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# Dissipation of Insecticidal Cry1Ac Protein and Its Toxicity to Nontarget Aquatic Organisms

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Supporting Information

**ABSTRACT:** The widespread cultivation of *Bacillus thuringiensis* crops has raised public concerns on their risk to nontarget organisms. Persistence of Cry1Ac protein in soil, sediment and water and its toxicity to nontarget aquatic organisms were determined. The dissipation of Cry1Ac toxin was well described using first order kinetics, with the half-lives ($DT_{50}$) ranging from 0.8 to 3.2, 2.1 to 7.6 and 11.0 to 15.8 d in soil, sediment and water, respectively. Microbial degradation played a key role in the dissipation of Cry1Ac toxin and high temperature accelerated the processes. Cry1Ac toxin was more toxic to the midge *Chironomus dilutus* than the amphipod *Hyalella azteca*, with the median lethal concentration ($LC_{50}$) of *C. dilutus* being 155 ng/g dry weight and 201 ng/mL in 10-d sediment and 4-d water bioassays, respectively. While Cry1Ac toxin showed toxicity to the midges, risk of *Bt* proteins to aquatic nontarget organisms was limited because their environmentally relevant concentrations were much lower than the $LC_{50}$s.

**KEYWORDS:** *dissipation, Bt cotton, Cry1Ac protein, toxicity, aquatic nontarget organisms*

## INTRODUCTION

The insecticidal Cry proteins target major lepidopteran and coleopteran pests by perforating membranes of the midgut of insect larvae, causing the loss of cell metabolism balance and eventually causing death.[1,2] Since its first commercialization in 1996, worldwide cultivation of transgenic crops expressing Cry toxins from *Bacillus thuringiensis* (*Bt*) has increased dramatically, from approximate $1.7 \times 10^6$ ha to more than $6.6 \times 10^7$ ha in 2011.[3] China is the second largest *Bt* cotton planting country in the world and 3.9 million ha of transgenic *Bt* cottons were planted in 2011, counting 71.5% of total areas of cottons in China, as shown in Figure S1 of the Supporting Information, SI.[4,5]

Planting *Bt* cottons has benefitted the environment due to the tremendous reduction in the usage of broad spectrum chemical pesticides.[4,6−8] Nevertheless, widespread cultivation of *Bt* crops has raised public concerns on their ecological risk.[9−11] *Bt* crops may release insecticidal Cry proteins to the soil through root exudates, tasseling, or plant debris, causing the accumulation of Cry proteins in soil.[12,13] Moreover, it has been reported that Cry proteins entered into aquatic ecosystems through tasseling and runoff of *Bt* crop detritus and particle-associated proteins.[14,15] The debate continues on the issues of the persistence and adverse effects of *Bt* proteins to nontarget organisms.[1]

Whereas Head et al.[16] reported no detection of Cry1Ac protein in soils after planting *Bt* cottons for multiple years, Saxena et al.[13] demonstrated that Cry proteins accumulated in soil and remained effective with regard to insecticidal activity for over 180 d. The sorption to clay minerals[17] and humic substances[18,19] reduced the degradation of Cry proteins, resulting in its persistence.[20] There were many factors affecting the fate of Cry proteins in soil, such as temperature, pH, moisture, contents of organic matters, and the presence of soil microorganisms.[21−23] At the same time, the unrelenting debate on ecological risk of *Bt* crops also requires more scientific evidence. Previous study suggested the toxicity of *Bt* crops to nontarget organisms including microorganisms and invertebrates in terrestrial environment.[24] A recent meta-analysis of field experiments concluded that planting *Bt* crops reduced the use of insecticides and resulted in more abundant nontarget invertebrates, but in comparison with insecticide-free non-transgenic fields, the abundance of some nontarget taxa was less in the fields of *Bt* crops.[1] So far, most studies regarding *Bt* toxins were conducted using soil as matrix. Instead, the dissipation dynamics and adverse effects of *Bt* proteins in sediment and water were scarcely investigated although *Bt* proteins were found in aquatic systems.[14,15,25]

The aims of the present study were to understand the dissipation kinetics of Cry1Ac protein from *Bt* cotton in different matrixes including soil, sediment and water; to

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determine the effects of temperature and sterilization conditions on the dissipation processes; and to evaluate the toxicity of Cry1Ac protein to two aquatic nontarget invertebrates, *Chironomus dilutus* and *Hyalella azteca*, using both sediment and water-only toxicity tests.

## MATERIALS AND METHODS

**Chemicals and Reagents.** Two cotton varieties, including a transgenic *Bt* cotton GK-12 cultivar and a nontransgenic cotton isoline Simian-3 (Cotton Research Institute of Chinese Academy of Agricultural Sciences, Anyang, China), were used. The GK-12 cultivar expresses a synthetic version of Cry1Ac toxin genes from *Bt* subsp. *kurstaki* and targets Lepidoptera. This transgenic *Bt* cotton was produced by importing the synthetic GFMCry1A insecticidal genes into the nontransgenic cotton isoline Simian-3 using a pollen tube pathway method. Both GK-12 and Simian-3 cotton varieties have been commercially planted in northern China and were collected from the fields where no *Bt* insecticides had ever been applied throughout the entire cotton-growing seasons. Additionally, Cry1Ac protein standard was purchased from EnviroLogix Incorporation (Portland, ME, U.S.).

Chemical grade Tween-20, hydrochloric acid, and concentrated sulfuric acid were obtained from Guangzhou Chemical Company (Guangzhou, China). Analytical grade sodium dodecyl sulfate (SDS), dichloromethane, and methanol were purchased from Tianjin Chemical Reagent Company (Tianjin, China). Additionally, clean sands were purchased from Etan Industrial and Investment Company (Dongguan, China) and sequentially washed by sonication with dichloromethane, methanol and distilled water before use. The Milli-Q water (Millipore, Bedford, MA, U.S.) was used to prepare the buffers.

