Science of the Total Environment 592 (2017) 97–105

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

# Occurrence, leaching, and degradation of Cry1Ab protein from transgenic maize detritus in agricultural streams

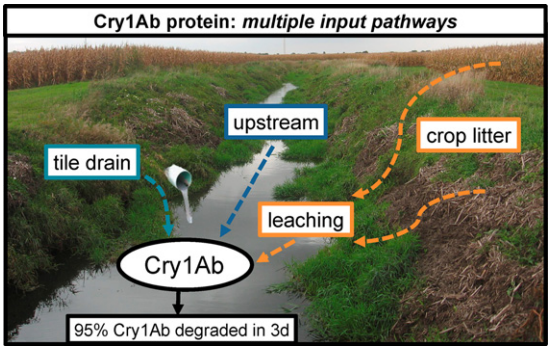
Natalie A. Griffiths [a,*], Jennifer L. Tank [a], Todd V. Royer [b], Emma J. Rosi [c,1], Arial J. Shogren [a], Therese C. Frauendorf [a,2], Matt R. Whiles [d]

[a] Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA
[b] School of Public and Environmental Affairs, Indiana University, 1315 East Tenth Street, Bloomington, IN 47405, USA
[c] Department of Biology, Loyola University Chicago, 6525 N. Sheridan Road, Chicago, IL 60626, USA
[d] Department of Zoology and Center for Ecology, Southern Illinois University, Carbondale, IL 62901-6501, USA

## HIGHLIGHTS

- Bt maize with Cry1Ab protein enters aquatic ecosystems, but fates are understudied.
- Examined occurrence, leaching, and degradation of Cry1Ab in agricultural streams
- Cry1Ab protein concentration in streams and tile drains was 3–60 ng/L.
- 99% of Cry1Ab leached from submerged Bt maize leaves into water over 70 d.
- Cry1Ab protein degraded rapidly in microcosms with water-column microorganisms.
- Cry1Ab may be pseudo-persistent at watershed scales due to multiple input pathways.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 24 January 2017
Received in revised form 7 March 2017
Accepted 7 March 2017
Available online 15 March 2017

Editor: Jay Gan

*Keywords:*
Genetically engineered crops
Corn

## ABSTRACT

The insecticidal Cry1Ab protein expressed by transgenic (Bt) maize can enter adjacent water bodies via multiple pathways, but its fate in stream ecosystems is not as well studied as in terrestrial systems. In this study, we used a combination of field sampling and laboratory experiments to examine the occurrence, leaching, and degradation of soluble Cry1Ab protein derived from Bt maize in agricultural streams. We surveyed 11 agricultural streams in northwestern Indiana, USA, on 6 dates that encompassed the growing season, crop harvest, and snowmelt/spring flooding, and detected Cry1Ab protein in the water column and in flowing subsurface tile drains at concentrations of 3–60 ng/L. In a series of laboratory experiments, submerged Bt maize leaves leached Cry1Ab into stream water with 1% of the protein remaining in leaves after 70 d. Laboratory experiments suggested that dissolved Cry1Ab protein degraded rapidly in microcosms containing water-column microorganisms, and light did not enhance breakdown by stimulating assimilatory uptake of the protein by autotrophs. The common detection of

* Corresponding author at: Climate Change Science Institute and Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6351, USA.
*E-mail address:* griffithsna@ornl.gov (N.A. Griffiths).
[1] Present address: Cary Institute of Ecosystem Studies, Millbrook, NY 12545-0129, USA.
[2] Present address: Department of Biology, University of Victoria, Victoria, BC V8W 3N5, Canada.

http://dx.doi.org/10.1016/j.scitotenv.2017.03.065

*Bacillus thuringiensis*
Agriculture
Lotic
Pseudo-persistent

Cry1Ab protein in streams sampled across an agricultural landscape, combined with laboratory studies showing rapid leaching and degradation, suggests that Cry1Ab may be pseudo-persistent at the watershed scale due to the multiple input pathways from the surrounding terrestrial environment.



## 1. Introduction

Transgenic crops are developed to express desirable traits, such as pest resistance, disease resistance, and herbicide tolerance. Maize (*Zea mays* L.) that expresses Cry proteins derived from strains of the bacterium *Bacillus thuringiensis* (Bt) is insecticidal to susceptible pests, and one of the most commonly planted varieties of Bt maize expresses the Cry1Ab protein. Cry1Ab is present in maize tissues throughout the growing season (Nguyen and Jehle, 2007; USEPA, 2008) and in maize detritus after harvest (Baumgarte and Tebbe, 2005; Hopkins and Gregorich, 2005; Zwahlen et al., 2003a). Cry1Ab protein can enter soils through roots (Saxena et al., 1999; Saxena and Stotzky, 2001a), pollen inputs (Dutton et al., 2003; Obrist et al., 2006; Romeis et al., 2008; Whiting et al., 2014), and via leaching from detritus (Baumgarte and Tebbe, 2005; Hopkins and Gregorich, 2005; Zwahlen et al., 2003a).

Terrestrial and aquatic ecosystems are closely linked, and agricultural materials (i.e., crop detritus) can enter adjacent water bodies, providing a pathway for movement of Cry proteins from terrestrial to aquatic environments. Maize detritus enters headwater streams draining agricultural fields via wind and surface runoff (Jensen et al., 2010; Rosi-Marshall et al., 2007). Inputs and standing stocks of maize pollen and detritus (e.g., leaves, husks, and cobs) can reach 1.0 g $m^{-2}$ $y^{-1}$ and 7.9 g ash-free dry mass [AFDM] $m^{-2}$ $y^{-1}$, respectively, and the fate of this material includes retention and subsequent decomposition via physical breakdown, microbial degradation, invertebrate consumption (Chambers et al., 2010; Griffiths et al., 2009; Jensen et al., 2010; Swan et al., 2009), or downstream transport (Griffiths et al., 2012; Rosi-Marshall et al., 2007).

In midwestern US streams, submerged Bt maize leaves decay over a few months, and the associated Cry1Ab protein concentration declines exponentially over time, with 20% of the initial protein remaining in maize leaves after ~70 d (Griffiths et al., 2009). This exponential decline of Cry1Ab suggests that the protein leaches from submerged detritus into the water column (Griffiths et al., 2009). Cry1Ab protein has been measured in stream water (Douville et al., 2005, 2007; Tank et al., 2010) as well as in sediments (Douville et al., 2005, 2007). Sediment-bound proteins are susceptible to downstream transport if agricultural soils wash into aquatic systems (e.g., Carstens et al., 2012; Madliger et al., 2011; Strain and Lydy, 2015), and dissolved Cry1Ab has been measured in surface runoff (Strain and Lydy, 2015; Whiting et al., 2014). Despite known terrestrial-aquatic linkages, the fate of dissolved Cry1Ab in aquatic ecosystems has only recently been considered (e.g., Douville et al., 2005, 2007; Strain and Lydy, 2015; Whiting et al., 2014).

In this study, we used a combination of field sampling and laboratory experiments to examine the occurrence, leaching, and degradation of Cry1Ab protein in streams. First, we investigated whether dissolved Cry1Ab concentration in streams and tile drains (subsurface drainage pipes that underlay agricultural fields) varied spatially (across an agriculturally dominated region) and temporally (among 6 time periods encompassing the crop growing season, crop harvest, and snowmelt/spring flooding) in 11 low-order streams in northwestern Indiana, USA. Tile drainage water was sampled for Cry1Ab, as subsurface tile drains are ubiquitous across the midwestern US landscape (Schilling and Libra, 2003; Skaggs et al., 1992), and may be a subsurface input pathway of Cry1Ab into streams. Second, recirculating, artificial streams were used to quantify the rate at which Cry1Ab protein leaches from maize leaves into the water column. Although several studies have examined the leaching dynamics of Cry1Ab under controlled conditions (e.g., Strain et al., 2014; Strain and Lydy, 2015), recirculating artificial streams more realistically represent conditions in flowing waters. Finally, 3 laboratory microcosm experiments were conducted to determine the influence of microorganisms, light, and water collected from 3 different agricultural headwater streams on Cry1Ab degradation in the water column. We hypothesized that the presence of microorganisms in stream water would increase degradation rates of Cry1Ab, and that light would stimulate degradation of Cry1Ab through assimilatory uptake of the protein by autotrophs. We predicted that Cry1Ab degradation rates would not differ in water collected from 3 agricultural streams due to the similar physical and chemical characteristics of these streams. We also hypothesized that Cry1Ab degradation rates would be similar among the 3 streams assuming that protein degradation is a common process carried out by microorganisms (Valldor et al., 2015).

