

ENVIRONMENTAL FACTORS THAT AFFECT THE SUSCEPTIBILITY OF  
*SIMULIUM VITATTUM* LARVAE TO INSECTICIDAL PROTEINS PRODUCED BY  
*BACILLUS THURINGIENSIS* SUBSP. *ISRAELENSIS*

by

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(Under the Direction of Raymond Noblet)

ABSTRACT

Application of insecticidal crystalline proteins (ICPs) produced by *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) to the larval habitats is the method of choice for black fly (Diptera: Simuliidae) suppression. There have been occasional reports of less than optimum control when applying the *Bti* ICPs to certain larval habitats. The effects of antibiotics, seston, and time on larval susceptibility to ICPs were investigated.

*Simulium vittatum* Zetterstedt larvae were exposed to enrofloxacin, tylosin, sulfamethoxazole, and trimethoprim followed by exposure to *Bti* ICPs. Anthropogenic concentrations of a mixture of these antibiotics and individual antibiotic concentrations 10,000-80,000 times higher than those found in contaminated rivers were unable to reduce the activity of ICPs.

Some components of seston from problematic sites impaired the effectiveness of ICPs. Exposure of larvae to clay minerals prior to ICP exposure resulted in no significant decrease in mortality. Exposure of larvae to cellulose, viable diatoms, and purified diatom frustules prior to *Bti* ICP exposure resulted in significant reductions in mortality.

Larvae were exposed to various materials and water-insoluble Neon Red particles (NRP) to determine if selected materials interfered with larval feeding. The quantity of NRP consumed by larvae in each medium was determined by spectrophotometric analysis. The number of times the larvae extended and retracted their cephalic fans per min (flick rate) in each medium was calculated. The flick and ingestion rates of larvae were not significantly affected by clay material or viable *Chlorella vulgaris* Pratt cells. *Scenedesmus quadricauda* (Turp) Bréb colonies, *Cyclotella meneghiniana* Kütz cells, and frustules caused significant decreases in larval flick and ingestion rates.

The time necessary for ICPs to cause mortality in larvae is poorly understood. Larvae were exposed to *Bti* ICPs and mortality was monitored over time. Larvae exposed to operational concentrations of ICPs exhibited maximum mortality after 4 h. Exposure of larvae to 1/3 of that concentration resulted in similar mortality after 8 h. Clay material had no effect on larval mortality or time to achieve maximum mortality. When cellulose was present in the medium the time to maximum mortality was increased 50% and overall mortality was reduced by 40%.

INDEX WORDS: Black Fly, *Simulium vittatum*, Seston, Feeding, *Bti*, Biological Larvicide, Antibiotics, Toxicity, Mortality

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## DEDICATION

I would like to dedicate this dissertation to my family. To my wife Brittany, for the love and support she has given me throughout the years and to my parents and sisters for their support and encouragement.

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CHAPTER 1  
INTRODUCTION AND LITERATURE REVIEW

**Black Flies**

Black flies (Diptera: Simuliidae) are common semi-aquatic insects whose larval stage occurs in freshwater rivers and streams throughout the world. The larvae of most species are considered indiscriminate filter feeders (Williams et al. 1961, Adler et al. 2004). A select few genera of black flies do not have cephalic fans and feed by scraping periphyton and debris from the substrate (Currie and Craig 1987). The majority of Simuliidae larvae feed by capturing seston (suspended biotic and abiotic material) with their cephalic fans (Chance 1970). The fans periodically close and are inserted into the cibarium where the mandibles and labrum remove captured material from the fans during fan retraction (Merritt et al. 1996). Black fly larvae typically capture and ingest materials ranging in size from 0.09 to 250  $\mu\text{m}$  in length (Wotton 1977, 2009).

Some black fly species are considered pests due to their blood feeding and nuisance swarming behaviors. When populations of black flies are uncontrolled, they can become a severe nuisance pest in rural, urban, and recreational areas. Populations can transmit a variety of pathogens to humans and livestock (Adler et al. 2010). The swarming and biting behaviors of some species have been attributed to economic loss as well (Gray et al. 1996). As a result of the pestiferous nature of some black fly species, suppression programs are conducted at various locations throughout the world. The

preferred approach for suppressing black fly populations is to target the rivers and streams where the rheophilic larval stages occur (Barjac and Sutheland 1990).

### ***Bti* Toxins**

*Bacillus* is a genus of rod-shaped bacteria that is ubiquitous in nature. *Bacillus thuringiensis* and *Bacillus sphaericus* are two species which have been found to produce toxins that are entomopathogenic (Barjac and Sutheland 1990). *Bacillus thuringiensis* is a Gram positive soil bacterium that produces proteinaceous-parasporal crystals during its growth cycle. When these proteins are ingested by certain insect larvae, the insecticidal crystalline proteins (ICPs) are activated by midgut proteases. Different subspecies of *B. thuringiensis* produce unique crystalline proteins that exhibit specificity toward different orders of insects (Bravo et al. 2007). Insecticidal crystalline proteins produced by *B. thuringiensis* subsp. *israelensis* (*Bti* ICPs) are formulated commercially and applied to bodies of water for the control of black flies (Barjac and Sutheland 1990). Numerous studies have demonstrated the high efficacy of *Bti* ICPs toward black flies while the toxins are virtually harmless to non-target populations at typical larvicidal concentrations (Jackson et al. 1994, Hershey et al. 1998). Based on a high degree of larvicidal activity and low toxicity to non-target species, *Bti* ICPs are the most widely used black fly larvicides throughout the world (Adler et al. 2004).

### **Rationale and Purpose of Dissertation**

Despite the high efficacy of *Bti* ICPs in controlling black fly populations, occasionally lower than expected mortality is achieved following larvicide applications

(Bob Fusco, Valent BioSciences Corp., personal communication). Currently, the world's largest black fly suppression program is conducted by the Pennsylvania Department of Environmental Protection, targeting nuisance black fly populations in habitats across the state. A large portion of this program is associated with suppressing members of the *Simulium jenningsi* species group in the Susquehanna River and has been highly successful. However, for over 10 years, less than optimal larval mortality has sporadically been observed in specific areas of the Susquehanna River following the application of *Bti* ICPs. There have been occasional reports of less than adequate control in other areas as well. The cause for this occasional low efficacy is unclear, however, it has been postulated that certain components of the environment could occasionally impair the larvicidal activity of *Bti* ICPs. The purpose of this study is to identify factors that can contribute to a reduction in *Bti* ICP activity towards larvae of the test organism, *Simulium vittatum* Zetterstedt.

In Chapter 2, the effects of antibiotics on the susceptibility of *S. vittatum* to *Bti* ICPs was investigated. Speculation has arisen that antibiotic contamination of waterways could be responsible for the mitigation of ICP activity. For example, Broderick et al. (2006) demonstrated that lepidopteran insect larvae reared on antibiotic-containing diets were less susceptible to the insecticidal proteins derived from *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*). Numerous studies have found low levels of antibiotics in rivers and streams on a world-wide basis (Kolpin et al. 2002, Loper et al. 2007). A series of experiments was conducted to determine the response of black fly larvae to *Bti* ICPs in the presence and absence of various antibiotics that are commonly found in contaminated

rivers. My hypothesis was that antibiotics would not interfere with *Bti* ICP activity as the mechanism of toxicity differs from that of Btk ICPs (Ferreira and Silva-Filha 2013).

In Chapter 3, the effects that seston components had on the susceptibility of black fly larvae to *Bti* ICPs were evaluated. Wilson et al. (2005) found that commercial products performed poorly in West Africa when the river turbidity was above 4.2 Nephelometric Turbidity Units (NTU). Gray et al. (2012) did not find any reduction in ICP activity in a river with a turbidity exceeding 250 NTU. These conflicting studies indicated that the components may be more important than the quantity of seston in regards to ICP activity. Experiments evaluated the effects of seston and soluble components in the river on the susceptibility of larvae to *Bti* ICPs. The composition of the seston was analyzed using a variety of techniques. Individual components of the seston were then evaluated for their effect on the susceptibility of larvae to *Bti* ICPs.

*Bti* ICPs must be ingested by larvae to cause mortality (Ferreira and Silva-Filha 2013). The experiments in Chapter 4 were conducted to develop a novel technique for measuring particle ingestion by black fly larvae. This technique was developed to test the hypothesis proposed in Chapter 3 that select materials interfered with *Bti* ICP activity by altering larval feeding behavior. Numerous methods have been used to characterize the feeding behavior of Simuliidae. In several studies the flick rate of the labral fans has been examined in relation to particle ingestion (Gaugler and Molloy 1980, Hart and Latta 1986, Merritt et al. 1996, Stoops and Adler 2009). In other studies, various types of markers such as dyes, beads, and charcoal were added to the environment of the larvae and the movement of the marker through the gut was measured (Mulla and Lacey 1976, Ladle and Hansford 1981, Thompson 1987, Miller et al. 1998, Wotton 2009). These

methods were developed to determine particle size selection and time needed for material to pass through the gut of the larvae; however, they were not suitable for measuring the precise quantity of larval ingesta.

*Simulium vittatum* Zetterstedt larvae were exposed to inert, water-insoluble particles of Neon Red Particles (NRP), a synthetic organic colorant. A spectrophotometric assay was developed for the quantification of NRP within the alimentary tract of larvae. The length of larval gut occupied by NRP was also determined. These measurements provided a unique insight into black fly larvae feeding behavior. The method developed in Chapter 4 was utilized in Chapter 5 to evaluate the feeding activity of *Simulium vittatum* Zetterstedt larvae following exposure to a variety of materials. In addition to the analysis of NRP ingestion in the presence of these materials, larvae were recorded feeding during the exposure. The number of times that the larvae extended and retracted their cephalic fans per unit time was calculated (flick rate). The average amount of NRP ingested by a larva per min was calculated for larvae in each medium. The average quantity of NRP captured by larvae in each medium per flick was also recorded. The materials used were chosen based upon the findings in Chapter 3 and from the work by Stephens et al. (2004). Clay material and viable *Chlorella vulgaris* Pratt cells were used because these materials were not found to have an influence on ICP activity at the concentrations used. Our hypothesis was that these materials would not have a significant effect on NRP ingestion or flick rate of larvae. Cellulose fibers, purified diatom frustules, viable *Cyclotella meneghiniana* Kütz cells, and viable *Scenedesmus quadricauda* (Turp) Bréb were utilized because the fore-mentioned studies found that these materials had a significant effect on ICP activity at the concentrations

used. Our hypothesis was that these materials would significantly affect the flick rate and/or NRP ingestion rate of larvae.

The experiments in Chapter 6 were conducted to evaluate larval mortality over time following exposure to *Bti* ICPs. There have been few studies related to the time needed for mortality to manifest in larvae after exposure to *Bti* ICPs. Back et al. (1985) found that the majority of larval mortality occurred within the first 3 h after exposure to a high label concentration of ICPs, and that the final mortality could take over 24 h. Gaugler and Molloy (1980) evaluated larval mortality following exposure to ICPs for four days and found that 95% of the mortality occurred within the first 24 h. Studies looking at the effects of *Bti* ICPs on larvae have evaluated mortality at 24 h (Lacoursiere and Charpentier 1988, Boisvert et al. 2002), 9 h (Car and De Moor 1984), and 6 h (Wilson et al. 2005). Iburg et al. (2011) and Overmyer et al. (2006) evaluated larval mortality after 5 h. These studies utilized various formulations containing *Bti* ICPs both in the field and in the laboratory. The conflicting evaluation times indicated that a more thorough understanding of larval mortality following exposure to *Bti* ICPs is necessary.

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## CHAPTER 2

# INFLUENCE OF SELECTED ANTIBIOTICS ON THE RESPONSE OF BLACK FLY (*SIMULIUM VITTATUM*) LARVAE TO INSECTICIDAL PROTEINS PRODUCED BY *BACILLUS THURINGIENSIS* SUBSP. *ISRAELENSIS*<sup>1</sup>

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<sup>1</sup> **Iburg, J. P., E. W. Gray, R. D. Wyatt, and R. Noblet. 2010.** Environ. Toxicol. Chem. 29: 1849-1853.  
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## Abstract

A controlled current toxicity test (CCTT) was developed to evaluate the response of black fly (*Simulium vittatum*) larvae to insecticidal proteins following exposure to various antibiotics. The bacterium, *Bacillus thuringiensis* subsp. *israelensis* (*Bti*), produces proteins that are toxic to Nematoceran Diptera, such as black flies and mosquitoes, when ingested. These insecticidal crystalline proteins (ICPs) are highly efficacious in controlling black flies; however, speculation has arisen regarding the potential for antibiotic contamination of waterways to mitigate the toxicity of these proteins. A series of experiments was conducted with the CCTT in which black fly larvae were exposed to enrofloxacin, tylosin, sulfamethoxazole, and trimethoprim followed by exposure to *Bti* ICPs. These antibiotics were selected based on their use in agricultural and documented anthropogenic contamination of rivers. Anthropogenic concentrations of a mixture of these four antibiotics did not affect the response of the larvae to *Bti* ICPs. Subsequent experiments were conducted with antibiotic concentrations 10,000-80,000 times higher than those found in contaminated rivers. Exposure of black fly larvae to high levels of enrofloxacin (0.5 mg/L) had no effect upon the susceptibility to *Bti* ICPs; however, exposure to high levels of tylosin (8 mg/L) resulted in a significant increase in the susceptibility of the larvae to *Bti* ICPs at 72 h of exposure, but not at 48 h. Exposure of black fly larvae to high concentrations of a mixture of sulfamethoxazole and trimethoprim resulted in a significant increase in the efficacy of the larvicide after 48 and 72 h of exposure. These results suggest that impairment of the efficacy of *Bti* ICPs to black fly larvae is not due to antibiotic contamination of the larval environment.

## Introduction

Black flies (Diptera: Simuliidae) are common aquatic insects whose larval stage occurs in freshwater rivers and streams. When populations of black flies are uncontrolled, they can become a severe nuisance pest in rural, urban, and recreational areas. In addition to being a notorious pest, adult black flies, due to their blood feeding behavior, can serve as vectors of disease-causing agents in both humans and animals (Adler et al. 2004).

*Bacillus* is a genus of rod-shaped bacteria that is ubiquitous in nature. *Bacillus thuringiensis* and *Bacillus sphaericus* are two species which have been found to produce toxins that are entomopathogenic (Barjac and Sutheland 1990). *Bacillus thuringiensis* is a Gram positive soil bacterium that produces proteinaceous-parasporal crystals during its growth cycle. When these proteins are ingested by insect larvae, the insecticidal crystalline proteins (ICPs) are activated by midgut proteases. Different subspecies of *B. thuringiensis* produce unique crystalline proteins which exhibit specificity toward different orders of insects (Bravo et al. 2007).

Insecticidal crystalline proteins produced by *B. thuringiensis* subsp. *israelensis* are formulated commercially and applied to bodies of water for the control of black flies (Simuliidae) and other Nematoceran Diptera. Numerous studies have demonstrated the high efficacy of *Bti* ICPs toward black flies while the toxins are virtually harmless to non-target populations at typical larvicidal concentrations (Jackson et al. 1994, Hershey et al. 1998). Based on a high degree of larvicidal activity and low toxicity to non-target species, *Bti* ICPs are the most widely used black fly larvicides throughout the world (Adler et al. 2004).

Despite the high efficacy of *Bti* ICPs in controlling black fly populations, occasionally less than expected mortality is achieved following larvicide applications (Bob Fusco, Valent BioSciences Corp., personal communication). The cause for this occasional low efficacy is unclear, however, it has been postulated that certain components of the environment could occasionally impair the larvicidal activity of *Bti* ICPs. For example, Broderick et al. (2006) demonstrated that lepidopteran insect larvae reared on antibiotic-containing diets were less susceptible to the insecticidal proteins derived from *Bacillus thuringiensis* subsp. *kurstaki*. These findings have led to speculation that water-contaminating antibiotics could be involved in impaired efficacy of *Bti* ICPs toward black fly larvae. Black fly larvae are characterized as indiscriminate filter feeders and typically attach to the outer surface of leaf packs, sticks and rocks for their larval substrates. As a result of this attachment, larvae would be exposed continually to any substance in their aquatic habitat, including contaminating antibiotics.

Numerous studies have found low levels of antibiotics in rivers and streams on a world-wide basis (Kolpin et al. 2002, Loper et al. 2007). This contamination is most often attributable to agricultural runoff and discharge from wastewater treatment facilities (Hirsch et al. 1999, Haggard et al. 2006). Contemporary environmental monitoring equipment and techniques are highly sensitive, thereby measuring concentrations of various pharmaceuticals in water that were previously undetectable (Kolpin et al. 2002).

A series of experiments was conducted to determine the response of black fly larvae to *Bti* ICPs in the presence and absence of various antibiotics. In our first experiment, black fly larvae were exposed to a combination of four antibiotics, enrofloxacin, tylosin, sulfamethoxazole and trimethoprim at in vitro concentrations

approximating those commonly found in freshwater bodies of water (Hirsch et al. 1999, Kolpin et al. 2002, Haggard et al. 2006, Loper et al. 2007). These antibiotics have been found in the Susquehanna River in the North-Eastern United States (Loper et al. 2007). One of the world's largest black fly control programs involves the treatment of this river with *Bti* ICPs. Following exposure to the antibiotics, the larvae were exposed to *Bti* ICPs and susceptibility to the larvicide was determined by measuring acute larval mortality. In subsequent experiments, black fly larvae were exposed to enrofloxacin and tylosin, singularly and a combination of sulfamethoxazole and trimethoprim. In these experiments, the in vitro concentration of the antibiotics was approximately 10,000 to 80,000 times higher than typically measured in natural settings. Following exposure to the antibiotic, larvae were exposed to *Bti* ICPs and susceptibility to the larvicide was determined by measuring acute larval mortality.

## **Materials and Methods**

### Chemicals

Tylosin tartrate (92%), sulfamethoxazole (100%), and trimethoprim (98%) were obtained from Sigma Life Science. Enrofloxacin was obtained as a multi-dose solution (100 mg/mL) from Bayer Pharmaceuticals. Stock solutions of all antibiotics were corrected for purity, and dissolved in Milli-Q water (ThermoFisher). Solubility of the sulfamethoxazole and trimethoprim was facilitated by adding a minimal volume of 0.1N NaOH to the antibiotics prior to preparation of the stock solution.

*Bti* ICPs were obtained as a commercial larvicidal product from Abbott Laboratories, Abbott Park, IL, USA. This commercial product (Vectobac® 12AS) is an

aqueous suspension containing 1.2% *Bti*-ICPs (1200 International Toxic Units (ITU)/mg). Serial dilutions of this product were made such that the addition of 5 ml of the final dilution to 195 mL of larval medium resulted in a concentration of 4.5 ng of *Bti* ICPs per ml in each treated flask.

### *Simulium vittatum* larvae

Experiments were conducted using approximately 5<sup>th</sup> and 6<sup>th</sup> instar *S. vittatum* Zetterstedt cytospecies IS-7 larvae obtained from the University of Georgia (Athens, GA) colony. The larvae were grown in a modified version of the Cornell Automated Rearing System which incorporates a closed circulation, trough system to create a larval habitat (Gray and Noblet 1999). This system provided a weekly supply of larvae of similar age and reared under similar conditions.

### Controlled Current Toxicity Test

The controlled current toxicity test (CCTT) is an in vitro test that entails the creation of multiple microcosms that simulate the natural Simuliidae larval habitat. The CCTT provides *S. vittatum* larvae a nutrient-rich medium, a surface upon which the larvae can attach, and a controlled current of circulating water to ensure the larvae can filter food material from the larval medium. The water and room temperature for all experiments was 20-22° C. The pH in each microcosm was 6.0.

The vessels used to create the replicate microcosms were 250 ml flat-bottomed extraction flasks (NDS Technologies). Each flask received 195 ml of the larval medium consisting of suspended organic nutrients in deionized water. The suspended organic

nutrients consisted of a 1:1 mixture of rabbit feed (Advanced Nutrition Brand Rabbit Feed, Professional Formula, Purina Mills) and soybean meal (University of Georgia Poultry Feed Mill). Prior to the addition of this feedstuff, the material was finely ground to a particle size of  $\leq 53 \mu\text{m}$ . This mixture was added to deionized water in 100 mg aliquots until a turbidity reading of 4.5 to 5.5 NTU was attained. The turbidity of the solution was measured using a Hach Turbidometer (Hach Company). Larvae were removed from the colony rearing unit and thirty larvae were placed into the larval medium in each flask with soft-tipped forceps. Immediately after the introduction of the larvae to the medium, the antibiotic solution under investigation was added to achieve the specified in vitro concentration. All flasks were capped with aluminum foil to prevent evaporation of the medium during the course of the experiment. A total of 10 replicate flasks were prepared for each treatment.

The flasks were then placed onto G10 Gyrotory® shakers (New Brunswick Scientific). Each shaker was surrounded by a Fisher Flexiframe (ThermoFisher) which was attached to the laboratory bench. This frame holds a syringe support board. This board is constructed of plywood (2 x 79 x 109 cm) which has holes drilled in alignment with the center of each flask and is positioned 3 cm above the top of the flasks. The shakers are then surrounded by an opaque covering to exclude any light from the individual microcosms for the duration of the experiment.

Once the covering was in place, the speed of the gyrotory shakers was gradually increased. To acclimate the larvae to a current, and to create a current within each flask similar in velocity to that encountered by the larvae in nature, the following procedure was used. The initial speed of the shakers was gradually increased from 0 rpm to 100 rpm

over 30 sec. This speed was maintained for 10 min, and then increased to 125 rpm over 30 sec. After 10 additional min, the speed was increased to 150 rpm and maintained for the duration of the experiment. Without this acclimation period the larvae would become entangled in their silk, attachment to the flask surface would be impaired, and feeding behavior would be non-uniform.

Immediately prior to the introduction of *Bti* ICPs, the opaque covering was removed from around each shaker and the aluminum foil cover of each flask was removed. The opaque cover was then replaced and the *Bti* ICP stock solution was added to the appropriate flasks. The flasks not receiving ICPs received 5 ml of deionized water. These applications were achieved simultaneously by filling 10 ml plastic syringes fitted with 20 gauge needles with 5 ml of either the stock solution containing ICPs or deionized water. The filled syringes with needles were inserted through the holes in the syringe support board directly above the appropriate flasks. A separate board (2 x 61 x 92 cm) was positioned on top of the syringe plungers and slowly depressed to simultaneously deliver the contents of the syringes into the respective flasks. Ten min after dosing with *Bti* ICP or deionized water, the shakers were stopped and remained stationary for an additional 4 hours and 50 min. At the completion of the specified time period, all larvae were transferred from the flasks to white enamel pans (5 x 23 x 35 cm). Visual inspection and assessment of all surviving larvae determined that the larvae exhibited normal attachment and taxis. Mortality and moribundity was determined if the larvae did not attach or exhibit vigorous taxis. The number of surviving and dead or moribund larvae from each flask was determined, recorded, and mortality calculated.

## Experiment 1

In Experiment 1, 80 flasks were utilized. No additive was initially made to 40 flasks. To the remaining 40 flasks, an appropriate volume of the respective antibiotic stock solution was added to attain an *in vitro* concentration of 0.05 µg/L of enrofloxacin, 0.05 µg/L of tylosin, 1.34 µg/L of sulfamethoxazole (SMZ), and 0.268 µg/L of trimethoprim (TMP). The flasks were then placed onto two shakers, each shaker receiving 20 antibiotic treated flasks and 20 flasks with no additive.

After 43 hours, *Bti* ICPs were added to 10 flasks containing no antibiotics and to 10 flasks containing the mixture of antibiotics. The *Bti* ICPs were applied so that each treated flask contained 1.08 ITU/ 200 ml. Ten minutes after the introduction of the *Bti* ICPs the shaker was turned off so that the larvae would cease to feed. This parameter imitates natural settings where larvae would be exposed to *Bti* ICPs post-treatment for a limited amount of time before the toxin is carried down-stream. After 48 h, all larvae were removed from each ICP-treated flask and placed into a white enameled pan and visually assessed for viability. In addition, 10 flasks with no antibiotic added and not treated with *Bti* ICPs (negative control) and 10 flasks treated with *Bti* ICPs only, with no antibiotics added (positive control) were also assessed for viability. The number of alive and dead larvae from each flask was recorded and mortality calculated.

The remaining 40 flasks were treated in a similar fashion as described above; however, the larvae were exposed to the *Bti* ICPs at 67 hours post-initiation. After 72 h, the larval mortality was recorded in identical fashion. This protocol afforded the maximum exposure time of the larvae to the antibiotics prior to exposure to *Bti* ICPs.