**Sample Collection and Spiking.** Control soil was collected from a garden in Nanjing Institute of Environmental Sciences, Jiangsu, China, and the area has no history of transgenic crop cultivation nor *Bt* insecticide application. The top 5 cm of soil was collected using a stainless steel spade, and the soil was air-dried at room temperature after removing the stones and plant debris. Then the soil was passed through a 300 $\mu$m sieve, homogenized, and stored at 4 °C in the darkness. The soil pH was 7.47 ± 0.01, and the soil contained 11.5 ± 0.15, 1.0 ± 0.10, and 0.94 ± 0.02 g/kg organic carbon (OC), total nitrogen, and total phosphorus, respectively.

Control sediment was collected from a drinking water reservoir in Conghua, Guangdong, China, where neither transgenic crops have been planted nor *Bt* insecticides have been used. The top 5 cm of sediment was collected using a spade shovel and then sieved through a 2 mm sieve to remove the large debris. The sediment was stored with ice and immediately transported to the laboratory, homogenized, and stored at 4 °C in the darkness. Chemical analysis and bioassay showed that this sediment had limited contamination and did not exhibit toxicity to benthic organisms.[26] Total OC content of the sediment was determined using an Elementar Vario ELIII (Hanau, Germany) after removing inorganic carbonates with 1 mol/L HCl, and the OC content was 2.75 ± 0.07%.

The soil and sediment were spiked with appropriate amounts of crude *Bt* proteins which were extracted from GK-12 transgenic cotton seeds using a PBST solution following the method recommended by EnviroLogix Company. The PBST solution was a phosphate-buffered saline (PBS: 1.9 mmol/L $NaH_2PO_4$, 8.1 mmol/L $Na_2HPO_4$, 150 mmol/L NaCl, pH 7.4), which contained 0.55% of Tween-20. The extraction of *Bt* proteins from the GK-12 seeds were performed following the method described by Li et al.[27] After extraction, concentrations of Cry1Ac protein in the crude *Bt* protein solution were quantified by enzyme-linked immunosorbent assay (ELISA) method as described below. This method showed good reproducibility with relative standard deviations at 7.3% and high sensitivity with a method detection limit of 0.8 ng/g dry weight (dw).[27]

After being spiked with appropriate amounts of the freshly prepared crude *Bt* proteins, the soil and sediment samples were thoroughly mixed on a vortex (IKA-Werke GmbH & Co. KG, Staufen, Germany). Throughout the experiments, Milli-Q water was added daily to the soil and sediment samples to compensate for the water evaporation and ensure that the moisture contents were 20% and 48%, respectively. Meanwhile, the water samples were prepared by directly adding appropriate amounts of fresh crude *Bt* proteins to Milli-Q water with pH value of 7.0 and thoroughly mixing on the vortex. The lost water in the water samples was replenished daily to keep constant weights of samples throughout the experiments.

**Quantification of Cry1Ac Protein.** Concentrations of Cry1Ac protein in the samples were quantified by double-antibody sandwich ELISA using a commercially available AP 003 Quantiplate kit (EnviroLogix), according to manufacturer's instructions. In brief, the 96-well microplate was precoated polyclonal as primary antibodies and detected by HRP-conjugated antibodies. Absorbance was measured at the wavelength of 450 nm using a Varioskan flash spectral scan multimode plate reader (Thermo Fisher Scientific, Waltham, MA). External standard calibration was applied to quantify concentrations of CrylAc protein in the extracts and the calibration curves were linear ($r^2$ = 0.996, $n$ = 3) within the range of 0.05 to 5 ng/mL.[27]

**Dissipation Processes.** The impacts of sterilization status and temperature on the dissipation of Cry1Ac protein in soil, sediment and water were evaluated (SI Table S1). The dissipation experiments were conducted at three temperatures of 4, 24, and 34 °C for three types of matrixes (soil, sediment, and water). Two sterilization conditions (sterilized and not sterilized) were used to assess the impact of microbial degradation on the dissipation of Cry1Ac protein, and sterilization was conducted by heating the matrixes at 121 °C for 30 min. The experiments were performed in triplicate and 0.4 g soil, 0.5 g sediment, or 1.5 mL water was used in each replicate. At the predetermined time intervals (0, 1, 3, 7, 15, 30, 45, and 60 d), three replicates were terminated, and concentrations of Cry1Ac protein in the substrates were measured. The tests were conducted in HPX-9052 incubators (Boxun Incorporation, Shanghai, China) to maintain constant temperature.

The Cry1Ac protein was extracted from the soil and sediment samples using a PBST/SDS solution following a previously developed method, and the extraction efficiency was 46.5 ± 3.4%.[27] Briefly, after adding 1.2 mL PBST/SDS extraction solution, the soil or sediment sample was vortexed for 30 s, and the extraction was conducted at 21 °C for 2 h at a rotation speed of 630 rpm. At the end of extraction, the samples were centrifuged at 13 000 rpm and 4 °C for 2 min using a ST16R refrigerated centrifuge (Thermo Scientific, Rockford, IL). The supernatant was decanted and analyzed using ELISA. Alternatively, no further extraction was needed for water samples and the protein was directly analyzed using ELISA after vortexing the water samples for 30 s.