## 2. Methods

### 2.1. Detection of Cry1Ab protein in streams and tile drains in the field

We sampled 11 headwater streams located in northwestern Indiana, USA, an intensively cultivated region with ~97% of land planted in a maize-soybean rotation (NASS, 2012). The study streams are typical of low-gradient, midwestern agricultural streams in that they have sand/silt-dominated beds with mainly run/pool sequences, and high nitrate concentrations as a result of fertilizer runoff from fields (Table 1). These streams are managed for effective water drainage and conveyance through frequent dredging and the use of subsurface tiles drains (Blann et al., 2009; Zucker and Brown, 1998). The riparian zones adjacent to these streams consist primarily of grass buffer strips, which vary in width (typically 10–20 m); however, we observed crops planted up to the stream edge along some sections of the study reaches. Further, the lack of riparian trees allows for high light penetration to streambeds, resulting in large diel swings in stream water temperature (Griffiths et al., 2013).

On 6 sampling dates that encompassed 3 seasonally important time periods, we collected water from 11 streams (stream names: 1A-F, 2B-F, after Griffiths et al., 2013 and Rosi-Marshall et al., 2007) and associated flowing tile drains as well as any maize detritus present in the active stream channel. These sampling dates were targeted to include potentially different modes of Cry1Ab entry to streams. Sampling in July and August encompassed the growing season when Cry1Ab protein is released from maize roots into soils (Saxena et al., 1999; Saxena and Stotzky, 2001a, 2001b; Whiting et al., 2014) and may enter streams via overland flow (Carstens et al., 2012) or through subsurface tile drains. Sampling in September and November encompassed the periods before and after crop harvest, respectively; the latter is when maize detritus can wash or blow into streams and leach Cry1Ab protein into the water column (e.g., Griffiths et al., 2009; Rosi-Marshall et al., 2007; Viktorov, 2011). Finally, sampling in March and May the following year encompassed the period of snowmelt and spring flooding, as precipitation events can transport large pulses of maize detritus into streams (Tank et al., 2010; N.A. Griffiths, personal observation). Four of the study streams were adjacent to at least one Bt maize field for a distance of >200 m, and 7 of the study streams were adjacent to non-Bt maize or soybean fields (Table 1); however, we were unable to determine which crops were planted in the watershed upstream of our sampling sites.

**Table 1**
Measured ranges in physical and chemical characteristics of 11 agricultural streams in northwestern Indiana, USA, in July, August, September, and November 2008, and March and May 2009.

| Stream | Crops planted adjacent to stream | Tile drains (#/200 m stream reach) | Stream gradient (m/m) | Stream discharge (L/s) | Temperature (°C) | Specific conductivity (μS/cm) | $NO_3^-$-N (mg/L) | $NH_4^+$-N (μg/L) | SRP (μg/L) |
|---|---|---|---|---|---|---|---|---|---|
| 1A | Soy/Soy | 0 | 0.0011 | <1–36 | 6.6–27.7 | 553–658 | 0.6–10.6 | 7.5–59.9 | 4.5–8.7 |
| 1B | Non-Bt maize/Soy | 3 | 0.0011 | <1–36 | 4.1–18.7 | 665–1314 | 0.1–10.3 | 8.1–299.0 | 4.8–92.5 |
| 1C | Bt maize/Soy | 0 | 0.0020 | 79–1581 | 9.3–24.9 | 582–678 | 1.5–8.9 | 5.2–44.6 | 2.0–5.7 |
| 1D | Bt maize/Bt maize | 3 | 0.0010 | 14–381 | 5.1–20.5 | 554–645 | 0.3–8.6 | <1–30.1 | 2.5–6.1 |
| 1E | Soy/Soy | 2 | 0.0008 | <1–478 | 3.8–21.8 | 574–717 | 0.4–9.3 | 1.6–42.1 | 1.8–8.4 |
| 1F | Bt maize/Bt maize | 4 | 0.0019 | <1–303 | 11.1–25.9 | 522–597 | 1.3–9.6 | 7.4–27.0 | 2.5–6.0 |
| 2B | Bt maize/Non-Bt maize | 1 | 0.0010 | 2–484 | 10.3–24.3 | 522–697 | 0.3–9.8 | <1–21.3 | 2.3–6.4 |
| 2C | Soy/Soy | 0 | 0.0022 | <1–312 | 10.9–27.4 | 439–638 | <0.01–10.6 | 7.5–41.5 | 2.4–7.2 |
| 2D | Non-Bt maize/Non-Bt maize | 0 | 0.0006 | 37–1870 | 12.4–27.4 | 569–756 | 0.1–10.1 | 13.2–26.1 | 3.2–7.6 |
| 2E | Non-Bt maize/Soy | 3 | 0.0029 | <1–99 | 10.3–22.2 | 584–779 | 5.2–10.9 | 5.8–38.1 | 2.6–10.2 |
| 2F | Non-Bt maize/Non-Bt maize | 0 | 0.0020 | 3–406 | 7.0–23.2 | 561–681 | 0.1–10.3 | 9.4–24.9 | 3.5–10.3 |

Note: stream discharge (i.e., stream flow), water temperature, specific conductivity, and water samples for nitrate ($NO_3^-$-N), ammonium ($NH_4^+$-N), and soluble reactive phosphorus (SRP) analyses were collected during each sampling period. Stream gradient (i.e., stream slope) was measured once in each stream. Tile drains indicate the number of drain outlets that were observed along each 200-m study reach.

In each stream, water samples were collected from 3 locations that spanned a distance of ~200 m. Each water sample was filtered through a 0.7 μm glass-fiber filter (GF/F, Whatman, Florham Park, New Jersey, USA) into an acid-washed, 25 mL polyethylene bottle. Filtered water samples were also collected from all flowing tile drains ($n = 2$–15 depending on the sampling date). If present, a composite sample of maize leaf and husk detritus was collected from the active stream channel. All water and maize detritus samples were stored on ice, and upon return to the laboratory, were immediately frozen at −30 °C until Cry1Ab protein analysis (described in Section 2.5). At each stream and on each sampling date, water velocity was measured using a velocity meter (Marsh-McBirney Flow-Mate, Frederick, Maryland, USA) and discharge was calculated using the velocity-area protocol (Gore, 2006). Replicate water samples were also collected for analysis of stream water nutrient concentrations. Water samples were filtered as described above, and frozen at −30 °C until analysis. Nitrate-N concentrations were measured using the cadmium reduction method (APHA, 2005), ammonium-N concentrations were measured using the phenol-hypochlorite method (Solorzano, 1969), and soluble reactive phosphorus (SRP) concentrations were measured using the molybdate-antimony method (Murphy and Riley, 1962) on a Lachat QC8500 Flow Injection Autoanalyzer (Lachat Instruments, Loveland, Colorado, USA).

### 2.2. Experimental leaching of Cry1Ab protein from maize leaves into stream water

We examined the long-term leaching dynamics of Cry1Ab protein from submerged maize leaves using recirculating artificial streams. The experiment took place over a 70-d period in order to quantify Cry1Ab leaching throughout the entire decomposition process (Griffiths et al., 2009). We placed 100 g of dried, senesced Bt maize leaves into 6 replicate recirculating streams each containing 40 L of groundwater (no sediments), and sampled maize leaves and stream water on days 3, 7, 13, 25, 37, and 70 for analysis of Cry1Ab protein. On each date, ~0.5 g of maize leaves was removed, and water samples were filtered through 0.7 μm Whatman GF/F filters into acid-washed, 60 mL polyethylene bottles. Both maize leaves and stream water samples were frozen at −30 °C until Cry1Ab analysis. Prior to water column sampling, the stream volume was brought up to 40 L to account for any evaporation that otherwise would have inflated the Cry1Ab protein concentration in stream water relative to previous sampling dates.

