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EFFECTS OF ALGAE ON THE EFFICACY OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* AGAINST LARVAL BLACK FLIES

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ABSTRACT. Personnel from several black fly control programs have reported that the efficacy of *Bacillus thuringiensis* var. *israelesis* (*Bti*) is reduced during periods when algal concentrations are high in the waterways. Although the reduction in *Bti*-induced mortality in black fly larvae is presumed to be related to the presence of algae, no scientific data support this theory. In this study, 4 genera of algae (*Microcytis, Scenedesmus, Dictyosphaerium,* and *Chlorella*) commonly detected in Pennsylvania rivers where *Bti*-induced mortality in black fly larvae has been reduced were assessed to determine their respective effects on *Bti*-induced mortality by using an orbital shaker bioassay with laboratory-reared black fly larvae (*Simulium vittatum* cytospecies IS-7). A significant reduction in *Bti*-induced mortality was observed when *Scenedesmus* was present in the flasks at concentrations ≥ 16,000 cells/ml. The *Bti*-induced mortality of larvae was not significantly reduced when *Chlorella, Dictyosphaerium,* or *Microcytis* was present in the flasks, even at concentrations ≥ 250,000 cells/ml. These results indicate that the presence of certain types of algae can reduce the mortality of black flies exposed to *Bti*. Although not clearly defined, the mechanisms involved may be related to algal morphology due to overall size and structures associated with certain types of algae, and possible interference with feeding.

KEY WORDS *Bti* efficacy, *Bacillus thuringiensis* var. *israelensis,* black fly control, *Simulium vittatum,* algae

INTRODUCTION

Black flies (Diptera: Simuliidae) are serious bloodfeeding pests of humans, birds, and other animals. Black flies cause immense agitation by their persistent swarming, crawling, and biting behavior (Burnham 1998). In addition, they are the vector causal agents of disease in humans and other animals throughout the world (Fredeen 1977). Thus, effective control of black flies is needed to reduce vector and nuisance populations.

Bacillus thuringiensis var. *israelensis* (De Barjac) (*Bti*) has proven to be highly effective in black fly treatment and control programs around the world (Margalit 1990). Black fly larvae filter *Bti* particles from the water column with their cephalic fans. After ingestion, the *Bti* is solubilized in the midgut and releases delta-endotoxins (Gill et al. 1992). Proteases in the midgut activate the toxins, which then interact with the midgut epithelium causing the cells to swell and burst due to the influx of ions through toxin-induced channels (Gill et al. 1992, Lambert and Perferoen 1992). Larval mortality soon follows. Although *Bti* provides excellent control for larval simuliids, several biotic and abiotic factors may affect the efficacy of *Bti.* Discharge, stream profile, turbidity, pollutants, water temperature, pH, degree of vertical mixing in the water column, and adsorption of *Bti* to benthic substrates or sediments can affect efficacy and disperson of *Bti* (Molloy 1990). Other factors may include turbulence, density of filter-feeding organisms, larval feeding behavior, larval instar, and presence of seston (Gaugler and Molloy 1980, Molloy 1990, Coupland 1993).

Personnel from black fly control programs in Pennsylvania and West Africa (Arbegast, Pennsylvania Department of Environmental Protection [DEP], personal communication; Kurtak, World Health Organization [retired], personal communication) have reported that the level of *Bti*-induced mortality in black fly larvae is reduced at times when high algae concentrations are detected in the waterways. Although reduced mortality was presumably related to the increased algal concentration in the water, no scientific evidence exists that these 2 situations are correlated. However, limited data provided from the Pennsylvania DEP indicate that although similar species of algae are present during periods of both good and poor efficacy, substantially higher cell counts are common when poor efficacy is observed. Thus, it was our objective to determine if algae have a negative effect on *Bti*-induced mortality and if so, what types of algae are responsible. In this study, 4 genera of algae (*Microcystis, Scenedesmus, Dictyosphaerium,* and *Chlorella*) were screened by using an orbital shaker bioassay to determine their respective effects on *Bti*-induced mortality in laboratory-reared larval *Simulium vittatum* Zetterstedt cytospecies IS-7. Algae were chosen based on their presence in Pennsylvania waterways when reduced black fly mortality after application of *Bti* was reported, commercial availability, and physical characteristics.