**Acute Toxicity Testing.** The bioassays were conducted with benthic invertebrates *C. dilutus* and *H. azteca* using either sediment or water-only exposure. Control sediment and the reconstituted water were spiked with the freshly prepared crude *Bt* proteins using the PBST solution as the carrier at five concentrations of 1.6, 5.2, 23.7, 81.4, and 157.3 ng/g dw and 3.7, 23.1, 77.8, 211.5, and 456.3 ng/mL, respectively. After spiking, the sediment was thoroughly mixed using a drill with a rotating stainless-steel blade for 4 h, and the water samples were thoroughly mixed using a stirrer for 2 h. Three controls were employed concurrently in both sediment and water-only bioassays, including a negative control (NC), a solvent control (SC), and a control check (CK). The NC was clean sediment or water, the SC was sediment or water spiked with the PBST solution, and the CK was sediment or water spiked with crude protein solution extracted from nontransgenic Simian-3 cotton seeds.

The test species have been cultured at Guangzhou Institute of Geochemistry, Chinese Academy of Sciences (GIGCAS), in accordance with U.S. Environmental Protection Agency (USEPA) protocols (2000). Acute bioassays were in five replicates carried out following USEPA standard protocols using 10-d sediment and 4-d water-only toxicity testing.[28] The sediment exposure was performed with 60 g wet sediment and 250 mL overlying water. After the sediment was settled overnight, 10 third instar midge larvae or 10 juvenile amphipods (7−14 d old) were introduced into the beakers. The overlying water was renewed twice a day with 150 mL each time using an automated water exchange system. Water-only exposures

**Table 1. Residues of Cry1Ac Protein in Sediment at Different Time, Temperature and Sterilization Conditions[a,b]**

| sterilization condition | time (d) | concentration (ng/g dry weight) 4 °C | | 24 °C | | 34 °C | |
|---|---|---|---|---|---|---|---|
| not sterilized | 0 | 243 ± 7.6 a | A | 243 ± 7.6 a | A | 243 ± 7.6 a | A |
| | 1 | 160 ± 7.7 a | B | 133 ± 8.6 b | B | 107 ± 5.1 c | B |
| | 3 | 118 ± 6.5 a | C | 95 ± 6.3 b | C | 83 ± 2.9 c | C |
| | 7 | 89 ± 5.3 a | D | 76 ± 7.5 b | D | 63 ± 2.6 c | D |
| | 15 | 44 ± 5.8 a | E | 48 ± 5.4 a | E | 22 ± 1.9 b | E |
| | 30 | 28 ± 2.8 a | F | 31 ± 3.1 a | F | 14 ± 0.7 b | F |
| | 45 | 18 ± 3.4 a | G | 23 ± 2.3 b | G | 4 ± 0.5 c | G |
| | 60 | 8 ± 2.9 a | H | 12 ± 3.3 a | H | 2 ± 0.4 b | H |
| sterilized | 0 | 243 ± 7.6 a | A | 243 ± 7.6 a | A | 243 ± 7.6 a | A |
| | 1 | 201 ± 5.7 a | B | 187 ± 2.4 b | B | 157 ± 3.6 c | B |
| | 3 | 135 ± 7.6 a | C | 125 ± 6.2 a | C | 111 ± 5.5 b | C |
| | 7 | 104 ± 4.5 a | D | 94 ± 7.9 a | D | 74 ± 3.9 b | D |
| | 15 | 65 ± 9.2 a | E | 55 ± 2.4 a | E | 35 ± 2.1 b | E |
| | 30 | 42 ± 9.7 a | F | 37 ± 3.1 a | F | 24 ± 2.0 b | F |
| | 45 | 29 ± 4.0 a | G | 19 ± 0.6 a | G | 7 ± 0.8 b | G |
| | 60 | 19 ± 3.3 a | G | 11 ± 1.4 b | G | 4 ± 0.6 c | G |

[a]Concentrations of Cry1Ac protein were shown as means ± standard deviation of three replicates. [b]Different letters indicated significant difference ($p < 0.05$, Duncan's Multiple Range Test). The lowercase letters were indicative the difference among the treatments at differing temperature under the same sterilization condition at the same time interval, while the uppercase letters implied the difference among concentrations of CrylAc protein at different time under the same testing conditions.

**Table 2. Residues of Cry1Ac Protein in Soil at Different Time, Temperature and Sterilization Conditions[a,b]**

| sterilization condition | time (d) | concentration (ng/g dry weight) 4 °C | | 24 °C | | 34 °C | |
|---|---|---|---|---|---|---|---|
| not sterilized | 0 | 176 ± 4.5 a | A | 176 ± 4.5 a | A | 176 ± 4.5 a | A |
| | 1 | 83 ± 1.9 a | B | 75 ± 2.7 b | B | 58 ± 3.9 c | B |
| | 3 | 77 ± 4.1 a | C | 55 ± 1.8 b | C | 42 ± 3.9 c | C |
| | 7 | 30 ± 1.4 a | D | 27 ± 2.0 b | D | 16 ± 0.9 c | D |
| | 15 | 12 ± 1.6 a | E | 10 ± 0.5 b | E | 7 ± 0.6 c | E |
| | 30 | 7 ± 0.6 a | EF | 3 ± 0.2 b | F | 1 ± 0.3 c | F |
| | 45 | 3 ± 0.6 a | FG | 2 ± 0.2 b | F | 1 ± 0.2 c | F |
| | 60 | 1 ± 0.2 a | G | 1 ± 0.2 a | F | 0 ± 0.3 a | F |
| sterilized | 0 | 176 ± 4.5 a | A | 176 ± 4.5 a | A | 176 ± 4.5 a | A |
| | 1 | 109 ± 4.8 a | B | 98 ± 2.9 b | B | 90 ± 1.3 c | B |
| | 3 | 85 ± 3.4 a | C | 80 ± 1.2 b | C | 72 ± 1.3 c | C |
| | 7 | 41 ± 1.7 a | D | 35 ± 1.7 b | D | 28 ± 2.2 c | D |
| | 15 | 18 ± 1.2 a | E | 16 ± 0.6 b | E | 14 ± 0.5 c | E |
| | 30 | 10 ± 0.5 a | F | 9 ± 0.6 b | F | 5 ± 0.3 c | F |
| | 45 | 4 ± 0.2 a | G | 3 ± 0.3 b | G | 2 ± 0.2 c | G |
| | 60 | 2 ± 0.3 a | G | 1 ± 0.1 b | G | 1 ± 0.2 c | G |