### 2.3. Degradation of Cry1Ab protein in stream water

Laboratory microcosm experiments were used to examine the degradation of dissolved Cry1Ab protein in stream water. Three experiments were conducted to examine: 1) the role of aquatic microorganisms in Cry1Ab degradation, 2) the role of light in potentially stimulating autotrophic uptake of Cry1Ab protein, and 3) whether Cry1Ab degradation differed in stream water (with associated water-column microbial communities) collected from 3 agricultural headwater streams. The source of Cry1Ab protein for all degradation experiments was from air-dried, senesced Bt maize leaves that were collected from one maize field just prior to crop harvest. The Cry1Ab source was created by placing 14 g of dried Bt maize leaves into 800 mL of de-ionized (DI) water for 3 h. The solution was then filtered through a 0.7 μm filter to remove detrital particles, and the filtrate was used as the Cry1Ab source for the degradation experiments. For the first two experiments, stream water was collected from Juday Creek, a headwater stream located 6 km from the University of Notre Dame that flows through a mosaic of land-use types (agricultural, forested, and suburban). For the third experiment, stream water was collected from 3 typical agricultural streams (stream 2B, 2C, and 2D) in Indiana to examine whether spatial variability in stream water affects Cry1Ab degradation rates. The initial concentrations of Cry1Ab protein used in the degradation experiments ranged from 1280 to 2195 ng Cry1Ab/L, and degradation results were reported as a percentage of Cry1Ab remaining in the water column based on these initial concentrations. The initial concentrations of Cry1Ab used in the degradation experiments were much higher than the maximum concentration of Cry1Ab protein measured in stream water in the field, and background concentrations of Cry1Ab protein in stream water used in the degradation experiments were assumed to be negligible.

To examine the role of stream-water microorganisms in Cry1Ab degradation, 5 mL of Cry1Ab solution was added to Erlenmeyer flasks filled with 40 mL of deionized water (DI), stream water filtered through a 0.2 μm filter (no microorganisms), stream water filtered through a 0.7 μm filter (microorganisms), or unfiltered stream water (microorganisms and organic particles). To promote water circulation within each flask and maintain constant environmental conditions, flasks were placed on a rotating shaker table located inside an environmental chamber (air temperature: 21.5 °C, light:dark cycle: 14 h:10 h). Water samples were collected every 24 h for a total of 72 h with $n = 5$ replicates per filtration treatment and collection period. Samples were poured into acid-washed, 25 mL polyethylene bottles and immediately frozen at −30 °C until Cry1Ab analysis.

To examine whether light influenced Cry1Ab uptake by autotrophs, 5 mL of Cry1Ab solution was added to Erlenmeyer flasks filled with 40 mL of DI water or unfiltered stream water, and half of the samples were placed under a shade cloth (dark) and half under

growth lamps (light). As in the previous experiment, flasks were placed on a rotating shaker table under the same environmental conditions described above, and water samples were collected every 24 h for a total of 72 h with $n = 5$ replicates per treatment and collection period.

Because the two previous degradation experiments used water from Juday Creek, which is influenced by a variety of land-use types, the third experiment was designed to examine whether Cry1Ab degradation varied in water collected from different agricultural streams. Unfiltered stream water was collected from streams 2B, 2C, and 2D (Table 1). In the laboratory, 40 mL of stream water was placed into Erlenmeyer flasks, using DI water as a control, and 5 mL of Cry1Ab solution was added to each flask. Flasks were placed on a rotating shaker table under the same environmental conditions described above, and water samples were collected every 24 h for a total of 72 h, with $n = 5$ replicates per treatment and collection period.

### 2.4. Analysis of Cry1Ab protein in maize detritus

The concentration of Cry1Ab protein in maize leaf and husk detritus was determined using a commercial double-antibody sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA; Strategic Diagnostics Inc., Newark, Delaware, USA) as described in Griffiths et al. (2009) and Zwahlen et al. (2003a). Maize detritus was dried at 60 °C for 48 h and then ground into fine particles using an electric grinder. To extract Cry1Ab protein, 1 g of ground maize detritus was placed into 50 mL of 1X Phosphate Buffered Saline Tween-20 (PBST). Next, the maize detrital sample was homogenized in PBST using a hand-held tissue homogenizer (BioSpec Products, Inc., Bartlesville, Oklahoma, USA). The sample was then centrifuged at 10,000 rpm for 10 min, and the supernatant was used for the ELISA analysis. The concentration of Cry1Ab in maize detritus was determined based on a 10-point calibration curve, ranging from 0.5 ng/mL to 100 ng/mL, which was created from the serial dilution of purified Cry1Ab protein (Abraxis, Warminster, Pennsylvania, USA) dissolved in 1X PBST. Five PBST blanks were included to identify any potential contamination among samples and to account for matrix effects associated with PBST. Samples, standards, and buffer blanks were aliquoted in triplicate into a 96-well ELISA plate and absorbance was read at 450 nm and 650 nm on a SpectraMax M2 microplate reader (Molecular Devices Corporation, Sunnyvale, California, USA). The absorbance at 450 nm was subtracted from the absorbance at 650 nm to correct for turbidity, and then the mean buffer absorbance was subtracted to account for PBST matrix effects. The concentration of Cry1Ab protein was expressed as μg Cry1Ab/g dry maize leaf (or detritus). The minimum detection limit (MDL) of our method (MDL = 0.56 ng/mL, equivalent to 0.03 μg/g dry maize leaf) was determined by multiplying the standard deviation of a low standard (0.72 ng/mL, $n = 7$ replicates) by 3.14 (APHA, 2005).

### 2.5. Analysis of Cry1Ab protein in stream water

For stream water samples with much lower Cry1Ab concentrations than Bt maize detritus, Amicon® Ultra-15 mL centrifugal filter units (30 K Nominal Molecular Weight Limit, Millipore, Billerica, Massachusetts, USA) were used to concentrate dissolved Cry1Ab protein for subsequent analysis. The centrifugal extraction method and ELISA assay for stream water samples were field validated with high recovery of Cry protein by Strain et al. (2014) using PBST. Centrifugal filter units were filled with 14.5 mL of stream water and 0.5 mL of 1X PBST. Samples were spun using a swinging-bucket centrifuge at 2500 rpm for 30 min, and the retentate (concentrated Cry1Ab) was weighed in a microcentrifuge tube to determine retentate recovery. The concentration of Cry1Ab in stream water samples was determined from an 8-point calibration curve, which was created from the serial dilution of purified Cry1Ab protein dissolved in DI water. The calibration curve ranged from 3 ng/L to 400 ng/L and was run in triplicate on the ELISA plate. Standards were prepared in DI water (after Strain et al., 2014) and 5 DI water blanks were included to identify any potential contamination between samples. All standards and blanks went through the Cry1Ab extraction procedure along with the stream water samples (i.e., centrifugal filter units, addition of 0.5 mL of 1X PBST). One hundred μL of retentate from samples, standards, and blanks was pipetted into a 96-well ELISA plate. The absorbance of each well was read at 450 nm and 650 nm and corrected for turbidity as described above. The concentration of Cry1Ab protein was expressed as ng Cry1Ab/L stream water. The MDL for this method was calculated using the concentrations of our 2 lowest standards of 3 ng/L and 6 ng/L. For each standard, the concentration of 7 samples was measured and the standard deviation of those measurements was multiplied by 3.14 (APHA, 2005). The average of the 2 MDLs was used to determine our overall MDL of 3 ng/L.

### 2.6. Statistical analyses

To examine spatial patterns of Cry1Ab protein in the field, Pearson's chi-square tests were used to determine whether there were differences in the detection of Cry1Ab protein in stream or tile drain water between Bt-maize fields and non-Bt maize or soybean fields, and to assess whether streams in which the sampling sites were adjacent to maize fields were equally as likely to have maize detritus in the active channel as streams in which the sampling sites were adjacent to soybean fields. Simple linear regression was used to determine whether mean stream discharge influenced the frequency of Cry1Ab detection among streams, and correlation analysis was used to examine whether there was an association between the frequency of Cry1Ab detection in streams and the frequency of Cry1Ab detection in the associated tile drains. Kruskal-Wallis tests were used to assess differences in Cry1Ab concentrations in stream water, tile drain water, and maize detritus among sampling dates, to determine if stream or tile drain Cry1Ab concentrations differed between Bt-maize and non-Bt maize or soybean fields, and to test whether concentrations of dissolved Cry1Ab differed between stream and tile drain water.

