cry3Bb1 susceptibility.

Materials and Methods

Tissue Input and Biomass Standing Stock. To measure tissue input, we deployed chicken-wire mesh (2.5 cm diameter) cages measuring 100 cm wide, 50 cm high, and 30 cm deep beside the stream at the Central Maryland Research and Education Center (CMREC), University of Maryland, near Clarksville, MD, over 2 yr. Starting at the western-most extent of the stream-cornfield interface, one cage was placed every 20 m beside the stream, with a total of eight cages deployed. Cages were deployed at least 2 wk before harvest. Harvest occurred 12 October 2006 and 19 October 2007, and in both cases there was no tillage, with stubble and debris remaining on the field. The cages were placed on the stream bank within 1 m of the stream, with the open side facing the corn field to catch tissue as it moved from the field toward the stream. The distance from the edge of the corn field to the stream edge varied from 20 to 30 m, with a slope ranging from 3.2 to 4.5%, and the vegetation consisted of perennial grasses up to 0.5 m in height. Cages were checked weekly between September 2006 and May 2007 and again between September 2007 and April 2008. Corn tissue found in cages was removed, dried at 60°C for 24 h, and the dry mass was recorded. The dried tissue was placed in a muffle furnace for 2 h at 550°C, the mass of the ash was recorded, and the ash-free dry mass (AFDM) was calculated (AFDM = dry mass of tissue − ash mass). The determination of AFDM allowed direct comparison to standing stock (described below) and eliminated the effect of deposition of minerals on corn leaves (Benfield 2006).

We performed biweekly benthic standing stock surveys for corn tissue at the stream site from October 2006 to May 2007 and from November 2007 until April 2008. The benthic surveys involved placing a meter stick across the 0.5 m wide streambed at eight ran-

domly chosen locations between the eight cages. Any identifiable corn tissue visible on the streambed or caught on submerged vegetation within 25 cm of either side of the meter stick was collected (0.5 m^2), dried for at least 48 h at 65°C, weighed for dry mass, and ashed for at least 3 h at 550°C in a muffle furnace, and AFDM was determined.

Corn Leaf Source for Bioassays. As sources of senesced corn tissue for both the time-course bioassay and the nontarget bioassay, we used plants from three hybrid families (1, 2, 3) with different genetic backgrounds, each with the same maturation time (115 d) and well adapted for the northeastern U.S. growing region. Within each hybrid family we used three near-isolines, all of which contained the Roundup Ready gene (NK603): (1) a non-*Bt* expressing near-isoline, (2) a near-isoline expressing the Cry1Ab gene (MON 810 'YieldGard'), and (3) a near-isoline stacked with both Cry1Ab and Cry3Bb1 genes (MON 810 and MON 863 'YieldGard Plus') for a total of nine near-isolines (three near-isolines for each hybrid). For hybrid families 1 and 2, the designation was an experimental number for each near-isoline. For hybrid family 3, the near-isoline names were Dekalb DKC 63–80 (non-*Bt*), Dekalb DKC 63–81 (Cry1Ab expressing), and Dekalb DKC 63–74 (Cry1Ab + Cry3Bb1 expressing).

All nine near-isolines were grown in three locations (I, II, III) in field plots at the Western Maryland Research and Education Center, University of Maryland, near Keedysville, MD, until plant senescence. Three locations were used to ensure that plot-specific environmental factors did not confound our treatments. All plots were cultivated on no-till or minimum-till Hagerstown silt loam (mesic Typic Haplualf, pH 7) with fertilizer applied on the basis of soil tests and herbicides applied for pre- and postemergence weed suppression based on standard practices. Plots were planted during the first week of May 2005 with a vacuum planter in 76-cm rows. When grain moisture reached 22% during senescence in late September, leaves from all nodes were removed and air dried under greenhouse conditions to remove surface moisture.