## Experiments 2-4

Experiments 2-4 were designed to determine if high levels of the antibiotics were able to affect black fly larval susceptibility to *Bti* ICPs. The concentration of each antibiotic used for these experiments was determined by the suggested dose for treating infections. Enrofloxacin (0.5 mg/L) and tylosin (8 mg/L) were tested separately. Sulfamethoxazole and TMP were tested in combination in a 5:1 ratio (30 mg/L). This ratio is the most commonly used by manufacturers, consequently the ratios found in the environment are similar. Each experiment followed the same protocol as Experiment 1 with a 48 and a 72 hour evaluation of larval response to *Bti* ICPs after exposure to the antibiotic. The flasks receiving *Bti* ICPs contained 1.08 ITU/200 mL.

## Statistical Analysis

One-way analysis of variance with Tukey-Kramer multiple comparison test was performed using GraphPad InStat version 3.06 for Windows XP, GraphPad Software, San Diego California USA. The use of the term “significant” in this manuscript has a statistical meaning and is based on  $p \leq 0.05$ .

## **Results**

The controlled current toxicity test (CCTT) as described herein provided an acceptable environment for maintaining black fly larvae in a viable state for up to 72 h without appreciable larval mortality. Mortality values in the control treatments ranged from 2.0-3.4% at 48 h and 1.3-5.1% at 72 h for the experiments conducted. Exposure of black fly larvae to either a mixture of antibiotics at anthropogenic concentrations (Table

2.1) or individual antibiotics at very high concentrations (Tables 2.2-2.4) did not result in larval mortality above that observed in the control treatment. Mortality values ranged from 2.4-5.0% at 48 h to 2.3-6.5% at 72 h of exposure to the antibiotics.

The CCTT provided a suitable environment in which to assess the toxicity of *Bti* ICPs on black fly larvae. Five hours after the introduction of *Bti* ICPs into the larval medium at a concentration of 1.08 ITU/200mL, ensuing mortality ranged from 75.5-89.5% after 48 h of total time in the flasks and 78.2-93.5% at 72 h total time in the flasks. Exposure of black fly larvae to this mixture of antibiotics for 48 or 72 h, followed by exposure to *Bti* ICPs, resulted in mortality that was not significantly different than exposure to *Bti* ICPs without prior exposure to the antibiotic mixture (Table 2.1).

The remaining experiments were conducted to determine the response of black fly larvae to *Bti* ICPs following exposure to the same antibiotics used in Experiment 1. However, larvae were exposed to enrofloxacin (0.5 µg/L) and tylosin (8.0 mg/L) individually and to SMZ (25.0 mg/L) and TMP (5.0 mg/L) in combination. All antibiotics were utilized at relatively high *in vitro* concentrations.

Exposure of black fly larvae to enrofloxacin at 0.5 mg/L at either 48 or 72 h followed by exposure of the larvae to *Bti* ICP did not significantly alter the mortality compared to the treatment with *Bti* ICPs only (Table 2.2). Black fly larvae exposed to tylosin (8.0 mg/L) and then exposed to *Bti* ICP exhibited mortality at 72 h that was significantly greater than the mortality recorded for the *Bti* ICP only treatment (Table 2.3).

Sulfamethoxazole and trimethoprim are frequently used in combination for treatment of bacterial infections in animals and humans and natural contamination of

water bodies will frequently reveal contamination with both SMZ and TMP (Hirsch et al. 1999, Kolpin et al. 2002, Haggard et al. 2006, Loper et al. 2007). Consequently, these two antibiotics were used in combination to determine their impact on susceptibility of black fly larvae to *Bti* ICPs. Black fly larvae that were exposed to the combination of these antibiotics for either 48 or 72 hs followed by exposure to *Bti* ICPs showed a significant increase in larval mortality (Table 2.4).

### **Discussion**

The insecticidal crystalline proteins produced by *Bacillus thuringiensis* subsp. *israelensis* have long been recognized as highly efficacious, cost-effective and environmentally safe control material for larval black fly populations. Despite the attributes of this biological black fly control agent, routine application of this agent has occasionally been reported to yield less than anticipated results. For example, targeting the larvae inhabiting an ever changing river or stream can present some unique challenges. *Bti* toxins must be ingested by the larvae to be effective. Water conditions such as velocity, temperature, or suspended solids are some of the factors that can influence the efficacy of *Bti* toxins (Boisvert et al. 2001, Ben-Dov et al. 2003). It has been shown that antibiotics impair the efficacy of *Bacillus thuringiensis* subsp. *kurstaki* towards lepidopteran insects (Broderick et al. 2006). Because of this, one proposed reason for impaired efficacy of *Bti* toxins has been contamination of the stream environment with antibiotics due to widespread use in agricultural settings and subsequent run-off.

Crystalline proteins from *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) are used to control lepidopteran insects in forestry and agricultural applications. Broderick et al. (2006) demonstrated that enteric midgut bacteria were necessary for *Btk* ICPs to be lethal to lepidopteran larvae. The experiment involved the rearing of lepidopteran insect larvae on an antibiotic-containing diet followed by a dosing of *Btk* ICPs. *Btk* ICPs were unable to kill these insects until the enteric bacteria were reintroduced into the midgut. These authors concluded that previous models of *Bt* mode of action failed to explain the necessity of enteric bacteria for the product to be lethal.

The results of Broderick et al. (2006) suggest that *B. thuringiensis* permeabilizes the gut epithelium allowing enteric bacteria to enter the hemolymph and cause septicemia. *Btk* contains Cry proteins which are thought to bind to protein receptors in the midgut of susceptible insect orders such as Coleoptera and Lepidoptera. *Bti* produces Cry and Cyt protein toxins specific for Nemotoceran Diptera (Bravo et al. 2007). The activated Cyt proteins do not bind to receptors but directly interact with membrane lipids. Numerous studies suggest that the activated Cyt proteins then form pores (Thomas and Ellar 1983, Gill et al. 1987, Promdonkoy and Ellar 2003) or destroy the membrane by a detergent-like reaction (Butko 2003). *Bti* ICPs clearly cause mortality much faster in Nemotoceran Diptera larvae (<5 h) than do *Btk* ICPs toward lepidopteran larvae (>24-72h). The enteric organisms are unlikely to be as important in the mode of action of *Bti* ICPs as that of *Btk* ICPs.

Numerous studies have found low levels of antibiotics in rivers and streams on a world-wide basis (Hirsch et al. 1999, Kolpin et al. 2002, Haggard et al. 2006, Loper et al. 2007). Review of analyses of water indicates that those antibiotics that most often

contaminate bodies of water where *Bti* toxins might be employed are typical of agricultural use and subsequent run-off into streams and rivers. Sewage treatment plant effluents and aquaculture seepage into ground water have also been found to contain biologically active antibiotics (Hirsch et al. 1999). This observation, coupled with the findings of Broderick et al. (2006) suggested that the role of water-contaminating antibiotics on *Bti* toxin efficacy be examined.

From the published literature three of the most commonly found antibiotics in contaminated bodies of water were used in our experiments. Enrofloxacin is a broad spectrum fluoroquinolone antibiotic used in the treatment of bacterial infections in most agriculturally important animals. Tylosin is a macrolide antibiotic that is used in livestock as a treatment for infections caused by mycoplasma and numerous Gram-positive bacteria. Sulfamethoxazole is a sulfonamide antibiotic which is commonly used in conjunction with trimethoprim. Trimethoprim is typically used in conjunction with sulfonamides as a synergist based upon the fact that both the sulfonamides and trimethoprim exert their effect in the pathway leading to synthesis of folate (Hitchings 1973). This common antibiotic mixture is used in veterinary medicine as well as for the treatment of human infections.

Results from our experiments indicate that a combination of antibiotics, at environmentally relevant concentrations, have no impact on *Bti* ICP efficacy toward black fly larvae. The results of this investigation indicate that neither enrofloxacin, tylosin, or SMZ:TMP at *in vitro* concentrations up to 10,000- 80,000 times that observed in anthropogenic contamination possess the ability to impair the larvicidal activity of *Bti* ICPs. Furthermore, exposure of black fly larvae to enrofloxacin did not have any

detectable effect upon the susceptibility of black fly larvae to *Bti* toxins. Conversely, when black fly larvae were exposed to tylosin or SMZ:TMP prior to exposure to *Bti* ICPs, an enhancement of *Bti* ICP efficacy was observed. In the case of tylosin, the assumed mechanism of action is binding to the 50s subunit of the bacterial 70s rRNA complex. When such binding occurs, protein synthesis is inhibited and numerous physiological processes required to sustain viability are impaired. Perhaps certain members of the microflora associated with the gut of the black fly larvae are involved in the synthesis of a factor or factors that are essential for optimum larval physiology. Consequently, without the normal microflora, and normal synthesis of those factors required or needed by the black fly larvae, their susceptibility to *Bti* ICPs would be enhanced.

Exposure of black fly larvae to SMZ:TMP resulted in a marked enhancement in the susceptibility of the larvae to *Bti* ICPs. A possible explanation for this enhancement in susceptibility to *Bti* ICPs could be related to the known mechanism of action of the sulfonamides, such as SMZ and a synergist, TMP. This antibiotic combination is known to impair tetrahydrofolate biosynthesis. SMZ is an analogue of p-aminobenzoic acid and acts as a competitive inhibitor of dihydropteroate synthetase. Trimethoprim interferes with the action of dihydrofolate reductase, another enzyme in this same biosynthetic pathway leading to the formation of tetrahydrofolate. Consequently, synthesis of folic acid, in the presence of SMZ:TMP is drastically reduced. Insects have been described as organisms that require folic acid (Fraenkel and Blewett 1947, Sedee 1958, Genc 2006). Folic acid is either obtained from the insect's diet or is derived from a symbiotic relationship between the insect and the bacterial microflora. Symbiotic bacteria in

*Pediculus humanus* lice provide the insect with B- vitamins including folic acid. When lice are exposed to sulfonamide antibiotics, there is impairment in the health of the insect, most likely due to a reduction in folic acid (Burns 1987). Exposure of black fly larvae to SMZ: TMP would most likely deprive the insect of an ample source of folic acid, regardless of the biosynthetic source of the vitamin. Hence, following exposure to SMZ:TMP it seems reasonable to hypothesize that the insect would be folic acid-deficient and would therefore be more sensitive to a larvicide such as *Bti* ICPs. This hypothesis is further substantiated by the generally accepted fact that folic acid is most needed by rapidly dividing cells, typical of rapidly growing larvae and especially those cells of the digestive tract, the known site of action of *Bti* ICPs.

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## Tables

**Table 2.1.** The response of *Simulium vittatum* larvae to *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) insecticidal crystalline proteins (ICPs) after exposure to an antibiotic mixture for 48 and 72 h (Experiment 1)

Treatment	Mortality (%)	Mortality (%)
	at 48 h.	at 72 h.
Control	2.4 ± 0.9 A	5.1 ± 1.3 A
Antibiotic mixture only <sup>1</sup>	2.4 ± 0.7 A	6.5 ± 1.5 A
<i>Bti</i> ICP only <sup>2</sup>	89.5 ± 2.6 B	93.5 ± 2.0 B
Antibiotic mixture <sup>1</sup> + <i>Bti</i> ICP <sup>2</sup>	93.8 ± 3.5 B	93.9 ± 1.4 B

A,B Values (mean ± SEM) with different superscripts differ significantly within a column ( $p \leq 0.05$ ).

<sup>1</sup> The antibiotic concentrations in the larval medium were: tylosin, 0.05 µg/L,

Sulfamethoxazole:Trimethoprim, 1.34 µg/L:0.268 µg/L, enrofloxacin, 0.05 µg/L.

<sup>2</sup> The concentration of *Bti* ICPs was 1.08 ITU/200 ml.

**Table 2.2.** The response of *Simulium vittatum* larvae to *Bacillus thuringiensis* subsp.*israelensis* (*Bti*) insecticidal crystalline proteins (ICPs) after exposure to enrofloxacin for 48 and 72 h (Experiment 2)

Treatment	Mortality (%)	Mortality (%)
	at 48 h.	at 72 h.
Control	2.0 ± 0.6 A	2.0 ± 0.5 A
Enrofloxacin only <sup>1</sup>	3.4 ± 0.7 A	2.7 ± 1.2 A
<i>Bti</i> ICP <sup>2</sup> only	80.4 ± 2.8 B	88.4 ± 1.3 B
Enrofloxacin <sup>1</sup> + <i>Bti</i> ICP <sup>2</sup>	83.0 ± 3.0 B	92.9 ± 1.6 B

A,B Values (mean± Standard error of the mean) with different superscripts differ significantly within a column ( $p \leq 0.05$ ).

<sup>1</sup> The concentration of enrofloxacin in the larval medium was 0.5 mg/L.

<sup>2</sup> The concentration of *Bti* ICPs was 1.08 ITU/200 ml.

**Table 2.3.** The response of *Simulium vittatum* larvae to *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) insecticidal crystalline proteins (ICPs) after exposure to tylosin for 48 and 72 h (Experiment 3)

Treatment	Mortality (%)	Mortality (%)
	at 48 h.	at 72 h.
Control	3.4 ± 0.9 A	1.3 ± 0.7 A
Tylosin only <sup>1</sup>	3.4 ± 1.1 A	2.3 ± 1.2 A
<i>Bti</i> ICP only <sup>2</sup>	76.4 ± 3.8 B	78.2 ± 2.8 B
Tylosin <sup>1</sup> + <i>Bti</i> ICP <sup>2</sup>	76.8 ± 3.3 B	85.80 ± 1.2 C

A,B,C Values (mean ± SEM) with different superscripts differ significantly within a column ( $p \leq 0.05$ ).

<sup>1</sup> The concentration of tylosin in the larval medium was 8.0 mg/L.

<sup>2</sup> The concentration of *Bti* ICPs was 1.08 ITU/200 ml.

**Table 2.4.** The response of *Simulium vittatum* larvae to *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) insecticidal crystalline proteins (ICPs) after exposure to Sulfamethoxazole (SMZ):Trimethoprim (TMP) for 48 and 72 h (Experiment 4).

Treatment	Mortality (%)	Mortality (%)
	at 48 h.	at 72 h.
Control	3.4 ± 0.8 A	2.5 ± 1.2 A
SMZ:TMP only <sup>1</sup>	5.0 ± 1.4 A	4.0 ± 1.0 A
<i>Bti</i> ICP only <sup>2</sup>	75.5 ± 2.6 B	81.8 ± 5.7 B
SMZ:TMP <sup>1</sup> + <i>Bti</i> ICP <sup>2</sup>	92.8 ± 2.3 C	93.5 ± 2.4 C

A,B,C Values (mean ± SEM) with different superscripts differ significantly within a column ( $p \leq 0.05$ ).

<sup>1</sup> The concentration of SMZ:TMP in the larval medium was 25.0 mg/L:5.0 mg/L.

<sup>2</sup> The concentration of *Bti* ICPs was 1.08 ITU/200 ml.

CHAPTER 3

THE EFFECT OF SESTON ON MORTALITY OF *SIMULIUM VITTATUM* (DIPTERA:  
SIMULIIDAE) FROM INSECTICIDAL PROTEINS PRODUCED BY *BACILLUS*  
*THURINGIENSIS* SUBSP. *ISRAELENSIS*<sup>2</sup>

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<sup>2</sup> **Iburg, J. P., E. W. Gray, R. D. Wyatt, J. E. Cox, R. A. Fusco, and R. Noblet. 2011.** Environ. Entom. 40: 1417-1426.  
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## Abstract

Water was collected from a site on the Susquehanna River in Eastern Pennsylvania where less than optimal black fly larval mortality had occasionally been observed following treatment with *Bacillus thuringiensis* subsp. *israelensis* de Barjac insecticidal crystalline proteins (*Bti* ICPs). A series of experiments was conducted with *Simulium vittatum* Zetterstedt larvae to determine the water related factors responsible for the impaired response to *Bti* ICPs (Vectobac<sup>®</sup> 12AS, strain AM 65-52). Seston in the water impaired the effectiveness of the ICPs, whereas the dissolved substances had no impact on larval mortality. Individual components of the seston were then exposed to the larvae followed by exposure to *Bti* ICPs. Exposure of larvae to selected minerals and nutritive organic material prior to ICP exposure resulted in no significant decrease in mortality. Exposure of larvae to silicon dioxide, cellulose, viable diatoms, and purified diatom frustules prior to *Bti* ICP exposure resulted in significant reductions in mortality. Exposure of larvae to purified diatom frustules from *Cyclotella meneghiniana* Kützing resulted in the most severe impairment of mortality following *Bti* ICP exposure. Frustule-induced impairment of feeding behavior is postulated as being responsible for the impairment of larval mortality.

## Introduction

Adult black flies (Diptera: Simuliidae) are serious pests to both humans and domestic livestock due to their blood feeding and nuisance swarming behaviors (Gray et al. 1996, Adler et al. 2004). Vector species transmit a variety of parasites in birds and mammals. As a result of the pestiferous nature of some black fly species, suppression programs are conducted at various locations throughout the world. The preferred approach for suppressing black fly populations is to target the rivers and streams where the rheophilic larval stages occur (Adler et al. 2004).

Application of products containing the insecticidal crystalline proteins (ICPs) produced by *Bacillus thuringiensis* subsp. *israelensis* de Barjac to the larval habitat is the method of choice for black fly population suppression (Barjac and Sutheland 1990). The ICPs have proven highly efficacious against black fly larvae, while exhibiting a high degree of safety for non-target organisms (Jackson et al. 1994). To be effective the ICPs must be ingested by the filter-feeding larvae to cause mortality. Consequently, environmental factors that influence feeding behavior of larvae could adversely affect ICP performance.

Water turbidity is one environmental factor that has been studied in relation to *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) ICP activity. Gaugler and Molloy (1980) found that the feeding behavior of *Simulium vittatum* Zetterstedt larvae was altered following exposure to high concentrations of food particles. This altered behavior was attributed to rapid gut filling followed by a decreased rate of ingestion. Experiments conducted by Hart and Latta (1986) demonstrated that the efficiency of particle ingestion by *Prosimulium mixtum* Syme and Davies was very low (<0.1% of available material)

and declined with increasing food concentration. Guillet et al. (1985) suggested that when the larval gut is full of particles, there may be reduced ICP ingestion.

Wilson et al. (2005) found that favorable performance of a commercial product in West Africa was achieved with river turbidities below 4.2 NTU. They speculated that either the fine mud particles were blocking the mid-gut receptors from the ICPs or that the ICPs were being adsorbed onto the surface of the suspended particles. Previous studies have demonstrated that *Bti* ICPs can bind to clay particles (Ohana et al. 1987, Lee et al. 2003). Both studies have found that despite binding, the ICPs retain their toxicity. Considering the variety of concepts and speculation concerning turbidity and *Bti* ICP efficacy, the need to apply a more analytical approach to this operational problem is apparent.

Currently, the world's largest black fly suppression program is conducted by the Pennsylvania Department of Environmental Protection, targeting nuisance black fly populations in habitats across the state. A large portion of this program is associated with suppressing members of the *Simulium jenningsi* species group in the Susquehanna River and has been highly successful. However, for over 10 years, less than optimal larval mortality has sporadically been observed in specific areas of the Susquehanna River following the application of *Bti* ICPs.

Experiments were conducted with *S. vittatum* larvae from the University of Georgia Black Fly Colony to characterize the response of larvae to *Bti* ICPs found in Vectobac® 12AS (Valent BioSciences, Libertyville, IL) following exposure to various materials typically found in the Susquehanna River. Experiments evaluated the effects of seston and soluble components in the river on the susceptibility of larvae to the *Bti* ICPs.

The composition of the seston was analyzed using a variety of techniques. Individual components of the seston were then evaluated for their effect on the susceptibility of larvae to *Bti* ICPs.

## **Materials and Methods**

### River Water Samples

Water samples were obtained from the Susquehanna River in August 2009 and July 2010. The collection sites have been occasionally characterized as exhibiting less than optimal performance of *Bti* ICPs for the suppression of black fly populations. The first site was on the North Branch of the Susquehanna River near Sunbury, PA. The second sampling site was also on the North Branch near Lime Ridge, PA. Water samples were collected from undisturbed river flow by tilting a clean 20 liter carboy (Nalgene, Rochester, NY) into the water flow and allowing the carboy to gently fill. The water being collected was upstream of the person collecting the sample. Approximately 20 liters of water from each of the two sites were collected and transported to the University of Georgia for analysis. Water samples were maintained at 5°C until analyzed.

### Determination of Turbidity of Water Samples

Upon arrival at the laboratory, the turbidity was measured for all samples in Nephelometric Turbidity Units (NTU). Five replicate determinations were made on each water sample using a Hach Turbidimeter (Hach Company, Loveland, CO). The average of all samples was recorded. Turbidity was also determined for the media utilized in Experiments 1 through 7.

### Separation of Seston from River Water

The seston in the river water samples was recovered by filtration of the water through a 0.20  $\mu\text{m}$  filter (Corning 250 ml Filter System, Corning, New York, NY). The membrane filters were removed from the filter unit and placed into a clean, sonic bath (FS20, Thermo-Fisher Scientific, Waltham, MA) containing 250 mL of deionized water. After sonication for 5 min, the recovered material was quantitatively removed from the sonic bath, and the water with seston was made to a volume equal to the original volume with deionized (Experiment 1) or moderately hard water (Experiment 2).

### Elemental Analysis

The filtrates of the 2009 and 2010 samples of water, after passing through a 0.20  $\mu\text{m}$  filter, were analyzed for elemental content to determine the predominant soluble cations. The dissolved elements were determined by inductively coupled plasma-atomic emission spectroscopy (ICP-OES) utilizing Environmental Protection Agency Method 6010C (Revision 3, February 2007).

### X-Ray Diffraction and Carbon/Nitrogen Determination

Powder X-ray diffraction (XRD) of the collected seston was performed in order to determine mineral composition. The seston sample was treated with hydrogen peroxide to remove organic material, air-dried, and mounted on glass. The sample was placed in a Bruker diffractometer (D8 Advance, Bruker Corporation, Billerica, MA). Reflections from directed X-rays were measured with the instrument detector moving in an arc over the sample in the angle range 2-40 degrees 2-theta. A second sample was saturated with

ethylene glycol prior to XRD in order to detect expandable clay minerals (i.e. smectites) and was similarly measured. Total carbon and nitrogen content of the organic portion of the seston was determined using a micro-Dumas combustion system (Costech CHN Analyzer, Costech, Valencia, CA).

### Scanning Electron Microscopy

Samples of the seston recovered from the Susquehanna River, viable diatoms, and diatom frustules were viewed using scanning electron microscopy. Viable algal cells were fixed in a solution of 2% glutaraldehyde in 0.2 M phosphate buffered saline (PBS), pH 7.2, for 1 h at 4°C. The cells were post-fixed in 1% OsO<sub>4</sub> in 0.2 M PBS for 1 h at room temperature, and then washed in distilled water. Once washed, the cells were dehydrated with ethanol. Cells were dried (Critical Point Drier, Tousimis model 850, Rockville MD) and filters with the samples were transferred to SEM stubs and sputter coated with gold (SPI Sputter coater, Structure Probe Inc., West Chester PA) to a thickness of approximately 15 nm. Susquehanna River seston and laboratory produced diatom frustules were filtered through Whatman Nuclepore® polycarbonate filters (Whatman, Kent, UK). Portions of the filters with the sample of interest were transferred to SEM stubs and sputter coated with gold. The samples were viewed with a Zeiss 1450 EP scanning electron microscope (Carl Zeiss MicroImaging, Inc., CA) at 20 kV and 150 pA iprobe with the backscatter detector.

## Algae Cultivation and Frustula Preparation

*Navicula pelliculosa* (Brébisson et Kützing) Hilse was obtained from Wards Scientific (Rochester, NY) and *Cyclotella meneghiniana* Kützing was obtained from the University of Texas Culture Collection (Austin, TX). Each diatom species was cultivated in separate vessels. Growth medium (DM - diatom medium) was prepared according to the formula as outlined by the Scottish Marine Institute, Argyll, Scotland, UK. The medium was prepared in 8 liter batches in 9 liter serum bottles (Ace Glassware, Vineland, NJ), the pH adjusted to 6.9, capped with aluminum foil, and sterilized at 121°C for 30 min. The medium was allowed to cool to room temperature, and then the stock solution containing cyanocobalamin, thiamine HCl, and biotin was added to the medium by passing the solution through a sterile 0.20 µm filter and a 10 ml sterile plastic syringe. The inoculum consisted of 2 ml of an actively growing culture of the algae. Following inoculation, the vessels were constantly aerated with room air using an aquarium pump equipped with flexible tubing and a 25 mm gas dispersion tube, with a 145-175 µm glass frit (Ace Glassware, Vineland, NJ). The cultures were incubated at 23°C on a 16:8 h light:dark cycle. Cell counts were monitored weekly by aseptically removing a 10 ml aliquot of the culture and performing a total cell count with a hemacytometer. After the cultures had attained stationary growth, they were centrifuged, the supernatant discarded, and the cells recovered. The viable cells utilized in Experiments 6 and 7 were then re-suspended in moderately hard water and the final cell concentration was confirmed by conducting a total cell count with a hemacytometer.