MATERIALS AND METHODS

Algae: Four types of algae were used in this study: a blue-green alga (*Microcystis*), and 3 green algae (*Scenedesmus, Dictyosphaerium,* and *Chlorella*). *Microcystis* has a mucilaginous sheath surrounding each spherical cell. *Microcystis* exists in colonies with millions of cells per milliliter in the

stock culture. Each individual cell has a length of approximately 5 μm; however, because of their existence in colonies, the colony length is approximately 50 μm (Graham and Wilcox 2000). *Scenedesmus* exists in cylindrical colonies consisting of 4 or more cigar-shaped cells adjoined with terminating spines on the corners. The spines are thought to give protection to the colonies by their general morphology and by increasing the overall size of each colony. The length of each *Scenedesmus* cell is approximately 25 μm. Therefore, a typical colony has a length of approximately 100 μm (Graham and Wilcox 2000). *Dictyosphaerium* is colonial, usually with a gelatinous coat. Four daughter cells are connected with filaments derived from their maternal cell wall. Cells are small, ranging from 3 to 10 μm in diameter, and have 1 or 2 parietal chloroplasts. The cell body is spherical, elliptical, or heart-shaped (Graham and Wilcox 2000). *Chlorella* is spherical in shape and the size of each cell is between 2 and 12 μm in diameter. Under favorable growth conditions, *Chlorella* can multiply rapidly. The complete reproduction cycle can take less than 24 h (Bold and Wynne 1985).

Cultures of algae were obtained from commercial sources (*Microcystis* and *Dictyosphaerium* from Carolina Science and Math, Burlington, NC; *Scenedesmus* and *Chlorella* from Ward's Biology, Rochester, NY). Cultures were maintained by mixing commercial aliquots with 1 liters of freshwater algae culture medium (Ward's Biology) with mechanical agitation and sterile techniques. To prepare suspensions for experiments, cell densities per milliliter were determined with a hemacytometer at 200× and appropriate dilutions were made with distilled water.

Bti: The *Bti* formulation used in all trials was VectoBac® 12AS (lot 65-841-BA, Valent Biosciences, North Chicago, IL). The formulation was stored at 4°C and stock solutions were prepared at the beginning of each experiment by weighing 3 g of the liquid formulation on a plastic weigh-boat with an AB204 Mettler Toledo analytical balance (Hightstown, NJ). The *Bti* was rinsed into a 125-ml beaker bottle with 100 ml of distilled water and shaken with a Burrell wrist-action shaker (Pittsburgh, PA) for 20 min to ensure that the stock solution was homogenous. All dilutions used on the day of the experiment were prepared from this original stock. Dilutions for each trial were prepared by adding the appropriate amount of the *Bti* stock solution to distilled water to produce the desired concentration. The concentration of *Bti* used for these experiments was determined by baseline median lethal concentrations obtained from laboratory bioassays that tested potencies of various *Bti* formulations during the course of this research. Concentrations of *Bti* used in these experiments were between 0.5 and 0.75 ppm.

Test organisms: The test organisms used in this study were 6th- to 7th-instar *S. vittatum* IS-7 reared in colony in the laboratory at the University of Georgia (Gray and Noblet 1999). This colony was established with eggs collected in Flaxmill Brook north of Cambridge, NY, in September 1981. The rearing tanks function as artificial streams, with each equipped with a runway capable of producing >100,000 larvae, and were scheduled to ensure that 1 runway had larvae at the appropriate stage of development each week.

