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Processing of transgenic crop residues in stream ecosystems

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Summary

1. Research in agricultural ecosystems is uncovering how the management of crop fields leads to the delivery of transgenic crop residues to adjacent waterways. Aquatic consumers encountering this material may be reduced in abundance and/or limit their feeding activity, subsequently altering organic matter breakdown rate, which is a key ecosystem process in streams.

2. We investigated the effect of the transgenic nature of senesced corn (*Zea mays* L.), tissue on breakdown rates, invertebrate abundance and invertebrate community composition in nine streams draining agricultural fields over 2 years (2004–2006). We studied corn tissue modified to express protein toxins from the bacterium *Bacillus thuringiensis* (Bt) from four hybrid families, each with its single, stacked and non-Bt near-isoline.

3. In 2004, we identified two instances whereby Bt leaf litter degraded slower (67–68%) than corresponding near isolines. At one site this was associated with significantly fewer individuals of *Pycnopsyche* sp., a leaf-chewing caddisfly. In 2005–2006, no differences in breakdown were found between Bt and non-Bt near isolines. Multivariate analysis of invertebrate communities found no difference associated with Bt treatment.

4. Principle components analysis identified important abiotic factors as explanatory variables influencing breakdown, but no interaction was found between these and Bt treatment. Breakdown was strongly related to total invertebrate abundance occurring on experimental litter bags, but this did not interact with Bt treatment across all hybrid × isoline × site combinations.

5. *Synthesis and applications*. Ecological interactions facilitate breakdown of allochthonous detritus, and understanding the potential disruption of these interactions is important to the management of ecosystem processes. The results from our study suggests that corn tissue breakdown is unlikely to be altered by Bt, but more so by hybrid- and site-specific factors such as nutrients. Management of agricultural streams will need to consider multiple sources of stress at larger scales, such as nutrient loading and temperature, which probably overwhelm the potential for consumer mediation of ecosystem processes in these ecosystems.

Key-words: agricultural streams, *Bacillus thuringiensis* (Bt), detritivores, ecosystem processes, genetically engineered corn (*Zea mays* L.), non-target effects, organic matter processing

Introduction

Ecologists are becoming increasingly aware of the implications of the cross-ecosystem movement of organisms and resources, and the cascading effects the presence of such subsidies can have on food web dynamics (e.g. Wallace *et al.* 1999; Greenwood *et al.* 2006). Research has documented that degradation of streams draining agricultural fields can be explained in part by elevated nutrient delivery (e.g. Peierls *et al.* 1991; Delong & Brusven 1998), and this has resulted in the implementation of best management practices (e.g. grass buffers strips) to ameliorate high nutrient loads (Lyons, Thimble & Paine 2000). Still, there exist novel sources of allochthonous material to these streams generated by no-till agriculture (Rosi-Marshall *et al.* 2007). These activities, whereby crop detritus is left on the field to reduce soil erosion, can result in the delivery of vegetative debris to streams (up to 40% identifiable non-woody organic matter; Stone *et al.* 2005).

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Genetically modified crops transformed for insect resistance and herbicide tolerance are increasingly employed worldwide. In 2008, 25 countries were producing transgenic crops on a global area of 125 million hectares, with 40% planted in corn (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.) expressing genes from the bacterium *Bacillus thuringiensis* (Bt) (GMO Compass 2009). The US is the largest producer of transgenic crops, with 63% of all corn planted modified to produce Bt proteins (USDA 2009). Hybrid families of Bt corn have been produced since 1996, including (i) hybrids from gene transformation events MON810, BT11 and TC1507, expressing *cry1Ab*, *cry1Ac* or *cry1F* endotoxins, respectively, for control of European corn borer *Ostrinia nubilalis* Hübner and other Lepidopterans; (ii) hybrids produced from gene transformation events MON863, MON88017, DAS-59122-7 and MIR604, expressing *cry3Bb1*, *cry34/35Ab1* and modified *cry3A* toxins targeted for the corn rootworm, *Diabrotica* spp. (Coleoptera), complex and (iii) stacked hybrid families expressing both *cry1*- and *cry3*-type toxins. The advantage of Bt crops over conventional insecticides is their high specificity (MacIntosh *et al.* 1990). However, transgenic crops may present a risk to non-target, beneficial insects (Obrycki *et al.* 2001). Although most types of Bt proteins are biologically active on one order of insects, few assessments have been made on aquatic consumers, and any associated ecosystem processes.

The breakdown of organic matter (e.g. senesced leaves) in streams is a key ecosystem process mediated by a suite of abiotic (e.g. nutrient content, flow) and biotic (e.g. microbial enzymes, invertebrate feeding) factors (Webster & Benfield 1986). The relative role of these factors can change depending on the environmental context (Paul, Meyer & Couch 2006), and in agricultural streams invertebrate consumers are thought to play an inferior role to the bacteria and fungi that proliferate in the presence of warmer water and higher nutrient levels (Hagen, Webster & Benfield 2006). However, invertebrate consumers can actually be quite abundant and diverse in agricultural streams, (e.g. Moore & Palmer 2005; Menninger & Palmer 2007) and most probably come into contact with *cry* proteins by feeding directly on decaying transgenic corn tissue washed or blown into aquatic systems. Detritivores inhabiting streams (e.g. leaf-chewing 'shredders', *sensu* Cummins & Klug 1979) rely on terrestrial leaf litter to meet their energy demands and have adapted with specialized mouthpart morphology and gut physiology to assimilate leaf litter (Cummins *et al.* 1989). Variation in plant tissue nutrient content and secondary/structural compounds is known to alter consumer feeding behaviour, and thus influence organic matter breakdown, which is a key ecosystem process in streams (Iversen 1974; Webster & Benfield 1986; Campbell & Fuchshuber 1995).