Cells from each culture were also used to furnish frustules for Experiment 8. After centrifugation, the cells were immediately re-suspended in 50 mL of H<sub>2</sub>O<sub>2</sub> (30% w/w)

and heated to 85°C in a water bath for 24 h. After 24 h, the cell-H<sub>2</sub>O<sub>2</sub> suspension was centrifuged, the H<sub>2</sub>O<sub>2</sub> was decanted and discarded, and 100 ml of fresh H<sub>2</sub>O<sub>2</sub> added to the cells. This process was repeated three additional times. After the final H<sub>2</sub>O<sub>2</sub> treatment, the resulting white material (frustules) was re-suspended in 50 ml of absolute methanol, centrifuged, and the methanol decanted and discarded. A minimal volume of methanol was then added to the white material in the bottom of the centrifugation vessel to re-suspend the frustules and the contents transferred to a glass Petri dish. The methanol was allowed to evaporate at 45°C in a forced-draft oven, and the dry frustules, free of all color and obvious organic material, were scraped from the glass dish with a new single-edged razor blade and transferred to a clean glass vessel for storage.

### Minerals

Minerals representing those found in the 2010 river water sample were obtained. Kaolinite clay was obtained from VWR International (Radnor, PA). The minerals chlorite (ripidolite) and illite (Cambrian shale) were obtained from The Source Clays Repository (Purdue University, West Lafayette, IN). Potassium feldspar was collected from granite pegmatite in Fremont County, CO. The solid minerals were crushed into a powder with a 200 ml ball mill containing tungsten carbide balls on an off-axis shaker (SPEX model 8000-115, SPEX Industries Inc., Edison, NJ). All mineral powders were passed through a 53µm sieve prior to use.

### Simulium vittatum Larvae

All experiments were conducted using late instar *S. vittatum* cytospecies IS-7 larvae. The larvae were grown in aquatic rearing units incorporating a closed circulation, trough system described by Gray and Noblet (1999). Larvae were removed from the trough of the rearing unit immediately prior to each experiment and placed in enamel pans with 1 liter of rearing unit water. Larvae were then transferred individually with soft-tipped forceps into 250 ml, flat bottomed extraction flasks containing the designated medium.

### Bti ICPs

A commercial biological larvicide containing *Bti* ICPs was obtained (Vectobac<sup>®</sup> 12AS *Bacillus thuringiensis* subsp. *israelensis*, strain AM 65-52, Valent BioSciences Corporation, Lot # 64-396-BA82). This product is an aqueous suspension containing 11.61% fermentation solids and solubles and has a potency of 1200 International Toxic Units (ITU) per mg (Oestergaard et al. 2007).

### Controlled Current Toxicity Test

The controlled current toxicity test (CCTT), originally developed by Barton et al. (1991) and modified by Iburg et al. (2010), was used throughout this study. The CCTT employs 250 ml, flat bottomed extraction flasks as the experimental vessels. The appropriate medium is added to each flask followed by the addition of 30 late-instar larvae. The flasks are placed on G10 Gyrotory<sup>®</sup> shakers (New Brunswick Scientific, Edison, NJ) and the speed increased in a stepwise fashion over 30 min to a final speed of

150 rpm. The shaker is maintained at 150 rpm for 30 additional min to allow acclimation of the larvae (i.e., allow larvae to attach to the flasks and consume available particles from the medium). Following acclimation, the larvae are then exposed to the *Bti* ICPs. The larvicide formulation was diluted in deionized water such that 5 ml of the final dilution, when added to 195ml of the medium, resulted in 0.375 ppm of Vectobac<sup>®</sup> 12AS (0.45 ITU/ml) in each treated flask. This concentration of *Bti* ICP's was used in all experiments. After a 10 min exposure, the shaker was stopped and mortality was assessed 5 h later. The short duration of the experiments eliminated the need to supplement each microcosm with a food source.

#### Media for Experiment 2-8

Moderately hard water was prepared as described by Weber (1993). Water is characterized as being moderately hard when the CaCO<sub>3</sub> concentration is 80-100 ppm (Weber 1993). For every 20 liters, a carboy was first filled with 19 liters of deionized water, followed by the addition of 1.2 g MgSO<sub>4</sub>, 1.92 g NaHCO<sub>3</sub>, and 0.80 g KCL. The solution was aerated for 24 h. This was followed by the addition of 1 liter of deionized water containing 1.2 g of dissolved CaSO<sub>4</sub> · 2 H<sub>2</sub>O. The solution was aerated for another 24 h. Moderately hard water was used for the negative control medium in Experiments 2 through 8 as well as the basal medium to which any additives were suspended.

#### Experiment 1

The river water utilized in this experiment was from the August 2009 collection site. Each treatment consisted of 10 flasks with 30 larvae in each flask. The first

treatment used water “as collected” as the medium. The second treatment used the filtrate (free of all seston) as the medium. The third treatment used re-suspended seston in deionized (DI) water as the medium. The last treatment used deionized water as the medium. Following the 1 h acclimation period, five flasks containing each medium received *Bti* ICPs (0.45 ITU/ml). The other five flasks received an equivalent amount of DI water. Five h post-exposure, larval mortality was determined based on the lack of tactile response (Iburg et al. 2010).

### Experiment 2

The water used in this experiment was collected from the July 2010 site. The protocol used here was the same as Experiment 1, however moderately hard water was used for the basal media with re-suspended particles and as the negative control media.

### Experiment 3

Experiments 3 through 8 were conducted to test materials that are similar to those found in the seston from the 2010 river water sample. In Experiment 3, the minerals chlorite, illite, kaolinite, and feldspar were evaluated separately in moderately hard water for their impact on *Bti* ICP activity toward black fly larvae. Each treatment contained 10 ppm of the designated mineral. The turbidity of each media was determined. The CCTT was implemented utilizing 10 treatments, six flasks per treatment, and 30 larvae per flask. After a 1 h acclimation period in the presence of each mineral, six flasks of larvae in each medium received *Bti* ICPs, whereas, the other six flasks received an equivalent amount of

moderately hard water. Larvae were exposed to the *Bti* ICPS for 10 min and then mortality was assessed after 5 h.

#### Experiment 4

In this experiment, two media were used. The first contained 10 ppm of nutritive organic material (Advanced Nutrition Brand Rabbit Feed™, Professional Formula, Purina Mills, St. Louis, MO). This material is a major component of the daily *S. vittatum* colony diet. This material was ground with a household blender and then passed through a 53 µm sieve. Moderately hard water was used as the control medium. The CCTT was implemented utilizing four treatments, 10 flasks per treatment, with 30 larvae per flask. Ten flasks of larvae in each medium received *Bti* ICPS after a 1 h acclimation, whereas, the other 10 flasks received an equivalent amount of moderately hard water. Larvae were exposed to the *Bti* ICPS for 10 min and mortality was assessed after 5 h.

#### Experiment 5

Silicon dioxide (.014µm) was obtained from Sigma Life Science (St. Louis, MO) as fumed silica. Purified cellulose (Solka-Floc®) was obtained from the International Fiber Corporation (North Tonawanda, NY). The cellulose material was passed through a 53µm sieve. Media were prepared with 10 ppm of either substance using moderately hard water as a control. The CCTT was implemented utilizing three treatments, 10 flasks per treatment, and 30 larvae per flask. Each flask received *Bti* ICPS after a 1 h acclimation. Larvae were exposed to the *Bti* ICPS for 10 min and mortality was assessed after 5 h.

### Experiment 6

Viable *C. meneghiniana* cells were removed from an actively growing culture and used as a medium additive. The cells were centrifuged, separated from the growth medium, and re-suspended in moderately hard water. The cell count was determined using a hemacytometer. After re-suspension, the cell count was 9,000 cells/ml. The control medium was moderately hard water. The CCTT was implemented using the same protocol as described in Experiment 4.

### Experiment 7

Viable *N. pelliculosa* cells were removed from an actively growing culture and used as the medium additive. An appropriate amount of cells were centrifuged, separated from the growth medium, and re-suspended in moderately hard water. The cell count was determined using a hemacytometer. After re-suspension the cell count was 102,000 cells/ml. The control medium was moderately hard water. The CCTT was implemented using the same protocol as described in Experiment 6.

### Experiment 8

Purified frustules from *N. pelliculosa* and *C. meneghiniana* were evaluated for their effect on *S. vittatum* susceptibility to *Bti* ICPs. The frustules were suspended individually in media at a concentration of 2 and 5 ppm. The control medium was moderately hard water. The CCTT was implemented utilizing 10 treatments, eight flasks per treatment, and 30 larvae per flask. Eight flasks of larvae in each medium received *Bti* ICPs after a 1 h acclimation, whereas, the other eight flasks received an equivalent

amount of moderately hard water. Larvae were exposed to the *Bti* ICPS for 10 min and mortality was assessed after 5 h.

### Statistical Analysis

One-way analysis of variance with Tukey-Kramer multiple comparison tests were performed comparing all treatments in each experiment. Individual treatments within an experiment were compared using unpaired t-tests. GraphPad InStat version 3.06 for Windows XP was used for these analyses (GraphPad Software, San Diego, California USA, [www.graphpad.com](http://www.graphpad.com)).

## **Results**

### ICP-OES Analysis

The cations calcium, sodium, magnesium, and potassium were present in concentrations greater than 2 ppm in the Susquehanna River filtrate in 2009 and 2010 (Table 3.1). Dissolved silicon was present in 2009 but was undetectable in water collected in 2010. All other remaining elements were in the filtrate in concentrations below 0.1 ppm. The results from Experiments 1 and 2 demonstrate that larvae respond similarly to *Bti* ICPs in control media and in river water filtrate. Hence, the dissolved cations did not alter *S. vittatum* susceptibility to ICPs in this study.

### X-Ray Diffraction and Carbon/Nitrogen

Powder X-ray diffraction of the dried seston revealed the presence of the silicate minerals illite, chlorite, and kaolinite, (all layered hydrous aluminum silicates) in addition

to quartz (SiO<sub>2</sub>) and feldspar (an aluminum silicate). These mineral species are commonly found in stream seston. Expandable clay minerals were not detected. The seston contained 18.5% carbon and 2.7% nitrogen.

### Scanning Electron Microscopy

Analysis of the recovered seston by scanning electron microscopy revealed the presence of a variety of viable diatoms along with diatom frustules in the seston from the 2010 river sample. An abundance of radial centric and pennate diatoms representing numerous taxa were present in the seston (Figure 3.1A). The viable cells of *C. meneghiniana* and *N. pelliculosa* (Figure 3.1B, 3.1C) and the frustules produced from cultures of *C. meneghiniana* and *N. pelliculosa* (Figure 3.1D, 3.1E) were used in Experiments 6,7, and 8, respectively. The size of the frustules and cells ranged from 5-18 µm.

### Experiment 1

Water collected from the North Branch of the Susquehanna River near Sunbury, PA in 2009 was used to determine whether seston or a soluble component in the river water filtrate was responsible for a reduction in larval mortality following *Bti* ICP exposure (Table 3.2). Larval mortality in DI water after ICP exposure was significantly different from larval mortality observed in the unfiltered river water after ICP exposure ( $t = 8.53$ ;  $df = 1, 18$ ;  $P < .0001$ ). When river water was filtered through a 0.20 µm filter, and the recovered seston re-suspended in DI water, the larval mortality from *Bti* ICPs was not significantly different from the mortality in unfiltered river water ( $t = 1.69$ ;  $df = 1, 18$ ;  $P$

= 0.11). The response of larvae in river water filtrate from the North Branch of the Susquehanna to *Bti* ICPs was not significantly different from that seen with larvae exposed to ICPs in deionized water ( $t = 0.24$ ;  $df = 1, 18$ ;  $P = 0.81$ ).

### Experiment 2

This experiment utilized water collected from the Susquehanna River in July 2010 from the North Branch at the Lime Ridge, PA site. The control media used in this experiment was moderately hard water. No significant difference were found between the mortalities of larvae in each media in the absence of ICPs ( $F = 18.57$ ;  $df = 3, 16$ ;  $P = 0.70$ ). Exposure to *Bti* ICPs in the moderately hard water as opposed to the unfiltered river water resulted in significantly different mortalities ( $t = 4.53$ ;  $df = 1, 8$ ;  $P < .01$ ; Table 3.2). Larval mortality in the river water filtrate was not significantly different from the mortality in moderately hard water ( $t = 1.001$ ;  $df = 1, 8$ ;  $P = 0.35$ ). No significant difference was found between larval mortality in the water with re-suspended seston and the unfiltered river water ( $t = 0.06$ ;  $df = 1, 8$ ;  $P = 0.57$ ).

### Experiment 3

Larvae acclimated in media containing either 10 ppm of chlorite, illite, kaolinite, or feldspar without exposure to *Bti* ICPs resulted in no significant difference in mortality as compared to the mortality of larvae in moderately hard water ( $F = 1.08$ ;  $df = 4, 25$ ;  $P = 0.39$ ; Table 3.3). The larval mortality in moderately hard water due to *Bti* ICPs was 88.8%. The addition of any mineral did not significantly influence this mortality ( $F = 0.90$ ;  $df = 4, 25$ ;  $P = 0.98$ ).

#### Experiment 4

In the absence of *Bti* ICPs no significant difference in mortality was found between larvae in media containing 10 ppm of nutritive organic material and larvae in moderately hard water ( $t = 0.35$ ;  $df = 1, 18$ ;  $P = 0.73$ ; Table 3.4). The larval mortality in moderately hard water due to *Bti* ICPs was 87.4%. The addition of organic material prior to ICPs did not significantly influence this mortality ( $t = 1.39$ ;  $df = 1, 18$ ;  $P = 0.18$ ).

#### Experiment 5

Larvae were acclimated in media containing 10 ppm of cellulose, 10 ppm of silicon dioxide, or moderately hard water (Table 3.5). All flasks then received *Bti* ICPs. Larvae exposed to cellulose material prior to the ICPs exhibited a significant reduction in mortality compared to that of larvae in moderately hard water ( $t = 8.52$ ;  $df = 1, 18$ ;  $P < 0.0001$ ). Larvae exposed to silicon dioxide prior to the ICPs also exhibited a significant reduction in mortality as compared to larvae in moderately hard water ( $t = 36.43$ ;  $df = 1, 18$ ;  $P < 0.0001$ ).

#### Experiment 6

Larvae were acclimated in either moderately hard water or a medium containing 9,000 cells/ml of viable *C. meneghiniana* (Table 3.6). No significant difference was found between mortality in either media in the absence of *Bti* ICPs ( $t = 0.33$ ;  $df = 1, 18$ ;  $P = .75$ ). The larvae in the medium containing viable diatom cells exhibited a significant reduction in mortality compared to those in moderately hard water after exposure to *Bti* ICPs ( $t = 10.74$ ;  $df = 1, 18$ ;  $P < 0.0001$ ).

### Experiment 7

Larvae were acclimated in either moderately hard water or a medium containing 102,000 cells/ml of viable *N. pelliculosa* (Table 3.7). No significant difference in mortality was found in either media in the absence of *Bti* ICPs ( $t = 1.28$ ;  $df = 1, 18$ ;  $P = 0.22$ ). The larvae in the medium containing viable diatom cells prior to ICPs exhibited a significant reduction in mortality compared to those in moderately hard water after exposure to ICPs ( $t = 2.82$ ;  $df = 1, 18$ ;  $P = 0.01$ ).

### Experiment 8

*N. pelliculosa* and *C. meneghiniana* frustules were used in the larval media. This was done because frustules similar to *Navicula* and *Cyclotella* were found in the seston from the 2010 river sample. No significant mortality was found in any media in the absence of *Bti* ICPs ( $F = 0.65$ ;  $df = 4, 34$ ;  $P = 0.63$ ; Table 3.8). Larvae exposed to 2 ppm of *N. pelliculosa* frustules prior to *Bti* ICPs exhibited a significant increase in mortality compared to the larvae in moderately hard water ( $t = 2.60$ ;  $df = 1, 13$ ;  $P = 0.02$ ). When the concentration of *N. pelliculosa* frustules was increased to 5 ppm, mortality was significantly decreased ( $t = 8.33$ ;  $df = 1, 13$ ;  $P < 0.0001$ ). Larvae exposed to 2 and 5 ppm of *C. meneghiniana* frustules prior to *Bti* ICPs also exhibited significant reductions in mortality (2 ppm:  $t = 24.07$ ;  $df = 1, 14$ ;  $P < 0.0001$ ; 5 ppm:  $t = 25.57$ ;  $df = 1, 13$ ;  $P < 0.0001$ ).

## Turbidity

The turbidity of all media used in Experiments 1 through 8 was measured. The turbidities ranged from 0.03 to 18.5 NTU (Tables 3.2-3.8). The correlation coefficient ( $r$ ) between turbidity of media containing additives and *Bti* ICP mortality was -0.05, which was determined to be not significantly different from zero (points = 19;  $P = 0.83$ ).

## **Discussion**

This is apparently the first study in which individual components of the seston from a larval black fly habitat have been systematically fractionated, identified, and evaluated for their ability to influence the susceptibility of larvae to *Bti* ICPs. Water was obtained from sites at the Susquehanna River, where on occasion less than optimum mortality has been achieved following the application of *Bti* ICPs. The dissolved substances in the water had no effect on the susceptibility of larvae to the ICPs; however the seston was responsible for a reduction in ICP induced mortality. The seston was characterized, and representatives of the identified components were obtained for experimentation. Some materials significantly reduced *Simulium vittatum* susceptibility to ICPs, whereas, other components of the water did not.

A variety of particulate materials have been found to cause feeding inhibition of *S. vittatum* larvae (Gaugler and Molloy 1980). Gaugler and Molloy (1980) revealed that the larvae changed their feeding behavior based on the types of materials in the larval media, however, the materials used in the study were chosen arbitrarily. Simuliidae larvae must ingest *Bti* ICPs for the larvicide to express its intended effect. The study by Gaugler and Molloy (1980) surprisingly did not provoke the need to examine components

of larval habitats and their potential effects on feeding behavior and subsequent *Bti* ICP exposure. Results from our study demonstrate that the susceptibility of larvae to *Bti* ICPs can be significantly altered depending on the composition of seston in their environment. The mechanism behind this phenomenon is beyond the scope of this study; however, altered feeding behavior like that observed in (Gaugler and Molloy 1980) could explain our results.

The application of *Bti* ICPs to the larval habitat has been avoided during times characterized by high turbidity by convention. This convention may increase the probability of success; however, data from these current experiments demonstrate that the success or failure of a *Bti* ICP application rests not in turbidity alone, but in what constitutes that turbidity. The CCTT has proven to be a useful technique for determining the effects of various materials on *S. vittatum* susceptibility to *Bti* ICPs.

Results from Experiments 1 and 2 clearly demonstrate that seston in the North Branch of the Susquehanna River is responsible for the reduction of larval susceptibility to *Bti* ICPs. No significant differences were found between larval mortalities in control water (deionized or moderately hard) or river water filtrate after ICP exposure. Also, no significant difference was found between larval mortality in unfiltered river water and control water with re-suspended seston after ICP exposure.

Analysis by ICP-OES revealed that the Susquehanna River water contained certain dissolved elements in concentrations ranging from 1.9 to 30.13 ppm in 2009 and 2010. These dissolved substances, along with any other undetected dissolved substances, were unable to reduce *Bti* ICP efficacy. It has been documented that an increase in diatom populations is correlated with a decrease in dissolved silica, which could explain the lack

of detectability of silicon in the 2010 filtrate (Wang and Evans 1969). Interestingly, the 2009 river water had a turbidity of 17.1 NTU as compared to the 2010 turbidity of 9.5 NTU, yet the mortality was reduced 26% more with re-suspended seston from the 2010 river sample than with that from 2009. More diatom cells or frustules may have been present in seston collected in 2010 as compared to the seston collected in 2009.

Various minerals are typically found in the seston of large, free-flowing bodies of freshwater. A sample of seston from the Susquehanna River near Lime Ridge, PA was analyzed for mineral content. Chlorite, illite, kaolinite, and feldspar were present in the seston. Larvae were exposed to the various minerals and then susceptibility to *Bti* ICPs was determined. Each substance was in the various larval media at 10 ppm so that an adequate comparison could be made as to the response of larvae to ICPs after exposure to each mineral. No reduction in ICP-induced mortality after larval exposure of larvae to these minerals was found. The turbidities of the various media in this experiment ranged from 0.3 to 7.7 NTU, illustrating again that a measurement of turbidity alone is insufficient for predicting the outcome of a treatment. This also tends to negate the possibility of kaolinite clay binding midgut receptors or the proteins thereby reducing efficacy as suggested by Wilson (2005).

In Experiment 4, 10 ppm of nutritive organic material did not significantly impair *Bti* ICP toxicity. The carbon and nitrogen concentration of the 2010 river seston suggested that a large portion of the material was organic. The nitrogen content of the seston was 2.7%. The estimated protein in the seston was 13.9-17.2% (Merrill and Watt 1973). The organic material used was chosen because it had similar protein content (18%). Gaugler and Molloy (1980) looked at the influence of multiple substances on

Simuliidae feeding behavior. The greatest feeding inhibition was observed when larvae were exposed to organic substances. The authors attributed the inhibition to be related more to the particle morphology than particle composition.

In Experiment 5, the larvae were exposed to cellulose prior to *Bti* ICP exposure. Cellulose was chosen as an additive due to the Susquehanna River receiving an abundance of organic carbon (McGonigal 2009). Multiple paper mills are also located along the Susquehanna River. The response of larvae to ICPs following exposure to cellulose was intriguing since a previous study demonstrated that *Simulium* retract their cephalic fans after exposure to secondary fiber waste water from paper mills (Illesova 1988). The larvae did not resume feeding in the presence of the cellulose fibers for 24 to 36 h.

Scanning electron microscopy revealed a prevalence of diatoms, both viable cells and frustules, in the seston from the 2010 Susquehanna River sample. The two species of diatoms cultivated in the laboratory, *N. pelliculosa* and *C. meneghiniana*, were chosen because the seston contained a high concentration of cells and frustules similar in size and morphology to these species. Furthermore, *Navicula* spp. and *Cyclotella* spp. have been reported as members of the microflora of the Susquehanna River (Lowe and Gale 1980). Experiments 6 and 7 were conducted to address the viable radial centric and pennate diatoms in the seston. Frustules observed in the seston prompted the need to produce frustules for Experiment 8 that represented both shapes.

The viable *C. meneghiniana* cells used in Experiment 6 significantly reduced susceptibility of larvae to *Bti* ICPs. Media containing *C. meneghiniana* (9,000 cells/ml) reduced ICP activity by 56%. In Experiment 7, *N. pelliculosa* reduced ICP efficacy by

16% with 102,000 cells/ml. Stephens et al. (2004) demonstrated that the green algae *Scenedesmus quadricauda* (Turpin) Brebisson was able to significantly reduce *Bti* ICP efficacy at concentrations greater than 16,000 cells/ml. Other types of green algae used in the study were unable to reduce ICP efficacy even at concentrations greater than 250,000 cells/ml.

Diatoms, as well as other algae, are part of Simuliidae diet (Schröder 1987, Martin and Edman 1993). If the diatoms affect feeding behavior, then the inhibition must be intermittent because larvae in these areas still complete their life cycles in a normal time frame. Diatom cells may be more difficult to move through the mid- or hindgut, and that the larger (~18 µm) *Cyclotella* cells are more difficult to pass than the smaller (~6 µm) *Navicula* cells. When supplied with an abundance of nutrients, the larvae may feed at a slower rate. In either case, results from our study as well as that of Stephens et al. (2004) indicate that algae species with large cells (*Cyclotella* spp., *Scenedesmus* spp.) affect susceptibility of larvae to *Bti* ICPs more so than species with smaller cells (*Navicula* spp., *Chlorella* spp.)

In Experiment 8, low concentrations of diatom frustules (2 and 5 ppm) significantly reduced susceptibility of larvae to *Bti* ICPs. *Bti* ICP efficacy was reduced over 90% in media with turbidities as low as 1.5 NTU. The response of larvae to ICPs changes depending on the species of diatom frustule encountered. This variance is most likely attributable to the differences in size and shape of the frustules. For example, exposure of larvae to the larger *C. meneghiniana* frustules prior to introducing ICPs resulted in a greater than 90% reduction in mortality at only 2 ppm, whereas the smaller *N. pelliculosa* frustules in the media at 5 ppm reduced mortality by 69%.

Silicon dioxide was used in Experiment 5 to represent another form of silicon that may be in the river in addition to diatom frustules. The presence of silicon dioxide in the medium at 10 ppm resulted in an 82% reduction in ICP induced mortality. The silicon dioxide most likely affects the larvae in the same manner as the diatom frustules. Diatomaceous earth is well-known as an abrasive substance to insect cuticle (Athanasios 2006). *S. vittatum* larvae possibly react to diatom frustules and silicon dioxide by retracting their fans for a period of time in response to the abrasive property of these materials.