[a]Concentrations of Cry1Ac protein were shown as means ± standard deviation of three replicates. [b]Different letters indicated significant difference ($p < 0.05$, Duncan's Multiple Range Test). The lowercase letters were indicative the difference among the treatments at differing temperature under the same sterilization condition at the same time interval, while the uppercase letters implied the difference among concentrations of CrylAc protein at different time under the same testing conditions.

were performed using 200 mL reconstituted water. To prevent cannibalism among the midges, 10 g of clean sand were added to each beaker for *C. dilutus* testing. Instead, a small piece of gauze was placed in the beakers for *H. azteca* testing. Similar to sediment bioassays, 10 organisms were used for each replicate. No water renewal was conducted throughout the 4-d exposure. Both sediment and water-only bioassays were conducted at 23 ± 1 °C with a 16:8 light-to-dark photoperiod. The conductivity, pH, dissolved oxygen, and temperature were measured daily, and ammonia concentrations were measured every two days. The organisms in sediment and water-only toxicity tests were fed once every day and once on alternate days with 1 mL of 6 g/L ground fish food, respectively. At the termination of bioassays, mortality of the organisms was assessed by sieving the organisms from the substrates using a 500 $\mu$m sieve.

**Data Analysis.** First order kinetics model shown in eq 1 was used to describe the dissipation processes of Cry1Ac protein by plotting the residue concentrations against time.

$$C_t = C_0 e^{-kt} \quad (1)$$

Where, $C_0$ represents the initial concentration of Cry1Ac protein in the matrix at time zero (d), $C_t$ stands for the concentration of Cry1Ac protein at time $t$ (d), $k$ and $t$ are the first-order rate constant ($d^{-1}$) and dissipation time (d), respectively.

The dissipation of Cry1Ac protein was generally expressed in terms of the time at which concentration of the protein reduced to one-half and 90% of its initial concentration ($DT_{50}$ and $DT_{90}$, respectively). The equations to calculate $DT_{50}$ and $DT_{90}$ are given in eqs 2 and 3 from $k$ value using the Hoskins formula.

Table 3. Residues of Cry1Ac Protein in Water at Different Time, Temperature and Sterilization Conditions[a,b]

| sterilization condition | time (d) | concentration (ng/mL) 4 °C | | 24 °C | | 34 °C | |
|---|---|---|---|---|---|---|---|
| not sterilized | 0 | 250 ± 8.8 a | A | 250 ± 8.8 a | A | 250 ± 8.8 a | A |
| | 1 | 233 ± 7.6 a | B | 227 ± 6.4 a | B | 228 ± 3.7 a | B |
| | 3 | 210 ± 6.9 a | C | 203 ± 9.3 ab | C | 203 ± 8.5 b | C |
| | 7 | 182 ± 3.2 a | D | 176 ± 2.8 b | D | 167 ± 6.3 c | D |
| | 15 | 108 ± 5.4 a | E | 95 ± 5.4 b | E | 83 ± 5.3 c | E |
| | 30 | 65 ± 1.9 a | F | 52 ± 3.5 b | F | 42 ± 4.7 c | F |
| | 45 | 37 ± 3.1 a | G | 30 ± 5.1 ab | G | 24 ± 4.7 b | G |
| | 60 | 25 ± 2.6 a | H | 17 ± 1.3 b | H | 12 ± 1.7 c | H |
| sterilized | 0 | 250 ± 8.8 a | A | 250 ± 8.8 a | A | 250 ± 8.8 a | A |
| | 1 | 234 ± 8.7 a | B | 235 ± 4.4 a | B | 241 ± 8.0 a | B |
| | 3 | 210 ± 8.7 a | C | 199 ± 11.4 b | C | 207 ± 8.0 c | C |
| | 7 | 190 ± 9.4 a | D | 170 ± 9.0 b | D | 169 ± 1.5 b | D |
| | 15 | 116 ± 11.3 a | E | 94 ± 8.7 b | E | 83 ± 4.1 b | E |
| | 30 | 69 ± 4.4 a | F | 50 ± 3.2 b | F | 44 ± 1.3 b | F |
| | 45 | 39 ± 0.9 a | G | 28 ± 1.8 b | G | 23 ± 1.4 c | G |
| | 60 | 19 ± 2.7 a | H | 14 ± 1.2 b | H | 10 ± 0.7 c | H |

[a]Concentrations of Cry1Ac protein were shown as means ± standard deviation of three replicates. [b]Different letters indicated significant difference ($p < 0.05$, Duncan's Multiple Range Test). The lowercase letters were indicative the difference among the treatments at differing temperature under the same sterilization condition at the same time interval, while the uppercase letters implied the difference among concentrations of CrylAc protein at different time under the same testing conditions.

$$DT_{50} = \ln(2)/k = 0.693/k \quad (2)$$

$$DT_{90} = \ln(10)/k = 2.301/k \quad (3)$$

Data fitting was conducted using SPSS 18.0 (SPSS Inc., Chicago, IL, U.S.) and SigmaPlot 12.0 (Systat Software Inc., San Jose, CA, U.S.). The median lethal concentration ($LC_{50}$) for toxicity tests was determined using Probit analysis with SAS 9.2 software (SAS Institute Inc., Cary, NC, U.S.). Student's *t*-test was used to determine the difference between two treatments. Moreover, ANOVA and Duncan's multiple range tests were used to determine the significant differences among the means of the treatments. Statistically significant differences were determined if $p$ values were <0.05.