Most Bt corn currently planted expresses the lepidopteran-active *cry1Ab* protein. Because of the close phylogenetic relationship between Trichoptera (caddisflies) and Lepidoptera (Grimaldi & Engel 2005), caddisflies may be impacted by the input of Bt residue into streams. Caddisflies are a dominant group within both undisturbed and agricultural streams (Moore & Palmer 2005; Menninger & Palmer 2007) and serve important trophic roles including decomposition of allochthonous inputs of vegetation (Allan 1995). The Bt endotoxin may be one anthropogenic means by which shredder feeding behaviour might be altered, or abundance on corn tissue reduced, thus changing organic matter processing in agricultural streams.

In a multi-year effort to uncover the potential effects of transgenic corn detritus on in-stream decomposition of this material, we asked: (i) Does senesced Bt corn leaf tissue decompose more slowly than non-Bt tissue in the aquatic environment? (ii) Is aquatic invertebrate abundance reduced on Bt vs. non-Bt corn in streams? (iii) Does the taxonomic composition of detritivorous invertebrates differ on leaf packs made of Bt or non-Bt corn? and (iv) Are there significant differences in the chemistry of the Bt and non-Bt corn isolines?

Materials and methods

CORN LEAF TISSUE TREATMENTS

Two studies were performed in the autumn of 2004 and autumn–winter 2005–2006, utilizing senesced plant tissue collected from field plots established during the summer of each year. Corn hybrids were planted no-till during early May in 0·76 m rows, using recommended seeding rates, fertility inputs and herbicide treatments. In 2004, we planted single plots of three hybrids at the Central Maryland Research and Education Center, Beltsville, MD facility: (i) the non-expressing near isoline (Pioneer cv. 3394), (ii) a MON810 event hybrid (Pioneer cv. 33V08Bt) expressing the *cry1Ab* gene and (iii) a stacked hybrid (Doeblers cv. 525BW) expressing both *cry1Ab* and *cry3Bb1* genes from events MON810 and MON863. In 2005–2006, we expanded the project to three hybrid groups (A, B, C) with the same base genetics and the Roundup Ready gene (NK603). Replicate sets of the hybrid groups were grown in three blocks of field plots at the Western Maryland Research and Education Center near Keedysville, MD. Blocks were employed to ensure plot-specific environmental factors did not confound our treatments. Each hybrid group included: (i) a non-expressing isoline, (ii) a hybrid expressing the *cry1Ab* gene (MON 810) and (iii) a stacked hybrid with both *cry1Ab* and *cry3Bb1* genes (MON 810 and MON 863). For groups A and B, an experimental number was given as the hybrid designation. For group C, the hybrids were Dekalb DKC 63–80 (non-Bt), Dekalb DKC 63–81 (*cry1Ab*) and Dekalb DKC 63–74 (*cry1Ab* + *cry3Bb1*). In both studies, leaves were removed from the senesced plants when grain moisture reached 22% in late September and air dried.

CORN LEAF TISSUE CHEMISTRY

Fibre concentrations have been reported higher in Bt corn hybrids compared with their non-Bt isolines (Saxena & Stotzky 2001). Nitrogen is also a known limiting nutrient for stream detritivores. To test for tissue differences, leaf samples from each hybrid family and treatment were sent to Cumberland Valley Analytical Services (Maugansville, MD) and analysed for nitrogen (crude protein) and fibre fractions (Goering & Van Soest 1970; AOAC 2000). Values of the acid-detergent fibre (ADF) estimated the lignin and cellulose fraction, while neutral-detergent fibre (NDF) estimated the lignin, cellulose and hemicellulose content.

STUDY SITES

The studies were performed across seven stream sites in Maryland. The 2004 study was carried out in three Piedmont streams (FLD, FQC, MPX) and three Coastal Plain streams (GBR, HHC, WEH) (Table 1). In 2005–2006, three new Piedmont streams were studied in addition to FQC (WIL, HOW, GAR) (Table 1). All sites were chosen because they drain agricultural land where corn is routinely planted adjacent to streams. Measurements were taken at each sample date (below) for baseflow discharge, dissolved oxygen, temperature, conductivity, nitrate and phosphate. The seven sites encompass a range in drainage area and nutrient levels, which were high as is typical for agricultural streams (Table 1).

LITTER BREAKDOWN AND INVERTEBRATE ABUNDANCE

We designed the two studies to determine if breakdown rates and invertebrate abundance patterns differed between non-, single-gene and stacked-gene Bt isolines and if such effects were consistent across sites within the region. For both studies, we employed a standard litter-bag technique by placing a known mass of corn tissue in a mesh bag (7 × 11 mm mesh) in the stream, and retrieving replicate bags over time to estimate the mass loss rate, k (day^{-1}), the exponent of the exponential decay function (Benfield 2006).

In 2004, *c.* 1·5 g fresh mass of senesced corn tissue from the non-, single-gene and stacked-gene Bt isolines were placed into mesh bags. An ash-free dry mass (AFDM) to fresh mass conversion was estimated to calculate initial AFDM of each leaf pack. A total of 24 replicate bags were created per site × hybrid combination so that $n = 4$ bags could be retrieved on six sample dates. Bags were placed in the streams by tethering each to galvanized wire staked to the streambed in a riffle-run area. Bags were placed on 8 October, 2004 for the Piedmont sites, and 7 October, 2004 for the Coastal Plain sites, and retrieved 7, 14, 21, 28, 36 and 42 days thereafter. A total of $n = 4 \times 3$ isolines × 6 sites × 6 sample dates = 432 litter bags were deployed.

In 2005–2006, *c.* 3·0 g fresh mass of corn tissue from the non-, single-gene and stacked-gene Bt isolines each from three hybrids were placed in mesh bags and deployed in four Piedmont streams. Twelve replicate litter bags were created for each isoline × hybrid × site combination (432 bags total), so that $n = 3$ litter bags could be retrieved four times over ~120-day period. The sampling period was expanded in this study to estimate as close to the full period of mass loss as possible, whereas in 2004 the goal was to reach more than 50% mass loss, as is often standard for litter decay studies. Litter bags were placed in streams in October (FQC and HOW, 23rd; GAR, 24th, WIL, 25th). Samples were retrieved at 14, 45, 80 and 109 days for FQC; 14, 48, 80 and 119 for HOW; 14, 47, 79 and 118 for GAR and 12, 45, 77 and 120 for WIL.