In this study, river water was collected from a problematic site. *Simulium vittatum* larvae were found to be less susceptible to *Bti* ICPs due to the seston and not the dissolved minerals in the water. The Susquehanna River receives multiple discharges from sewage treatment plants, industrial waste, and acid mine drainage above the locations where our water samples were collected (McGonigal 2009). These conditions could lead to excessive blooms of problematic diatom species. This study did not look at the influence of bacteria on *Bti* ICP activity; however, they may be of interest because they are common in Simuliidae diets (Fredeen 1964). Diatoms, as well as other components of the seston present in the river, could impair the susceptibility of larvae to *Bti* ICPs by any or all of the previously mentioned mechanisms. Future research will focus on better understanding how the seston affects the larvae and how to prevent or predict these occurrences.

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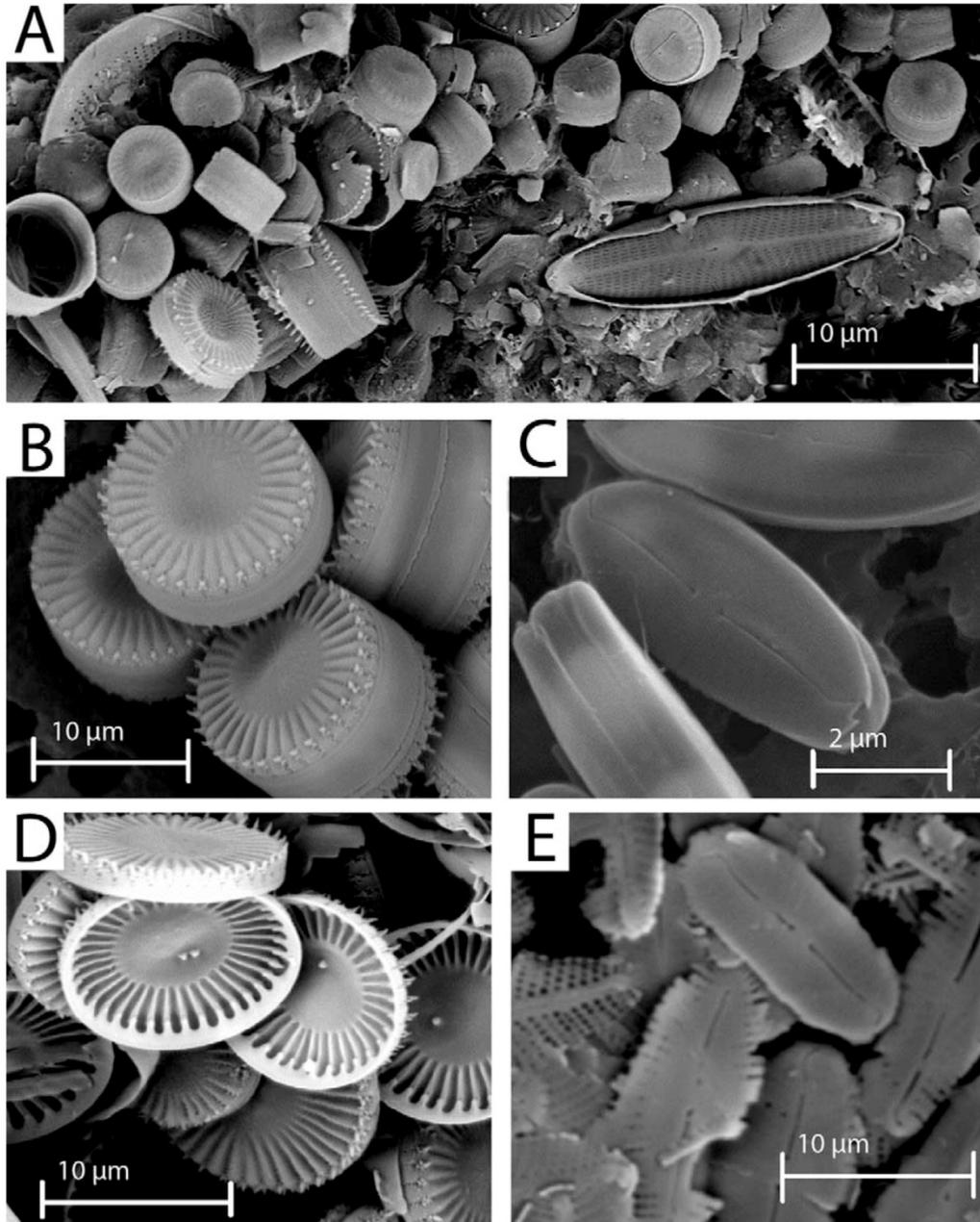
## Tables and Figures

**Table 3.1.** Concentration (ppm) of the 5 most predominant cations in the Susquehanna River filtrates in 2009 and 2010

Cation	2009 River filtrate <sup>a</sup>	2010 River filtrate <sup>b</sup>
Calcium	17.67	30.13
Sodium	12.46	29.14
Magnesium	4.37	7.85
Potassium	2.08	2.43
Silicon	1.92	None detected

<sup>a</sup> Water from Sunbury, PA collection site (17.3 NTU)  
after filtration through a 0.2 µm filter.

<sup>b</sup> Water from Lime Ridge, PA collection site (9.5 NTU)  
after filtration through a 0.2 µm filter.



**Figure 3.1.** Scanning electron microscopy of river seston and laboratory cultivated diatoms. (A) Seston from the Susquehanna River recovered near Lime Ridge, PA containing radial centric and pennate diatoms. (B) Viable *C. meneghiniana* cultivated in the laboratory. (C) Viable *N. pelliculosa* cultivated in the laboratory. (D) Purified *C. meneghiniana* frustules. (E) Purified *N. pelliculosa* frustules.

**Table 3.2.** The percentage mortality of *Simulium vittatum* larvae after exposure to *Bti* ICPs in various media

Medium	Experiment 1 Turbidity (NTU)	Experiment 1 2009 <i>Bti</i> <sup>a</sup> Exposure	Experiment 2 Turbidity (NTU)	Experiment 2 2010 <i>Bti</i> <sup>a</sup> Exposure
Water <sup>b</sup>	0.05	65.5 ± 3.7 a	0.03	58.3 ± 2.4 a
River water filtrate <sup>c</sup>	0.06	71.3 ± 4.3 a	0.03	59.6 ± 3.5 a
Unfiltered river water <sup>d</sup>	17.3	44.6 ± 2.7 b	9.5	30.0 ± 2.3 b
Re-suspended seston <sup>e</sup>	18.5	48.5 ± 6.0 b	8.6	37.1 ± 3.5 b

Values (mean ± SEM) within a column followed by the same letter are not significantly different (ANOVA,  $P \leq 0.05$ ).

<sup>a</sup> The concentration of *Bti* ICPs was 0.45 ITU/ml.

<sup>b</sup> Deionized water used in 2009, moderately hard water was used in 2010.

<sup>c</sup> Water from the North Branch of the Susquehanna River after passing through a 0.20 µm filter.

<sup>d</sup> Water from the North Branch of the Susquehanna River.

<sup>e</sup> Seston was re-suspended in deionized water in 2009 and in moderately hard water in 2010.

**Table 3.3.** The percentage mortality of *S. vittatum* larvae from *Bti* ICPs after exposure to 10 ppm of selected clay minerals

Medium	Medium Turbidity (NTU)	Control	<i>Bti</i> <sup>a</sup> Exposure
Water	0.03	0.6 ± 0.6 a	88.8 ± 1.8 b
Chlorite	3.9	2.4 ± 1.5 a	89.8 ± 2.0 b
Illite	6.4	2.8 ± 1.8 a	89.0 ± 2.2 b
Kaolinite	7.7	0.0 ± 0.0 a	87.8 ± 3.0 b
Feldspar	2.9	1.7 ± 0.7 a	89.0 ± 2.7 b

Values (mean ± SEM) followed by the same letter are not significantly different (ANOVA,  $P \leq 0.05$ ).

<sup>a</sup> The concentration of *Bti* ICPs was 0.45 ITU/ml.

**Table 4.4.** The percentage mortality of *S. vittatum* larvae from *Bti* ICPs after exposure to nutritive organic material

Medium	Turbidity (NTU)	Control	<i>Bti</i> <sup>a</sup> Exposure
Water	0.03	0.9 ± 0.5 a	87.4 ± 1.1 b
Organic material <sup>b</sup>	2.3	0.7 ± 0.5 a	90.6 ± 1.3 b

Values (mean ± SEM) followed by the same letter are not significantly different (ANOVA,  $P \leq 0.05$ ).

<sup>a</sup> The concentration of *Bti* ICPs was 0.45 ITU/ml.

<sup>b</sup> Organic material (10 ppm) blended and passed through a 53 µm sieve.

**Table 4.5.** The percentage mortality of *S. vittatum* larvae from *Bti* ICPs after exposure to 10 ppm of cellulose or silicon dioxide

Medium	Turbidity (NTU)	<i>Bti</i> <sup>a</sup> Exposure
Water	0.04	80.3 ± 1.1 a
Cellulose	1.9	61.8 ± 1.9 b
Silicon dioxide	0.8	14.2 ± 1.4 c

Values (mean ± SEM) followed by the same letter are not significantly different (ANOVA,  $P \leq 0.05$ ).

<sup>a</sup> The concentration of *Bti* ICPs was 0.45 ITU/ml.

**Table 4.6.** The percentage mortality of *S. vittatum* larvae from *Bti* ICPs after exposure to viable *Cyclotella meneghiniana* cells

Medium	Turbidity (NTU)	Control	<i>Bti</i> <sup>a</sup> Exposure
Water	0.03	1.0 ± 0.5 a	68.0 ± 1.7 b
<i>C. meneghiniana</i> <sup>b</sup>	2.3	1.3 ± 0.5 a	29.9 ± 3.1 c

Values (mean ± SEM) followed by the same letter are not significantly different (ANOVA,  $P \leq 0.05$ ).

<sup>a</sup> The concentration of *Bti* ICPs was 0.45 ITU/ml.

<sup>b</sup> Viable cells in moderately hard water at an *in vitro* concentration of 9,000 cells/ml.

**Table 4.7.** The percentage mortality of *S. vittatum* larvae from *Bti* ICPs after exposure to viable *Navicula pelliculosa* cells.

Medium	Turbidity (NTU)	Control	<i>Bti</i> <sup>a</sup> Exposure
Water	0.03	2.4 ± 0.9 a	75.2 ± 2.7 b
<i>N. pelliculosa</i> <sup>b</sup>	0.86	1.1 ± 0.5 a	63.0 ± 3.4 c

Values (mean ± SEM) followed by the same letter are not significantly different (ANOVA,  $P \leq 0.05$ ).

<sup>a</sup> The concentration of *Bti* ICPs was 0.45 ITU/ml.

<sup>b</sup> Viable cells in moderately hard water at an *in vitro* concentration of 102,000 cells/ml.

**Table 4.8.** The percentage mortality of *S. vittatum* from *Bti* ICPs after exposure to selected diatom frustules

Medium	Turbidity (NTU)	Control	<i>Bti</i> <sup>a</sup> Exposure
Water	0.04	0.8 ± 0.6 a	58.8 ± 1.8 b
2 ppm <i>N. pelliculosa</i>	1.3	1.9 ± 0.8 a	68.5 ± 3.1 c
5 ppm <i>N. pelliculosa</i>	2.7	1.2 ± 0.8 a	18.0 ± 4.3 d
2 ppm <i>C. meneghiniana</i>	1.5	1.0 ± 1.0 a	5.6 ± 1.3 a
5 ppm <i>C. meneghiniana</i>	3.4	0.3 ± 0.3 a	3.2 ± 1.2 a

Values (mean ± SEM) followed by the same letter are not significantly different (ANOVA,  $P \leq 0.05$ ).

<sup>a</sup> The concentration of *Bti* ICPs was 0.45 ITU/ml.

## CHAPTER 4

### A SPECTROPHOTOMETRIC TECHNIQUE FOR MEASURING PARTICLE INGESTION BY BLACK FLY (DIPTERA: SIMULIIDAE) LARVAE <sup>3</sup>

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<sup>3</sup> **Iburg, J. P., E. W. Gray, R. D. Wyatt, and R. Noblet. 2013.** Ent. Exp. App. 146: 293-301.  
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### **Abstract**

A spectrophotometric technique was developed to provide insight into the feeding behavior of *Simulium vittatum* Zetterstedt (Diptera: Simuliidae) larvae. Larvae were exposed to water insoluble pigmented particles (DayGlo® Neon Red) in a controlled current. The insoluble particles were available for capture by the cephalic fans of the larvae and subsequent ingestion. The length of the gut occupied by the particles after a given exposure time was determined by visual inspection with a dissecting microscope. Larvae were then homogenized in acetone to solubilize the particles. After filtration, the quantity of pigmented particles in the alimentary tract of the larvae was determined by spectrophotometric analysis. The quantity of particles per unit length of the alimentary tract was calculated. An experiment was conducted to demonstrate the utility of this technique. This experiment revealed that the consumption of these particles by larvae was influenced by various naturally occurring substances in the larval media.

## Introduction

Black fly larvae are indiscriminate filter feeders that thrive throughout the world in streams and rivers (Adler et al. 2004). The majority of Simuliidae larvae feed by capturing seston (suspended biotic and abiotic material) with their labral fans (Chance 1970). The fans periodically close and are inserted into the cibarium where the mandibles and labrum remove captured material from the fans during fan retraction (Merritt et al. 1996).

In areas where black flies thrive, populations can transmit a variety of pathogens to humans and livestock (Adler et al. 2004). The adult female flies can cause economic problems due to their nuisance and biting behavior (Gray et al. 1996). Currently, the only products used to suppress black fly larvae contain insecticidal crystalline proteins (ICPs) produced by *Bacillus thuringiensis* subsp. *israelensis* de Barjac. Because the ICPs must be ingested for the protoxin to become toxic to the larvae, a comprehensive understanding of black fly feeding behavior is necessary.

Numerous methods have been used to characterize the feeding behavior of Simuliidae. In several studies the flick rate of the labral fans has been examined in relation to particle ingestion (Gaugler and Molloy 1980, Hart and Latta 1986, Merritt et al. 1996, Stoops and Adler 2009). In other studies, various types of markers such as dyes, beads, and charcoal were added to the environment of the larvae and the movement of the marker through the gut was measured (Mulla and Lacey 1976, Ladle and Hansford 1981, Thompson 1987, Miller et al. 1998, Wotton 2009). These methods were developed to determine particle size selection and time needed for material to pass through the gut of the larvae.

Hart and Latta (1986) estimated the quantity of material ingested by black fly larvae by calculating how many particles of a marker could occupy the volume of space in the midgut that contained the marker. This estimate relied on the assumption that material was packed tightly and homogeneously within the midgut. Morin et al. (1988) and Ladle et al. (1972) determined the quantity of material ingested by weighing larval midgut contents. This method is time consuming and prone to error due to the difficulty of removing the entirety of the midgut contents.

To reduce error and the time involved in quantifying larvae ingesta, a spectrophotometric method was developed that permits precise and efficient quantification of the marker. Retnakaran (1983) determined the ingestion rates of *Chrostoneura fumiferana* Clemens larvae by measuring the absorbance of a water-soluble dye ingested along with food material. Similar methods have been developed to study the feeding behavior of adult *Drosophila melanogaster* Meigen by adding a water soluble dye to the food material (Edgecomb et al. 1994, Wong et al. 2009). These methods are suitable for terrestrial insects; however, water-soluble dyes cannot be used to study the ingestion behavior of aquatic insects. A suitable marker was needed that could be captured by aquatic filter feeding larvae and subsequently solubilized and measured using spectrophotometry.

*Simulium vittatum* Zetterstedt larvae were exposed to inert, water-insoluble particles of Neon Red pigment, a synthetic organic colorant. A spectrophotometric assay was developed for the quantification of particles within the alimentary tract of larvae. The length of larval gut occupied by inert particles was also determined. These measurements provide a unique insight into black fly larvae feeding behavior.

## **Materials and Methods**

### Pigment Particles

Neon Red pigment (AX-12-5, Lot #5641) was obtained from Day-Glo® Color Corp., Cleveland, OH. Neon Red is water insoluble yet readily soluble in certain organic solvents. This thermoplastic fluorescent pigment has an average particle size of 4.5-5 µm and is biologically inert (Dayglo® Color Corp, A/AX pigment Technical Bulletin, www.dayglo.com). This pigment was selected based upon the chemical characteristics of the pigment, particle morphology, and ease of recognition when in the alimentary tract of the larvae.

### Spectral Analysis and Absorption Maximum of Neon Red

The absorbance maximum of the Neon Red particles (NRP) was determined by adding 50 mg of the particles to 100 ml of acetone and measuring the absorbance from 400-600 nm using a Spectra Max Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA). The light path was 1 cm and the cuvettes used were 1.0 ml quartz. Acetone was used as the blank.

### Standard Curve for Neon Red

A stock solution of NRP (50.0 mg/100.0 ml acetone) was prepared and serially diluted with acetone (1:1) to a final concentration of 0.0488 mg/100 ml. Thirty larvae, free of NRP were homogenized in 3.0 ml of each dilution. The resulting homogenate was filtered through a 0.20 µm PTFE syringe filter (Thermo-Fisher Scientific, Waltham, MA). Following filtration, the absorbance of each dilution was determined at 528 nm.

Thirty larvae, free of NRP, were homogenized in 3.0 ml of acetone and subsequently filtered. This filtrate was used as the blank.

### *Simulium vittatum* Larvae

All experiments were conducted using late instar *S. vittatum* cytospecies IS-7 larvae. The larvae were reared as described by Gray and Noblet (1999). Larvae were removed from the rearing unit and placed in enameled pans with 1 liter of rearing unit water immediately prior to each experiment. Individual larvae were placed in exposure vessels with soft-tipped forceps. The sizes of the larvae chosen for the experiments were uniform in size to aid in comparing the lengths of gut containing NRP.

Several late instar larvae were randomly selected for photographic purposes. Some had no NRP within the gut, whereas others had ingested NRP such that approximately 30% of the gut was occupied by NRP. The larvae were killed by transferring them to a vial containing 3.0 ml of EtOH. The larvae were removed from the EtOH and placed into saline (0.85%) immediately prior to photographing. All larvae were photographed with a Leica dissecting microscope with a 1.5X Wild objective, 10X Leica objectives and a camera attachment (Leica Microsystems Inc., Buffalo Grove, IL). The larvae were photographed with either a Volpi NCL fiber optic light source (Volpi USA, Auburn, NY) or this same light augmented with a hand-held long-wave UV light (HQRP<sup>®</sup>, Harrison, NJ). The camera used was a Canon Digital Rebel (Canon USA, Inc, Lake Success, NY).

### Particulate Suspension

A suspension of NRP was prepared by adding 300 mg of particles to 500 ml of deionized water containing 0.1 mg of Triton™ X-100/ml. When 5 ml of this solution was added to 195 ml of larval media, the resulting NRP and Triton™ X-100 concentrations in each flask were 15 ppm and 2.5 ppm, respectively. Samples of particles from the suspension were viewed using scanning electron microscopy (SEM) to characterize the morphology of the particles. SEM analysis was performed using a Zeiss 1450 EP scanning electron microscope (Carl Zeiss MicroImaging, Inc., CA) following the method of Iburg et al.(2011).

### CCTT

The controlled current toxicity test was used to examine feeding behavior in black fly larvae (Iburg et al. 2010). Thirty larvae were gently placed into 250 ml flat bottom extraction flasks containing 195 ml of larval medium consisting of moderately hard water (Weber 1993). Moderately hard water was the basal medium for all experiments in this study. The flasks were then placed on G10 Gyrotory® shakers (New Brunswick Scientific, Edison, NJ). The initial speed was 100 rpm (0.14 x g) and was gradually increased in a stepwise fashion to 150 rpm (0.32 x g) over a 30 min time frame. This gradual increase in current velocity permits the larvae to attach to the side of the vessels with their silk pads and to orient their labral fans to the current. After thirty additional minutes, 5ml of either a suspension of NRP or *Bti* ICPs were simultaneously introduced into flasks using 10 ml disposable syringes. The shaker was turned off after the exposure

period. Larvae exposed to NRP were immediately removed and stored. Larvae exposed to *Bti* ICPS were assessed for mortality 5 h post-exposure.

### *Bti* ICPS

A commercial larvicide (Vectobac<sup>®</sup> 12AS, *Bacillus thuringiensis* subsp. *israelensis*, strain AM 65-52, Lot # IS131) containing insecticidal crystalline proteins (ICPs) produced by *Bacillus thuringiensis* subsp. *israelensis* was obtained from Valent BioSciences Corp., Libertyville, IL. This product is an aqueous suspension containing 11.61% fermentation solids and solubles and has a potency of 1200 International Toxic Units (ITU) per mg. This aqueous suspension contains particles with an average diameter of 2 to 9 microns (Valent Biosciences 2003). The potency is related to the activity of a standard assessed in bioassays using *Aedes aegypti* as a target host (Oestergaard et al. 2007).

### Removal and Storage of the Larvae

After exposure of the larvae to NRP for the appropriate time, the flasks were immediately removed from the shaker and the larvae gently detached from the vessel and emptied into enameled pans. The larvae were then recovered and placed into vials containing 10% neutral buffered formalin. To minimize release of the particles from the alimentary tract, larvae remained in formalin for no more than 24 h prior to analysis.

### Gut Passage

Larvae were removed from the formalin and viewed with the aid of a dissecting microscope (10X) equipped with a reticle micrometer. The length of the band from NRP ingestion was measured from the head capsule to the distal terminus of the band. The length of this band was determined individually on the thirty larvae in each vessel.

### NRP Analysis

After each gut passage measurement, the larvae were placed on a paper towel and blotted of excess formalin. All larvae recovered from each vessel were transferred to 4 ml (15x45 mm) glass vials with screw caps and a Teflon cap liner. Three ml of acetone was added to each vial of larvae. The vials were then capped and placed in a -80°C freezer for 5 min to minimize volatilization of the acetone. The cap of the vial was removed and, while partially immersing the vial in an ice bath, the probe of a Sonic Dismembrator (Model 1501, Thermo-Fisher Scientific, Waltham, MA) was placed approximately 1.5 cm below the surface of the acetone. Each vial containing larvae in acetone received 40-1 sec pulses (slope was 1.06 with an amplitude of 75%). The resulting homogenate was recovered from the homogenizing vessel with a 5 ml plastic syringe with a 20 gauge needle. After recovery of the acetone homogenate, the needle was removed from the syringe and replaced with a 33 mm, 0.20 µm PTFE syringe filter. The homogenate was then passed through the filter directly into a 1 ml quartz cuvette. Reference acetone was prepared by homogenizing 30 larvae that had not been exposed to NRP in acetone followed by filtration through a 0.20 µm PTFE filter. The absorbance of the filtered larval homogenate was determined at 528 nm using the reference acetone as a blank. The

average quantity of particulate material consumed per larvae per unit of time was calculated. This data was combined with the data from the length of the band to calculate the quantity of particulate material per unit of gut length.

#### Experiment 1: The Effect of a Surfactant on the Susceptibility of Larvae to *Bti* ICPs

This experiment was conducted to determine the effect of Triton™ X-100 at a concentration of 2.5 ppm on larval feeding behavior utilizing larval mortality following exposure to *Bti* ICPs as an indirect measure of larval ingestion. A total of 40 flasks containing 30 larvae each were utilized in this experiment. Twenty flasks contained moderately hard water as the larval medium and served as controls. The remaining 20 flasks contained moderately hard water containing Triton™ X-100 at a concentration of 2.5 ppm. Ten flasks of each medium received 0.45 ITU/ml of *Bti* ICPs, while the other 10 received an equivalent volume of water. After a 10 min exposure, the shaker was stopped and mortality was assessed 5 h post-exposure. Larval mortality was determined by visual inspection and was based upon the lack of any tactile response.

#### Experiment 2: The Effect of NRP on the Susceptibility of Larvae to *Bti* ICPs

Larval mortality following exposure to *Bti* ICPs was used as the indirect measure of larval ingestion. The three media used were moderately hard water and moderately hard water with either 15 or 25 ppm of NRP. A total of 60 flasks containing 30 larvae each were utilized in this experiment. Ten flasks of each medium received 0.45 ITU/ml of *Bti* ICPs, while the other 10 received water as a control. After a 10 min exposure, the

shaker was stopped and mortality was assessed 5 h post-exposure as described in Experiment 1.

### Experiment 3: The Influence of the Experimental Matrix on the Measured Absorbance of NRP

Four solutions were prepared with 5 replicates each. Each solution contained 100 ppm of NRP in acetone. No additions were made to the first solution. To the second, 20 ppm of Triton™- X 100 was added. The third solution contained NRP and 30 larvae that had been stored in formalin for 24 h. The fourth solution contained NRP, Triton™ X-100, and 30 larvae. The replicate samples containing larvae were homogenized with the sonic dismembrator and passed through a 0.20 µm filter as previously described. The absorbance at 528 nm was determined and the concentration of the pigment was calculated for all replicates.