## RESULTS AND DISCUSSION

**Factors Influencing Dissipation of Cry1Ac Protein.** Concentrations of Cry1Ac protein residues in sterilized and nonsterilized sediment, soil, and water at different incubation temperatures throughout 60-d experiments were shown in Tables 1−3, respectively. The dissipation of Cry1Ac protein was affected by various factors, and first-order,[29] shift-log[30] and three-parameter kinetic models[31] have been used to describe the dissipation processes of *Bt* proteins. The dissipation of CrylAc protein was fitted with the first order kinetic model in the present study. Similar to the classical dissipation profiles of Cry toxins,[32,33] Figure 1 showed two-phase profiles composing with a rapid decline in the early stage and a slow decline in the late stage. The kinetic equations and parameters, including coefficients of determination ($R^2$), $p$ values, $k$, $DT_{50}$, and $DT_{90}$, were summarized in Table 4.

As shown in Figure 1 and Table 4, the dissipation of CrylAc protein in soil was faster than that in sediment and water, with $DT_{50}$ values ranged from 0.8 to 3.2 d. The $DT_{50}$ values were in the range of previously reported $DT_{50}$ values of 0.6 d[30] to 10.9 d,[22] and the maximum value of 3.2 d in the present study was proximal to the results of 4.0 d by Bai et al.[21] The variations in $DT_{50}$ values were the results of different geochemical characteristics of the soils and various experimental conditions. The influences of sterilization and temperature on the dissipation of Cry1Ac toxin were discussed below with a focus on sediment.

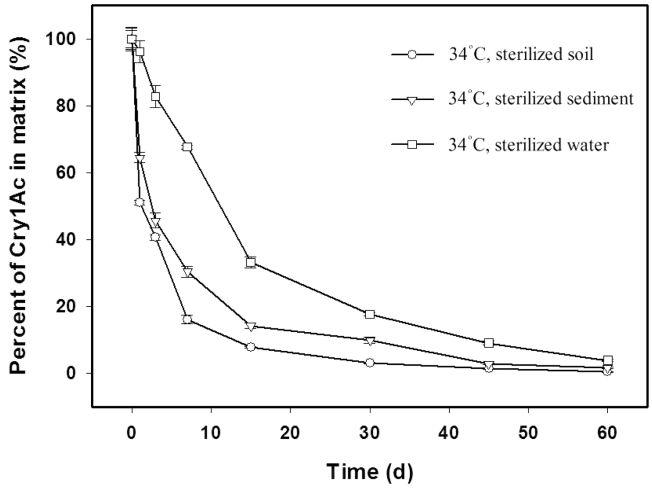


**Figure 1.** Dissipation curves of Cry1Ac protein in the sterilized soil, sediment and water at 34 °C. The first order dissipation kinetic equations were $C_t = 161e^{-0.312t}$ ($r^2 = 0.949$, $DT_{50} = 2.2$, and $DT_{90} = 7.4$), $C_t = 218e^{-0.181t}$ ($r^2 = 0.946$, $DT_{50} = 3.8$, and $DT_{90} = 12.7$), and $C_t = 252e^{-0.063t}$ ($r^2 = 0.994$, $DT_{50} = 11.0$, and $DT_{90} = 36.5$) in soil (○), sediment (▽), and water (□), respectively. The symbols are the means of three replicates and the error bars represent the standard deviations.

Palm et al.[32] suggested that microbial degradation contributed considerably to the dissipation of *Bt* proteins. To study the effect of microbial degradation, that dissipation kinetics of Cry1Ac protein in the sterilized and nonsterilized matrixes were compared (Tables 1−3). Concentrations of residual toxin in the nonsterilized sediment and soil at any time during the course of 60-d incubation period were always less than those in their sterilized counterparts ($p < 0.05$). In comparison with the sterilized conditions, the dissipation rates of Cry1Ac protein were faster under the nonsterilized conditions regardless of the


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Table 4. First Order Kinetic Equations, The Rate Constant ($k$) and the Times When Concentration of Cry1Ac Protein Reduced to the Half ($DT_{50}$) and 90% ($DT_{90}$) of Its Initial Concentration in Three Matrixes under Different Testing Conditions[a]