At sampling, leaf packs were cut from the wire and placed in separate plastic bags. Bags were stored on ice, returned to the laboratory and stored at 4°C until processing (within 48 h). Invertebrates were removed from the corn tissue in each leaf bag after rinsing the contents into a 500 μm sieve, and the remaining tissue dried at 60°C for 24 h in aluminum pans, weighed, then combusted at 550°C for 2 h to determine the ash-free dry mass (Benfield 2006). The macroinvertebrates were preserved in an 80% ethanol solution. Total invertebrates were counted, and all trichopterans and other shredder taxa identified to the lowest possible taxonomic level.

DATA ANALYSIS

All analyses were carried out in SAS version 9.1 (SAS Institute 2002, Cary, NC, USA). Invertebrate data were $\log_{10}(x + 1)$ transformed to improve normality. The assumption of normality of residuals were met for all analyses (Shapiro-Wilkes test), but we did observe unequal variances among some treatment combinations. When necessary, we grouped residual variances by treatments using the GROUP option in PROC MIXED using the method of Littell *et al.* (1996), and denominator degrees of freedom adjusted using the Satterthwaite approximation. Statistical significance was evaluated at $\alpha = 0{\cdot}05$ for all analyses.

Differences in nitrogen and fibre content between single-gene Bt, stacked Bt and the non-Bt near-isoline were assessed separately for each study year. For 2004, one-way ANOVAS were conducted on the

Table 1. Site descriptions. FQC was studied in both 2004 and 2005–2006

Study year	Site	Latitude, longitude	Drainage (km^2)	Discharge (m^3 s^{-1})	Dissolved O^2 (mg L^{-1})	Temperature (°C)	Conductivity (MS)	Nitrate, NO3$^-$ (mg L^{-1})	Phosphate, PO$_4^{3-}$ (μg L^{-1})
2004	GBR	N 39° 00·55′ W 75° 56·19′	50·7	0·553	11·90	5·8	112·9	37·9	–
	HHC	N 39° 03·22′ W 75° 58·19′	3·2	0·039	12·01	5·9	115·4	31·8	91·0
	WEH	N 38° 57·81′ W 76° 02·93′	7·8	0·065	12·79	2·9	80·5	21·9	86·0
	FLD	N 39° 15·74′ W 76° 56·03′	< 1	0·004	13·77	4·7	107·6	14·5	–
	FQC	N 39° 15·26′ W 76° 55·62′	< 1	0·017	10·17	9·8	398·5	20·7	79·0
2005–2006	MPX	N 39° 15·34′ W 76° 55·59′	57·8	0·491	14·96	4·6	148·7	3·7	70·9
	WIL	N 39° 33·71′ W 76° 52·36′	8·9	0·077	11·05	9·9	162·9	54·8	17·6
	HOW	N 39° 32·72′ W 76° 49·74′	1·2	0·151	12·13	9·0	223·7	30·5	30·1
	GAR	N 39° 34·85′ W 76° 52·36′	26·0	0·268	12·53	7·9	250·8	21·2	14·9
	FQC	N 39° 15·26′ W 76° 55·62′	< 1	0·026	8·43	11·3	526·5	36·4	25·8

nitrogen and fibre fractions separately. For 2005–2006, we used two-way ANOVAS (3 hybrid families × 3 Bt treatments) to determine differences in Bt isolines within family. For both sets of analyses, significance of pairwise comparisons was determined after adjusting *P*-values with the Tukey's HSD method.

We tested for differences in breakdown rate between each litter treatment using a multi-factorial indicator variables regression analysis, which is essentially an ANCOVA with day as a covariate and relaxing the assumption of identical slopes. Breakdown of plant litter generally follows a negative exponential decay process (Webster & Benfield 1986), so the natural logarithm of the fraction of AFDM remaining was tested as a function of time in the stream and the interaction between day, Bt treatment, site and, in the case of the 2005–2006 study, hybrid. No intercept was fitted since the fraction of AFDM at the beginning of the experiment (i.e. day 0) was defined as 1. Any significant interaction term of Bt treatment with day was taken as evidence that loss rate differed between at least two of the Bt treatments. The breakdown rate, k (day^{-1}), was estimated from the output of the analysis using the ESTIMATE statement. Comparisons of breakdown rates between Bt treatments within a site (and hybrid for 2005–2006) were performed, and the *P*-values adjusted for inflation of error using the sequential Bonferroni procedure (Rice 1989).

We examined abundance patterns for all shredder taxa in addition to all non-shredder Trichoptera, and taxon-specific responses separately. Total invertebrate abundance (g^{-1} AFDM per litter bag) was analysed identically to litter mass loss, but with intercept terms included. Any significant interaction term with Bt treatment was interpreted as evidence that invertebrate abundance differed between at least two of the Bt treatments. To understand taxon-specific responses to the Bt treatments, two-way ANOVAS were performed for each taxon within a sample year, with site and Bt treatment (pooled across hybrids for 2005–2006) as main effects. Time was not included as a factor since focusing on one taxon at a time resulted in many litter bags with no individuals for the taxon being analysed and, thus no linear relationship could be assumed between abundance and time. Furthermore, if a taxon was not found in three or more litter bags across sample dates at a site, that site was excluded from the analysis since an error estimate could not be assigned to each Bt treatment for that site × taxon combination. After identifying a significant treatment effect, pairwise comparisons were made and significance evaluated by adjusting the *P*-values using the sequential Bonferroni procedure (Rice 1989).

To test if the invertebrate community was influenced by Bt treatment, we summarized all taxa using partial redundancy analysis (pRDA; Legendre & Legendre 1998). This linear method of direct ordination detects compositional differences in the colonizing communities. The analyses were conducted with CANOCO 4.5 (ter Braak & Smilauer 2002), with Bt treatments coded as categorical explanatory variables, and study site and sampling time as covariables. pRDA removed the variation accounted for by the covariables before determining the variation in the taxa matrix explained by the Bt treatments. In all analyses, we used average abundances of taxa pooled over replicate litter bags for each treatment combination. The null hypothesis that differences of taxa composition were not related to the Bt treatments was tested using Monte Carlo permutations. This was performed by shuffling data in a specific way to remove site and sampling time effects as sources of error from the residual, and generating 499 new sets of data that were equally likely under the null hypothesis.