European Corn Borer Bioassays. Because of our interest in the bioactivity of the *Bt* protein within leaf tissue, we used a bioassay approach instead of using an enzyme-linked immunosorbent assay (ELISA) to detect sublethal effects of *Bt* in corn leaves on the European corn borer, *Ostrinia nubilalis* (Hübner). This target pest has been used as a sensitive indicator of the toxin since *Bt* corn was first developed (Chaufaux et al. 2001). The corn leaf material was freeze-dried and ground in a tissue grinder using a 1-mm sieve plate and kept at −80°C until used for bioassay.

A meridic diet for European corn borer (Southland Products, Lake Village, AR) was mixed with the lyophilized corn leaf powder at a concentration of 6 g/liter of diet. Initial range-finding assays determined that this concentration of senesced *Bt* leaf tissue at harvest resulted in 40–60% growth inhibition of first instars after 7 d of feeding. For each bioassay treatment and representing one experimental unit, cohorts

of 16 pairs of neonates were reared individually in 97.5-ml Solo plastic cups containing 15 ml of diet. Previous studies had shown no interference between pairs of larvae within cups and that this arrangement optimized the allocation of resources in bioassays. Eggs were obtained from the USDA–ARS Corn Insects and Crop Genetics Research Laboratory, Ames, IA, and shipped overnight, usually 5–7 d before each assay. Eggs were incubated in a growth chamber under temperature regimens manipulated to schedule a supply of first-instar larvae for bioassay.

After 7 d, each cup was examined to record larval survival, instar, and larval weight. Surviving larvae of each cohort were pooled and weighed together. The average weight gain per larva was calculated by dividing the pooled weight by the number of larvae in the cohort minus the average initial weight and used in our analysis of larval growth.

To determine initial sublethal responses of European corn borer larvae to harvested corn tissue, we used senesced corn tissue at 0 wk from all three near-isolines of each of the three hybrids for a total of nine treatments. Plot locations (I, II, III) were used as replicates through time in the laboratory for a total of 27 experimental units in the experiment. The *Bt* and non-*Bt* tissue of each hybrid group sampled directly from the corn plots served as positive and negative controls to compare with relative differences in sublethal responses after environmental exposure.

For the time course bioassay, we used senesced leaf tissue from one location (I) because preliminary analysis indicated no differences in insect responses from the three locations. Replicates were subsampled from location I tissue. Leaf tissue was cut into sections of ≤10 cm and placed in standard plastic produce bags (7 by 11-mm mesh, 15 by 35 cm overall) in packets of ≈5 g. One hundred eight (3 hybrid groups × 3 near-isolines × 3 replicates × 4 sampling periods) bags were used that were set in a full-sun riparian terrestrial habitat, as well as another 108 in a headwater-stream, aquatic habitat at the CMREC site. Three replicate bags of each near-isoline from each hybrid group were collected from each habitat at each sampling time (2, 4, 6, and 8 wk), and remaining leaf tissue was removed from bags, freeze-dried, and ground as described above. Leaf powder from one replicate bag representing each near-isoline and hybrid group by habitat and time combination was incorporated with diet and fed to one group of 16 pairs of neonates.

Nontarget Bioassays. Bioassays of four test species were performed in 250-ml aerated Erlenmeyer flasks filled to the neck with a 50:50 mixture of Clarksville site stream water and deionized water. Because the Clarksville site has elevated dissolved nutrients concentrations, this water ratio was a used as a result of pilot testing with a goal of closely approximating the stream water quality but avoiding harmful nutrient concentrations in the bioassay chambers as a result of overnight evaporation. Water lost to evaporation was replaced daily with deionized water. All assays were performed in a walk-in growth chamber maintained at 14°C on a 16:8 L:D cycle. Each assay consisted of 60 flasks, with 20 flasks assigned to each of the three near-isolines from hybrid one grown in location I. All corn tissue was "conditioned" for 2 wk before bioassay initiation in 20:80 filtered Clarksville site stream water to deionized water to ensure bacterial and fungal growth on the leaf tissue, which is an integral component of detritus for shredder feeding (Bärlocher and Kendrick 1975, Benfield 2006). This low stream water ratio was used for corn tissue conditioning because higher concentrations of stream water caused excessive bacterial and fungal growth in pilot testing. All organisms were collected from headwater streams located at the CMREC site between July and September of 2006. The bioassays consisted of no-choice feeding of conditioned corn leaf tissue ad libitum representing the three near-isolines for 30 d.