| matrix | treatment[b] | kinetic equation[c] | $r^2$ | $p$ value | dissipation parameter | | |
|---|---|---|---|---|---|---|---|
| | | | | | $k$ ($d^{-1}$) | $DT_{50}$ (d) | $DT_{90}$ (d) |
| soil | 4 °C, NS | $C_t = 157e^{-0.293t}$ | 0.926 | <0.0001 | 0.293 | 2.4 | 7.9 |
| | 4 °C, S | $C_t = 161e^{-0.216t}$ | 0.966 | <0.0001 | 0.216 | 3.2 | 10.7 |
| | 24 °C, NS | $C_t = 164e^{-0.475t}$ | 0.934 | <0.0001 | 0.475 | 1.5 | 4.8 |
| | 24 °C, S | $C_t = 160e^{-0.251t}$ | 0.952 | <0.0001 | 0.251 | 2.8 | 9.2 |
| | 34 °C, NS | $C_t = 171e^{-0.830t}$ | 0.946 | <0.0001 | 0.830 | 0.8 | 2.8 |
| | 34 °C, S | $C_t = 161e^{-0.312t}$ | 0.949 | <0.0001 | 0.312 | 2.2 | 7.4 |
| sediment | 4 °C, NS | $C_t = 212e^{-0.137t}$ | 0.928 | <0.0001 | 0.137 | 5.1 | 16.8 |
| | 4 °C, S | $C_t = 219e^{-0.091t}$ | 0.926 | <0.0001 | 0.091 | 7.6 | 25.3 |
| | 24 °C, NS | $C_t = 205e^{-0.179t}$ | 0.839 | <0.0001 | 0.179 | 3.9 | 12.9 |
| | 24 °C, S | $C_t = 219e^{-0.116t}$ | 0.936 | <0.0001 | 0.116 | 6.0 | 19.8 |
| | 34 °C, NS | $C_t = 214e^{-0.324t}$ | 0.882 | <0.0001 | 0.324 | 2.1 | 7.1 |
| | 34 °C, S | $C_t = 218e^{-0.181t}$ | 0.946 | <0.0001 | 0.181 | 3.8 | 12.7 |
| water | 4 °C, NS | $C_t = 245e^{-0.046t}$ | 0.993 | <0.0001 | 0.046 | 15.1 | 50.0 |
| | 4 °C, S | $C_t = 246e^{-0.044t}$ | 0.995 | <0.0001 | 0.044 | 15.8 | 52.3 |
| | 24 °C, NS | $C_t = 244e^{-0.054t}$ | 0.992 | <0.0001 | 0.054 | 12.8 | 42.6 |
| | 24 °C, S | $C_t = 246e^{-0.057t}$ | 0.994 | <0.0001 | 0.057 | 12.2 | 40.4 |
| | 34 °C, NS | $C_t = 246e^{-0.062t}$ | 0.994 | <0.0001 | 0.062 | 11.2 | 37.1 |
| | 34 °C, S | $C_t = 252e^{-0.063t}$ | 0.994 | <0.0001 | 0.063 | 11.0 | 36.5 |

[a]Statistical parameters, including coefficient of determination ($r^2$) and $p$ value for the first order kinetic model, were attained using nonlinear regression with SigmaPlot. [b]NS represents not sterilized whereas S represents sterilized. [c]Data are presented as the means of three replicates.

temperature. In contrast, the drop in dissipation rate due to sterilization did not occur for Cry1Ac toxin in the water (Table 4). Because sterilization decreased enzymes activity of the microorganisms, the smaller dissipation rates due to sterilization in the solid substrates implied that the presence of microorganisms played a key role in the dissipation of Cry1Ac toxin. This was consistent to the study by Palm et al.,[32] who reported a decline in the levels of extractable Cry1Ab protein from a soil after $\gamma$-irradiated sterilization and microbial activity was considered as the reason of the degradation of the protein. Alternatively, Helassa et al.[33] suggested that the decline of extractable Cry1Aa toxin in soil with prolonged time was mainly due to physicochemical interactions between the protein and the soil matrix. Therefore, the reduction in the extractable Cry1Ac protein from the soil and the sediment may be the combination of microbial degradation and sequestration to solid matrixes.

Additionally, greater difference in dissipation rates between sterilized and nonsterilized matrixes was observed in the beginning stage of dissipation experiment. As shown in Table 1, concentrations of Cry1Ac protein in nonsterilized sediments decreased for 34%, 45%, and 56% at the first day at 4, 24, and 34 °C, respectively, yet the decreases were only 17%, 23%, and 35% in sterilized sediments after 1-d incubation at the respective temperatures. Similar trends were also observed for the dissipation of Cry1Ac protein in soil (Table 2). When incubation time continued to increase, the difference in concentrations of Cry1Ac protein between the sterilized and nonsterilized matrixes minimized. The microbe reinfestation in the sterilized matrixes at the late stage of experiments may explain the diminished difference in dissipation rates with or without sterilization. This observation also supported the findings that microbial degradation played a dominant role in the dissipation of Cry1Ac toxin in sediment and soil.

The influence of temperature on the dissipation of Cry1Ac protein was also evaluated at 4, 24, and 34 °C (Tables 1−3). In general, the higher the temperature, the faster Cry1Ac protein dissipated, no matter the sterilization conditions or types of matrixes. The lowest concentration of Cry1Ac protein was detected in sediment which was incubated at 34 °C (Table 1). As shown in Table 4, $DT_{50}$ values were 5.1, 3.9, and 2.1 d in nonsterilized sediments and 7.6, 6.0, and 3.8 d in sterilized sediments at 4, 24, and 34 °C, respectively, with the corresponding $DT_{90}$ values being 16.8, 12.9, and 7.1 d, and 25.3, 19.8, and 12.7 d, respectively.