Orbital shaker bioassay: Strips of nylon screen with larvae attached were removed from the runway of the rearing tank and placed into an enamel pan containing approximately 1 liter of water from the rearing tank. Thirty larvae were carefully transferred with soft-tipped forceps into round, flat-bottomed, 250-ml extraction flasks containing 170 ml of bioassay water. Bioassay water was prepared by adding black fly food material (1:1 homogenized rabbit chow and soybean meal washed through a 53-μm sieve) to distilled water in sufficient amounts to produce a turbidity of 4.5–5.5 nephelometric turbidity units. Mean water temperature ± standard error during the experiments was 21.5 ± 0.5°C. A 25-ml aliquot of the appropriate algal dilution was added to the experimental flasks. Control flasks received 25 ml of bioassay water. The flasks were placed on a New Brunswick Scientific G-10 Gyratory® shaker (Edison, NJ). The larvae were acclimated for 1 h starting at 100 rpm and gradually increased to 150 rpm. The action of the orbital shaker creates a current in the flask simulating stream flow that allows the larvae to select an optimal attachment site and subsequently to filter and ingest the algae and *Bti* particles. During the acclimation period, 10-ml syringes with 20-gauge needles (Becton Dickinson, Franklin Lakes, NJ) were filled with 5 ml of *Bti* or bioassay water (controls) and inserted into a dosing board that was supported above each shaker by an aluminum-rod frame. The dosing board was designed with the syringe insertion hole centered over the opening of each flask so the syringe did not hit the wall of the flask during dosing and in the normal rotation of the orbital shaker. After the 1-h acclimation period, the flasks were dosed in unison by using a flat board to depress the syringe plungers, bringing the final volume in the flasks to 200 ml. After a 10-min exposure period, the shaker was turned off and flasks were held at room temperature for 5 h for mortality assessment. Contents of the flasks were poured into white enamel pans; larvae that did not respond to a gentle probing or were unable to attach to the bottom of the pan were considered dead.

Each test consisted of 4 treatments, a standard control (no *Bti* or algae added), an algal control (highest concentration of algae but no *Bti* added), a standard (*Bti* but no algae added), and an algal treatment (algae and *Bti* added) at several concentrations. The algal concentrations used were based on the cell counts of the stock cultures. The stock culture of the 4 genera of algae were: *Microcystis,*