Breakdown of organic matter in streams is a function of a suite of biotic and abiotic factors, including invertebrate feeding activity/abundance, microbial activity, nutrients, flow, etc. (Webster & Benfield 1986). To interpret inter-site patterns of breakdown, we performed two analyses. First, we analysed breakdown rate for both studies as a function of average invertebrate abundance and interactions with isoline separately for each study year. This tested if invertebrate abundance could explain variation in breakdown and if such an effect interacted with Bt isoline. If so, this would lend support to the hypothesis that Bt altered breakdown via changes in invertebrate dynamics. However, there could also be multiple and inter-correlated environmental factors important to breakdown that varied across sites and potentially interacted with the presence of Bt. Given this, we then performed a principle components analysis (PCA) of environmental factors known to be influential on litter breakdown (Webster & Benfield 1986). These variables included average baseflow discharge, dissolved oxygen, temperature and nitrate; phosphate was excluded because of limited data at two sites. The first two axes explained 84% of the variation in those data. The scores from the first two axes were used in a multi-factor indicator variables regression for each study separately, where breakdown rate was made a function of each PCA score, Bt treatment (single, stacked, near-isoline) and the interaction of each PCA score with Bt treatment. Any significant interaction with Bt treatment and PCA axis score was taken as evidence that these environmental factors differentially influenced breakdown of Bt corn tissue.

Results

CORN TISSUE CHEMISTRY

We observed different patterns in both nutrient and fibre chemistry across the two harvest years. For 2004, we observed differences between isolines for both nutrient (N; $F_{2,6} = 41{\cdot}0$, $P < 0{\cdot}001$) and NDF ($F_{2,6} = 36{\cdot}2$, $P < 0{\cdot}001$) fractions, but not the ADF fraction ($F_{2,6} = 2{\cdot}0$, $P = 0{\cdot}22$). The single-gene Bt isoline had 33% higher N content than both the isoline and the stacked Bt combined (Table 2). Both Bt isolines had statistically lower NDF fractions compared with the isoline. In 2005, no differences in nutrient or fibre fractions were observed between isolines across (all insignificant Bt isoline main effects; $F_{2,16} < 2{\cdot}2$, $P > 0{\cdot}10$) or within hybrids (Bt isoline × Hybrid effect; all $F_{4,16} < 0{\cdot}6$, $P > 0{\cdot}66$; Table 2). No fraction (N, ADF, NDF) for any of the three isolines deviated from the mean for that hybrid by more than 5%.

LITTER BREAKDOWN

Tissue breakdown rates varied significantly across sites in both study years and among hybrid types in 2005–2006 (Table 3). Furthermore, we observed isoline effects in 2004 at two sites (WEH, FQC; Fig. 1a). In 2004, the greatest difference in breakdown was observed at FQC. Both the single and stacked Bt isolines lost mass at a rate <67% of that of the non-Bt isoline (Fig. 1a). A significant effect was also observed at WEH, where the stacked Bt breakdown rate was ~68% of the rate estimated for the non-Bt isoline. Of the remaining sites, all but one displayed slower loss rates for the Bt isolines compared with the non-Bt isoline; the opposite was true for GBR (Fig. 1a). None of these trends was statistically significant.

Table 2. Summary of litter nitrogen and fibre fractions

Study year	Hybrid	Isoline	N	ADF	NDF
2004		Non-Bt	1·17 (0·038)[a]	41·7 (0·71)[a]	68·1 (0·54)[a]
		Single-gene Bt	1·65 (0·038)[b]	40·0 (0·71)[a]	62·3 (0·54)[b]
		Stacked Bt	1·31 (0·038)[a]	41·8 (0·71)[a]	62·5 (0·54)[b]
2005–2006	A	Non-Bt	1·77 (0·105)[a]	35·6 (0·76)[a]	63·8 (0·86)[a]
		Single-gene Bt	1·75 (0·105)[a]	34·6 (0·76)[a]	63·6 (0·86)[a]
		Stacked Bt	1·68 (0·105)[a]	34·9 (0·76)[a]	63·8 (0·86)[a]
	B	Non-Bt	1·44 (0·056)[a]	36·5 (0·76)[a]	65·7 (0·86)[a]
		Single-gene Bt	1·36 (0·056)[a]	35·3 (0·76)[a]	66·7 (0·86)[a]
		Stacked Bt	1·49 (0·056)[a]	35·9 (0·76)[a]	64·5 (0·86)[a]
	C	Non-Bt	1·17 (0·056)[a]	35·7 (0·76)[a]	66·5 (0·86)[a]
		Single-gene Bt	1·20 (0·069)[a]	36·5 (0·93)[a]	68·0 (1·06)[a]
		Stacked Bt	1·25 (0·069)[a]	35·8 (0·93)[a]	66·4 (1·06)[a]

Results are the mean (SE) percentage relative to total dry mass ($n = 3$). Values with the same letter are not significantly different. Comparisons are restricted to within isoline for each year and hybrid (for 2005). N, nitrogen; ADF, acid-detergent fibre; NDF, neutral-detergent fibre.

Inter-isoline variation in breakdown was not observed for any of the three hybrids at any of the sites in 2005–2006 (non-significant Bt interaction terms, Table 3). Loss rates were faster for hybrid A compared with B and C ($P = 0·005$ after Bonferroni adjustment), with the fastest occurring at FQC ($P = 0·001$ vs. all other sites; Fig. 2, left). Unlike the 2004 patterns, there was no clear trend showing lower breakdown of Bt isolines compared with non-Bt tissue.