For nontarget bioassays, we selected four aquatic leaf-chewing invertebrates (hereafter "shredders", sensu Klug and Cummins 1979) common in headwater streams in Maryland during and after the corn harvest as test organisms for this study. Two of these nontarget taxa were trichopterans: *Lepidostoma* spp. and *Pycnopsyche* cf. *scabripennis* (Rambur). No key exists for *Lepidostoma* larvae, so species identification is not possible. Because of the close phylogenetic relationship between Trichoptera (caddisflies) and Lepidoptera (Wiggins 1995, Grimaldi and Engel 2005), the caddisflies may be especially impacted by the input of tissue containing lepidopteran-specific *Bt* Cry1Ab protein into streams. Caddisflies are one of the dominant groups within streams, serve important trophic roles including decomposition of allochthonous inputs of vegetation, and are susceptible to changes in water quality (Klug and Cummins 1979, Allan 1995, Wiggins 1995). Rosi-Marshall et al. (2007) found two trichopteran species to be susceptible to Cry1Ab-containing corn tissue, although several other studies have shown no effect of other sources of *Bt* on trichopterans (Merritt and Wipfli 1991 as *Bt*i, Eidt 1985 as Thuricide).

The third test species was a crane fly larva [Diptera: Tipulidae, *Tipula (Nippotipula)* cf. *abdominalis* (Say)] and was selected because of the high volume of detritus that these larvae process during their maturation (Vannote and Sweeney 1985). The fourth test organism selected was a crustacean, the aquatic isopod, *Caecidotia communis* (Williams), because of its ubiquity and high numbers in agricultural headwater streams in Maryland. Although only the trichopterans were expected to respond to the Cry1Ab toxin, high doses of *Bt* applied under laboratory conditions have caused a decrease in detritus decomposition (Kreutzweiser et al. 1993), and thus we included a wider range of nontarget taxa.

For the trichopterans, five larvae of *Lepidostoma* spp. (300 total across the three near-isolines) and three larvae of *P. scabripennis* (180 total across the three near-isolines) were randomly picked for each experimental unit and photographed for digital measurement of initial head capsule width. Different test subject quantities were used based on ad libitum feeding requirements over the test duration determined in

pilot studies. Larval length was not used as a metric because of the tendency of the larvae to not reclaim their cases once extracted in pilot studies, with changes in their behavior ensuing in the bioassays. Larvae were placed in flasks with 10 g (ww) of leaf tissue placed in each flask. Survival was recorded after 30 d. Larvae were photographed again, removed from their case, and dried at 60°C for 24 h. Average dry mass was recorded for each replicate. Head capsule width measurements were made using ImageJ 1.36b (National Institutes of Health, Bethesda, MD).

For *T. abdominalis*, one randomly picked larva was used in each experimental unit for a total of 60 larvae across the three near-isolines. Additional larvae could not be included in each bioassay chamber because of conspecific predation. After blotting on paper towels, initial wet mass was recorded, and larvae were placed in flasks with 10 g (ww) of leaf tissue in each flask. Survival and wet mass (after blotting on paper towels) were recorded for each larva after 30 d. At the termination of the bioassay, larvae were dried at 60°C for 24 h, and dry mass was recorded and used in a wet-dry regression to calculate initial dry mass (dw = 0.0676ww, $r^2 = 0.81$, $n = 47$, range = 0.077–0.533 g).