For the laboratory recirculating stream results, simple linear regression was used to determine whether Cry1Ab protein concentration (natural-log transformed) in maize leaves and stream water decreased over time. For the degradation experiment results, analysis of covariance (ANCOVA) was used to examine whether degradation of Cry1Ab protein (natural-log transformed) was influenced by filtration, light, and stream water source over time. When there was a significant interaction between time and the main factors, a Tukey's Honestly Significant Difference (HSD) post-hoc test was used to determine which groups were different from each other. When necessary, data were transformed using natural-log, square-root, or arcsine-square root to meet parametric assumptions. Significance was defined as $P \leq 0.05$ and all statistical analyses were performed using SYSTAT v.12 (SYSTAT, 2007).

## 3. Results

### 3.1. Detection of Cry1Ab protein in streams and tile drains in the field

In the field, each of the 11 streams sampled in this study tested positive for Cry1Ab protein on at least one of the six sampling dates (Table 2). Cry1Ab protein was detected in 73% of streams in September (start of harvest) and May (end of snowmelt/spring floods). In July (start of the growing season) and March (start of snowmelt/spring floods), Cry1Ab was detected in 27% of streams. Finally, in August near the end of the growing season and in November after crop harvest, Cry1Ab was detected in 18% of streams. Differences in stream size may have accounted for the variation in the frequency of Cry1Ab-positive water; however, mean stream discharge was not related to the frequency of Cry1Ab detection among streams (simple linear regression, $r^2 = 0.29$, $P = 0.09$). Furthermore, there was no difference in the

**Table 2**
Detection of Cry1Ab protein in stream water, tile drain water, and maize detritus collected in July, August, September, and November 2008, and March and May 2009.

| Season | Sampling date | Stream water samples positive for Cry1Ab (%) | Tile drain water samples positive for Cry1Ab (%) | Streams with maize detritus present (%) | Streams with Cry1Ab-positive maize detritus present (%) |
|---|---|---|---|---|---|
| Crop growing season | 7/7/2008 | 27% | 17% | 0% | 0% |
| | 8/13/2008 | 18% | 50% | 0% | 0% |
| Crop harvest | 9/29/2008 | 73% | 40% | 0% | 0% |
| | 11/3/2008 | 18% | 0% | 82% | 73% |
| Spring floods/snowmelt | 3/5/2009 | 27% | 40% | 73% | 36% |
| | 5/2/2009 | 73% | 47% | 18% | 0% |

Note: within each stream, 3 water samples were collected along a 200-m reach; when one or more water samples tested positive for Cry1Ab (above the detection limit of 3 ng/L), the stream was considered to be positive for Cry1Ab.

detection of Cry1Ab protein in streams in which the sampling site was adjacent to Bt maize fields (e.g., 46% of the samples were Cry1Ab-positive) compared to streams where we sampled adjacent to non-Bt maize or soybean fields (e.g., 45% of the samples were Cry1Ab-positive) (Pearson's chi-square test, $\chi^2 = 0.002$, $df = 1$, $P = 0.96$).

Six of the 11 study streams had at least one tile draining into each ~200 m study reach (Table 1). Tiles draining into 5 of those streams were positive for Cry1Ab protein on at least one sampling date. However, none of the 10 tile drain samples collected from the 3 tile drains in stream 1D were positive for Cry1Ab, even though the sampling site was located adjacent to fields planted in Bt maize. Dissolved Cry1Ab was also detected in water flowing from at least one tile drain on all sampling dates, except in November after crop harvest (Table 2). Cry1Ab was detected in 17% of flowing tile drains in July, 40% in September and March, 47% in May, and 50% in August. There was no relationship between the frequency of Cry1Ab detection in streams and the frequency of Cry1Ab detection in the associated tile drains (Pearson's correlation, $R = 0.64$, $P = 0.17$). Furthermore, there was no difference in the occurrence of Cry1Ab protein in tiles that drained Bt maize fields (35% of the samples were Cry1Ab-positive) compared to tiles that drained non-Bt maize or soybean fields (32% of the samples were Cry1Ab-positive) (Pearson's chi-square test, $\chi^2 = 0.04$, $df = 1$, $P = 0.84$).

Overall, the concentration of Cry1Ab protein in both stream and tile drain water was generally low, and ranged from our detection limit of 3 ng/L up to 60 ng/L, and there was no difference in Cry1Ab concentration between stream water and tile drain water (Kruskal-Wallis, $P = 0.16$). Of the samples that were above the detection limit, Cry1Ab concentrations were variable over time (Fig. 1). There were no differences in Cry1Ab concentrations in stream or tile drain water among seasons (streams: Kruskal-Wallis, $P = 0.21$; tile drains: Kruskal-Wallis, $P = 0.41$) or between Bt maize fields and other fields (streams: Kruskal-Wallis, $P = 0.87$; tile drains: Kruskal-Wallis, $P = 0.09$) (Fig. 1).

Maize detritus was found in the active channels of 82% of streams sampled in November at the end of harvest, in 73% of streams sampled in March at the start of snowmelt/spring floods, in 18% of streams sampled in May at the end of snowmelt/spring floods. We did not find maize detritus in streams sampled during all other time points (e.g., start and end of the growing season, start of crop harvest) (Table 2). Streams in which the sampling site was adjacent to maize fields were equally as likely to have maize detritus in the active channel as streams in which the sampling site was adjacent to fields planted in soybeans (November: Pearson's chi-square test, $\chi^2 = 0.64$, $df = 1$, $P = 0.43$; March: Pearson's chi-square test, $\chi^2 = 1.55$, $df = 1$, $P = 0.21$). The percentage of streams containing Cry1Ab-positive maize detritus (above the detection limit of 0.03 μg/g dry maize detritus) varied over time: 73% of streams in November, 36% of streams in March, and none of the streams in May contained Cry1Ab-positive maize detritus (Table 2). Furthermore, half of the Cry1Ab-positive maize detrital samples collected in November and March were found in streams with sampling sites adjacent to Bt maize fields, and the other half were collected from streams with sampling sites adjacent to non-Bt maize or soybean fields. The mean concentration of Cry1Ab protein in maize detritus was higher in November (1.85 ± 0.35 μg/g dry maize detritus) directly after crop harvest than in March ~4 months after harvest (0.34 ± 0.19 μg/g dry maize detritus) (Kruskal-Wallis, $P = 0.02$).

### 3.2. Experimental leaching of Cry1Ab protein from maize leaves into stream water

In the artificial stream experiment, the concentration of Cry1Ab in submerged, Bt maize leaves decreased over time (Fig. 2a; simple linear regression on natural-log transformed data, $r^2 = 0.88$, $P < 0.0001$) from 4.76 ± 0.05 μg/g dry maize leaf on day 0 to 0.04 ± 0.02 μg/g dry maize leaf on day 70, resulting in the loss of 99% of Cry1Ab from Bt maize leaves over this time period. Cry1Ab protein was detectable in the water column after Bt maize leaves were submerged, and the concentration of Cry1Ab in stream water also decreased over time (Fig. 2b; simple linear regression on natural-log transformed data, $r^2 = 0.44$, $P = 0.0001$). Water-column Cry1Ab concentrations were highest on day 3 (201.6 ± 13.1 ng/L), slightly above the detection limit on day 25 (4.5 ± 4.5 ng/L), and below the detection limit of 3 ng/L on days 13, 37, and 70. Using a mass balance approach, the total amount of Cry1Ab protein lost from Bt maize leaves after leaching for 3 days was 312.4 ± 25.8 μg/-stream, while the amount of protein measured in the water column was much lower (8.1 ± 0.5 μg/stream), suggesting that Cry1Ab degraded over the experiment.