INVERTEBRATE ABUNDANCE

For both study years, invertebrate abundance on litter bags varied significantly through time and with site, but these effects did not interact with Bt isoline except at one site (Table 4). Average abundance (g^{-1} AFDM) in 2004 ranged from 5·4 at HHC for each of the hybrids to 170·8 on the non-Bt isoline at the FLD site. Abundance on the stacked Bt isoline was significantly lower compared with the non-Bt isoline at the FLD site (Fig. 1b). In 2005–2006, abundances on litter bags were within the same range as 2004, with the highest estimates occurring for all three hybrids at FQC, and the lowest observed at HOW on the stacked Bt isoline for hybrid B (Fig. 2, right). There was more than 34-fold variation in abundance across the 18 isoline × hybrid combinations in 2004 (Fig. 1b), and 101-fold variation in abundance across all 36 hybrid × isoline × site combinations in 2005–2006 (Fig. 2, right).

We did observe some taxon-specific responses to Bt treatment, but the pRDA analysis did not reveal shifts in community composition with Bt treatment after controlling for both site and sampling time effects. In 2004, 69·9% of the variation in community structure was explained by site and sampling time, but the ordination did not extract a significant portion of the variance (0·8%) in the community data of 10 taxa in 2004

Table 3. Multi-factorial regression analysis of corn litter breakdown (k, day^{-1}) for both 2004 and 2005–2006 studies

Study year	Source	d.f.	F	P
2004	Day	1	1519·6	< 0·001
	Day × Site	5	61·2	< 0·001
	Day × Bt	2	9·9	< 0·001
	Day × Site × Bt	10	3·7	< 0·001
2005–2006	Day	1	3826·0	< 0·001
	Day × Site	3	28·8	< 0·001
	Day × Hybrid	2	6·6	0·002
	Day × Bt	2	0·6	NS
	Day × Site × Hybrid	6	0·7	NS
	Day × Site × Bt	6	2·1	NS
	Day × Hybrid × Bt	4	2·2	NS
	Day × Site × Hybrid × Bt	12	1·3	NS

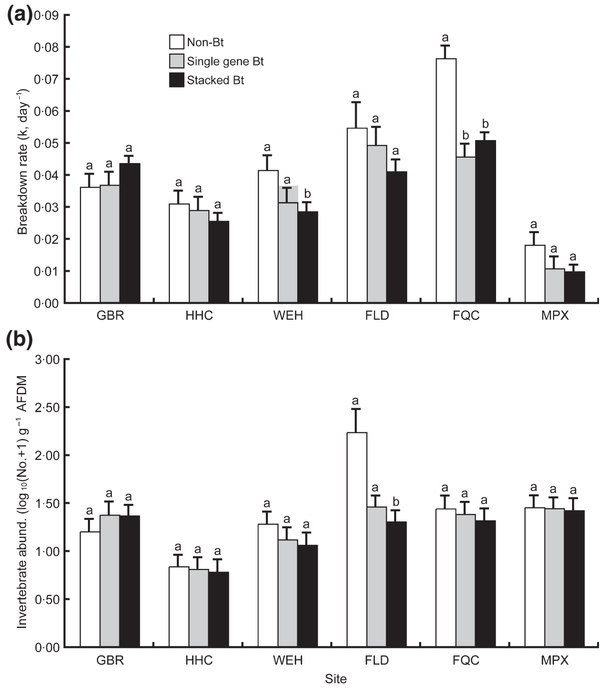

Fig. 1. (a) 2004 breakdown rates and (b) invertebrate abundance in experimental litter bags. Bars are the least-squares means + 1 SE. For litter breakdown rates, comparisons are restricted to isolines within a site. Bars with the same letter are not significantly different ($\alpha = 0·05$).

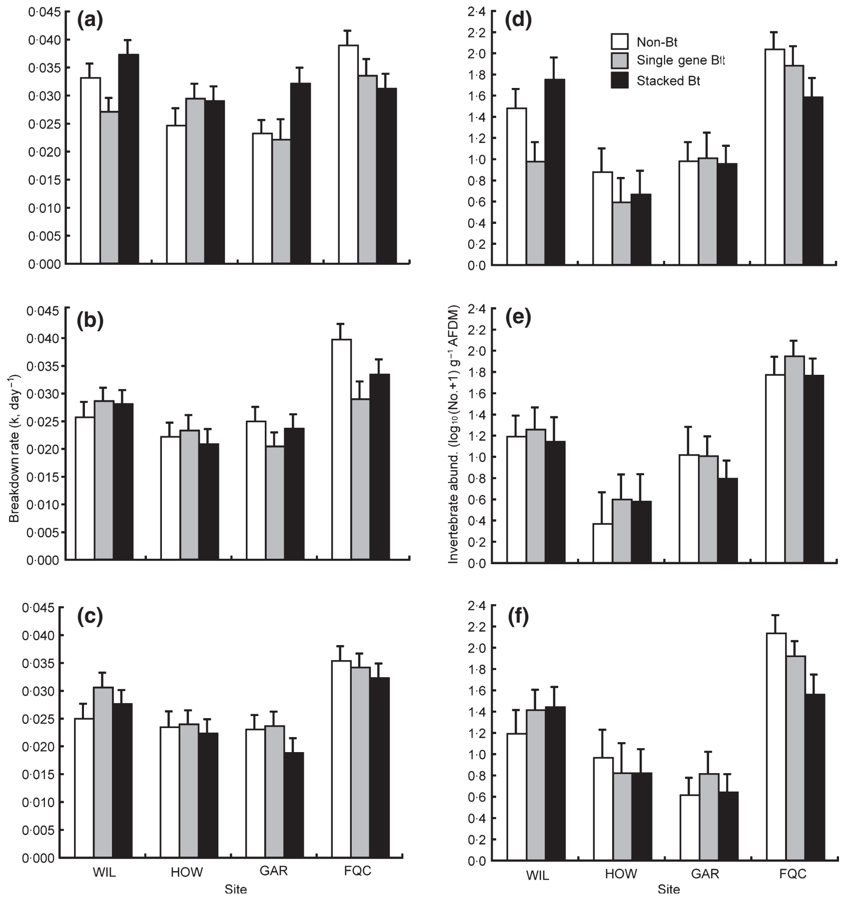

Fig. 2. Results of the 2005–2006 study. Panel (a,b) refers to hybrid A, (c,d) hybrid B and (e,f) hybrid C. For each site × isoline treatment combination, breakdown rates are left, invertebrate abundance on the right. Bars are the least-squares means + 1 SE.