For *C. communis*, gravid adult females were held in a beaker with stream water and leaf debris and checked daily for newly emerged first-instar larvae. Five randomly collected larvae were measured for individual length and placed in a flask with 5 g (ww) of leaf tissue. With 20 flasks for each of the three near-isolines, this experiment included a total of 300 organisms. We used less tissue in these bioassay chambers because the extremely small larvae were otherwise almost impossible to find at the conclusion of the bioassay. Individual length and average mass of each larva were recorded after 30 d, and all larvae for each replicate were dried at 60°C for 24 h to determine average dry mass.

Statistical Analyses. All statistical analyses were performed using SAS 9.1 (SAS Institute, Cary, NC). The European corn borer bioassay results were divided into three analyses: (1) assays performed with tissue before environmental exposure (at harvest, time = 0), (2) assays performed using tissue exposed to the terrestrial environment, and (3) assays performed using tissue exposed to the stream environment. For the first analysis at harvest, analysis was with a two-way analysis of variance (ANOVA; Proc Mixed), with hybrid group and near-isoline as fixed factors, and blocked by bioassay replication to evaluate main and interactive effects. This analysis indicated that hybrid group was not significant. For the other analyses through time, a two-way ANOVA was used with time and near-isoline treatments as fixed factors, blocked by bioassay, and replicated by hybrid group. The repeated option in Proc Mixed was used to adjust for autocorrelation among sampling times. Post hoc differences were determined using a Tukey-Kramer adjustment with $\alpha = 0.05$. Data compliance with model assumptions of normality and variance heterogeneity were checked using Shapiro-Wilk's test and by using rank correlation between absolute residuals and predicted values, re-

spectively (Proc Univariate). All survival results were arcsine square-root transformed before analysis. A one-way ANOVA was used to evaluate differences between near-isoline treatments for growth and survival for all nontarget bioassays. A probit analysis (Proc Probit) was used to evaluate differences in survival between treatments for *Lepidostoma* spp. and *T. abdominalis* because of a violation of the assumption of normality in the ANOVA model.

Results

Tissue Input and Biomass Standing Stock. We measured the standing stock and lateral input of corn debris into an agricultural stream adjacent to a corn field from October (before harvest) through March for 2 yr. In April of both years, corn debris gradually stopped moving toward the stream because of new vegetation growth trapping the tissue in place. Corn debris was nearly always found in the stream through the observation period, although the amounts varied widely with date and location in the stream (Fig. 1). Input occurred just after harvest, but peak input was delayed until February (2008) or March (2007).

European Corn Borer Bioassay. To obtain a robust estimate of Cry1Ab protein activity in senesced leaves before environmental exposure, we measured the growth response of the target European corn borer on corn leaves at time of harvest. Near-isoline was a significant main effect in determining instar ($F_{(2,24)} = 7.3$; $P = 0.003$), larval weight gain ($F_{(2,24)} = 9.68$; $P = 0.001$), and percent growth inhibition ($F_{(2,24)} = 8.3$; $P = 0.002$). European corn borer larvae gained 33.3 and 43.3% less body weight when feeding on tissue expressing Cry1Ab and Cry1Ab+Cry3Bb1 corn tissue, respectively, compared with senesced non-*Bt* corn tissue (Fig. 2a). Stadial development was also delayed 10.5–14.5% by feeding on *Bt* corn tissue.

To test the hypothesis that the Cry proteins remain biologically active in corn leaves after harvest in the environment, leaf tissue collected from the two habitats at 2-wk intervals was tested using the same diet-incorporated feeding bioassay. No significant differences were found in growth parameters across near-isolines or the interaction of near-isoline with the duration of environmental exposure to the elements in the terrestrial or stream environments (Table 1; Fig. 2b and c). These results indicate that Cry1Ab protein bioactivity was not detectable in any of the tissue after 2 wk of exposure in either environment. Considered in tandem with the corn tissue input results above, a relatively small portion of the corn debris with active *Bt* entered the stream at harvest time.