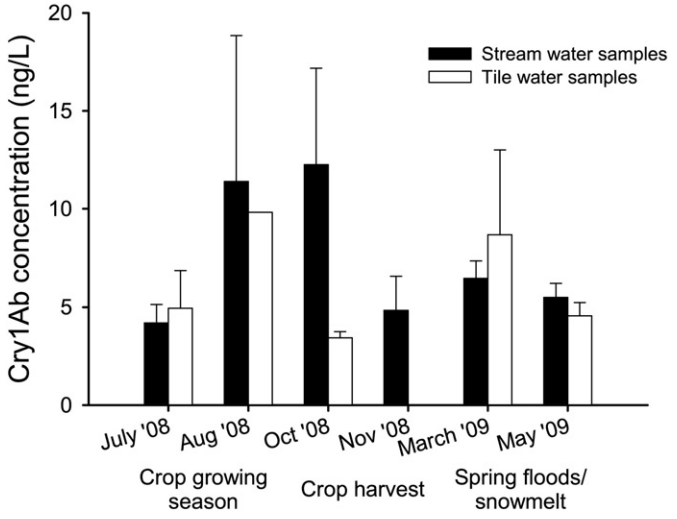



**Fig. 1.** Mean concentration (±SE) of Cry1Ab protein (ng/L) in stream (black bars) and tile drain (white bars) water samples that were above the detection limit of 3 ng/L. Water samples were collected for Cry1Ab protein analysis on 6 dates (July and August 2008 [summer growing season], October and November 2008 [crop harvest], and March and May 2009 [snowmelt/spring flood period]) from 11 streams and associated flowing tile drains ($n = 12$ in July, $n = 2$ in August, $n = 5$ in October, $n = 4$ in November, $n = 10$ in March, and $n = 15$ in May).

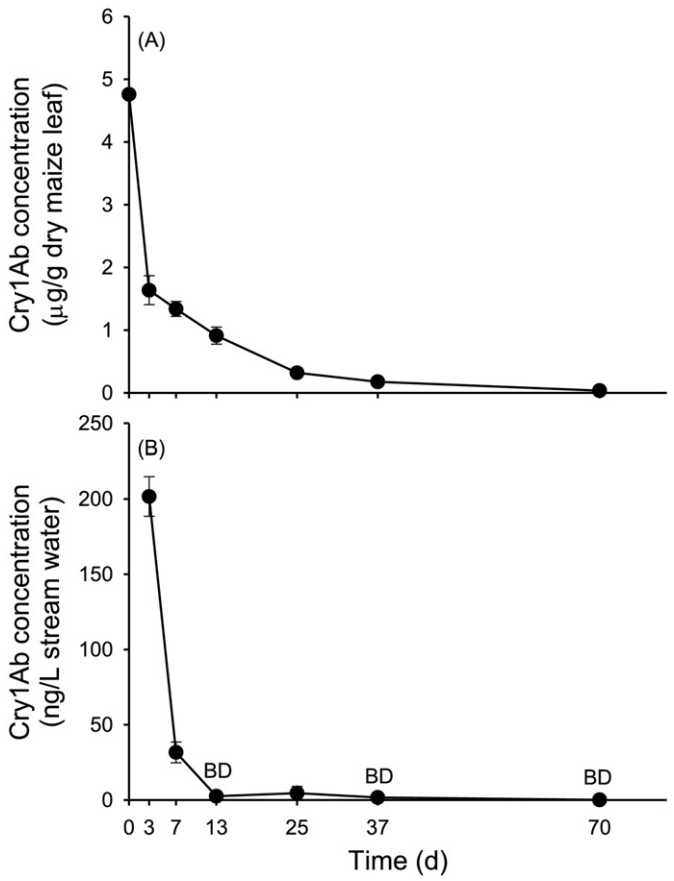



**Fig. 2.** Mean Cry1Ab concentration (±SE) in (a) submerged Bt maize leaves (µg/g dry weight) and (b) stream water (ng/L) measured in artificial, recirculating streams over a period of 70 d ($n = 6$ per data point). Note that the mean Cry1Ab concentrations in stream water on days 13, 37, and 70 were below the detection limit of 3 ng/L ('BD'), and mean Cry1Ab concentrations in stream water were above the detection limit on days 3, 7, and 25.

### 3.3. Degradation of Cry1Ab protein in stream water

Degradation of Cry1Ab differed among experimental filtration treatments over the 72 h microcosm experiment (Fig. 3a, ANCOVA, $P < 0.0001$), as Cry1Ab degraded more quickly in the presence of water-column microorganisms compared to the treatments without microorganisms (all Tukey's HSD $P < 0.0001$). After 72 h, >99% of Cry1Ab had degraded in microcosms containing microorganisms, and Cry1Ab concentrations in these treatments were near the detection limit of 3 ng/L. In microcosms without microorganisms (i.e., 0.2 µm filtered stream water and DI water), 56.0 ± 2.4% and 29.4 ± 6.5% of the Cry1Ab protein had degraded after 72 h, respectively.

Light did not influence autotrophic uptake of Cry1Ab as there was no significant difference in the percentage of Cry1Ab remaining between light and dark treatments over time (Fig. 3b; ANCOVA, $P = 0.46$). Similar to the experiment described above, Cry1Ab degradation differed among filtration treatments over time (ANCOVA, $P < 0.0001$), with faster degradation in microcosms containing water-column microorganisms. After 72 h, regardless of light treatment, 99.8 ± 0.2% of Cry1Ab protein had degraded in microcosms containing microorganisms compared to 31.2 ± 5.9% degradation in microcosms without microorganisms.

There was also no influence of stream water source on Cry1Ab degradation as Cry1Ab degraded similarly in unfiltered water collected from streams 2B, 2C, and 2D (Fig. 3c; Tukey's HSD, all $P > 0.05$). As in the previous two experiments, degradation was faster in stream water with microorganisms than in DI water (ANCOVA, $P = 0.007$; Tukey's HSD, all $P < 0.05$).