(P = 0·81). In 2005–2006, 71·3% of the variation was explained by site and sampling time effects, and 2% of the variation in the community data of 6 taxa (P = 0·33).

Table 4. Multi-factorial regression analysis of invertebrate abundance (No. g^{-1} AFDM) on plant litter

Study year	Source	d.f.	F	P
2004	Day	1	24·5	< 0·001
	Site	5	4·2	0·001
	Bt	2	1·2	NS
	Day × Site	5	4·5	0·001
	Day × Bt	2	3·4	0·036
	Site × Bt	10	1·4	NS
	Day × Site × Bt	10	2·4	0·010
2005–2006	Day	1	103·0	< 0·001
	Site	3	3·3	0·023
	Hybrid	2	0·0	NS
	Bt	2	0·4	NS
	Day × Site	3	6·1	0·001
	Day × Hybrid	2	0·2	NS
	Day × Bt	2	0·7	NS
	Site × Hybrid	6	0·2	NS
	Site × Bt	6	0·5	NS
	Hybrid × Bt	4	0·7	NS
	Day × Site × Hybrid	6	0·5	NS
	Day × Site × Bt	6	0·9	NS
	Day × Hybrid × Bt	4	1·4	NS
	Site × Hybrid × Bt	12	0·8	NS
	Day × Site × Hybrid × Bt	12	1·4	NS

The results of the ANOVA performed on each taxon within site separately for each study year (47 combinations) revealed two instances of a Bt effect. In 2004, the trichopteran shredder *Pycnopsyche* sp. was very abundant at FQC, and was more than 11× more abundant on the non-Bt litter treatment compared with the single-gene Bt (P = 0·015, Table 5). While more than 4× more abundant on the non-Bt vs. the stacked Bt, that result was not significantly different (P = 0·085, Table 5). In 2005–2006, significantly fewer isopods (*Caecidotea communis*) at FQC were found on the stacked Bt treatment compared with the non-Bt (P = 0·018, Table 6).

LITTER BREAKDOWN, INVERTEBRATE ABUNDANCE AND ENVIRONMENTAL FACTORS

We identified a significant linear relationship (r^2 = 0·70) between breakdown rate (k, day^{-1}) and invertebrate abundance for the 2005–2006 study (Table 7; Fig. 3b), but not in 2004 (Fig. 3a). This suggests that in 2005–2006, invertebrate presence was important to breakdown, but this did not interact with Bt isoline treatment (Table 7). If the transgenic nature of senesced corn tissue should result in slower breakdown rates because of changes in invertebrate abundance and/or feeding behaviour, invertebrate abundance should differentially influence breakdown rates across isoline treatments. This was not the case. The distribution of values around the regression line (Fig. 3b) relating breakdown to abundance is statistically indistinguishable across isolines.

Many abiotic factors can influence litter breakdown, and one advantage of a multi-site, multi-year study is to capture the variability in such factors. The PCA resulted in axis 1 explaining 63·1% of the variation in the data set and was most strongly correlated with temperature (r = 0·55) and dissolved oxygen (r = −0·59). PCA axis 2 explained an additional 21·1% of the variation, and was strongly correlated with discharge (r = 0·90) and nitrate (r = 0·41). In both study years, axis 1 was highly significant in explaining breakdown rates (Table 7). However, there was no interactive effect of Bt treatment, and therefore no evidence that the transgenic nature of corn tissue interacted with abiotic factors to influence breakdown.

Discussion

Increasingly, transgenic crop residue will enter streams as new transformations generate new crop cultivars. Our goal was to learn if senesced transgenic corn leaves, engineered with a Bt gene and observed to occur in agricultural streams, decayed differently than leaves of non-Bt corn representing near genetic isolines. We found little evidence that Bt corn altered breakdown rates, but when effects were found, they (i) were consistent with the hypothesis that non-Bt corn breaks down faster, (ii) were site specific and (iii) occurred only in 2004. However, while tissue breakdown rate was positively related to invertebrate abundance in 2005–2006, as has been previously shown elsewhere (e.g. Sponseller & Benfield 2001), the response did not interact with Bt isoline, nor did abiotic variation across sites interact with Bt treatment to alter breakdown rate.

Breakdown of corn tissue in both of our study years was similar to, and sometimes higher than, values reported for plant tissue in other systems. Rosi-Marshall *et al.* (2007) reported decay values of k = 0·015 (non-Bt) to k = 0·020 (Bt), but the underlying genetics and hybrid families were not available. Griffiths *et al.* (2009) report a range of k = 0·012–0·024 for Bt and k = 0·015 for non-Bt corn tissue. Our values

Table 5. Mean invertebrate abundance (No. g^{-1} AFDM) for all site × Bt treatment combinations in 2004