Nontarget Organism Bioassays. We found no negative effects from the Cry1Ab near-isoline on the growth of the two caddisfly species. For *Lepidostoma* spp. larvae, we did not find any significant differences between the near-isoline treatments for initial head width, final head width, change in head width, or final dry weight (Table 2). No differences in survival among near-isolines were detected using a probit analysis (2df, Wald $\chi^2 = 0.037$, $P = 0.98$). For *P. scabrip-*
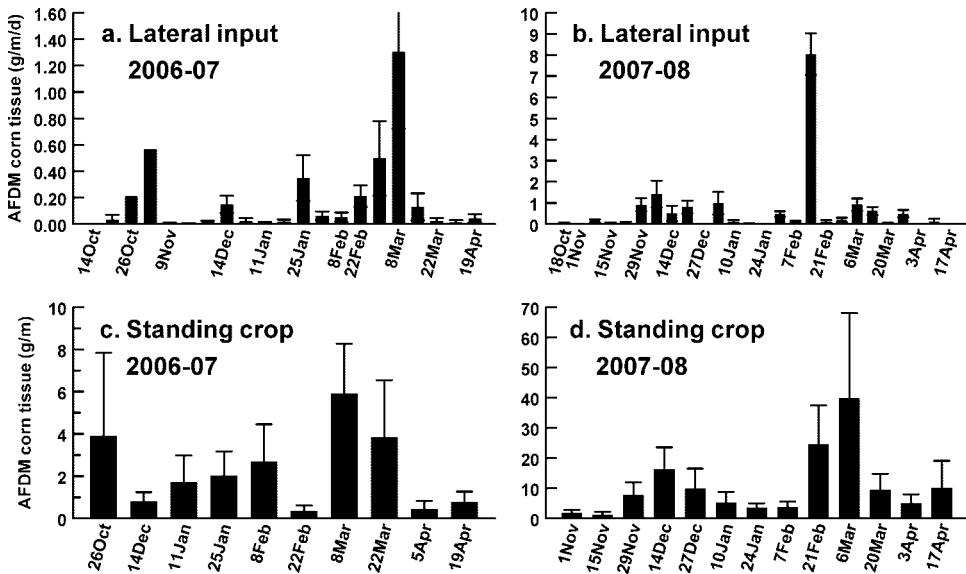
Fig. 1. Movement of corn tissue subsequent to harvest from a field to an adjacent stream expressed as means ± SE. (a) Lateral input during 2006–2007. (b) Lateral input during 2007–2008. (c) Standing stock biomass of corn during 2006–2007. (d) Standing stock biomass of corn during 2007–2008.

ennis larvae, final dry mass was significantly greater in the stacked near-isoline compared with either the non-*Bt* or the Cry1Ab near-isolines (Table 2).

We found significant negative effects in the crane fly larva and the isopod feeding on Cry1Ab near-isoline leaf tissue (Table 2). After a month of feeding on corn tissue, we found that the crane fly larvae on the Cry1Ab near-isoline treatment had the lowest change in mass compared with larvae on the control and stacked near-isoline treatments, with growth reduced by 19.6% in the Cry1Ab treatment compared with the non-*Bt* treatment. A probit analysis indicated that there was no significant difference in survival across the three near-isoline treatments (2 df, Wald $\chi^2 = 2.3336$, $P = 0.3114$). For the isopod, *C. communis*, we found significant differences in all final parameters, including final mass, change in length, and survival (Table 2). Similar to the *T. abdominalis* results, the isopods exposed to the Cry1Ab near-isoline treatment had significantly shorter length (49.7%), less mass (50.0%), and lower percent survival (43.3%) compared with isopods exposed to the non-*Bt* near-isoline treatment. Parameter values for the Cry1Ab+Cry3Bb1 stacked near-isoline treatment were similar to those values for the non-*Bt* near-isoline treatment.