### Experiment 4: The Effect of NRP Concentration on NRP Consumption

This experiment was conducted to determine if a lower *in vitro* concentration of NRP, typical of that noted near the end of an experiment, influenced the ingestion of this substance. Twelve flasks were used containing 30 larvae per flask. After an acclimation of 1 h, 6 flasks received 10 ppm of NRP and the other six received 15 ppm. The larvae were exposed to the NRP for 30 min then removed from the vessels and placed into formalin. The length of gut containing the pigment was measured. All larvae from each flask were then homogenized in acetone, the solution filtered as previously described, and the absorbance determined for each group of 30 larvae at 528 nm. The quantity of

particles consumed per larvae was determined by comparing the absorbance to the standard curve.

#### Experiment 5: The Effect of Medium Additives on NRP Consumption

Potential differences in larval feeding in various media were determined in this experiment. Ninety flasks were used with 30 larvae per flask. Thirty flasks had moderately hard water as the medium, 30 contained 10 ppm of kaolinite clay (5  $\mu\text{m}$  particle size) (VWR International, Radnor, PA), and 30 contained 10 ppm of cellulose (37  $\mu\text{m}$  particle size) (Solka-Floc®, International Fiber Corporation, North Tonawanda, NY). After a 1 h acclimation in the media, 10 flasks with each medium received *Bti* ICPs, 10 received NRP, and 10 received deionized water. The larvae were exposed to the *Bti* ICPs or NRP for 10 min. The larvae exposed to NRP were analyzed for length of gut occupied by particles and subsequently homogenized in acetone for spectrophotometric analysis. Larvae that had received *Bti* ICPs and those that had received deionized water were removed from the flasks after 5 h and mortality assessed.

#### Statistical Analysis

One-way analysis of variance tests with Tukey-Kramer multiple comparisons were performed comparing all treatments in each experiment. Individual treatments within an experiment were compared using unpaired *t*-tests. GraphPad Prism version 5.04 was used for all analyses (GraphPad Software, San Diego, California USA, [www.graphpad.com](http://www.graphpad.com)).

## Results

*Simulium vittatum* larvae with and without NRP in their alimentary canal can be seen in Figure 4.1. Larva (A) is situated dorsal side up with the cephalic fans extended. The other larva (B & C) is situated lying on its left side with contracted cephalic fans in both images. The NRP band in the larval gut is visible through the cuticle under incandescent light (B). Visualization of the NRP band was aided by augmenting incandescent light with long wave UV light (C).

Assessment of Neon Red particles (NRP) by scanning electron microscopy revealed that the material contained heterogeneous particles that varied in diameter from approximately 0.1 to 6  $\mu\text{m}$  (Figure 4.2). The absorbance maximum for NRP was 528 nm with a prominent shoulder from 550-553 nm (Figure 4.3). Serial dilutions of 50 mg NRP in 100 ml acetone resulted in a linear plot from 0.0488 to 50.0 mg /10 ml (Figure 4.4). These dilutions had been homogenized with 30 larvae and filtered prior to reading the absorbance. An additional standard curve was calculated using dilutions without the addition of larvae. The resulting curve had an identical slope and y-intercept indicating that the addition of larvae, the sonication of the larvae, and the filtration of the homogenate did not alter the absorbance of NRP. The minimum detectable concentration of NRP in acetone was determined to be 0.0488 mg/100ml. The absorbance of filtered homogenates of larvae in acetone at 528 nm ranged from 0.000 to 0.001 using acetone as a blank.

Results from Experiment 1 indicate that 2.5 ppm of Triton™ X-100 does not significantly alter larval susceptibility to *Bti* ICPs ( $t = 0.60$ ;  $df = 1, 18$ ;  $P = 0.56$ ; Table 4.1). Additionally, this surfactant did not significantly alter mortality in the absence of *Bti*

ICPs ( $t = 2.00$ ;  $df = 1, 18$ ;  $P = 0.06$ ). Results from Experiment 2 indicate that the presence of 25 ppm of NRP in the larval medium significantly reduced larval susceptibility to *Bti* ICPs ( $t = 3.77$ ;  $df = 1, 17$ ;  $P = 0.001$ ; Table 4.2). No significant difference was found between larvae exposed to *Bti* ICPs in the presence or absence of 15 ppm of NRP ( $t = 1.01$ ;  $df = 1, 18$ ;  $P = 0.33$ ).

The results from Experiment 3 indicate that the components of the experimental matrix did not significantly interfere with the absorbance of NRP ( $F = 2.79$ ;  $df = 3, 16$ ;  $P = 0.65$ ; Table 4.3). These results also indicate that the sonication and filtration of samples did not compromise the absorbance of the samples.

Results from Experiment 4 indicate that a 1/3 decrease in NRP concentration in the larval medium did not significantly alter the ingestion of NRP after 30 min ( $t = 0.63$ ;  $df = 1, 10$ ;  $P = 0.54$ ; Table 4.4). This indicates that the gradual decrease in particle concentration, due to feeding or entanglement in larval silk, is not sufficient to alter the ingestion of the particles by the larvae.

Results from Experiment 5 demonstrate that the gut passage, consumption, and distribution of NRP by larvae are significantly different in media containing various additives (Table 4.5). The length of gut containing this particulate was significantly different between larvae in moderately hard water and larvae in a medium containing 10 ppm of kaolinite or 10 ppm of cellulose (kaolinite:  $t = 2.48$ ;  $df = 1, 18$ ;  $P = 0.02$ ; cellulose:  $t = 8.82$ ;  $df = 1, 18$ ;  $P < 0.0001$ ). Larvae in medium containing 10 ppm of cellulose also had a significantly longer band than larvae in medium containing 10 ppm of kaolinite ( $t = 7.60$ ;  $df = 1, 18$ ;  $P < 0.0001$ ). Spectrophotometric analysis of larvae revealed that the average quantity of NRP ingested was significantly different in each of

the 3 media ( $F = 27.93$ ;  $df = 2, 27$ ;  $P = 0.04$ ). The quantity of NRP per mm of gut was also significantly different between larvae in each medium ( $F = 57.24$ ;  $df = 2, 27$ ;  $P = 0.0006$ ). No significant difference in *Bti* ICP induced mortality was found between larvae in moderately hard water and 10 ppm of kaolinite ( $t = 0.03$ ;  $df = 1, 18$ ;  $P = 0.97$ ). A significant reduction in larval mortality occurred in medium containing 10 ppm cellulose after *Bti* ICP exposure as compared to larvae in the other two media ( $F = 10.20$ ;  $df = 2, 27$ ;  $P = 0.0005$ ). Control mortality was not affected in any of the media ( $F = 0.08$ ;  $df = 2, 27$ ;  $P = 0.92$ ).

### Discussion

This method was developed specifically to determine both the location of a marker in the midgut of black fly larvae as well as the quantity of the marker ingested. Neon Red Pigment (NRP) is a useful marker for measuring ingestion in aquatic filter feeders. The size of this material approximates the size of particles typically found in seston, as defined as all of the biotic and abiotic material suspended in the waterway. Using this technique, one can determine not only the quantity of NRP ingested over time per larvae, but also the quantity of NRP present per unit length of the gut. Results from this study indicate that the ingestion of NRP can be affected by the quantity and composition of other particles in the surrounding water.

In this study, the spectrophotometric method was developed to evaluate the feeding behavior of *Simulium vittatum* larvae under controlled conditions. Consequently, materials used to assess feeding behavior should not interfere with feeding behavior of the larvae. Experiments 1 and 2 were designed to determine the influence of Triton™ X-

100 and NRP on feeding behavior as determined by their influence on *Bti* ICP induced mortality. *Bti* ICPs must be consumed by the larvae for the protoxin to manifest its toxicity (Barjac and Sutheland 1990, Ben-Dov et al. 2003). A reasonable assumption can be made that when all other conditions are the same (i.e. pH, dissolved oxygen, and temperature) a reduction in mortality is likely due to a disruption of normal feeding behavior. Mortality from *Bti* ICPs can then be considered an indirect measure of feeding behavior.

Results from Experiment 1 indicate that 2.5 ppm of Triton™ X-100 does not change feeding behavior sufficiently to alter larval susceptibility to *Bti* ICPs. Previous experiments (unpublished data) indicated that concentrations of NRP in the larval media below 10 ppm do not reduce *Bti* ICP induced mortality. Experiment 2 was conducted to determine the maximum concentration of NRP that could be used without altering *Bti* ICP induced mortality. Twenty five ppm of NRP in the larval medium resulted in significant reductions in larval mortality from *Bti* ICPs. Fifteen ppm of NRP in the larval environment did not interfere with larval susceptibility to *Bti* ICPs and was the maximum concentration used for assessing feeding.

Experiments 3 and 4 were conducted to determine the effects of the experimental matrix on the spectrophotometric results and to determine if the gradual decrease of NRP in the closed system affected ingestion. Results from Experiment 3 indicated that the absorbance of the larval homogenates in acetone accurately reflected the NRP consumed by the larvae. Results from Experiment 4 demonstrated that a 1/3 decrease in NRP concentration did not alter NRP consumption.

Results from Experiment 5 revealed the utility of the spectrophotometric assay for evaluating the feeding behavior of black fly larvae in various media. The quantity of NRP consumed by larvae was significantly different in each medium. These differences in NRP ingestion can then be compared with the *Bti* ICP mortality in each medium. Larvae in a medium containing 10 ppm of kaolinite consumed 23% less particles than the larvae in moderately hard water. The mortality of larvae from *Bti* ICPs was not influenced by 10 ppm of kaolinite as compared to moderately hard water. This demonstrates that when media additives cause small changes in NRP consumption, they do not necessarily cause reductions in the susceptibility of larvae to *Bti* ICPs. This also indicates that while mortality from *Bti* ICPs can be used as an indirect measure of feeding behavior, it is not as sensitive as the spectrophotometric NRP assay in determining small changes in larval ingestion.

Larvae in a medium containing 10 ppm of cellulose ingested 61% less NRP than larvae in the control medium. The susceptibility of larvae to *Bti* ICPs was significantly reduced in a medium containing cellulose. Interestingly, the portion of the gut containing NRP was longer in larvae exposed to cellulose as compared to larvae in the other two media. It appears that the larvae were more efficient in capturing the cellulose particles than the particles of Neon Red. If the larvae were more efficient at capturing the cellulose particles than *Bti* ICPs, this could also explain the decrease in mortality of larvae in this medium. These results indicate that while the larvae may be unable to discriminate between particles of different compositions, they do discriminate between particles of different sizes. This is most likely related to the size of the larval cephalic fans.

When particulate matter is present in the larval medium, this technique will only enable the quantification of the NRP consumed. The quantity of particulate matter consumed in addition to NRP will be unknown; however one can determine the effect the additional material had on NRP consumption. For example, NRP consumption in a medium containing cellulose was much lower than it would be if the larvae did not discriminate between particles of different sizes.

In Experiments 1,2, 4 and 5, the additions of Triton™ X-100, NRP, and other additives to the larval medium were evaluated for their impact on *Bti* ICP induced mortality or NRP consumption. The controls and treatments in these experiments had different densities of particles in the larval medium. The change in particle density is most likely responsible for the decreased activity of *Bti* ICPs.

The reported median size of NRP is 4.5-5  $\mu\text{m}$  (Dayglo® Color Corp, A/AX pigment Technical Bulletin, [www.dayglo.com](http://www.dayglo.com)). The larvicide Vectobac® 12AS used in this study contains *Bti* ICPs that are bound to particles with a reported particle range of 2-9  $\mu\text{m}$  (Valent Biosciences 2003). The similar dimensions of NRP and ICPs is fortuitous because if a media additive is known to reduce *Bti* ICP activity, then NRP can be used in place of the ICPs to determine if the reduction is due to particle size discrimination. Results from this study indicate that the larvae do not discriminate between the *Bti* ICP formulation particles and NRP. This is demonstrated by the results in Experiment 2 when an increase in NRP resulted in decreased *Bti* ICP induced mortality. The results from Experiment 5 indicate that the larvae preferentially capture the larger cellulose particles over the NRP and the larvicide particles.

Some pest species of black flies can transmit diseases to humans and livestock. Insecticidal crystalline proteins produced by *Bti* ICPs are highly toxic to black fly larvae. The preferred approach to suppress these insects is to apply larvicides containing the *Bti* ICPs to the larval habitat. The larvicide must be ingested by the insects during the period of time that the material is in the water column. A thorough understanding of the feeding behavior of the larvae, including ingestion and gut passage rates, is necessary for a complete understanding of the control of target species. It has been postulated that some components of river seston could alter normal feeding behavior, thus causing a reduction in mortality due to low ingestion of *Bti* ICPs (Iburg et al. 2011).

This spectrophotometric technique can be used to study the feeding behavior of black fly larvae in an environment containing problematic seston components. The addition of kaolinite to the larval medium resulted in a small decrease in NRP ingestion, but not a decrease in *Bti* ICP activity. A direct assay would be necessary to determine the effects of a medium additive on *Bti* ICP induced mortality. When medium additives cause a decrease in *Bti* ICP activity, the spectrophotometric assay is a useful tool for determining if changes in feeding behavior were the cause of the decreased activity. An understanding of black fly larvae feeding behavior will also enable control operators to limit larvicide applications to times when larvae are feeding optimally, thereby reducing unnecessary introduction of the larvicide to the environment.

The spectrophotometric assay can be used to describe many other aspects of Simuliidae feeding behavior. Changes in pH, dissolved oxygen, temperature, and other chemical characteristics of the larval medium can be evaluated for their effects on NRP consumption. The correlation between NRP consumption and larval growth and

development can also be studied by evenly incorporating NRP into the larval diet. These and other applications will be the objects of future studies.

### **Acknowledgments**

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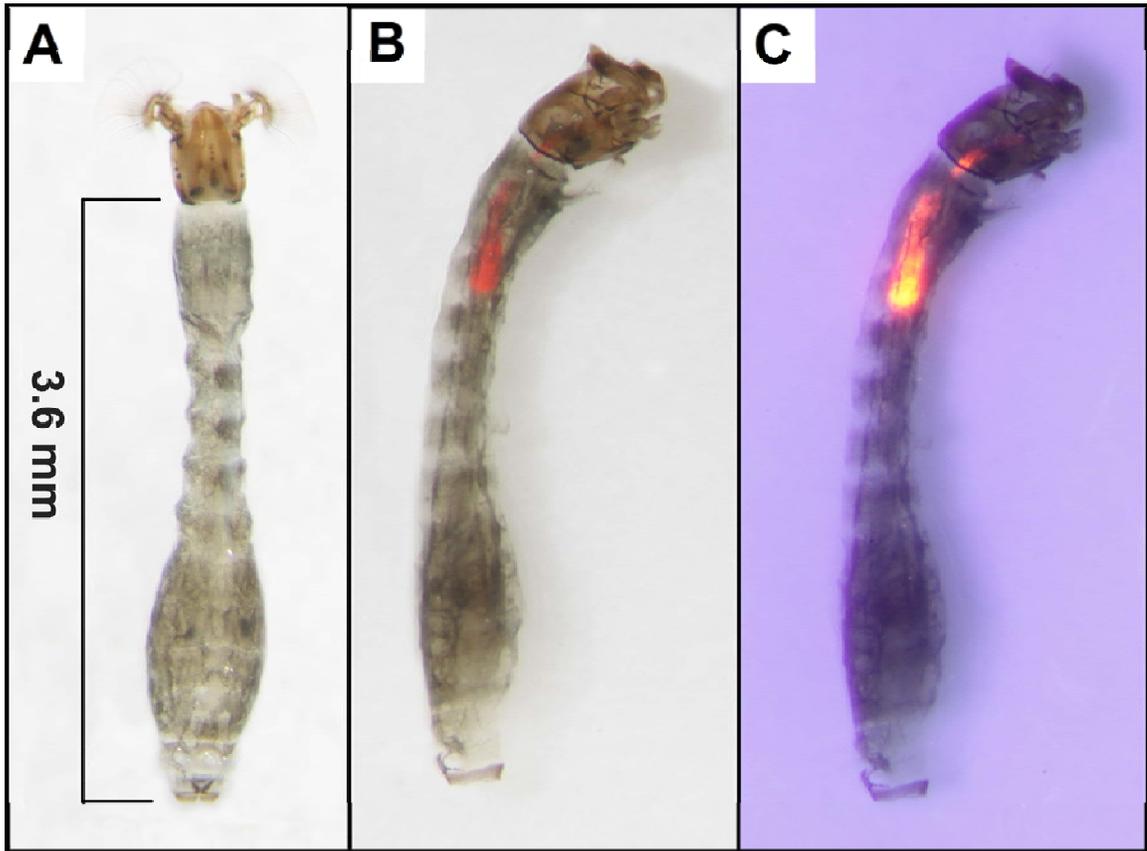
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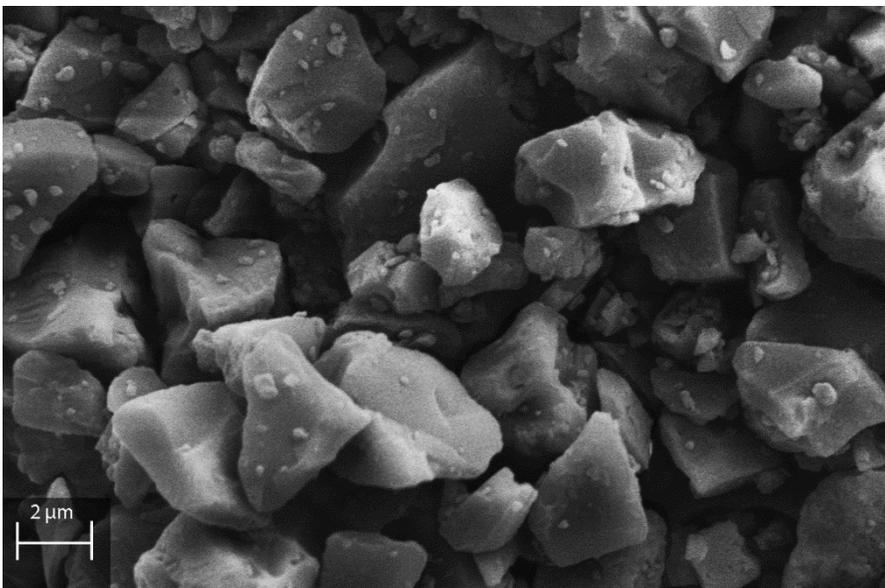
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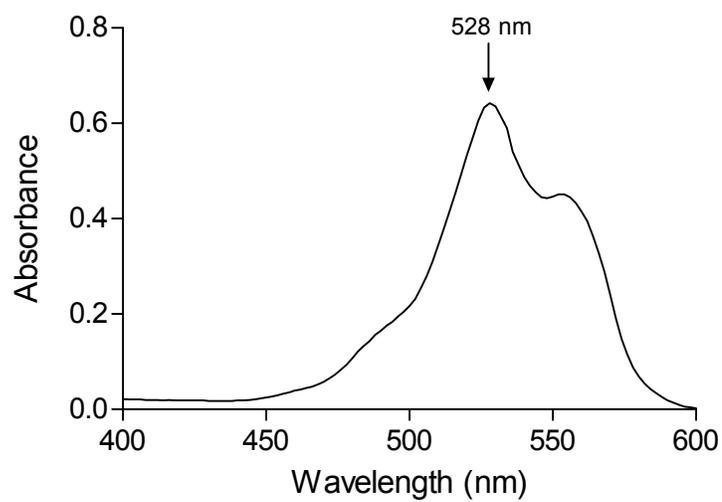
Tables and Figures



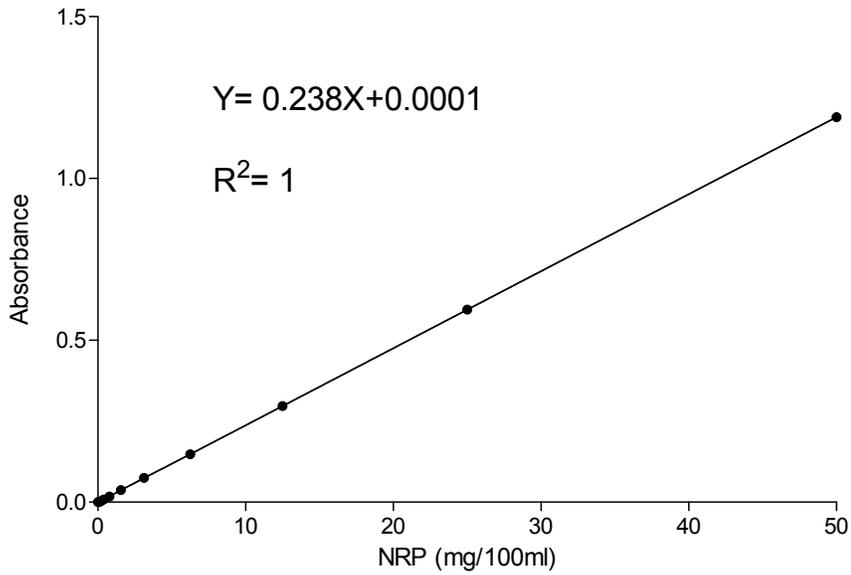
**Figure 4.1.** *Simulium vittatum* larvae. (A) Larva dorsal side up with extended cephalic fans. (B) Larva after ingesting Neon Red particles (NRP) under incandescent light. (C) Larva after ingesting NRP under incandescent light augmented with UV light.



**Figure 4.2.** Scanning electron microscope image of Neon Red particles.



**Figure 4.3.** Absorption spectrum of 50 mg Neon Red in 100 ml acetone.



**Figure 4.** Absorbance (at  $\lambda = 528$  nm) and standard curve of Neon Red particles (NRP) in acetone.

**Table 4.1.** The mortality of *S. vittatum* larvae from *Bti* ICPs with and without Triton™ X-100

Media	Control mortality (%)	<i>Bti</i> <sup>1</sup> mortality (%)
Water	1.3 ± 0.7 A	54.9 ± 3.7 A
Triton™ X-100 <sup>2</sup>	3.5 ± 0.8 A	52.3 ± 2.0 A

Values (mean ± SEM) within a column followed by the same letter are not significantly different ( $P \geq 0.05$ ).

<sup>1</sup> The concentration of *Bti* ICPs was 0.45 ITU/ml.

<sup>2</sup> Triton™ X-100 was in the larval medium at 2.5 ppm.

**Table 4.2.** The effect of two concentrations of Neon Red particles on *Bti* ICP activity

Media	Control mortality (%)	<i>Bti</i> <sup>1</sup> mortality (%)
Water	2.0 ± 1.1 A	86.4 ± 3.1 A
Neon Red (15 ppm)	0.0 ± 0.0 A	86.5 ± 1.9 A
Neon Red (25 ppm)	0.6 ± 0.4 A	67.8 ± 3.8 B

Values (mean ± SEM) within a column followed by the same letter are not significantly different ( $P \geq 0.05$ ).

<sup>1</sup> The concentration of *Bti* ICPs was 0.45 ITU/ml.

**Table 4.3.** Absorbance of Neon Red in the presence of the components of the experimental matrix

Suspension <sup>1</sup>	Absorbance @ 528 nm
Neon Red	0.269 ± 0.000 A
Neon Red + Triton™ X-100 <sup>2</sup>	0.270 ± 0.000 A
Neon Red + Larvae <sup>3</sup>	0.272 ± 0.000 A
Neon Red + Larvae + Triton™ X-100	0.273 ± 0.000 A

Values (mean ± SEM) followed by the same letter are not significantly different ( $P \geq 0.05$ ).

<sup>1</sup> All solutions contained 100 ppm of Neon Red in acetone.

<sup>2</sup> Triton™ X-100 in solution at 20 ppm.

<sup>3</sup> Thirty larvae sonicated in solution followed by filtration through a 0.2µm PTFE membrane.

**Table 4.4.** The gut passage and quantity consumed of two concentrations of Neon Red after 30 min

Neon Red concentration (ppm)	Gut passage (mm)	Neon Red consumption ( $\mu\text{g}/\text{larvae}$ )
10	2.1 $\pm$ 0.09 A	6.8 $\pm$ 0.50 A
15	2.2 $\pm$ 0.04 A	7.6 $\pm$ 0.70 A

Values (mean  $\pm$  SEM) within a column followed by the same letter are not significantly different ( $P \geq 0.05$ ).