Although our discussion focused on sediment, the impact of temperature on the dissipation of Cry1Ac protein was similar in soil and water (Tables 2 and 3). Temperature significantly affected the dissipation of Cry1Ac protein in all three matrixes, and generally the higher the temperature, the shorter the $DT_{50}$ and $DT_{90}$ (Table 4). This observation was consistent with previous studies on the degradation of Cry1Ab protein in soil.[21,22] Feng et al.[22] found that $DT_{50}$ and $DT_{90}$ of Cry1Ab protein in soil were significantly shorter at 35 °C than those at 15 °C. A relatively higher temperature accelerated Cry1Ac protein degradation because microorganisms had an optimum growth temperature and higher temperature stimulated microbial activity if it was still within the acceptable temperature range.[22]

**Dissipation of Cry1Ac Protein in Different Matrixes.** The dissipation of *Bt* proteins in soil has been previously investigated,[22,29] but the fate and effects of *Bt* proteins in aquatic ecosystems were scarcely studied.[14,15] The present study compared the dissipation processes of Cry1Ac protein in soil, sediment, and water and the dissipation kinetic equations and parameters were presented in Table 4 and Figure 1. As discussed early, while sterilization significantly reduced the dissipation of Cry1Ac in sediment and soil, it showed no impact on the dissipation process in water. The $DT_{50}$ values of Cry1Ac protein at different experimental conditions in soil, sediment and water varied from 0.8 to 3.2 d, 2.1 to 7.6 d and 11.0 to 15.8 d, respectively, with the corresponding $DT_{90}$ being from 2.8 to 10.7 d, 7.1 to 25.3 d, and 36.5 to 52.3 d, respectively (Table 4). Dissipation rates of Cry1Ac protein in the three matrixes

showed an order of water < sediment < soil. With the largest $DT_{50}$ and $DT_{90}$ values, Cry1Ac persisted in water for the longest time and the lack of sites for microbial growth in water may be the reason.[34] Organic matters could be used by microbe as carbon or nitrogen sources, and thus greater amounts of organic matters benefit the growth of microorganisms. Consequently, microbial degradation of *Bt* proteins in the matrix with larger amounts of organic matters elevated.[35] Compared to those in soil and sediment, organic matter content in water was negligible, so did the amounts of microorganisms. As a result, the dissipation of Cry1Ac protein in water was the slowest among the three matrixes. This observation also confirmed that microbial degradation was the major route for the dissipation of the protein.

However, the content and composition of OC also affected the bioavailability of contaminants.[36] Greater amounts of OC may provide more sorption sites for Cry1Ac protein and reduce its bioavailability to the microorganisms, subsequently reducing microbial degradation rate. The sediment had greater OC content (2.75 ± 0.07%) than the soil (1.15 ± 0.15%), and thereby less Cry1Ac protein was available to microbial degradation. Furthermore, higher OC may reduce extractability of Cry1Ac protein from solid matrixes. Consequently, the dissipation rate of Cry1Ac protein in sediment was less than that in soil. To fully understand the mechanisms of microbial degradation of *Bt* proteins in various environmental matrixes, more studies are required, but it is out of the scope of the present study.

Overall, the dissipation of CrylAc protein in various environmental matrixes was evaluated, and Cry1Ac was more stable in water and sediment than in soil. Rosi-Marshall et al.[14] demonstrated that *Bt* toxins could enter into stream ecosystems and cause unexpected consequences. The present study showed that trace amounts of CrylAc protein were still detectable in sediment and water after 60 d. Hence, it is needed to assess the risk of the residual *Bt* proteins to aquatic nontarget organisms.

**Toxicity of Cry1Ac Protein to Aquatic Nontarget Organisms.** Acute toxicity of *Bt* proteins to nontarget organisms in aquatic ecosystems were assessed using 10-d sediment and 4-d water-only bioassays with two aquatic invertebrates *C. dilutus* and *H. azteca*. Figure 2 presented results of sediment toxicity testing to both species. Throughout the toxicity tests, temperature, dissolved oxygen, pH, and conductivity ranged from 22 to 24 °C, 5.9 to 7.8 mg/L, 7.1 to 7.5, and 276 to 358 μS/cm, respectively, and ammonia remained a constant level at 0.6−1.0 mg/L. In order to verify if the observed toxicity was from other proteins and gossypol ingredients in cotton seeds, CK with the extracts from nontransgenic cotton seeds were also included in the bioassays besides of the commonly used NC and SC. Mortality was all less than 20% for both species in all of the controls (Figure 2). Since toxicity of CKs was not significantly different from those of NC and SC, the noted toxicity in sediment spiked with crude *Bt* proteins to the invertebrates was caused by Cry1Ac protein. The $LC_{50}$ of Cry1Ac crude protein was 155 ng/g dw to the midges after 10-d exposure. However, no $LC_{50}$ value was calculated for *H. azteca* because its mortality was <50% even at the highest concentration of 157 ng/g dw. Although toxicity was detected, the $LC_{50}$ was 3 orders of magnitudes higher than concentration of Cry1Ac protein found in soil from cotton fields where *Bt* transgenic cottons had been cultivated for several years (0.94 ± 0.08 ng/g dw).[27]

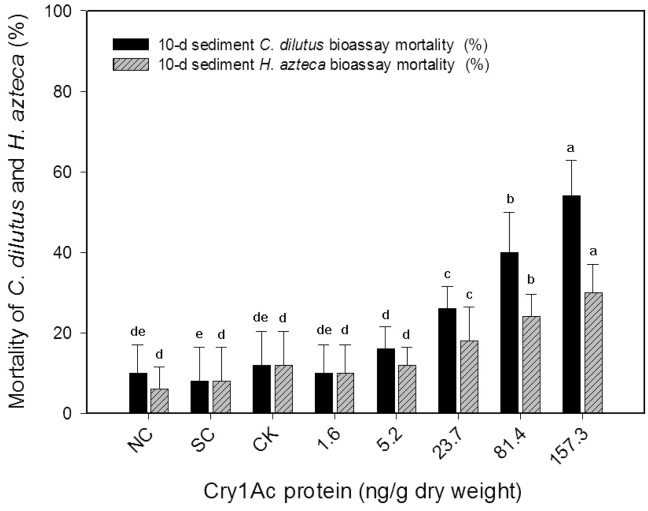


**Figure 2.** Toxicity of Cry1Ac crude protein to *Chironomus dilutus* and *Hyalella azteca* in 10-d sediment toxicity testing. The tests were conducted in five replicates, and the error bars represent the standard deviations. Measured concentrations of Cry1Ac in sediment were used and three controls were included in the testing, including the NC, which was control sediment; the SC, which was sediment spiked with the PBST solution; and the CK, which was sediment spiked with the crude protein solution extracting from nontransgenic Simian-3 cotton seeds. Different letters indicated significant difference.