Discussion

The scenario that we considered in this set of experiments is the movement of senesced corn leaf tissue from field to agricultural headwater stream after harvest. We observed a delayed input of senesced corn tissue into a stream adjacent to a corn field, with peak input occurring several months after harvest. When leaf debris finally enters the stream, it is colonized by fungi

and bacteria and consumers that are adapted for shredding the leaf material and feeding on the matrix of leaf components and associated microbes (Arsuffi and Suberkropp 1985, 1988). Through the enzymatic degradation of the leaf material and physical impact of the running water, the Cry1Ab proteins will continue to degrade over time (Yao-yu et al. 2007). Our goal was to determine whether the Cry1Ab protein remains active after entering streams and to determine potential lethal or sublethal impacts on local aquatic shredders.

The degradation of the Cry1Ab protein in the environment is an integral component of the exposure analysis in the assessment of risk to nontarget organisms from plant incorporated protectants in genetically modified crop tissue. Several studies have examined the degradation rates of the *Bt* Cry proteins associated with a soil matrix (Palm et al. 1994, 1996; Tapp and Stotzky 1998; Saxena and Stotzky 2000; Zwahlen et al. 2003) and found low levels of bioactive Cry proteins many months after tissue senescence (but see Margarit et al. 2008). However, the surface of the soil environment exhibits harsher conditions than the soil matrix with the addition of UV light to the list of biotic and abiotic degradation stressors (Koskella and Stotzky 1997), and the stream environment exhibits even harsher conditions including constant physical abrasion because of water flow that may lead to faster decomposition of the Cry protein activity.

The results from our bioassays using European corn borer verified the bioactivity of the Cry1Ab proteins in senesced corn before environmental exposure. The subsequent exposure of the senesced leaf tissue to environmental conditions in both terrestrial and aquatic environments eliminated any detectable bioactivity against the corn borer after 2 wk. This is especially relevant because it is a shorter interval than
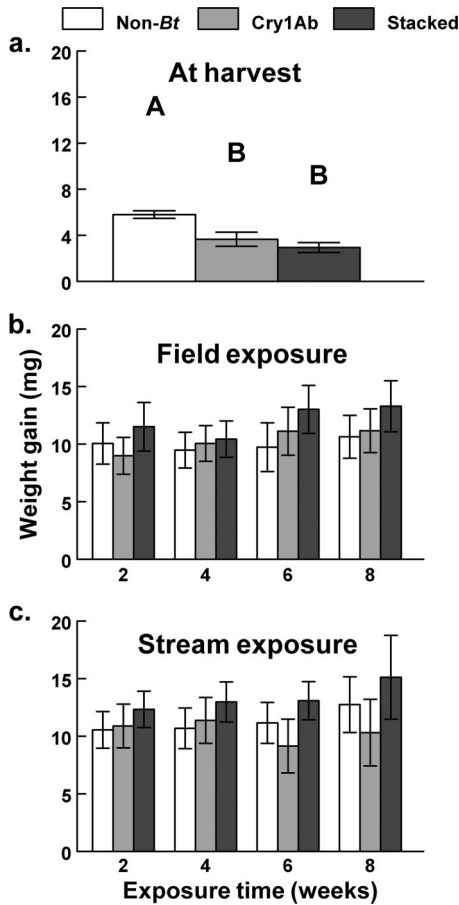
Fig. 2. Bioactivity of *Bt* in senesced corn tissue as determined by growth change (mean weight gain ± SE) of target European corn borer larvae after 7 d on diet incorporating tissue from three near-isolines. (a) Corn tissue at 0 wk (=time of harvest). (b) Corn tissue after exposure to the terrestrial environment in a riparian area between corn field and stream. (c) Corn tissue after exposure to the aquatic environment of an agricultural stream.