**Table 4.5.** Neon Red particle consumption and *Bti* ICP susceptibility of larvae in various media <sup>1</sup>

Media <sup>2</sup>	Neon Red gut passage (mm)	Neon Red consumption ( $\mu\text{g}/\text{larvae}$ )	Neon Red distribution ( $\mu\text{g}/\text{mm}$ )	Control mortality (%)	<i>Bti</i> ICP <sup>3</sup> mortality (%)
Water	0.71 $\pm$ 0.02 A	2.38 $\pm$ 0.17 A	3.35 $\pm$ 0.21 A	1.02 $\pm$ 0.73 A	69.34 $\pm$ 2.39 A
Kaolinite	0.78 $\pm$ 0.06 B	1.83 $\pm$ 0.16 B	2.36 $\pm$ 0.19 B	1.07 $\pm$ 0.55 A	69.23 $\pm$ 2.34 A
Cellulose	1.05 $\pm$ 0.03 C	0.92 $\pm$ 0.07 C	0.87 $\pm$ 0.05 C	1.34 $\pm$ 0.55 A	54.72 $\pm$ 3.10 B

Values (mean  $\pm$  SEM) within a column followed by the same letter are not significantly different ( $P \geq 0.05$ ).

<sup>1</sup> Larvae exposed to Neon Red or *Bti* ICPs for 10 minutes.

<sup>2</sup> Kaolinite and cellulose in the media at 10 ppm.

<sup>3</sup> The concentration of *Bti* ICPs was 0.45 ITU/ml.

CHAPTER 5  
FEEDING BEHAVIOR OF *SIMULIUM VITTATUM* LARVAE IN RESPONSE TO  
VARIOUS SUSPENDED MATERIALS: IMPLICATIONS FOR CONTROL USING  
BIOLOGICAL LARVICIDES <sup>4</sup>

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<sup>4</sup> **Iburg, J. P., E. W. Gray, R. D. Wyatt, R. Noblet.** To be submitted to Environmental Entomology

## Abstract

Biologically based larvicides used to suppress nuisance populations of black flies require that the larvae ingest the larvicide for mortality to occur. Materials present in the larval habitat that alter larval feeding behavior may reduce the effectiveness of the larvicide. *Simulium vittatum* Zetterstedt larvae were exposed to kaolinite clay, cellulose fibers, and purified diatom frustules; larvae were exposed to 10 ppm of the clay and cellulose materials and 4 ppm of the frustules. Larvae were also exposed to 10,000 cells or colonies per ml of viable green algae, *Chlorella vulgaris* Pratt and *Scenedesmus quadricauda* (Turp) Bréb, and the diatom *Cyclotella meneghiniana* Kütz in a current and under standardized conditions to determine the effects that these materials had on larval feeding behavior. Individual larvae were recorded using a digital camcorder to determine the average number of times the larvae extended and retracted their cephalic fans per min (flick rate) in each medium. Following exposure to the selected material, water insoluble Neon Red particles (NRP) (DayGlo®) were added to the larval medium. After a 20 min exposure to NRP, larvae were removed and homogenized in acetone to solubilize the NRP. A spectrophotometric analysis was conducted to determine the average quantity of NRP consumed by larvae per min in each medium. The quantity of NRP captured per flick was calculated. The flick rate and quantity of NRP consumed by larvae was not significantly affected by clay material or viable *Chlorella* cells. When *Scenedesmus* colonies or *Cyclotella* cells were present in the larval medium, larval flick rate and NRP ingestion was significantly reduced. Purified diatom frustules present in the medium at 4 ppm resulted in the most severe reduction in larval flick rate and NRP ingestion.

## Introduction

Black flies (Diptera: Simuliidae) are hematophagous insects that are found throughout the world. Some species are considered pests due to their blood feeding and nuisance swarming behaviors (Gray et al. 1996). Pathogens are transmitted by various species of black flies that can affect livestock and human health (Adler et al. 2004). The larval stages develop in fast moving rivers and streams and feed primarily by using cephalic fans to filter particles from the flowing water.

The application of products containing insecticidal crystalline proteins (ICPs) produced by *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) to the larval habitat is to date the most successful strategy for suppressing nuisance populations (Barjac and Sutheland 1990). *Bti* ICPs must be ingested by larvae to cause mortality (Ferreira and Silva-Filha 2013). Formulations that contain the ICPs typically contain larger particles to which the proteins are bound. The larger particles are captured by the cephalic fans of the larvae more efficiently resulting in higher larval mortality (Barjac and Sutheland 1990). The ICPs are solubilized in the gut of the larvae and acted on by proteases thus converting the pro-toxin into a toxin (Ferreira and Silva-Filha 2013). Black fly larvae are only exposed to the ICPs for a brief period of time following application due to the nature of the larval habitat. Formulations are typically labeled so that an application results in a 1 to 10 min exposure of larvae to the ICPs before they are carried downstream. The effectiveness of ICPs relies on black fly larvae actively feeding during this brief exposure period.

Personnel involved with black fly control programs have reported the occasional decrease in ICP activity in select locations (Stephens et al. 2004, Iburg et al. 2011). Coupland (1993) found that heavy loads of material in the water could cause reductions

in ICP activity. For example, Stephens et al. (2004) found that the presence of the green alga *Scenedesmus quadricauda* in larval medium caused a reduction in ICP activity. The effect of this alga might be important because it was detected in areas where occasional decreases in ICP activity have been reported. Iburg et al. (2011) investigated seston from these same locations and found that the presence of diatom cells and frustules in larval medium caused significant reductions in *Bti* ICP activity, while other components of the seston such as various clays had no significant effects on larval mortality. The results of these studies indicated that while the quantity of seston in the habitat is of some importance, the qualities of the seston may determine more accurately if there will be a reduction in ICP activity. Stephens et al. (2004) and Iburg et al. (2011) hypothesized that problematic materials caused a reduction in ICP activity either by particle competition or by interfering with larval feeding behavior.

Iburg et al. (2013) developed a method to test these hypotheses. The method utilized inert, water-insoluble neon red particles (NRP) as a surrogate for *Bti* ICPs to determine the ingestion rate of particles by larvae in the presence of various seston components. NRPs are similar in size to the particles found in Vectobac<sup>®</sup> 12AS (Valent Biosciences Corp., Libertyville, IL), the most widely used product at this date for black fly suppression. Iburg et al. (2013) exposed larvae to these particles for set amounts of time in medium containing various additives. The larvae were then homogenized in acetone, releasing the NRP into solution. The homogenate was filtered and subjected to spectrophotometric analysis to determine the average quantity of NRP ingested by larvae per unit time. Results indicated that a materials effect on NRP ingestion corresponded to

the expected mortality from exposure to *Bti* ICPs in the presence of said material (Iburg et al. 2013).

The purpose of this study was to evaluate the feeding behavior of larvae after exposure to clay, cellulose, diatom frustules, viable cells green algae, and viable diatom cells. We chose to use materials that have previously been studied in relation to the susceptibility of larvae to *Bti* ICPs to determine if the changes in susceptibility can be explained by changes in larval feeding behavior. The materials for this study were chosen based upon earlier findings of Stephens et al. (2004) and Iburg et al. (2011). Clay material and viable *Chlorella vulgaris* Pratt cells were used because these materials were not found to have an influence on ICP activity at the concentrations used. Our hypothesis was that these materials would not have a significant effect on NRP ingestion or flick rate of larvae. Cellulose fibers, purified diatom frustules, viable *Cyclotella meneghiniana* Kütz cells, and viable *Scenedesmus quadricauda* (Turp) Bréb were utilized because the fore-mentioned studies found that these materials had a significant effect on ICP activity. Our hypothesis was that these materials would significantly affect the flick rate and/or NRP ingestion rate of larvae.

The present study adapts the method described by Iburg et al. (2013) to evaluate the feeding activity of *Simulium vittatum* Zetterstedt larvae following exposure to a variety of materials. In addition to the analysis of NRP ingestion in the presence of these materials, feeding larvae were visually observed during the exposure. The number of times that larvae extended and retracted their cephalic fans per unit time was calculated (flick rate). The average amount of NRP ingested by a larva per min was calculated for

larvae in each medium. The average quantity of NRP captured by larvae in each medium per flick was also recorded.

## **Materials and Methods**

### Experimental Design

This experiment had 7 treatments. Larvae were exposed to moderately hard water, clay, cellulose, diatom frustules, *Chlorella* cells, *Cyclotella* cells, or *Scenedesmus* colonies. The average flick rate and ingestion of NRP by *S. vittatum* larvae in each medium was calculated. Five replicates were conducted with each treatment. At the beginning of each replicate, 4,750 ml of moderately hard water was added to the bottom reservoir of the experimental tank. The water was prepared using the methods described by Weber (1993) and Iburg (2011) and served as the basal media in all replicates. The pump was then turned on and the flow of water was kept at 2.4 liters per min providing a trickle of water flowing down the runway. Approximately 200 late instar larvae were removed from the colony rearing unit on nylon screen substrate that measured 2x 5 cm. The nylon substrate containing the larvae was then clipped to the lip of the experimental tank reservoir and as the water flowed over the substrate the larvae detached into the current. A majority of the larvae that detached from the substrate attached to the waterway using silk pads. The larvae that did not attach to the waterway before the drop to the bottom reservoir were captured by a temporary nylon screen for later removal. The current of water was kept flowing at a rate of 2.4 liters per min for 5 min, allowing the larvae to spread out and attach to the steel waterway. After 5 min, the current speed was increased to 8 liters per min and the nylon screen was removed. After a total time of 10

min, the medium additive was added to the tank. The control replicates received 125 ml of moderately hard water. Replicates that utilized dry materials or viable algal cells received the materials suspended in 125 ml of moderately hard water. Once added to the tanks, the concentration of clay or cellulose material in the experimental tank was 50 ppm. The concentration of frustules in the experimental tank was 4 ppm. Viable *Chlorella* or *Cyclotella* cells were present in the medium at a concentration of 10,000 cells per ml. *Scenedesmus* colonies were present in the medium at a concentration of 10,000 colonies per ml.

The larvae were exposed to the medium additives for 10 min prior to the addition of 125 ml of NRP. Individual larvae were recorded for 20 min following the application of NRP. Larvae were recorded for 1 min intervals resulting in 10-15 larvae being recorded during the 20 min exposure to NRP. Following the 20 min exposure, the pump was turned off and 3 groups of 30 larvae were randomly removed for spectrophotometric analysis. The average number of times the larvae flicked per min, the average quantity of NRP consumed per min, and the average amount of NRP captured per flick was calculated for larvae in each medium.

### Experimental Setup

The experimental tank used for this study was designed as a miniature version of the tanks described by Gray and Noblet (1999) that are used in the rearing facility. The tank was constructed of stainless steel and the dimensions of the entire unit were 51 x 27 x 47 cm (length, width, height). The tank was separated into two portions. The bottom portion, or reservoir, measured 51 x 27 x 32 cm. The top portion of the experimental tank

was fitted above the reservoir. This portion has a smaller reservoir in the rear that contained a lip measuring 10.5 x 4 cm. Situated below the lip is a waterway held at an angle of 15° that measures 35.5 x 15 cm. The waterway was constructed of stainless steel and was painted white to provide more contrast when viewing the larvae through the camera. A pump was connected to the rear of the tank to pump water from the bottom reservoir to the top reservoir. The flow of water was controlled by a valve and the flow rate was monitored by a flow meter. Once the water reached the top of the reservoir on the upper portion of the tank, it flowed through the lip and down the runway. At the end of the runway the water passed through an opening that re-circulates it into the bottom reservoir.

#### Medium Additives

Kaolinite clay was obtained from VWR International (Radnor, PA). The clay particles had a mean diameter of 4.7 µm as determined by a Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK.) Purified cellulose (Solka-Floc®) was obtained from the International Fiber Corporation (North Tonawanda, NY). The cellulose fibers had a mean diameter of 32 µm as determined by a Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK). Purified frustules from *Cyclotella meneghiniana* Kütz were prepared as described by Iburg et al. (2011). The frustule preparations consisted of fragments with particles ranging in diameter from approx. 1-10µm. Viable *Chlorella vulgaris* Pratt and *Scenedesmus quadricauda* (Turp) Bréb cells and the growth medium for the green algae were obtained from Ward's Biology (Rochester, NY, USA). The *Chlorella* cells were approx. 4µm in diameter and the *Scenedesmus* colonies were

approx. 30µm in diameter. Viable cells of *C. meneghiniana* Kützing were obtained from the University of Texas Culture Collection (Austin, TX). The *Cyclotella* cells were approx. 15 µm in diameter. The diatoms were cultivated in growth medium that was prepared as described by Iburg et al. (2011). The viable cells used in the experiment were centrifuged prior to use to remove growth medium and re-suspended in moderately hard water.

#### Pigment Particle Suspension

Neon Red pigment (AX-12-5, Lot #5641) was obtained from Day-Glo® Color Corp., Cleveland, OH. This pigment is water insoluble yet readily soluble in certain organic solvents. The average particle size of NRP is reported as 4.5-5 µm in diameter (Dayglo® Color Corp, A/AX pigment Technical Bulletin, [www.dayglo.com](http://www.dayglo.com)). A suspension of NRP was prepared by following the procedure outlined in Iburg et al. (2013). This suspension was prepared with a minimal amount of Triton™ X-100 to aid in particle dispersion. The suspension was left to settle for 1 h to allow the large agglomerates to sink to the bottom of the vessel. The vessel containing the suspension was then placed in a sonic bath for 30 sec to break up those larger agglomerates of particles. When 125 ml of this solution was added to 4875 ml of larval medium, the resulting NRP and Triton™ X-100 concentrations in the larval medium were 15 ppm and 2.5 ppm, respectively.

### Absorbance Maximum and Standard Curve of Neon Red Particles

The absorbance maximum and standard curve of Neon Red particles (NRP) in acetone was determined by Iburg et al. (2013). The absorbance of NRP was detected using a wavelength of 528 nm, the absorbance maximum. The quantity of NRP in acetone was calculated using the formula  $Y = 0.238X + 0.0001$ , where X is the quantity of NRP in acetone (mg/100 ml) and Y is the absorbance of the sample at 528 nm. Samples of acetone containing NRP were analyzed using a Spectra Max Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA).

### *Simulium vittatum* Larvae

Late instar *Simulium vittatum* Zetterstedt cytospecies IS-7 larvae were used for all replicates. The larvae were colony reared under conditions described by Gray and Noblet (1999). Each replicate utilized a new generation of larvae reared under similar conditions until the larvae had developed to the appropriate stage.

### Removal and Storage of the Larvae

Following the exposure of larvae to NRP and other medium additives for the appropriate time, the larvae were gently detached from the experimental tank using soft-tipped forceps. Three groups of 30 larvae were placed into vials containing 10% neutral buffered formalin. The larvae remained in formalin for no more than 24 h prior to analysis.

### NRP Analysis

The analysis of NRP within the alimentary tracts of larvae was conducted using the method outlined by Iburg et al. (2013). Larvae were removed from the formalin, placed on a paper towel, and blotted of excess formalin. Each group of 30 larvae was transferred to 4 ml (15x45 mm) glass vials with Teflon<sup>®</sup> lined screw caps containing 3 ml of acetone. Each vial containing larvae in acetone received 40-1 sec pulses (slope was 1.06 with an amplitude of 75%) from the probe of a Sonic Dismembrator (Model 1501, Thermo-Fisher Scientific, Waltham, MA) that was placed approximately 1.5 cm below the surface of the acetone. A 5 ml plastic syringe with a 20 gauge needle was used to recover the homogenate from the homogenizing vessel. The needle of the syringe containing the homogenate was removed and replaced with a 33 mm, 0.20 µm PTFE syringe filter. A 1 ml quartz cuvette was used to collect the filtered homogenate. Thirty larvae that had not been exposed to NRP were homogenized in acetone followed by filtration through a 0.20 µm PTFE filter to provide the reference acetone blank. The absorbance of filtered larval homogenates was determined after referencing the blank. The average quantity of NRP consumed per larvae per unit of time was calculated for larvae in each medium.

### Flick Rate Analysis

Individual larvae feeding on various media were recorded using a Canon VIXIA HF S30 A camcorder (Canon Inc., Tokyo, Japan). The camera was fitted with a custom micro lens adapter (Martin Microscope Company, Easley, SC, USA). The camera was positioned above the experimental tank with a custom stand (Martin Microscope

Company, Easley, SC, USA) that enabled recording of larvae along the waterway. Each larva was recorded for 1 min. The video was then viewed in slow motion (0.12-0.33 X original speed) to count the number of times the larva flicked its cephalic fans. A behavior was considered a flick when a cephalic fan was open to the current then closed and inserted into the cibarium to remove the collected material by the mandibles and labrum. The average number of flicks per min was calculated for larvae feeding on each medium.

### Statistical Analysis

One-way analysis of variance tests with Tukey-Kramer multiple comparisons were performed comparing all treatments in the experiment. GraphPad Prism version 5.04 was used for all analyses (GraphPad Software, San Diego, California USA, [www.graphpad.com](http://www.graphpad.com)).

## **Results**

### NRP Ingestion and Flick Rate

No significant difference was determined in NRP consumption between larvae in water or the medium containing kaolinite ( $q = 3.21$ ;  $df = 6, 28$ ;  $P > 0.05$ , Table 5.1), or between larvae in water or medium containing viable *Chlorella* cells ( $q = 0.72$ ;  $df = 6, 28$ ;  $P > 0.05$ ). Larvae exposed to *Cyclotella* cells consumed significantly less NRP than larvae in water ( $q = 5.83$ ;  $df = 6, 28$ ;  $P < 0.05$ ); however, they did not consume significantly less than larvae in medium containing kaolinite ( $q = 2.62$ ;  $df = 6, 28$ ;  $P > 0.05$ ). Larvae exposed to cellulose, frustules, and *Scenedesmus* colonies consumed

significantly less NRP than those in water ( $q = 9.79$ ;  $df = 6, 28$ ;  $P < 0.05$ ), ( $q = 10.28$ ;  $df = 6, 28$ ;  $P < 0.05$ ), ( $q = 8.92$ ;  $df = 6, 28$ ;  $P < 0.05$ ).

No significant differences were found in the flick rate of larvae in water as compared to those in medium containing kaolinite, cellulose, or *Chlorella* cells ( $q = 0.26$ ;  $df = 6, 28$ ;  $P > 0.05$ ), ( $q = 3.94$ ;  $df = 6, 28$ ;  $P > 0.05$ ), ( $q = 1.17$ ;  $df = 6, 28$ ;  $P > 0.05$ ). Larvae in medium containing *Chlorella* cells flicked significantly more than those in medium containing cellulose ( $q = 5.11$ ;  $df = 6, 28$ ;  $P < 0.05$ ). The flick rate of larvae in water was significantly higher than those in medium containing frustules, *Cyclotella* cells, and *Scenedesmus* colonies ( $q = 8.61$ ;  $df = 6, 28$ ;  $P < 0.05$ ), ( $q = 5.82$ ;  $df = 6, 28$ ;  $P < 0.05$ ), ( $q = 6.02$ ;  $df = 6, 28$ ;  $P < 0.05$ ). The flick rate of larvae in medium containing cellulose was not significantly different from larvae in medium containing *Cyclotella* cells or *Scenedesmus* colonies ( $q = 1.88$ ;  $df = 6, 28$ ;  $P > 0.05$ ), ( $q = 2.07$ ;  $df = 6, 28$ ;  $P > 0.05$ ). The flick rate of larvae in medium containing frustules was significantly less than that of larvae in medium containing cellulose ( $q = 4.67$ ;  $df = 6, 28$ ;  $P < 0.05$ ).

The average quantity of NRP captured per flick by larvae in water was not significantly different from those in medium containing kaolinite, *Chlorella* cells, *Cyclotella* cells, or *Scenedesmus* colonies ( $q = 2.38$ ;  $df = 6, 28$ ;  $P > 0.05$ ), ( $q = 0.33$ ;  $df = 6, 28$ ;  $P > 0.05$ ), ( $q = 0.51$ ;  $df = 6, 28$ ;  $P > 0.05$ ), ( $q = 4.14$ ;  $df = 6, 28$ ;  $P > 0.05$ ). The quantity of NRP captured per flick by larvae in water was significantly higher than that captured by larvae in medium containing cellulose or frustules ( $q = 6.52$ ;  $df = 6, 28$ ;  $P < 0.05$ ), ( $q = 5.34$ ;  $df = 6, 28$ ;  $P < 0.05$ ). The quantity of NRP captured per flick by larvae in medium containing kaolinite was not significantly different from that of larvae in medium containing cellulose or frustules ( $q = 4.14$ ;  $df = 6, 28$ ;  $P > 0.05$ ), ( $q = 2.96$ ;  $df =$

6, 28;  $P > 0.05$ ). The quantity of NRP captured per flick by larvae in medium containing *Scenedesmus* colonies was not significantly different from that of larvae in medium containing cellulose or frustules ( $q = 2.38$ ;  $df = 6, 28$ ;  $P > 0.05$ ), ( $q = 1.20$ ;  $df = 6, 28$ ;  $P > 0.05$ ).

### Observations of Feeding Behavior

The behavior of larvae in moderately hard water was consistent among larvae in this medium. Larvae in this medium fully extended their cephalic fans with each flick, the flicks were rapid, and very little time occurred between each flick. Occasionally a larva would retract its fans in response to the movement of another larva, or it would cease to feed for a brief period while moving to a new location. The majority of the larvae were evenly distributed on the waterway; however, the larvae occasionally formed small groupings. Larvae in medium containing kaolinite clay or *Chlorella* cells exhibited similar behavior. These materials did not appear to affect the feeding activity of the larvae (see Table 5.1).

Larvae in medium containing cellulose material, frustules, *Cyclotella* cells, and *Scenedesmus* colonies exhibited various types of altered feeding behavior (Table 5.1). Larvae in each of these media seemed to flick more often with one fan than the other. Larvae in each of these media also formed tighter groups on the waterway. Larvae in medium containing cellulose flicked slightly less often and most larvae did not fully extend their cephalic fans for each flick, extending them less than half the typical width of larvae in moderately hard water. Larvae exposed to cellulose also spent more time removing the material in the cibarium as if the material was difficult to dislodge from the

fans. Larvae exposed to *Cyclotella* cells and *Scenedesmus* colonies flicked less often with approximately equal numbers of larvae keeping their fans retracted between flicks and keeping their fans extended between flicks. Most of the larvae in medium containing frustules kept their cephalic fans retracted for the majority of the time, periodically flicking for a few seconds before retracting their fans again.

### **Discussion**

Some naturally occurring materials had significant effects on the feeding behavior of *Simulium vittatum* larvae, while others did not. The feeding behavior of Simuliidae larvae is important to consider as the current forms of biological larvicides require that nuisance populations of larvae be actively feeding for the control efforts to be successful. The predominant biological larvicides used to control nuisance populations of black flies contain insecticidal crystalline proteins produced by the bacterium *Bacillus thuringiensis* subsp. *israelensis* (*Bti* ICPs). The proteins must be ingested by larvae to cause mortality. Stephens et al. (2004) and Iburg et al. (2011) hypothesized that occasional reductions in larval mortality following exposure to ICPs may have occurred because certain components of the seston interfered with ingestion of the ICPs, either through competition or by altering the feeding behavior of the larvae. Results from our study support these hypotheses.

Feeding inhibition of larvae related to particles in the medium has been documented in the past. Gaugler and Molloy (1980) found a non-specific response of larvae to the presence of various particles in the larval medium. The larvae exhibited altered feeding behavior when confronted with 50 mg per liter or more of each material.

They postulated that the inhibition of feeding behavior occurred due to rapid gut filling and that the response was non-specific. The inhibition of feeding behavior by larvae in the present study in medium containing *Cyclotella vulgaris* cells or *Scenedesmus quadricauda* colonies may have been a result of rapid gut filling as it seemed that larvae in these media had guts tightly packed with algal cells. Larvae may not feed as rapidly when they have an abundance of material packed in the upper portion of the gut, or when the materials are relatively large particles. The feeding inhibition of larvae exposed to frustules or cellulose, however, is not explained by this hypothesis.

Stephens et al. (2004) evaluated the effects that algal cells had on the mortality of *Simulium vittatum* larvae following exposure to *Bti* ICPs. The presence of *Chlorella* cells did not have an effect on larval mortality at concentrations exceeding 200,000 cells per ml. The presence of 16,000 cells per ml of *Scenedesmus* resulted in a significant reduction in larval mortality. The results from the present study indicate that *Chlorella* cells do not alter the feeding behavior of the larvae whereas the presence of *Scenedesmus* colonies does. *Chlorella* cells are much smaller ( $\approx 4 \mu\text{m}$ ) than the colonies of *Scenedesmus* ( $\approx 30 \mu\text{m}$ ), and these colonies also have spines. The feeding inhibition from *Scenedesmus* in the present study may be due to rapid gut filling, and the larvae may not be able to remove the colonies from their cephalic fans because of the spines or the large size. The low quantity of NRP collected per larvae in medium containing *Scenedesmus* colonies indicates that the large colonies may also block the fans from being able to capture the smaller NRP particles.