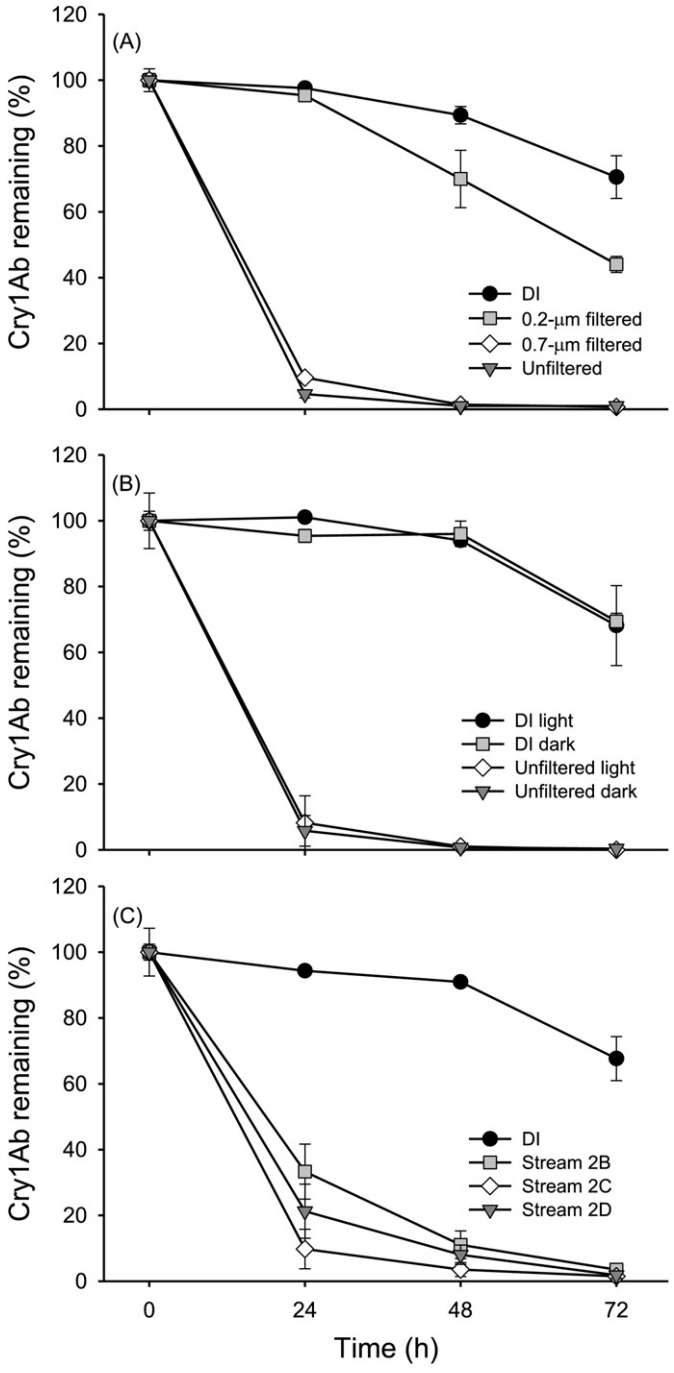



**Fig. 3.** Mean percentage (±SE) of Cry1Ab protein remaining in water in the degradation experiments ($n = 5$ per data point). (a) The microbial contribution to Cry1Ab degradation was examined by adding dissolved Cry1Ab to DI water (sterile), stream water that was filtered through a 0.2-µm filter (no microorganisms), a 0.7-µm filter (microorganisms), or was unfiltered (microorganisms and organic particles). (b) The influence of light on Cry1Ab degradation was examined by adding dissolved Cry1Ab to DI water or unfiltered stream water, with half of the treatments covered in a shade cloth ('dark') and half placed under growth lamps ('light'). (c) The effect of different agricultural stream water sources on Cry1Ab degradation was examined by adding dissolved Cry1Ab to DI water or stream water collected from 3 agricultural streams (streams 2B, 2C, or 2D, see Table 1).

## 4. Discussion

### 4.1. Detection of Cry1Ab protein in streams and tile drains in the field

In this study, we found that agricultural streams receive inputs of Cry1Ab protein from Bt maize throughout the year. The prevalence of

Cry1Ab protein in water samples collected from agricultural streams across space and time suggests that dissolved Cry1Ab protein may be widespread in headwater streams and ditches in the corn belt of the midwestern US, and that there are likely multiple mechanisms by which Cry1Ab can enter adjacent waterways. A survey of the spatial distribution of dissolved Cry1Ab protein in water-column samples collected from midwestern US streams conducted 6 months after crop harvest found that 50 of the 215 stream water samples (23%) were positive for Cry1Ab, with no obvious spatial aggregation across the maize-soybean dominated landscape (Tank et al., 2010). Similarly, our study did not find any spatial pattern in Cry1Ab protein across 11 streams sampled in this study, and the probability of detecting Cry1Ab protein was similar between streams adjacent to Bt maize fields and non-Bt maize or soybean fields. These detection patterns for dissolved Cry1Ab protein were consistent with a recent study that measured Cry1Ab protein in runoff from both Bt and non-Bt maize fields (Strain and Lydy, 2015). Because the majority of Indiana's maize crop was Bt maize at the time of sampling (NASS, 2008), it was likely that all stream sites had Bt maize planted somewhere in the watershed upstream of the sampling sites, and these fields could have influenced the detection of Cry1Ab in stream sampling sites not located directly adjacent to a Bt maize field.

One potential mechanism by which Cry1Ab can enter streams is through the input of Bt maize detritus (Douville et al., 2005; Rosi-Marshall et al., 2007) and subsequent detrital leaching of soluble Cry1Ab proteins (Griffiths et al., 2009). Given the rapid leaching of Cry1Ab protein after detritus is submerged (Griffiths et al., 2009; Strain and Lydy, 2015), the input of Bt maize detritus is a potentially significant source of dissolved proteins to agricultural streams. In our field survey, Bt maize detritus was found in 73% of streams sampled at the end of crop harvest in November, and in 36% of streams sampled at the beginning of the following year during snowmelt/spring floods. These findings are consistent with a spatially intensive survey carried out 6 months after crop harvest, that found 13% of 217 streams sites contained Bt maize detritus with no clear spatial pattern across the landscape (Tank et al., 2010). Overall, these findings, combined with results from this study showing the detection of dissolved Cry1Ab across seasons, suggest that Bt maize detritus may be a source of Cry1Ab protein to streams and drainage ditches for several months after maize crops are harvested.

A second mechanism by which Cry1Ab can enter streams is the input of Cry1Ab protein via surface runoff and subsurface flow paths, thus sourcing sediment-bound proteins or leached proteins into adjacent streams. Multiple studies have shown that dissolved Cry1Ab can enter streams via surface runoff (Strain and Lydy, 2015; Whiting et al., 2014), and sediment-bound proteins can be subjected to overland erosion into aquatic systems (Whiting et al., 2014). Our goal was to investigate a potential flow path for leached Cry1Ab protein through subsurface tile drains that are typically buried 0.6–1.2 m below the soil surface (Blann et al., 2009). Of the 48 samples collected from intermittently flowing tile drains over the 6 sampling dates, 32% were positive for Cry1Ab protein, but there was no difference in the occurrence of Cry1Ab protein in tile drains that were sampled from outlets adjacent to Bt maize fields compared to tile outlets located next to non-Bt maize or soybean fields. The distance and area drained by each tile was not known, and tile drains can underlay fields for hundreds of meters in an interconnected network. Thus, it is possible that tile drains we attributed to non-Bt maize or soybean fields may have drained Bt maize fields that were not directly adjacent to the streams. Overall, the detection of Cry1Ab protein in tile drains across seasons suggests that tiles may be a direct mechanism by which Cry1Ab can be transported from agricultural fields into streams, but due to the complexity of tile drain networks, it is not possible to attribute tile drainage to a particular field.

Natural populations of *B. thuringiensis* in soils and sediments could be a potential source of dissolved Cry1Ab protein in tile and stream water; however, in the field, the protein from *B. thuringiensis* populations would likely be in the insoluble, crystalline form (protoxin) rather than the soluble, activated form (endotoxin) that is expressed in Bt maize (Douville et al., 2005). Furthermore, the ELISA assay is optimized to extract the endotoxin via a neutral-pH buffer (1X PBST has a pH of 7.4; Strain et al., 2014), whereas the protoxin is extracted at a higher pH of 10–11 (Douville et al., 2005), suggesting that the ELISA assay used in this study is primarily detecting the activated Cry1Ab protein rather than the insoluble form expressed by natural populations of *B. thuringiensis*. While our study did not quantify *B. thuringiensis* populations in soils and sediments, a previous study found no correlation between bacterial cell counts of *B. thuringiensis* in the field and Cry1Ab concentrations in soils and sediments, and concluded that measured Cry1Ab protein was derived from Bt maize (Douville et al., 2005).

### 4.2. Degradation of Cry1Ab protein in stream water

Previous research in terrestrial environments has shown that unbound Bt proteins are rapidly degraded by microorganisms (Accinelli et al., 2008; Crecchio and Stotzky, 1998, 2001; Koskella and Stotzky, 1997; Palm et al., 1996; Valldor et al., 2015; Wang et al., 2007) and we predicted a similar fate for Cry1Ab in aquatic ecosystems. In a direct comparison of Cry1Ab degradation in sediments and surface waters, Douville et al. (2005) found that breakdown was faster in non-sterile surface water, perhaps because Bt proteins bound to soils and sediments are less susceptible to degradation (Crecchio and Stotzky, 1998, 2001; Stotzky, 2004; Strain and Lydy, 2015; Valldor et al., 2015). In our study, microbial degradation of unbound Cry1Ab protein in stream water was rapid, and >95% of added Cry1Ab degraded within 72 h. A similar result was found in our Cry1Ab leaching experiment, where a mass balance revealed that after 3 d, 97% of Cry1Ab leached from detritus was not detectable in the water column. In addition, light did not appear to influence degradation of Cry1Ab by stimulating autotrophic assimilatory uptake of the protein (Mulholland and Lee, 2009), suggesting that the open canopy and high autotrophic production in agricultural streams in the midwestern US (Griffiths et al., 2013) may not enhance degradation of Cry1Ab. There were no differences in Cry1Ab degradation among water collected from 3 different agricultural streams. The 3 streams had similar physical and chemical characteristics (Table 1), but we did not characterize the microbial communities in water collected from these streams. Thus, we cannot determine which factors resulted in the similar Cry1Ab degradation rates among streams. In terrestrial systems, no previous studies have examined how different microbial assemblages may influence degradation; however, previous research has shown that soil microbial community structure does not differ in fields planted with Bt vs. non-Bt maize (Baumgarte and Tebbe, 2005; Griffiths et al., 2005).