Table 1. ANOVA results for *Bt* bioassay using the growth response of European corn borer to corn debris in diet

Exposure	Parameter	Source	NDF, DDF	F	P
Terrestrial	Instar	Week (W)	3, 22.1	2.01	0.14
		Near-isoline (I)	2, 25.4	0.71	0.50
		W × I	6, 22.1	0.61	0.72
	Weight gain	Week (W)	3, 70.2	3.06	0.03
		Near-isoline (I)	2, 24.4	0.40	0.68
		W × I	6, 70.2	1.84	0.10
	Percent inhibition	Week (W)	3, 20.9	0.55	0.65
		Near-isoline (I)	2, 27.7	1.82	0.18
		W × I	6, 20.9	0.23	0.96
Stream	Instar	Week (W)	3, 17.9	1.32	0.30
		Near-isoline (I)	2, 24.1	0.67	0.52
		W × I	6, 17.8	0.45	0.83
	Weight gain	Week (W)	3, 18.1	1.23	0.33
		Near-isoline (I)	2, 24.7	0.71	0.50
		W × I	6, 18.4	0.53	0.78
	Percent inhibition	Week (W)	3, 14.4	0.07	0.98
		Near-isoline (I)	2, 24.5	1.65	0.21
		W × I	6, 14.3	0.36	0.89

NDF, numerator degrees of freedom; DDF, denominator degrees of freedom.

the delay that we observed between harvest and peak input into a stream adjacent to a corn field. The lack of detectable bioactivity against European corn borer after 2 wk is also relevant to the nontarget bioassay results because all of the corn tissue used in the nontarget bioassays had been "conditioned" in stream water for 2 wk before incorporation in the bioassay. Both of these factors (delayed input and conditioning time) suggest that any differences in the growth and survival of the nontarget taxa are not likely caused by exposure to the Cry proteins.

The sublethal effects exhibited by *T. abdominalis* and *C. communis* to the Cry1Ab treatment compared with the non-*Bt* near-isoline was surprising because of the taxonomic specificity of the Cry proteins (MacIntosh et al. 1990, Betz et al. 2000), as well as a previous study showing no response of two terrestrial isopod species to purified Cry1Ab protein (Clark et al. 2006). However, these negative responses to the Cry1Ab

treatment were tempered by the lack of a difference between the Cry1Ab+Cry3Bb treatment compared with the non-*Bt* near-isoline in both species. The lack of response in the stacked *Bt* near-isoline suggests that another factor beyond the Cry proteins is responsible for the negative responses. Although our results indicate that it is difficult to link Cry1Ab with the negative growth responses of common aquatic shredders, isolating the causal factor will require additional research. Because these lines are near-isolines, genetic differences may be responsible. We also suggest that, although the seeds of the tested hybrid represented three genetically similar lines, corn plants from each line likely responded differentially to abiotic and biotic stresses during the course of the growing season. Senesced corn leaves at the time of harvest are likely to have phenotypic characteristics (e.g., physical, chemical, microbial factors) that vary across near-isolines as a result of growing conditions and production effects (Reynolds et al. 2005). Thus, tissue-mediated differences may be responsible for the mixed responses of shredders among the near-isoline treatments. Even so, chemical analyses indicated no significant differences in nitrogen and NDF (lignin, cellulose, and hemicellulose) between any near-isolines within a hybrid (Swan et al. 2009). Micronutreint analyses were not performed, however, and may be one source of tissue-mediated effects at work in this study.

The trichopteran, *Lepidostoma* spp., did not respond significantly to the near-isoline treatments. This result contrasts with recent results from Rosi-Marshall et al. (2007), who showed significantly lower length measurements in *Bt* treatments versus non-*Bt* treatments using *Lepidostoma liba*. The discrepancy in the *Lepidostoma* spp. bioassay results may be attributed to differences in our methods, including a greater tissue conditioning time and the use of non-*Bt* near-isoline tissue within the same corn hybrid family.