Clay material has not had a significant effect on larval susceptibility to *Bti* ICPs in field or laboratory studies (Iburg et al. 2011, Gray et al. 2012). In the present study, clay

material did not have a significant effect on NRP ingestion or flick rates, which may explain why these materials did not interfere with ICP activity in previous studies. The cellulose fibers used in the present study and by Iburg et al. (2011) do not approximate naturally occurring materials; however, they are of concern in larval habitats that receive secondary effluent from paper-mills. Illesova (1988) found that larvae exposed to cellulose fibers in paper-mill effluent retracted their cephalic fans and ceased to feed. In our study, it appeared that the larvae still flicked their fans in medium containing cellulose; however, they flicked at a slightly slower rate and did not fully extend their fans. The number of larval flicks in medium containing cellulose was not significantly lower than that of larvae in water; however, the quantity of NRP ingested by larvae and captured per flick was lower. This indicates that while larvae are feeding in the presence of cellulose, they are not able to capture the NRP. The larvae in medium containing cellulose spent more time attempting to clear their fans than larvae in other media indicating that the fibers may become entangled in the rays of the cephalic fans, thus preventing proper removal.

Iburg et al. (2011) found that viable *Cylotella meneghiniana* Kütz cells and the purified frustules of the diatom both caused significant reductions in *Bti* ICP activity when present in the larval media. In the present study these materials also caused significant reductions in the capture of NRP by larvae and their flick rate. The suspension containing viable cells used in the present study and by Iburg et al. (2011) could possibly have contained small quantities of frustules that were the primary cause of the reductions in ICP activity, flick rate, and ingestion of NRP. The cells were collected from young cultures of the diatom to reduce the quantities of frustules that may be present, but we did

not confirm that they were completely absent from the viable cell suspensions used in the experiment. The larvae may feed at a reduced rate when ingesting highly nutritive material such as diatoms. Thompson (1987) found that larvae reared on diatoms exhibited higher rates of growth and survival than larvae reared on other materials. Martin and Edman (1993) also found that Simuliidae could easily assimilate nutrients from diatoms. Iburg et al. (2011) found that frustules caused the most significant reductions in larval mortality from *Bti* ICPs and the present study found that only 4 ppm of frustules in the larval medium resulted in the most severe reduction in flick rate and NRP ingestion among materials tested. Diatomaceous earth is a known abrasive to insect cuticle, and it is likely that abrasion causes the larvae to retract their cephalic fans (Athassiou et al. 2006).

Lacoursiere and Craig (1993) noticed that black fly larvae formed aggregates on substrate in a water column. They postulated that these aggregates were formed to maximize the flow of water through the cephalic fans of larvae. In the present study this behavior was exhibited somewhat by larvae that were in medium containing water, kaolinite, or *Chlorella* cells. Interestingly, the aggregates of larvae in medium containing cellulose, frustules, *Cyclotella* cells, or *Scenedesmus* colonies seemed to be even more pronounced. The aggregates of larvae in these media, particularly those in medium containing frustules, were not likely to have been formed to maximize the flow of water because these materials seemed to disturb the larvae. The larvae may also form dense aggregates to protect themselves from materials that may be abrasive or that overwhelm their ability to clear their fans in the cibarium.

The flick rate exhibited by larvae and their ability to capture smaller particles, such as NRP or *Bti* ICPs, appears to be related to the particle sizes of materials suspended in the waterway. The particles of clay and *Chlorella* cells were below 5  $\mu\text{m}$  in diameter and these materials did not significantly interfere with flick rate or capture of NRP. The cellulose fibers, *Cyclotella* cells, and *Scenedesmus* colonies were all larger than 10  $\mu\text{m}$  in diameter and each material had a significant effect on flick rate and NRP ingestion. Previous studies have found that most Simuliidae larvae are better able to capture larger particles (Schröder 1987, Barjac and Sutheland 1990). Our results indicate that the large particles cause larvae to slow their flick rate and block smaller particles from being captured by the cephalic fans. This slowing of flick rate may occur because it takes longer for the larger materials to be moved through the fore- and midgut. An exception to this would be the diatom frustules which were smaller than all other materials tested, present at concentrations that were over 12 times less than that of the other dry materials, yet caused the most severe reduction in flick rate and NRP ingestion. We hypothesized that the abrasive nature of this material to insect cuticle is responsible for this activity. It would be prudent for applicators of products containing *Bti* ICPs in operational settings to minimize applications when large blooms of diatoms are in the waterway as the frustules are also likely to be present.

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**Table****Table 5.1.** Neon Red<sup>1</sup> particle (NRP) consumption and flick rate of larvae in various media

Media	NRP consumption (ng/larva/min)	Flick rate (flicks/larva/min)	NRP/ Flick (ng)
Water <sup>2</sup>	186.0 ± 23.3 A	248.1 ± 18.9 AB	0.75 ± 0.07 A
50 ppm Kaolinite	134.1 ± 14.7 AB	253.4 ± 27.1 AB	0.54 ± 0.04 AB
50 ppm Cellulose	27.87 ± 4.56 C	168.9 ± 19.5 AC	0.16 ± 0.01 B
4 ppm Frustules <sup>3</sup>	19.96 ± 2.83 C	75.04 ± 8.9 D	0.27 ± 0.03 B
<i>C. vulgaris</i> <sup>4</sup>	197.6 ± 28.3 A	271.7 ± 23.3 B	0.78 ± 0.18 A
<i>C. meneghiniana</i> <sup>4</sup>	91.80 ± 14.6 BC	131.1 ± 15.4 CD	0.70 ± 0.08 A
<i>S. quadricauda</i> <sup>4</sup>	42.00 ± 5.42 C	127.2 ± 22.4 CD	0.37 ± 0.09 AB

Values (mean ± SEM) within a column followed by the same letter are not significantly different

( $P \geq 0.05$ , ANOVA, Tukey post-hoc)

<sup>1</sup> Neon Red particles were present in the media at a concentration of 15 ppm.

<sup>2</sup> Moderately hard water was used as the basal media in each treatment. Larvae were exposed to medium additives for 10 min prior to the addition of Neon Red particles.

<sup>3</sup> Purified frustules from *Cyclotella meneghiniana*.

<sup>4</sup> Medium containing *Chlorella vulgaris* and *Cyclotella meneghiniana* contained 10,000 cells/ml. Medium containing *Scenedesmus quadricauda* contained 10,000 colonies/ml.

CHAPTER 6

MORTALITY PATTERNS OF *SIMULIUM VITTATUM* LARVAE FOLLOWING  
EXPOSURE TO INSECTICIDAL PROTEINS PRODUCED BY *BACILLUS*  
*THURINGIENSIS* SUBSP. *ISRAELENSIS*<sup>5</sup>

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## Abstract

Products containing insecticidal crystalline proteins (ICPs) produced by *Bacillus thuringiensis* var. *israelensis* de Barjac (*Bti* ICPs) are used to suppress vector and nuisance populations of black flies. The efficacy of an application of these products is often determined by a post-treatment evaluation of larval mortality. Larvae are typically removed from the substrate at some point in time after application of the product and mortality is determined. The time necessary for the effects of *Bti* ICPs to cause mortality in exposed larvae can vary, and there is little consensus on how long operators should wait before evaluating larval mortality. This study was conducted to provide more information to larvicide applicators when conducting post-treatment evaluations. *Simulium vittatum* Zetterstedt larvae were exposed to *Bti* ICPs under controlled conditions and the mortality was monitored over time. Larvae exposed to operational concentrations of ICPs exhibited maximum mortality, approximately 87%, after 4 h. Exposure of larvae to 1/3 of that concentration resulted in similar mortality; however the maximum mortality was not reached until 8 h post-exposure. Additional experiments revealed that maximum mortality and time to maximum mortality can also be affected by components in the larval medium. Larval mortality was compared between larvae exposed to *Bti* ICPs in moderately hard water, medium containing 50 ppm of kaolinite, and medium containing 50 ppm of cellulose. The clay material had no significant effect on larval mortality or time to achieve maximum mortality. When cellulose was present in the medium the time to maximum mortality was increased 50% and overall mortality was reduced by more than 40%.

## Introduction

Black flies (Diptera: Simuliidae) are aquatic insects that are found throughout the world. Certain species of black fly are responsible for the transmission of various pathogens to both humans and livestock. The blood feeding and nuisance swarming behavior of some species can result in significant economic losses as well. Most black fly larvae are indiscriminate filter feeders that develop in running waters (Adler et al. 2004). Thus far the most successful strategy for suppressing problematic populations is to apply products that contain insecticidal crystalline proteins produced by *Bacillus thuringiensis* var. *israelensis* de Barjac (*Bti* ICPs) to the larval habitat (Barjac and Sutheland 1990).

Products containing *Bti* ICPs are the only commercial products available at this date for suppressing nuisance black fly populations in the United States. These products are known for the high degree of safety they exhibit to non-target organisms that are present in larval habitats (Gibbs et al. 1986, Hershey et al. 1998). The ICPs must be ingested by larvae for the protoxin to be converted to a toxin, and mortality follows soon after. Black fly suppression programs typically involve pre- and post-treatment evaluations of larval density and mortality to determine the success of the treatments. The post-treatment evaluation must be accurate to avoid additional and unnecessary application of larvicide to the environment.

Few studies have described the time needed for mortality to manifest in larvae after exposure to *Bti* ICPs. Back et al. (1985) found that the majority of larval mortality occurred within the first 3 h after exposure to a high label rate of ICPs, and that the final mortality could take over 24 h. Gaugler and Molloy (1980) evaluated larval mortality following exposure to ICPs for four days and found that 95% of the mortality occurred

within the first 24 h. Studies looking at the effects of *Bti* ICPs on larvae have evaluated mortality at 24 h (Lacoursiere and Charpentier 1988, Boisvert et al. 2002), 9 h (Car and De Moor 1984), and 6 h (Wilson et al. 2005). Iburg et al. (2011) and Overmyer et al. (2006) evaluated larval mortality after 5 h. These studies utilized various formulations containing *Bti* ICPs both in the field and in the laboratory. A more thorough understanding of larval mortality following exposure to *Bti* ICPs is necessary to standardize the time at which larval mortality is assessed.

We exposed *Simulium vittatum* larvae to various concentrations of *Bti* ICPs as well as high concentrations of ICPs in various media. Our first experiment was conducted to determine the length of time required for the onset and conclusion of mortality of larvae exposed to various concentrations of ICPs. This information is necessary for both a basic toxicological understanding of *Bti* ICPs and to aid larvicide applicators in conducting post-treatment evaluations. An additional experiment was conducted to determine the effects that the presence of clay or cellulose would have on larval mortality from ICPs. Clay material was used because Iburg et al. (2011) and Gray et al. (2012) found that clay material had no significant effect on larval mortality following exposure to *Bti* ICPs. Cellulose material was used because previous studies have demonstrated that this material can cause significant reductions in larval susceptibility to ICPs (Iburg et al. 2011). This material is not naturally occurring; however, the fibers are released as effluent by some paper-mills and Illesova (1988) found that the effluent can alter the feeding behavior of black fly larvae. The mortality of larvae from *Bti* ICPs in media containing either clay material or cellulose was characterized to determine if these components cause reductions and/or delays in mortality. The experimental design used in

the present study required that larvae be exposed to ICPs in a current for a set amount of time. Following the exposure, the larvae remained in media in the absence of a current until they were evaluated for mortality. *Bti* ICPs that were not ingested by larvae during the exposure time could have been present in the media. The final experiment was conducted to determine if larval mortality can be affected by ICPs in the absence of a current to test if this artifact of the experimental design could affect larval mortality.

## **Materials and Methods**

### Larvae

Late instar *S. vittatum* cytospecies 1S-7 larvae were used for all experiments. The larvae were colony reared as described by Gray and Noblet (1999). Larvae were removed from the rearing unit immediately prior to each experiment and placed in enameled pans with 1 liter of rearing unit water. Individual larvae were placed in exposure vessels with soft-tipped forceps.

### Medium

Moderately hard water was used as the basal medium in all experiments. This medium was prepared as described by APHA et al. (1995). Moderately hard water is defined by a hardness (as CaCO<sub>3</sub>) of 80-100 ppm (Weber 1993). Each medium was 20°C for the duration of each experiment.

### Bti ICPs

A commercial product containing *Bti* ICPs was obtained (Vectobac<sup>®</sup> 12AS *Bacillus thuringiensis* subsp. *israelensis*, strain AM 65-52, Valent BioSciences Corporation, Lot # 206-410-N9). This formulation is an aqueous suspension containing 11.61% fermentation solids and solubles and has a potency of 1200 International Toxic Units (ITU) mg<sup>-1</sup> (Oestergaard et al. 2007).

### Controlled Current Toxicity Test

The controlled current toxicity test described in Iburg et al. (2010) was used to evaluate larval mortality following exposure to *Bti* ICPs. Flat-bottom extraction flasks (250 ml) were filled with 195 ml of moderately hard water. Approximately 2,000 larvae were removed from a rearing tank containing the appropriate age larvae and placed in white enameled pans with 1 liter of rearing tank water. Thirty larvae were then transferred from the pans into each flask using soft-tipped forceps. The flasks were placed onto G10 Gyrotory<sup>®</sup> shakers (New Brunswick Scientific, Edison, NJ). The current speed was slowly increased in a stepwise fashion over a 1 h period producing a current that allows the larvae to attach to the sides of the flask and resume their normal feeding behavior. The final setting of the shaker was 150 rpm (0.32 x g). Following the 1 h acclimation period, 5 ml of *Bti* ICP solution was applied to the treated flasks. The control flasks received 5 ml of deionized water. The ICP mixture or control water was applied to each flask using syringes that had been vertically positioned above each flask. The syringes were held in place by a board with holes above each flask that allow the syringe needles to insert into each flask above the medium. A separate board was used to

simultaneously depress each syringe, emptying the contents into the flask. The current was maintained for 10 min and the shaker was then stopped.

### Experiment 1

Experiment 1 was conducted to determine larval mortality over time following exposure to multiple concentrations of *Bti* ICPs. First, 132 flasks were filled with 195 ml of moderately hard water. Thirty larvae were gently placed into each flask. The flasks were placed on the gyratory shakers and subjected to a 1- h acclimation to the current. The treated and control flasks then received either *Bti* ICPs or moderately hard water respectively. The larvae remained in a current for 10 additional min, and the shaker was turned off. Twenty flasks were used for the controls. Four of these flasks were removed every 2 h and the larvae were assessed for mortality. Twenty-four flasks received *Bti* ICPs at a concentration of 1.35 ITU ml<sup>-1</sup> in each flask. Four of these flasks were removed after 30 min, 4 flasks were removed after 1 h, and 4 more were removed after 2, 3, 4, and 5 h. Forty flasks received ICPs resulting in a concentration of 0.45 ITU ml<sup>-1</sup> in each flask. Four of these flasks were removed after 30 min, 4 flasks were removed after 1 h, and 4 more were removed hourly up to 9 h. The final 48 flasks received *Bti* ICPs at a concentration of 0.15 ITU ml<sup>-1</sup> in each flask. Four of the flasks were removed hourly for 12 h. Larvae from each flask were immediately assessed for mortality upon removal. The number of time points for each treatment at which larval mortality was analyzed in Experiment 1 was determined by preliminary experiments and available space on the gyratory shakers.

## Experiment 2

Experiment 2 was conducted to determine larval mortality over time when exposed to *Bti* ICPs in media containing various additives. Forty-eight flasks each received 195 ml of moderately hard water. Twenty-eight flasks each received 195 ml of moderately hard water containing 50 ppm of kaolinite clay (VWR International, Radnor, PA). Forty flasks each received 195 ml of moderately hard water containing 50 ppm of cellulose material (Solka-Floc®). The turbidity of the various media was measured in Nephelometric Turbidity Units (NTU) using a Hach Turbidimeter (Hach Company, Loveland, CO). Thirty late instar larvae were gently placed into each flask. The flasks were then placed onto the gyratory shakers and a current was initiated. The flasks received *Bti* ICPS or moderately hard water for the controls following a 1- h acclimation period. The treated flasks received ICPs at a concentration of 1.35 ITU ml<sup>-1</sup> in each flask. The shaker was turned off after a 10- min exposure. Twenty of the flasks containing moderately hard water received deionized water and served as the controls. Four of these flasks were removed every 2 h for 10 h. Twenty-eight flasks containing moderately hard water received ICPs. Four of these flasks were removed after 30 min and 4 were removed at 1 h and every subsequent h for a total of 6 h. Twenty-eight flasks containing medium with 50 ppm of kaolinite received *Bti* ICPs. Four of these flasks were removed after 30 min and 4 were removed at 1 h and every subsequent h for a total of 6 h. Forty flasks containing 50 ppm of cellulose received *Bti* ICPs. Four of these flasks were removed every h for a total of 10 h. Larvae from each flask were immediately assessed for mortality upon removal. The number of time points for each treatment at

which larval mortality was analyzed in Experiment 2 was determined by preliminary experiments and available space on the gyratory shakers.

### Experiment 3

This experiment was implemented to determine if larvae in the CCTT were able to consume a quantity of ICPs sufficient to produce mortality in the absence of a current. For the 4 treatments, 40 flasks containing 30 larvae and 195 ml of moderately hard water each were placed on a shaker. Twenty flasks received moderately hard water after the acclimation period. The other 20 flasks received *Bti* ICPs after the acclimation period. Upon receiving the *Bti* ICPs, the treated flasks contained 0.9 ITU of ICPs ml<sup>-1</sup>. The shaker was turned off after a 10 min exposure. Ten of the flasks that received deionized water and 10 of the treated flasks were rinsed after 10 min of exposure to ICPs to determine if rinsing larvae had an effect on the mortality. To rinse the flasks, the medium from each flask was carefully decanted and set aside. Moderately hard water was slowly added to the rinsed flasks and decanted, and then a final 200 ml of moderately hard water was added to and left in the flasks. Mortality was assessed after 5 h.

Three additional treatments were applied to 30 flasks on a second shaker. Ten flasks were filled with decanted medium from the flasks that had received *Bti* ICPs in the previous shaker. Thirty larvae were placed in each of these flasks and placed on the shaker. Ten flasks received the decanted medium from the control treatments on the previous shaker as well as 30 larvae each. The final 10 flasks were filled with moderately hard water, received 30 larvae each and were placed on the shaker. The larvae were then acclimated to the current by gradually increasing the shaker speed to 150 rpm during the

first 30 min. The shaker was turned off after an acclimation time of 1 h and 10 min. At this point the third set of 10 flasks received ICPs resulting in a concentration of 0.9 ITU ml<sup>-1</sup>. Mortality was evaluated after 5 h.

### Statistical Analysis

One-way analysis of variance with Tukey-Kramer multiple comparison tests were conducted to compare larval mortality over time and under various conditions. Normal distributions were assumed. Unpaired t-tests were used to compare two data points within or between treatments. GraphPad Prism® version 5.04 was used for all statistical analyses (GraphPad Software, San Diego, California USA, [www.graphpad.com](http://www.graphpad.com)).

## **Results**

### Experiment 1

The mortality of larvae following exposure to 3 concentrations of *Bti* ICPs was evaluated. No significant differences were found between the mortalities in the control group over the first 10 h of the experiment, and control mortality never exceeded 1% ( $F = 1.00$ ;  $df = 4, 15$ ;  $P = 0.4380$ , Figure 6.1). No significant differences were determined between larval mortality for the first h in all treatments that received ICPs ( $F = 2.22$ ;  $df = 4, 15$ ;  $P = 0.1158$ ). In the treatments that received 1.35 ITU ml<sup>-1</sup>, a significant difference developed between larval mortality in h 1 and h 2 ( $t = 5.39$ ;  $df = 1, 6$ ;  $P = 0.0017$ ) and h 2 and h 3 ( $t = 3.50$ ;  $df = 1, 6$ ;  $P = 0.0128$ ). There were no significant changes in larval mortality after h 3 ( $F = 0.28$ ;  $df = 2, 9$ ;  $P = 0.7629$ ). The mortality of larvae exposed to 1.35 ITU ml<sup>-1</sup> of ICPs reached a peak of 87.3% at 4 h.

The mortality of larvae exposed to 0.45 ITU ml<sup>-1</sup> was significantly different from those exposed to 1.35 ITU ml<sup>-1</sup> at h 2 ( $t = 5.05$ ;  $df = 1, 6$ ;  $P = 0.0023$ ). Larvae exposed to 0.45 ITU ml<sup>-1</sup> exhibited no significant differences in mortality after 6 h ( $F = 2.77$ ;  $df = 3, 12$ ;  $P = 0.0875$ ). The peak mortality of larvae exposed to this treatment was 83.3% at 8 h. No significant differences were seen between the mortality of larvae exposed to 1.35 ITU ml<sup>-1</sup> after 4 h and the mortality of larvae exposed to 0.45 ITU ml<sup>-1</sup> after 8 h ( $t = 1.09$ ;  $df = 1, 6$ ;  $P = 0.3168$ ).

The mortality of larvae exposed to 0.15 ITU ml<sup>-1</sup> gradually increased until h 9. After 9 h no significant changes in mortality were found ( $F = 0.50$ ;  $df = 3, 12$ ;  $P = 0.6893$ ). The peak mortality of larvae that received this treatment was 62.4% occurring at 9 h post-exposure. This peak mortality was significantly different from the peak mortalities of larvae exposed to 1.35 and 0.45 ITU ml<sup>-1</sup> ( $t = 5.481$ ;  $df = 1, 6$ ;  $P = 0.0015$ ), ( $t = 4.04$ ;  $df = 1, 6$ ;  $P = 0.0068$ ). The results are also plotted in two ways revealing the percentage mortality of larvae at each time point as well as the change in mortality at each time point as compared to the previous one (Figure 6.2).

## Experiment 2

Larvae were exposed to various medium additives followed by exposure to *Bti* ICPs, and mortality was evaluated over time. The change in mortality between larvae exposed to ICPs in water-only and those in medium containing clay in the first 30 min could not be determined with a t-test as all replicates of larvae in kaolinite at this time point had the same value of 0%. A significant difference was seen between mortality of larvae in water-only and those in medium containing clay at both h1 and h 2 ( $t = 6.198$ ;

df = 1, 6;  $P = 0.0008$ ), ( $t = 3.140$ ; df = 1, 6;  $P = 0.0201$ , Figure 6.3). Larvae exposed to ICPs in water-only or medium containing clay exhibited no change in mortality after 4 h ( $F = 2.759$ ; df = 5, 18;  $P = 0.0510$ ). The peak mortality of larvae in water-only occurred at 6 h (97.2%) and in medium containing clay at 6 h (97.2%).

No significant differences were found between the mortality of larvae exposed to ICPs in medium containing cellulose for the first 3 h ( $F = 3.317$ ; df = 2, 9;  $P = 0.0833$ ). A significant difference in mortality was found at h 4 as compared to h 3 ( $t = 3.861$ ; df = 1, 6;  $P = 0.0084$ ). Larvae exposed to ICPs in medium containing cellulose exhibited no significant difference in mortality during h 4 through 6 ( $F = 1.152$ ; df = 2, 9;  $P = 0.3584$ ). A significant increase in mortality was determined from h 6 to h 7 ( $t = 3.524$ ; df = 1, 6;  $P = 0.0125$ ). No significant differences were found in mortality from h 7 to h 10 ( $F = 2.268$ ; df = 3, 12;  $P = 0.1329$ ). The peak mortality of larvae in this treatment occurred at h 9 (52.9%). The peak mortality of larvae exposed to ICPs in medium containing cellulose was significantly different from the peak mortality of larvae in medium containing water only ( $t = 7.822$ ; df = 1, 6;  $P = 0.0002$ ) and larvae exposed to ICPs in medium containing clay ( $t = 7.203$ ; df = 1, 6;  $P = 0.0004$ ). The results are also plotted in two ways revealing the percentage mortality of larvae at each time point as well as the change in mortality at each time point as compared to the previous one (Figure 6.4)

### Experiment 3

Experiment 3 was conducted to test the artifact from the first two experiments in which larvae were left in the medium until evaluated for mortality. No significant differences were found between the mortality of larvae in the control medium, the control

medium after rinsing, in previously used control medium, in medium that had contained ICPs prior to a 10 min exposure to larvae, or medium that had ICPs added after the cessation of the current ( $F = 0.79$ ;  $df = 4, 45$ ;  $P = 0.5300$ , Table 6.1). Larvae exposed to *Bti* ICPs for 10 min in a current exhibited 86.5% mortality after 5 h. Larvae that had received the same concentration of ICPs followed by a rinsing of the flasks and the introduction of new medium exhibited a slightly higher mortality (91.3%) as compared to the larvae that had not been rinsed after exposure to ICPs (86.5%). This difference was considered significant when using a Tukey-Kramer post-hoc test following an analysis of variance between all treatments ( $q = 4.68$ ; Mean diff= -4.89,  $p < 0.05$ ). A t-test between the two ICP treatments did not reveal a significant difference ( $t = 1.94$ ;  $df = 1, 18$ ;  $P = 0.0690$ ).