Taxon	Isoline	GBR	HHC	WEH	FLD	FQC	MPX
Crustacea							
Amphipoda	Non-Bt	–	0·2[a]	12·5[a]	–	0·4[a]	–
	Single	0·7	0·7[a]	11·1[a]	–	0·2[a]	–
	Stacked	3·9*	0·4[a]	7·9[a]	0·5*	0·2[a]	2·5*
Decapoda	Non-Bt	0·2*	0·9*	–	–	–	–
	Single	2·2*	–	0·8*	–	–	–
	Stacked	–	–	0·6*	1·9*	–	–
Isopoda	Non-Bt	0·8[a]	0·3[a]	0·9[a]	0·0[a]	7·5[a]	0·4[a]
	Single	0·6[a]	0·6[a]	0·5[a]	0·2[a]	12·2[a]	0·2[a]
	Stacked	0·4[a]	0·4[a]	0·9[a]	0·1[a]	12·5[a]	0·1[a]
Diptera							
Tipulidae	Non-Bt	0·4[a]	0·9[a]	0·4[a]	3·8[a]	0·5[a]	0·2[a]
	Single	1·4[a]	1·7[a]	0·1[a]	2·9[a]	0·0[a]	0·4[a]
	Stacked	0·5[a]	1·5[a]	0·1[a]	1·9[a]	0·0[a]	0·2[a]
Plecoptera							
Taeniopteryx sp.	Non-Bt	0·0[a]	1·2*	–	11·3[a]	0·0[a]	5·4[a]
	Single	0·5[a]	–	–	8·5[a]	0·2[a]	4·6[a]
	Stacked	0·0[a]	–	–	5·4[a]	0·3[a]	6·2[a]
Trichoptera							
Hydropsychidae	Non-Bt	0·8[a]	1·7[a]	0·2[a]	5·8[a]	2·4[a]	11·3[a]
	Single	1·7[a]	0·5[a]	0·0[a]	4·0[a]	2·0[a]	18·2[a]
	Stacked	0·3[a]	0·9[a]	0·1[a]	1·8[a]	0·9[a]	13·2[a]
Lepidostomatidae							
Lepidostoma sp.	Non-Bt	–	–	–	–	–	–
	Single	–	–	–	1·7*	–	–
	Stacked	–	7·9*	–	–	–	–
Limnephilidae							
Frenesia sp.	Non-Bt	–	–	–	1·2*	2·5*	–
	Single	–	–	–	0·5*	–	–
	Stacked	–	–	–	0·4*	–	–
Pycnopsyche sp.	Non-Bt	–	–	–	0·0[a]	4·6[a]	–
	Single	1·4*	–	–	0·1[a]	0·4[b]	–
	Stacked	2·6*	–	–	0·1[a]	1·1[ab]	–
Mollusca							
Gastropoda	Non-Bt	11·8[a]	1·9[a]	0·1[a]	–	2·5[a]	1·2*
	Single	15·7[a]	1·1[a]	0·3[a]	2·0*	1·9[a]	1·0
	Stacked	20·0[a]	0·8[a]	0·4[a]	–	1·1[a]	–

Values are the back-transformed $\log_{10}(x + 1)$ least-squared means across all sample dates. An asterisk indicates that fewer than three leaf bags contained that taxon. Comparisons were restricted within site, and means with the same letter are not significantly different. Dashes indicate taxa were absent.

Table 6. Mean invertebrate abundance (No. g^{-1} AFDM) for all site × Bt treatment combinations for 2005–2006

Taxon	Isoline	WIL	HOW	GAR	FQC
Crustacea					
Amphipoda	Non-Bt	–	–	1·5*	1·4[a]
	Single	–	–	–	0·4[a]
	Stacked	61·2*	–	–	0·4[a]
Isopoda	Non-Bt	0·0[a]	0·1[a]	0·0[a]	22·0[a]
	Single	0·1[a]	0·1[a]	0·1[a]	14·1[a]
	Stacked	0·1[a]	0·1[a]	0·1[a]	6·3[b]
Plecoptera					
Taeniopteryx sp.	Non-Bt	2·9[a]	1·0[a]	0·2[a]	–
	Single	2·3[a]	0·6[a]	0·4[a]	0·2
	Stacked	2·9[a]	0·8[a]	0·1[a]	7·7*
Trichoptera					
Hydropsychidae	Non-Bt	8·9[a]	3·3[a]	6·1[a]	31·8[a]
	Single	5·9[a]	2·7[a]	7·1[a]	22·4[a]
	Stacked	10·1[a]	1·9[a]	4·8[a]	10·5[a]
Limnephilidae					
Pycnopsyche sp.	Non-Bt	–	–	–	0·3[a]
	Single	–	–	–	0·5[a]
	Stacked	–	–	–	0·2[a]
Mollusca					
Gastropoda	Non-Bt	0·8[a]	–	0·1[a]	1·9[a]
	Single	0·4[a]	–	0·0[a]	2·6[a]
	Stacked	0·7[a]	–	0·1[a]	1·6[a]

Means are across hybrid, and values are the back-transformed $\log_{10}(x + 1)$ least-squared means across all sample dates. An asterisk indicates fewer than three leaf bags contained that taxon. Comparisons were restricted within site, and means with the same letter are not significantly different. Dashes indicate taxa were absent.

fall within this range, although our use of various hybrids revealed higher decay rates than reported in either study. The differences could be explained by our sites having warmer temperatures and higher nutrient levels, both known to influence rates of litter breakdown, especially in agricultural streams (Hagen *et al.* 2006; Paul *et al.* 2006). Another explanation could be the large contribution of invertebrates to explaining variation in mass loss in the 2005–2006 study, as well as the taxon-specific responses we observed at two sites. This, in addition to other abiotic factors (e.g. geographic location), may explain our high estimates.

Table 7. Multi-factorial regression results testing the interaction between isoline and invertebrate abundance (top) and abiotic factors (bottom) on litter breakdown rate for both 2004 and 2005–2006 study years

Factor	Study year	Source	d.f.	*F*	*P*
Invertebrate abundance	2004	Bt	2	0·00	NS
		Invertebrate	1	0·62	NS
		Bt × Invertebrate	2	0·03	NS
	2005–2006	Bt	2	1·0	NS
		Invertebrate	1	64·3	< 0·001
		Bt × Invertebrate	2	1·7	NS
Abiotic factors	2004	Bt	2	0·84	NS
		PCA 1	1	9·15	0·014
		PCA 2	1	0·75	NS
		PCA 1 × Bt	2	0·16	NS
		PCA 2 × Bt	2	0·42	NS
	2005–2006	Bt	2	0·58	NS
		PCA 1	1	36·10	< 0·001
		PCA 2	1	3·36	NS
		PCA 1 × Bt	2	0·03	NS
		PCA 2 × Bt	2	2·12	NS

PCA 1 and PCA 2 refer to the axes scores calculated from the principle components analysis performed on discharge, dissolved oxygen, temperature and nitrate.