Table 2. ANOVA results for nontarget bioassays among the three corn near-isolines

Test organism (class: order)	Parameter	NDF, DDF	F	P	Means ± SE for each near-isoline		
					Non-*Bt*	Cry1Ab	Stacked
Lepidostoma spp. (Insecta: Trichoptera)	Initial head width (mm)	2, 45	0.06	0.945	0.42 ± 0.03	0.41 ± 0.04	0.42 ± 0.02
	Final head width (mm)	2, 45	0.05	0.956	0.94 ± 0.01	0.94 ± 0.01	0.94 ± 0.01
	Head change (mm)	2, 44	0.34	0.716	0.08 ± 0.01	0.09 ± 0.02	0.08 ± 0.01
	Final dry mass (mg)	2, 45	1.73	0.189	5.4 ± 0.7	4.6 ± 0.7	3.7 ± 0.7
Pycnopsyche scabripennis (Insecta: Trichoptera)	Initial head width (mm)	2, 57	0.44	0.646	1.14 ± 0.07	1.22 ± 0.06	1.15 ± 0.07
	Final head width (mm)	2, 51	0.32	0.726	2.21 ± 0.07	2.14 ± 0.07	2.13 ± 0.08
	Head change (mm)	2, 51	1.10	0.339	1.07 ± 0.07	0.91 ± 0.09	0.98 ± 0.06
	Final dry mass (mg)	2, 51	6.42	0.003	8.3 ± 1.0	10.2 ± 1.0	13.3 ± 1.0
	Survival (%)	2, 57	2.24	0.116	0.56 ± 0.07	0.77 ± 0.07	0.72 ± 0.08
Tipula abdominalis (Insecta: Diptera)	Initial mass (mg)	2, 56	0.66	0.521	54.7 ± 12.7	55.4 ± 12.7	65.2 ± 12.7
	Mass change (mg)	2, 45	3.93	0.027	406 ± 49	327 ± 47	402 ± 46
	Increase in mass (%)	2, 45	1.68	0.197	5.5 ± 0.6	4.2 ± 0.6	6.0 ± 0.9
Caecidotia communis (Malacostraca: Isopoda)	Initial length (mm)	2, 45	0.52	0.599	1.65 ± 0.03	1.68 ± 0.03	1.65 ± 0.03
	Length change (mm)	2, 36	9.97	<0.001	2.0 ± 0.2	1.0 ± 0.1	1.8 ± 0.1
	Final mass (mg)	2, 35	4.71	0.016	2.0 ± 0.3	1.2 ± 0.2	2.4 ± 0.3
	Survival (%)	2, 45	3.71	0.032	55 ± 7	31 ± 8	58 ± 8

The increasing rarity of true non-*Bt* isolines will make it difficult to untangle any existing causal relationships in similar studies in the future. Our results indicate a need for an aquatic artificial diet that would allow the partitioning and testing of individual tissue components, as well as the direct delivery of the Cry proteins to aquatic nontarget organisms in a tier 1 test consistent with Rose (2007).

Two caveats should be considered in the extrapolation of our results to existing populations in agricultural headwater streams. The presence of riparian buffers or filter strips in many production areas would likely decrease the input of corn tissue into headwater streams bordering agricultural production. USDA conservation programs and scientific evidence regarding water quality and nitrogen removal are encouraging the implementation of conservation buffers adjacent to streams to mitigate agricultural impact on waterways (Mayer et al. 2005). Second, the lack of food choice in our nontarget organism bioassays could overestimate exposure risks, because tissue containing bioactive Cry proteins may be actively avoided by the shredders in the stream. For example, Swan et al. (2009) found significantly fewer *P. scabripennis* larvae colonizing *Bt* corn litter in the same stream during a controlled litter breakdown study. To our knowledge, however, no choice studies have been performed with genetically engineered corn tissue and aquatic consumers.

We showed a rapid decrease in bioactivity of *Bt* protein with environmental exposure of the senesced transgenic corn tissue, resulting in target larval growth inhibition declining rapidly within 2 wk, and a delayed input of senesced corn tissue into a stream adjacent to a corn field. Degradation notwithstanding, we observed lethal and negative sublethal effects in two of our four nontarget test species caused by exposure to tissue from the Cry1Ab containing near-isoline, but similar effects were not observed by exposure to tissue from the Cry1Ab+Cry3Bb1 near-isoline. Taken as a whole, our results provide evidence that adverse effects to aquatic nontarget shredders involve complex interactions arising from plant genetics and environment that cannot be ascribed to the presence of Cry1Ab proteins.

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