In our field survey, Cry1Ab protein was commonly detected in streams and tile drains. Given that our laboratory studies show that Cry1Ab protein that leaches from submerged Bt maize leaves is rapidly degraded by water-column microorganisms, it is likely that there are multiple pathways by which Cry1Ab can enter stream water including direct leaching from submerged Bt maize detritus, and lateral inputs via subsurface tile drains, overland flow, or erosion of soils with bound Cry1Ab protein. Nevertheless, we acknowledge that the laboratory conditions under which the degradation experiments occurred may not reflect variation in field conditions. For example, temperature can influence degradation rates of Cry1Ab protein (Zwahlen et al., 2003b), and our microcosm experiments were carried out in an environmental chamber at 21.5 °C. Thus, degradation rates in the field may be slower, especially during the colder fall and winter seasons when microbial activity has slowed. Similarly, Cry1Ab concentration in maize leaves declined more quickly in the leaching experiment compared to our previous field experiments (Griffiths et al., 2009), which may be due to warmer temperatures in the laboratory (range = 16 to 33 °C) compared to the field (range = −1 to 15 °C), or the faster water velocities in the experimental recirculating streams. Furthermore, our degradation experiments were carried out under oxic conditions; however,

anoxic microsites are common in agricultural streams sediments, and Cry1Ab may degrade more slowly in the field when oxygen is depleted (Wang et al., 2007). The presence of sediments can also influence the persistence of Cry1Ab in water. Experiments conducted in aquatic microcosms found that Cry1Ab leached from maize leaves and concentrations increased in sediments over 60 d, suggesting that adsorption to sediments increased the persistence of Cry1Ab (Strain and Lydy, 2015).

## 5. Conclusions

Bt maize is common in the US, with 79% of the 2016 maize acreage planted as Bt maize (NASS, 2016). In the midwestern US, the widespread planting of Bt maize, combined with the growing use of conservation tillage practices that leave crop detritus (i.e., stover) on fields post-harvest, provides multiple pathways for maize detritus and associated Cry1Ab proteins to enter adjacent waterways. These input pathways include wind, overland flow, erosion, groundwater, and subsurface tile drainage. However, in comparison to terrestrial ecosystems, the effects and ultimate fate of Bt proteins in aquatic systems are less well known, primarily because it has only recently been recognized that this material enters adjacent water bodies (Chambers et al., 2010; Douville et al., 2005; Griffiths et al., 2009; Jensen et al., 2010; Rosi-Marshall et al., 2007; Strain and Lydy, 2015; Swan et al., 2009; Tank et al., 2010, Whiting et al., 2014). In this study, Cry1Ab protein was detected in streams and tile drains in the field throughout the year, despite confirmation of rapid protein degradation rates. These results suggest that there may be a reservoir of Cry1Ab in the terrestrial environment (e.g., soils, surface and buried detritus) and several input pathways of Cry1Ab protein to agricultural streams. These source pathways may vary seasonally as crops are growing (Strain and Lydy, 2015) and harvested (Tank et al., 2010). While Cry1Ab protein degraded quickly in stream water, the ubiquitous detection and multiple input pathways suggest that Cry1Ab could be considered "pseudo-persistent", meaning that the consistent annual production and continuous input of Cry1Ab replaces the rapidly degraded protein (Daughton, 2003). However, the spatial and temporal occurrence of Cry1Ab in stream and tile drain water at the stream scale was sporadic and not predictable; hence, we define Cry1Ab as exhibiting pseudo-persistent characteristics only at the watershed scale. The pseudo-persistent nature of Cry1Ab is supported by findings from soils showing that Cry1Ab can sorb to soils, but after Cry1Ab desorbs and is present in soil water, it can be rapidly degraded (Valldor et al., 2015). Due to the prevalent use of transgenic crops, a better understanding of the fates of transgenic detritus in aquatic ecosystems is needed.

## Acknowledgements

We thank A. Aubeneau, P. Champion, R. Conley, J. Hellmann, P. Hoppe, J. Kang, M. McDowell, and J. Whitcomb, for field and laboratory assistance, and E. Willows for help with the graphical abstract. We thank T. Mathews and two anonymous reviewers for comments on earlier versions of this manuscript. We also thank private land owners for facilitating access to study sites. This work was supported by a National Science Foundation Doctoral Dissertation Improvement Grant (DEB-0808480). N.A. Griffiths was supported by a Post-Graduate Scholarship (PGS-D) from the Natural Sciences and Engineering Research Council of Canada and a Bayer Predoctoral Research Fellowship from the Center for Environmental Science and Technology (CEST) at the University of Notre Dame. Funding sources were not involved in the study design, collection, analysis, and interpretation of the data, or manuscript writing.

## References

Accinelli, C., Koskinen, W.C., Becker, J.M., Sadowsky, M.J., 2008. Mineralization of the *Bacillus thuringiensis* Cry1Ac endotoxin in soil. J Agric Food Chem 56, 1025–1028.

APHA, 2005. Standard Methods for the Examination of Water and Wastewater. 21st ed. American Public Health Association, Washington D.C.

Baumgarte, S., Tebbe, C.C., 2005. Field studies on the environmental fate of the Cry1Ab Bt-toxin produced by transgenic maize (MON810) and its effect on bacterial communities in the maize rhizosphere. Mol Ecol 14, 2539–2551.

Blann, K.L., Anderson, J.L., Sands, G.R., Vondracek, B., 2009. Effects of agricultural drainage on aquatic ecosystems: a review. Crit Rev Environ Sci Technol 39, 909–1001.

Carstens, K., Anderson, J., Bachman, P., De Schrijver, A., Divley, G., Federici, B., Hamer, M., Gielkens, M., Jensen, P., Lamp, W., Rauschen, S., Ridley, G., Romeis, J., Waggoner, A., 2012. Genetically modified crops and aquatic ecosystems: considerations for environmental risk assessment and non-target organism testing. Transgenic Res 21, 813–842.

Chambers, C.P., Whiles, M.R., Rosi-Marshall, E.J., Tank, J.L., Royer, T.V., Griffiths, N.A., Evans-White, M.A., Stojak, A.R., 2010. Responses of stream macroinvertebrates to Bt maize leaf detritus. Ecol Appl 20, 1949–1960.

Crecchio, C., Stotzky, G., 1998. Insecticidal activity and biodegradation of the toxin from *Bacillus thuringiensis* subsp. *kurstaki* bound to humic acids from soil. Soil Biol Biochem 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 hydroxypolymers. Soil Biol Biochem 33, 573–581.

Daughton, C.G., 2003. Cradle-to-cradle stewardship of drugs for minimizing their environmental disposition while promoting human health. 1. Rationale for and avenues towards a green pharmacy. Environ Health Perspect 111, 757–774.

Douville, M., Gagne, F., Masson, L., McKay, J., Blaise, C., 2005. Tracking the source of *Bacillus thuringiensis* Cry1Ab endotoxin in the environment. Biochem Syst Ecol 33, 219–232.

Douville, M., Gagne, F., Blaise, C., Andre, C., 2007. Occurrence and persistence of *Bacillus thuringiensis* (Bt) and transgenic corn *cry1ab* gene from an aquatic environment. Ecotoxicol Environ Saf 66, 195–203.

Dutton, A., Romeis, J., Bigler, F., 2003. Assessing the risks of insect resistant transgenic plants on entomophagous arthropods: Bt-maize expressing Cry1Ab as a case study. BioControl 48, 611–636.

Gore, J.A., 2006. Discharge measurements and streamflow analysis. In: Hauer, F.R., Lamberti, G.A. (Eds.), Methods in Stream Ecology. Elsevier, New York, New York, pp. 51–78.

Griffiths, B.S., Caul, S., Thompson, J., Birch, A.N.E., Scrimgeour, C., Andersen, M.N., Cortet, J., Messéan, A., Sausse, C., Lacroix, B., Krogh, P.H., 2005. A comparison of soil microbial community structure, protozoa and nematodes in field plots of conventional and genetically modified maize expressing the *Bacillus thuringiensis* Cry1Ab toxin. Plant Soil 275, 135–146.