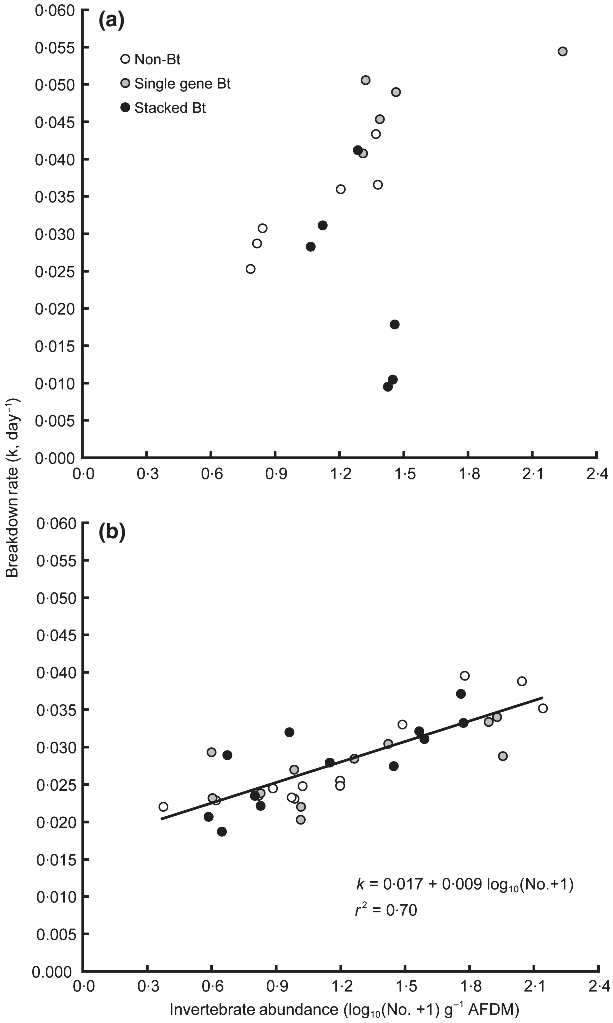

Fig. 3. Relationship between breakdown rate (k, day^{-1}) and average invertebrate abundance across all sample dates for each isoline × site combination for (a) 2004 (n = 12) and (b) 2005–2006 study (n = 36). Points are shaded differently for each Bt isoline to illustrate the spread of each treatment across ranges in invertebrate abundance and breakdown rate, but the regression line represents the relationship for all isolines together for 2005–2006 only.

Why did we see little response of breakdown and invertebrate abundance to transgenic tissue? Evidence from terrestrial systems suggests (i) high specificity of the Bt endotoxin, even among phylogenetically related species (e.g. O'Callaghan *et al.* 2005) and (ii) the short persistence time of the endotoxin in the environment following tissue deposition on the ground and/or the stream (Dubelman *et al.* 2005; P.D. Jensen, G.P. Dively, C.M. Swan & W.O. Lamp, Unpublished data; Zwahlen *et al.* 2003). Despite evidence from Rosi-Marshall *et al.* (2007)

whereby caddisfly larvae offered transgenic detritus in the laboratory suffered lower growth and survival, there exists no evidence to suggest the European corn borer (P.D. Jensen *et al.*, Unpublished data) or other taxa suffer from the endotoxin once tissue has been substantially exposed to the environment. Our results largely support this by showing little differential abundance patterns or contribution to explaining variation in decay between isolines. This is despite the most comprehensive set of studies to date (864 replicate litter bags were placed in the field for both studies).

The evidence we gathered from nine streams across 2 years suggests that the Bt isolines had little to no influence on organic matter breakdown. Since we hypothesized invertebrates would be responsible for any difference between treatments, one explanation could be that watershed-scale agricultural degradation overwhelmed any influence invertebrates could have on breakdown rates. In agricultural streams, it is known that invertebrate abundance and diversity is generally low, and leaf breakdown more because of microbial activity stimulated by high nutrients and warmer temperatures (Hagen *et al.* 2006; Paul *et al.* 2006). However, we did see a positive relationship between breakdown rate and total invertebrate abundance (comprised of leaf shredding detritivores and filter-feeding caddisflies). We also identified two instances of Bt isolines exhibiting slower breakdown than corresponding near isolines. At FQC in 2004, *Pycnopsyche* sp. abundance on the single-gene Bt treatment was reduced, although no differences in abundance the following year were found when three groups of hybrids were tested. At FQC in 2005–2006, the isopod *Caecidotea communis* was found in lower abundance on stacked Bt tissue, but this was not associated with a reduction in breakdown. At WEH in 2004, the stacked Bt tissue breakdown was slower than the non-Bt isoline's, but we found no concomitant pattern in invertebrate abundance within or across taxa. These examples, however, are few in comparison to the number of site $\times$ Bt hybrid combinations employed, and so it was reasonable to expect that after accounting for site and sampling time effects, our multivariate analysis revealed no change in invertebrate community structure across Bt treatments.

Two potential avenues of research to reconcile differences among studies involves examining (i) the temporal changes in corn leaf chemistry (e.g. N, fibre) as it degrades and the response of non-target taxa at various stages of decay and (ii) quantifying the relative role of water quality degradation on decay vs. invertebrate feeding. For example, early in the decay process when biologically active endotoxin may be present (Griffiths *et al.* 2009), invertebrates might exhibit the patterns in growth/feeding observed in the laboratory studies, but later when leaf litter can become more palatable, such negative effects might be negligible. The overall effect on decomposition of corn tissue in agricultural streams would not depend on the Bt endotoxin, but more so on hybrid- and site-specific factors as we show here. This might be best understood in the larger context of agricultural degradation of streams. Elevated nutrient levels and water temperatures because of landscape degradation might overwhelm any effect of Bt by reducing invertebrate abundance, eliminating sensitive leaf shredding taxa, or by enhancing the microbial contribution to the decay processes. Microbial decomposers are not susceptible to Bt, and respond positively to elevated nutrient levels and warmer temperatures (Paul *et al.* 2006). Streams degrading agricultural landscapes suffer from a plethora of stressors. We focused here on the breakdown of transgenic crop residues, yet high site-to-site variation and larger scale stressors are more important to how rates of key ecosystem processes are impacted by agricultural practices.

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