In addition to sediment bioassays, water-only toxicity testing was also conducted. Different from the sediment toxicity testing, CK exhibited slightly higher toxicity to both organisms in water than that of NC and SC, but they were still less than 20% (Figure 3). On the contrary, the increase of concentrations of Cry1Ac protein significantly increased water toxicity. Mortality was 72 ± 8.4% for *C. dilutus* and 42 ± 4.5% for *H. azteca* at the highest concentration of Cry1Ac protein (456 ng/

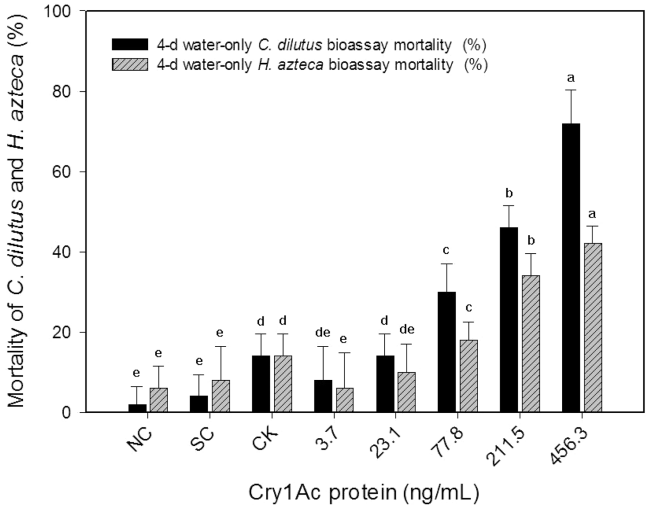


**Figure 3.** Toxicity of Cry1Ac crude protein to *Chironomus dilutus* and *Hyalella azteca* in 4-d water-only toxicity testing. The tests were conducted in five replicates and the error bars represent the standard deviations. Measured concentrations of Cry1Ac in water were used and three controls were included in the testing, including the NC which was control water, the SC which was water spiked with the PBST solution, and the CK which was water spiked with the crude protein solution extracting from nontransgenic Simian-3 cotton seeds. Different letters indicated significant difference.

mL). Owing to the relatively low toxicity of Cry1Ac protein to the amphipod, $LC_{50}$ value was only estimated for the midges, being 201 ng/mL.

Both sediment and water-only bioassays demonstrated that *C. dilutus* was more susceptible to Cry1Ac toxin than *H. azteca*. Like lepidopteran pests, the dipteran midges have an alkaline pH insect midgut where *Bt* toxins were activated and bound to the receptors on the epithelial cell wall, causing membrane perforations and eventually death.[5,6,25] Larvicidal active *Bt* toxins against midges have also been reported in previous studies. Kondo et al.[25] reported that the *B. thuringiensis* serovar *israelensis* (H14) exhibited activity against *Chironomus riparius* larvae. Prihoda and Coats[15] reported a significant decrease in the survival of *C. dilutus* which were fed with Cry3Bb1 protein extracted from MON863 root. Furthermore, *Bt* proteins were also toxic to mosquito larvae, including *Aedes* and *Culex* spp.[37] Instead, *H. azteca* is a freshwater amphipod. Although no study has reported the pH in the gut of *H. azteca*, a previous study showed that the digestive juices of shrimp were slightly acidic, with a pH of 6.7.[38] Therefore, the greater susceptibility of the midge to *Bt* proteins than the amphipod was reasonable.

The present study indicated that *Bt* proteins were more persistent in aquatic ecosystems at low temperature than those in soil and at high temperature. Compared to the amphipod, the midges were more susceptible to *Bt* proteins because of their likeness to lepidopteran pests, the target organisms of Cry1Ac toxin. While acute mortality was detected in laboratory bioassays, concentrations of Cry1Ac toxin were one-hundred times higher than those detected in the field, suggesting low risk of *Bt* proteins to nontarget aquatic organisms. As shown in SI Figure S1, *Bt* cotton acreage and the proportion of total cotton acreage in China increased year by year since its first cultivation in 1997. The majority of *Bt* cotton planting areas were located in northern China, where the temperature was ranged from 19 to 24 °C during the cotton growth seasons. In addition, *Bt* toxins from transgenic crops would enter into aquatic ecosystems and existed for a longer time. Therefore, more studies on the chronic toxicity of *Bt* toxins to susceptible aquatic nontarget organisms, e.g., midges, are needed.

In conclusion, dissipation of Cry1Ac protein in soil, sediment, and water were similar and well described by the first order kinetic equations. Microbial degradation contributed significantly to the dissipation, but the role of sequestration of the protein to solid substrates should not be ignored. High temperature accelerated the dissipation process. Cry1Ac toxin dissipated the fastest in soil, followed by sediment, and the slowest in water, which indicated *Bt* proteins may exist in aquatic systems for a long time. Bioassays showed *C. dilutus* was more susceptible than *H. azteca* to Cry1Ac protein, although acute toxicity of Cry1Ac toxin to both organisms was limited at environmentally relevant concentrations.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Cultivation areas of *Bt* cotton in China (Figure S1) and the influencing factors studied in the present study (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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