Griffiths, N.A., Tank, J.L., Royer, T.V., Rosi-Marshall, E.J., Whiles, M.R., Chamber, C.P., Frauendorf, T.C., Evans-White, M.A., 2009. Rapid decomposition of maize detritus in agricultural headwater streams. Ecol Appl 19, 133–142.

Griffiths, N.A., Tank, J.L., Royer, T.V., Warrner, T.J., Frauendorf, T.C., Rosi-Marshall, E.J., Whiles, M.R., 2012. Temporal variation in organic carbon spiraling in Midwestern agricultural streams. Biogeochemistry 108, 149–169.

Griffiths, N.A., Tank, J.L., Royer, T.V., Roley, S.S., Rosi-Marshall, E.J., Whiles, M.R., Beaulieu, J.J., Johnson, L.T., 2013. Agricultural land use alters the seasonality and magnitude of stream metabolism. Limnol Oceanogr 58, 1513–1529.

Hopkins, D.W., Gregorich, E.G., 2005. Decomposition of residues and loss of the δ-endotoxin from transgenic (Bt) corn (*Zea mays* L.) in soil. Can J Soil Sci 85, 19–26.

Jensen, P.D., Dively, G.P., Swan, C.M., Lamp, W.O., 2010. Exposure and nontarget effects of transgenic Bt corn debris in streams. Environ Entomol 39, 707–714.

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. Appl Environ Microbiol 63, 3561–3568.

Madliger, M., Gasser, C.A., Schwarzenbach, R.P., Sander, M., 2011. Adsorption of transgenic insecticidal Cry1Ab protein to silica particles. Effects on transport and bioactivity. Environ Sci Technol 45, 4377–4384.

Mulholland, M.R., Lee, C., 2009. Peptide hydrolysis and the uptake of dipeptides by phytoplankton. Limnol Oceanogr 53, 856–868.

Murphy, J., Riley, J.P., 1962. A modified single solution method for determination of phosphate in natural waters. Anal Chim Acta 26, 31–36.

NASS [National Agricultural Statistics Service], 2008. Acreage. Agricultural Statistics Board, United States Department of Agriculture, Washington, D.C. http://usda.mannlib.cornell.edu/usda/nass/Acre//2000s/2008/Acre-06-30-2008.pdf (Accessed January 2017).

NASS [National Agricultural Statistics Service], 2012. Census of Agriculture County Profile: Benton County, Indiana. Indiana Agricultural Statistics Service, United States Department of Agriculture, Washington D.C. https://www.agcensus.usda.gov/Publications/2012/Online_Resources/County_Profiles/Indiana/cp18007.pdf (Accessed January 2017).

NASS [National Agricultural Statistics Service], 2016. Acreage. Agricultural Statistics Board, United States Department of Agriculture, Washington, D.C. http://usda.mannlib.cornell.edu/usda/nass/Acre//2010s/2016/Acre-06-30-2016.pdf (Accessed January 2017).

Nguyen, H.T., Jehle, J.A., 2007. Quantitative analysis of the seasonal and tissue-specific expression of Cry1Ab in transgenic maize Mon810. Journal of Plant Diseases and Protection 114, 82–87.

Obrist, L.B., Dutton, A., Albajes, R., Bigler, F., 2006. Exposure of arthropod predators to Cry1Ab toxin in Bt maize fields. Ecol. Entomol. 31, 143–154.

Palm, C.J., Schaller, D.L., Donegan, K.K., Seidler, R.J., 1996. Persistence in soil of transgenic plant produced *Bacillus thuringiensis* var. *kurstaki* δ-endotoxin. Can J Microbiol 42, 1258–1262.

Romeis, J., Van Driesche, R.G., Barratt, B.I.P., Bigler, F., 2008. Insect-resistant transgenic crops and biological control. In: Romeis, J., Shelton, A.M., Kennedy, G.G. (Eds.),

Integration of Insect-resistant Genetically Modified Crops Within IPM Programs. Springer, Dordrecht, pp. 87–117.
Rosi-Marshall, E.J., Tank, J.L., Royer, T.V., Whiles, M.R., Evans-White, M., Chambers, C., Griffiths, N.A., Pokelsek, J., Stephen, M.L., 2007. Toxins in transgenic crop byproducts may affect headwater stream ecosystems. Proc Natl Acad Sci U S A 104, 16204–16208.
Saxena, D., Stotzky, G., 2001a. *Bacillus thuringiensis* (Bt) toxin released from root exudates and biomass of Bt corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil. Soil Biol Biochem 33, 1225–1230.
Saxena, D., Stotzky, G., 2001b. Bt toxin uptake from soil by plants. Nat Biotechnol 19, 199.
Saxena, D., Flores, S., Stotzky, G., 1999. Insecticidal toxin in root exudates from Bt corn. Nature 402, 480.
Schilling, K.E., Libra, R.D., 2003. Increased baseflow in Iowa over the second half of the 20th century. J Am Water Resour Assoc 39, 851–860.
Skaggs, R.W., Breve, M.A., Gillham, J.W., 1992. Environmental impacts of agricultural drainage. In: Engman, T. (Ed.), Irrigation and Drainage: Proceedings of ASCE Water Forum '92. ASCE, New York, New York, pp. 19–24.
Solorzano, L., 1969. Determination of ammonia in natural waters by phenolhypochlorite method. Limnol Oceanogr 14, 799–801.
Stotzky, G., 2004. Persistence and biological activity in soil of the insecticidal proteins from *Bacillus thuringiensis*, especially from transgenic plants. Plant Soil 266, 77–89.
Strain, K.E., Lydy, M.L., 2015. The fate and transport of the Cry1Ab protein in an agricultural field and laboratory aquatic microcosms. Chemosphere 132, 94–100.
Strain, K.E., Whiting, S.A., Lydy, M.L., 2014. Laboratory and field validation of a Cry1Ab protein quantification method for water. Talanta 128, 109–116.
Swan, C.M., Jensen, P.D., Dively, G.P., Lamp, W.O., 2009. Processing of transgenic crop residues in stream ecosystems. J Appl Ecol 46, 1304–1313.
SYSTAT, 2007. Systat Software, Version 12.0, San Jose, California.
Tank, J.L., Rosi-Marshall, E.J., Royer, T.V., Whiles, M.R., Griffiths, N.A., Frauendorf, T.C., Treering, D.J., 2010. Occurrence of maize detritus and a transgenic insecticidal protein (Cry1Ab) within the stream network of an agricultural landscape. Proc Natl Acad Sci U S A 107, 17645–17650.
USEPA [United States Environmental Protection Agency], 2008. Pesticides: Regulating Pesticides, Plant Incorporated Protectants, Currently & Previously Registered Section 3 PIP Registrations. Environmental Protection Agency, Washington D.C.
Valldor, P., Miethling-Graff, R., Martens, R., Tebbe, C.C., 2015. Fate of the insecticidal Cry1Ab protein of GM crops in two agricultural soils as revealed by $^{14}$C-tracer studies. Appl Microbiol Biotechnol 99, 7333–7341.
Viktorov, A.G., 2011. Transfer of Bt corn byproducts from terrestrial to stream ecosystems. Russ J Plant Physiol 58, 543–548.
Wang, H.Y., Ye, Q.F., Wu, L.C., Gan, J., 2007. Biodegradation of Cry1Ab protein from Bt transgenic rice in aerobic and flooded paddy soils. J Agric Food Chem 55, 1900–1904.
Whiting, S.A., Strain, K.E., Campbell, L.A., Young, B.G., Lydy, M.J., 2014. A multi-year field study to evaluate the environmental fate and agronomic effects of insecticide mixtures. Sci Total Environ 497–498, 534–542.
Zucker, L.A., Brown, L.C., 1998. Agricultural drainage: water quality impacts and subsurface drainage studies in the Midwest. Bulletin. The Ohio State University, Columbus, Ohio, pp. 871–898.
Zwahlen, C., Hilbeck, A., Gugerli, P., Nentwig, W., 2003a. Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the field. Mol Ecol 12, 765–775.
Zwahlen, C., Hilbeck, A., Howald, R., Nentwig, W., 2003b. Effects of transgenic Bt corn litter on the earthworm *Lumbricus terrestris*. Mol Ecol 12, 1077–1086.