## Discussion

Experiment 1 was conducted to determine the time until maximum mortality of *Simulium vittatum* larvae following exposure to various concentrations of insecticidal crystalline proteins produced by *Bacillus thuringiensis* subsp. *israelensis* (*Bti* ICPs). To achieve the high concentration of ICPs ( $1.35 \text{ ITU ml}^{-1}$ ), 1.125 ppm of larvicide product was used. The larvae were exposed to this concentration for 10 min, which is the equivalent of an application of 11.25 ppm of the product for 1 min. This is a concentration that is suggested on the product label. Larvae exposed to this concentration achieved the highest observed mortality 4h after exposure. Results from this experiment indicate that larvae exposed to 1/3 the concentration of *Bti* ICPs ( $0.45 \text{ ITU ml}^{-1}$ ) exhibited peak mortality that is not significantly different from larvae exposed to the

higher dose; however, the peak mortality did not manifest until 8 h after exposure. Larvae exposed to 0.15 ITU ml<sup>-1</sup> of ICPs exhibited a slow increase in mortality until 9 h had passed. The peak mortality of larvae exposed to this treatment was significantly lower than with the other two treatments.

In Experiment 2, the mortality of larvae following exposure to *Bti* ICPs was influenced by the presence of cellulose in the larval medium. The mortality of larvae in medium containing clay was significantly lower from those in moderately hard water following 2 h of exposure; however, the mortality was not significantly different between larvae in each of these media at exposure times  $\geq 3$  h. The medium containing kaolinite clay had a turbidity of 40.8 NTUs. Cellulose material in the medium caused both a reduction in final mortality and an increase in time needed to achieve this mortality. The medium containing cellulose had a turbidity of 6.5 NTUs. These results are important because the turbidity of the water in the larval habitat is usually considered before application of larvicides that contain *Bti* ICPs. Pesticide applicators are instructed on the product label to apply the larvicide when the turbidity in a river is low. While this may increase efficacy in some cases, results from Experiment 3 and observations made by Iburg et al. (2011) suggested that the components of the seston affect larvicide efficacy more than the turbidity of the water.

Kaolinite and cellulose were used in this study based upon previous observations. Kaolinite clay is naturally occurring in many waterways. Iburg et al. (2011) found that larval susceptibility to *Bti* ICPs was not significantly influenced by the presence of clay in the larval medium. Gray et al. (2012) also found that larval susceptibility to *Bti* ICPs was not influenced by clay material in the waterway. The cellulose material (Solka-

Floc<sup>®</sup>) used in the present study consists of small cellulose fibers that may not approximate naturally occurring materials; however, they are similar to fibers released into waterways as part of the secondary fiber waste water from paper mills. Illesova (1988) observed *Simulium* larvae retracting their cephalic fans and ceasing to feed after exposure to secondary fiber waste water from paper mills. Clay and cellulose were used in the present study to demonstrate the differences in larval susceptibility to ICPs resulting from prior exposure to these materials.

The biology of black fly larvae suggests that a current is required for the larvae to capture particles in their cephalic fans. Results from Experiment 3 demonstrate that in the absence of a current *S. vittatum* larvae are not able to capture a sufficient number of ICPs to cause significant mortality. We observed larvae attempting to flick their cephalic fans in the absence of a current. This suggests that a larva could possibly capture particles in its immediate vicinity; however no evidence was found that this would be sufficient to increase mortality. Results from Experiment 3 also indicate that the larvae were able to remove enough *Bti* ICPs from the medium containing 0.9 ITU ml<sup>-1</sup> of *Bti* ICPS in 10 min that any remaining concentration in the medium was not toxic towards additional larvae. Interestingly, a significant increase in the mortality of larvae exposed to ICPs was determined when the larvae were rinsed as opposed to the exposed larvae that were not rinsed. The increase in mortality indicates that mortality can be affected by the handling of the larvae. Larvae were possibly able to ingest additional ICPs while medium was being poured from the flasks.

An interesting pattern occurred in 3 of the treatments when evaluating the hourly changes in larval mortality (Figures 6.3, 6.4) . Larvae exposed to the two lower

concentrations of ICPs in Experiment 1 (Figure 6.2B and C) exhibited an initial rapid increase followed by a gradual increase and then a second rapid increase in mortality. Larvae in Experiment 2 exposed to the high concentration of ICPs in the presence of cellulose material exhibited the same pattern (Figure 6.4C). Multiple possible explanations can be made for these observations. The patterns could have been caused by the position of the larvae in the flat bottom flasks. Larvae attached to the bottom of the flasks experienced different current conditions than those attached to the sides of the flasks. This could have resulted in some larvae consuming a lower quantity of ICPs and perhaps mortality was delayed in these larvae. Larvae of different sexes or developmental states, such as those that recently molted or were preparing to molt, could have been feeding at different rates resulting in a delay in mortality in a portion of the larvae. The ICPs produced by *Bacillus thuringiensis* subsp. *israelensis* are comprised of both CYT and CRY proteins. The CYT proteins act synergistically with the CRY proteins (Ferreira and Silva-Filha 2013) and larvae possibly consumed various ratios of these proteins thereby causing differences in the time it takes to achieve mortality.

The observations from Experiments 1 and 2 occurred under standardized laboratory conditions. A field application of products containing *Bti* ICPs would require additional considerations. While the final mortality of larvae exposed to 0.45 ITU ml<sup>-1</sup> was the same as the mortality of larvae exposed to the higher concentration, the two concentrations may not achieve control over the same distances in the larval habitat. Larvae further downstream from the application point are exposed to lower concentrations of the larvicide, and high concentrations may be necessary to achieve ideal suppression over long distances. Future studies are necessary to determine the

effect of using lower concentrations in regard to the transport of the formulation in a larval habitat.

Results from Experiments 1 and 2 indicate that low concentrations of ICPs or cellulose in the larval medium can delay the time required to achieve maximum mortality. This complicates the task of conducting a post-treatment evaluation. Applicators in operational settings could wait to evaluate mortality for 8 or more h following an application; however, they would then have to take into account larval drift. Bach et al. (1985) observed 14-22% detachment of larvae at 6 h post-treatment. Car and De Moor (1984) noticed a 60-fold increase in larval drift at 43 min following treatment. One possible solution would be to deploy drift-nets prior to treatment as was done by Back et al. (1985) and Boisvert et al. (2002) to aid in post-treatment evaluations. An alternative would be to collect larvae from the habitat as soon as the river had carried the larvicide past all collection sites. The larvae could be placed in beakers with a supply of oxygen and the mortality evaluated after 24 h. Beakers containing larvae from upstream of the treatment site could be collected in the same manner and used as controls. This technique has been used with success by Gray et al. (1996).

The highest concentration of *Bti* ICPs used in this experiment is similar to that routinely used by larvicide applicators. The results from this study indicate that similar larval mortality can be observed using 1/3 the concentration of *Bti* ICPs if the mortality is evaluated 4 h later. Future experiments are required to determine the effects of these concentrations in the field; however, these results could be used to make more economical decisions regarding application rates. A reduction in application rates could also reduce unnecessary application of larvicide to the environment. Results also indicate

that seston components can delay and reduce the efficacy of *Bti* ICPs. This suggests that a delay in post-treatment evaluations of mortality may be prudent regardless of the concentration used. In this case the use of drift-nets or the removal larvae from the river may be necessary and the mortality should be assessed at a later time.

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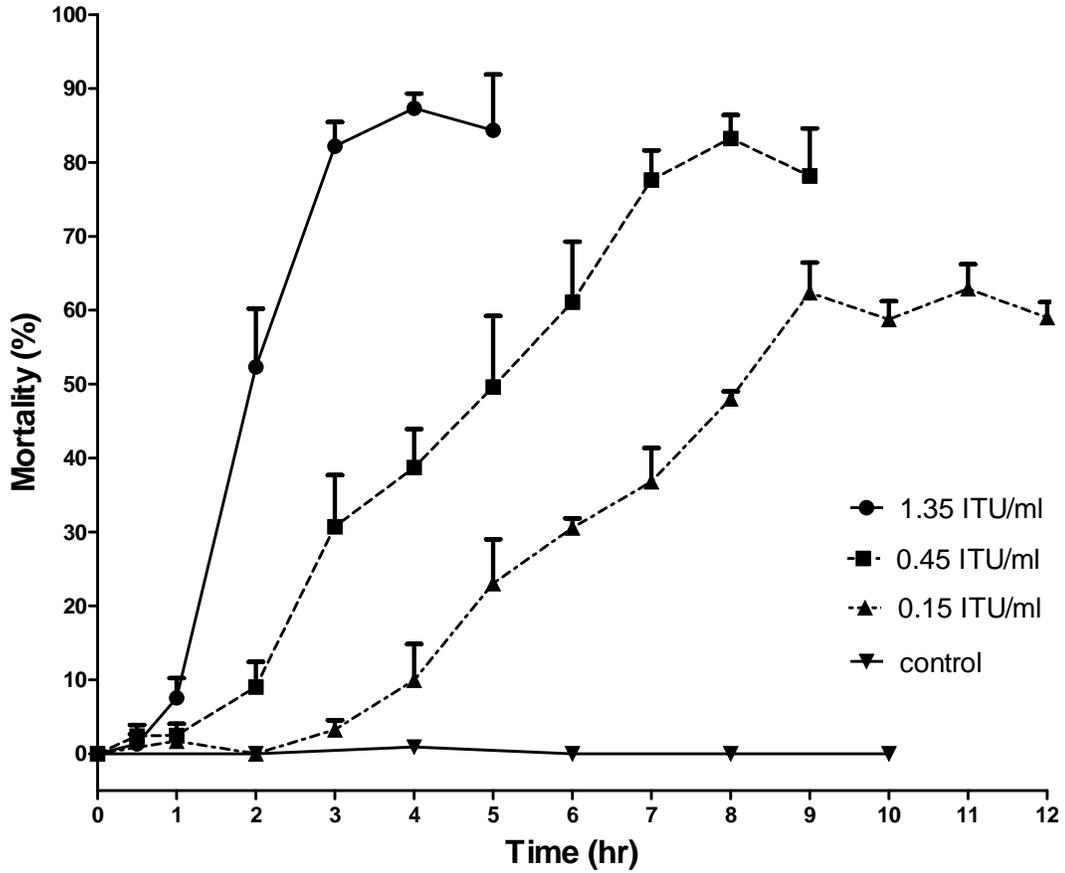
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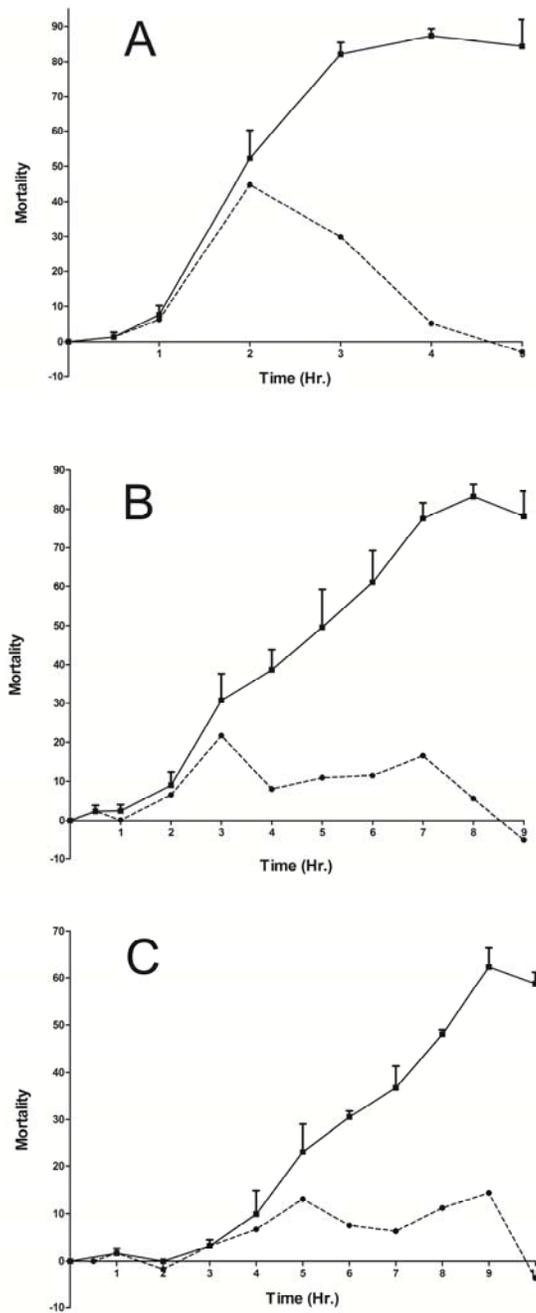
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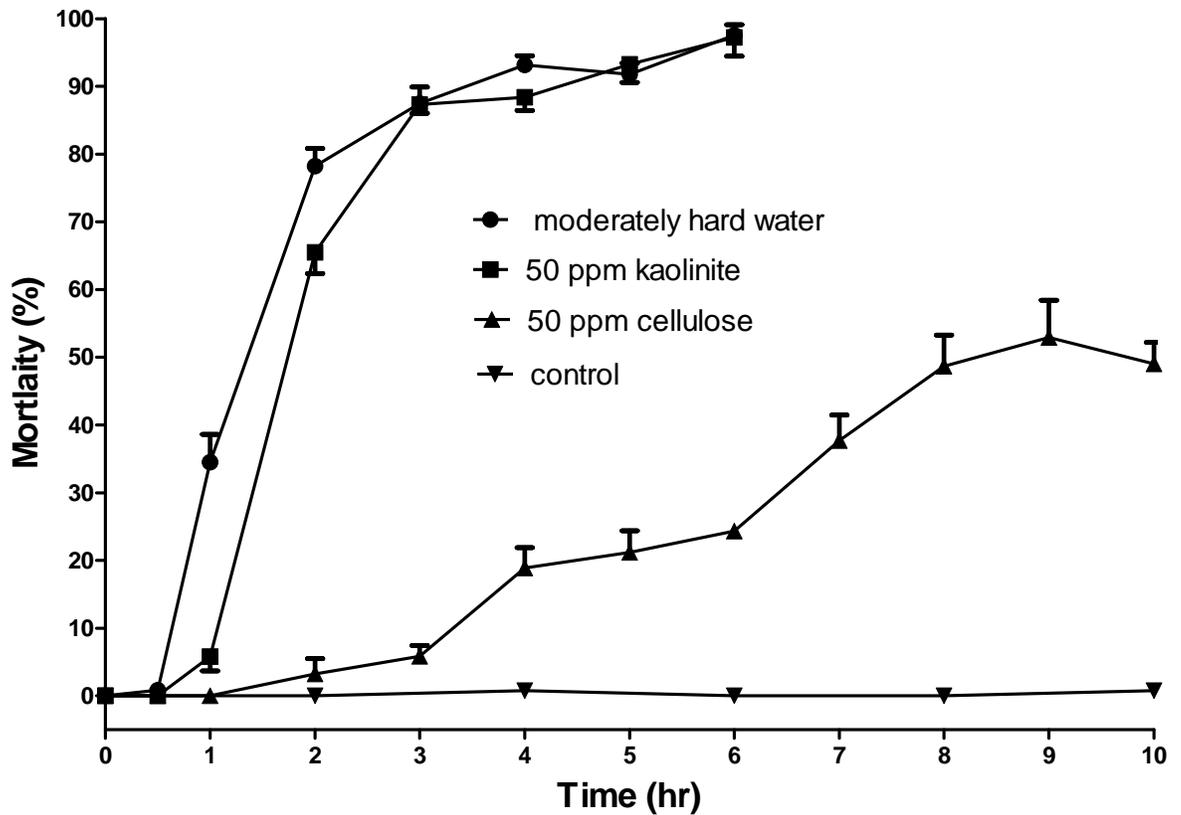
## Tables and Figures



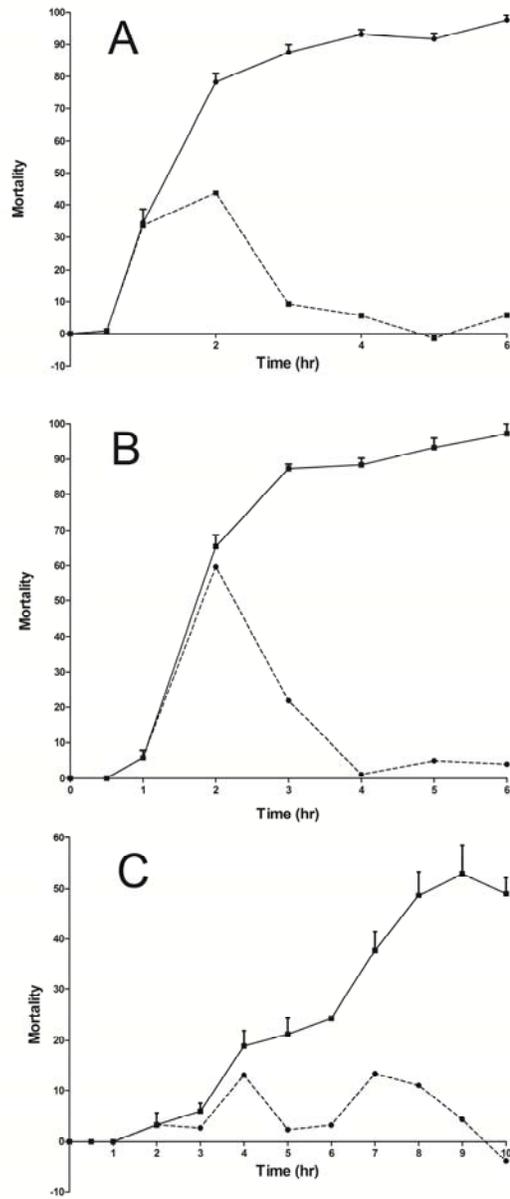
**Figure 6.1.** Mortality of *Simulium vittatum* larvae following exposure to various concentrations of insecticidal crystalline proteins produced by *Bacillus thuringiensis* subsp. *israelensis*. Bars above data points represent the SEM. Relevant statistical analyses are provided in the results.



**Figure 6.2.** The mortality of *Simulium vittatum* larvae following exposure to insecticidal crystalline proteins produced by *Bacillus thuringiensis* subsp. *israelensis*. (A) Larvae were exposed to 1.35 ITU ml<sup>-1</sup>. (B) Larvae were exposed to 0.45 ITU ml<sup>-1</sup>. (C) Larvae were exposed to 0.15 ITU ml<sup>-1</sup>. The solid lines represent the mean mortality (%) of larvae at each data point, the bars represent the SEM. The dotted line depicts the change in mortality at every data point from the previous one.



**Figure 6.3.** Mortality of *Simulium vittatum* larvae following exposure to insecticidal crystalline proteins produced by *Bacillus thuringiensis* subsp. *israelensis* (*Bti* ICPs). The concentration of *Bti* ICPs used in all treatments was  $1.35 \text{ ITU ml}^{-1}$ . Bars above or below data points represent the SEM. Relevant statistical analyses are available in the results. The medium containing clay had a turbidity of 40.8 NTU. The medium containing cellulose had a turbidity of 6.5 NTU.



**Figure 6.4.** The mortality of *Simulium vittatum* larvae in various media following exposure to insecticidal crystalline proteins produced by *Bacillus thuringiensis* subsp. *israelensis* (*Bti* ICPs). Larvae in all treatments were exposed to a concentration of 1.35 ITU ml<sup>-1</sup> for 10 min. (A) Larvae were exposed to ICPs in moderately hard water. (B) Larvae were exposed to ICPs in media containing 50 ppm clay. (C) Larvae were exposed to ICPs in media containing 50 ppm cellulose. The solid lines represent the mean mortality (%) of larvae at each data point, the bars represent the SEM. The dotted line depicts the percentage change in mortality at every data point from the previous one.

**Table 6.1.** Mortality of *Simulium vittatum* larvae 5 h after exposure to various media and current conditions. The concentration of insecticidal crystalline proteins produced by *Bacillus thuringiensis* subsp. *israelensis* (*Bti* ICPs) in exposed treatments was 0.9 ITU ml<sup>-1</sup>.

Treatment	Mortality (%)
Control	1.0 ± 0.7 A
Control Rinsed <sup>1</sup>	1.3 ± 0.5 A
<i>Bti</i> ICPs	86.5 ± 2.0 B
<i>Bti</i> ICPs Rinsed <sup>1</sup>	91.3 ± 1.5 C
Decanted control medium <sup>2</sup>	0.3 ± 0.3 A
Decanted exposure medium <sup>2</sup>	1.0 ± 0.5 A
<i>Bti</i> ICPs No current <sup>3</sup>	0.3 ± 0.3 A

Values (mean ± SEM) followed by the same letter are not significantly different ( $P \geq 0.05$ , ANOVA, Tukey post-hoc).

<sup>1</sup> Larvae in treatments 2 and 4 were acclimated to a current and exposed to the control water or *Bti* ICPs for 10 min. The medium was decanted from the flasks, rinsed once, and replaced with fresh control medium.

<sup>2</sup> Larvae were acclimated in the media that was decanted from treatments 2 and 4.

<sup>3</sup> Larvae were acclimated to a current to allow attachment to the flasks. The current was then stopped prior to the addition of *Bti* ICPs.

## CHAPTER 7

### CONCLUSIONS

Results from Chapter 2 indicate that a combination of antibiotics, at environmentally relevant concentrations, have no impact on the efficacy of insecticidal crystalline proteins produced by *Bacillus thuringiensis* subsp. *israelensis* (*Bti* ICPs) toward black fly larvae. The results of this investigation reveal that neither enrofloxacin, tylosin, or SMZ:TMP at *in vitro* concentrations up to 10,000- 80,000 times that observed in anthropogenic contamination possess the ability to impair the larvicidal activity of *Bti* ICPs. Exposure of black fly larvae to enrofloxacin did not have any detectable effect upon the susceptibility of black fly larvae to *Bti* toxins. Conversely, when black fly larvae were exposed to tylosin or SMZ:TMP prior to exposure to *Bti* ICPs, an enhancement of *Bti* ICP efficacy was observed. Exposure of black fly larvae to SMZ:TMP resulted in a marked enhancement in the susceptibility of the larvae to *Bti* ICPs.

Chapter 3 describes experiments in which river water was collected from a site where there have been occasional reductions in the activity of *Bti* ICPs towards black fly larvae. *Simulium vittatum* Zetterstedt larvae were found to be less susceptible to *Bti* ICPs due to the seston and not the dissolved minerals in the water. The Susquehanna River receives multiple discharges from sewage treatment plants, industrial waste, and acid mine drainage above the locations where our water samples were collected. These conditions could lead to excessive blooms of problematic diatom species. Diatoms and diatom frustules impaired the susceptibility of larvae to *Bti* ICPs.

The spectrophotometric technique described in Chapter 4 can be used to study the feeding behavior of black fly larvae in an environment containing problematic seston components. The addition of kaolinite to the larval medium resulted in a small decrease in Neon Red particle (NRP) ingestion, but not a decrease in *Bti* ICP activity. A direct assay would be necessary to determine the effects of a medium additive on *Bti* ICP induced mortality. When medium additives cause a decrease in *Bti* ICP activity, the spectrophotometric assay is a useful tool for determining if changes in feeding behavior were the cause of the decreased activity.

The results presented in Chapter 5 indicate that the particle sizes of materials suspended in the waterway appears to be related to the flick rate exhibited by larvae and their ability to capture smaller particles, such as NRP or *Bti* ICPs. The particles of clay and *Chlorella* cells were below 5 $\mu$ m in diameter and these materials did not significantly interfere with flick rate or capture of NRP. The cellulose fibers, *Cyclotella* cells, and *Scenedesmus* colonies were all larger than 10  $\mu$ m in diameter and each material had a significant effect on flick rate and NRP ingestion. Our results indicate that the large particles cause larvae to slow their flick rate and possibly block smaller particles from being captured by the cephalic fans. This slowing of flick rate may occur because it takes longer for the larger materials to be moved through the fore- and midgut. An exception to this would be the diatom frustules which were smaller than all other materials tested, present at concentrations that were over 12 x less that of the other dry materials, yet they caused the most severe reduction in flick rate and NRP ingestion. Our hypothesis is that the abrasive nature of this material to insect cuticle is responsible for this activity. It would be prudent for applicators of products containing *Bti* ICPs in operational setting to

minimize applications when large blooms of diatoms are in the waterway as the frustules are also likely to be present.

The highest concentration of *Bti* ICPs used in the experiments described in Chapter 6 is similar to that routinely used by larvicide applicators. The results from this study indicate that similar larval mortality can be observed using 1/3 the concentration of *Bti* ICPs if the mortality is evaluated 4 h later. Future experiments are required to determine the effects of these concentrations in the field; however, these results could be used to make more economical decisions regarding application rates. A reduction in application rates could also reduce unnecessary application of larvicide to the environment. Results also indicate that seston components can delay and reduce the efficacy of *Bti* ICPs. This suggests that a post-treatment evaluation of mortality should be delayed regardless of the concentration used. In this case the use drift-nets or the removal of larvae from the river may be necessary followed by an assessment of mortality at a later time