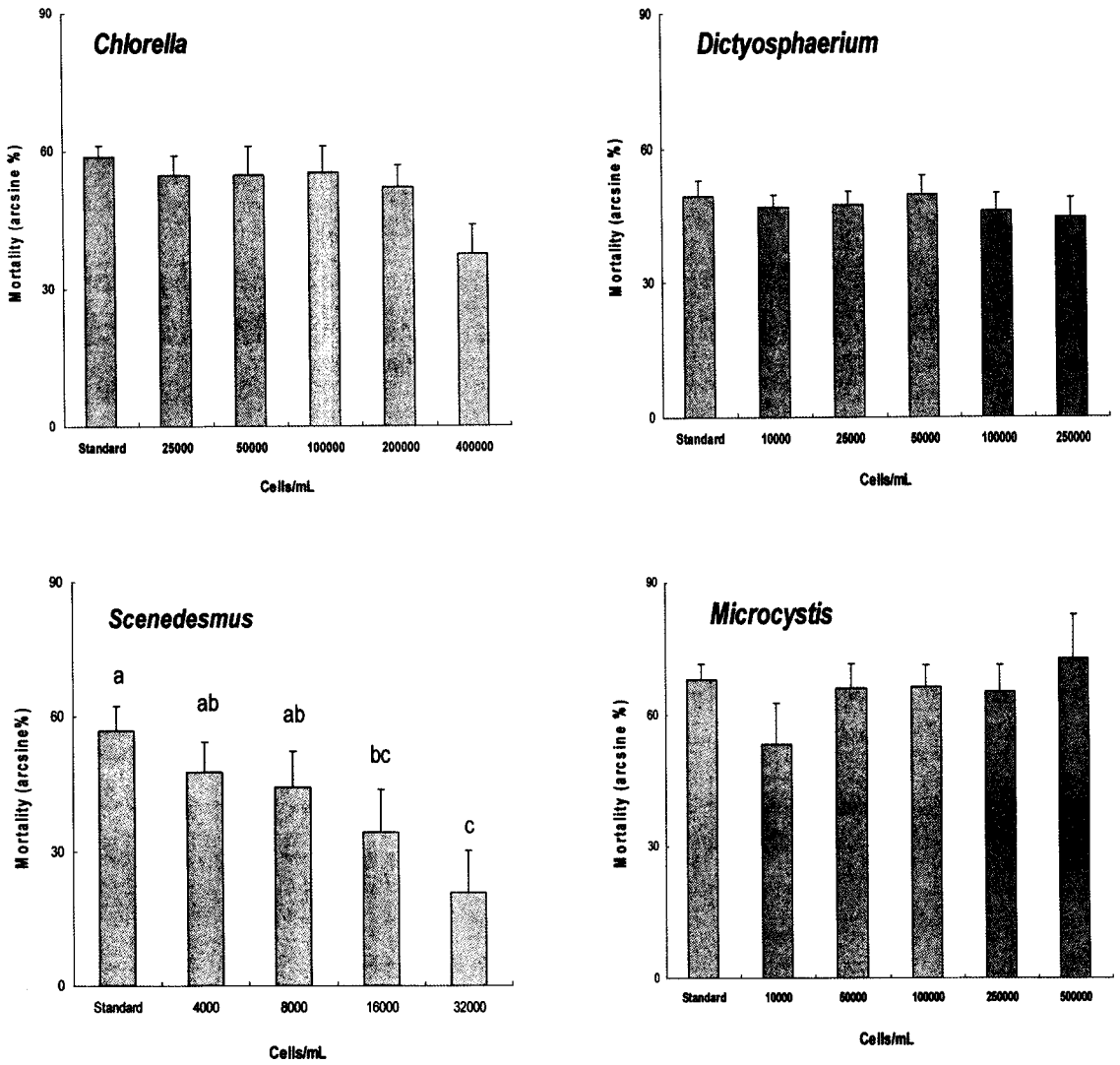

Fig. 1. Mean mortality ± standard error of larval *Simulium vittatum* IS-7 exposed to *Bacillus thuringiensis* var. *israelensis* and varying concentrations of algae. Mortality data were arcsine transformed before statistical analysis. Columns with different letters are significantly different ($\alpha = 0.05$).

approximately 2,000,000 cells/ml; *Scenedesmus,* approximately 100,000 cells/ml; *Dictyosphaerium,* approximately 2,000,000 cells/ml; and *Chlorella,* approximately 2,000,000 cells/ml. Five flasks per algal concentration, control, and standard were used for each experiment. Experiments with each genus of algae were replicated a minimum of 3 times.

Data analysis: Each trial compared percent mortality of the standard treatment to percent mortality of the algal treatments. Mortality data were adjusted by using Abbott's formula (Abbott 1925). Flasks with algae were adjusted with algal control mortality and the standard was adjusted with mortality data from the standard controls. Because percentages were used, transformation of the data was required. The data were transformed by taking the arcsine of the square root of the observed percent mortality. The data were analyzed for normality and homogeneity of variance with a Shapiro–Wilk's test and Bartlett's test, respectively, by using JMP SAS (SAS Institute, Cary, NC). A 1-way analysis of variance was used to determine statistical significance, followed by a Tukey studentized range test to determine differences among treatment means ($\alpha = 0.05$).

RESULTS

Control mortality was <3% for all trials. Of the 4 genera of algae used in this study, only the green alga *Scenedesmus* significantly reduced the efficacy of *Bti* ($F = 19.76$; df = 4,10; $P = 0.0003$; Fig. 1).

Larval mortality was significantly reduced when concentrations of *Scenedesmus* in the flasks were ≥16,000 cells/ml. The blue-green alga *Microcytis* ($F = 0.92$; df = 5,12; $P = 0.4853$) and the green algae *Chlorella* ($F = 2.78$; df = 5,12; $P = 0.0541$) and *Dictyosphaerium* ($F = 0.17$; df = 5,12; $P = 0.9685$) had no effect on *Bti* efficacy even at concentrations ≥ 250,000 cells/ml (Fig. 1).

DISCUSSION

Reduced mortality in black fly larvae after treatment with *Bti* during the warm summer months has plagued several black fly control programs around the world for many years. Technical personnel in these programs believed that increased algal concentrations might be causing this phenomenon. However, no scientific data were available to support this theory. Results of this study showed that the presence of algae can reduce black fly larval mortality and that the effects of algae on mortality are specific to the type of algae present.

Although we have demonstrated that algae can significantly reduce mortality of black fly larvae exposed to *Bti,* we have not clearly defined the mechanism. Gaugler and Molloy (1980) and Coupland (1993) have shown that increasing the concentration of seston results in decreased larval mortality from exposure to *Bti.* Reduced mortality was attributed to either feeding inhibition by the larvae or dilution of the material in the pool of particulates passing the larvae at a given time (Gaugler and Molloy 1980, Coupland 1993). These explanations for reduced efficacy could be applied to this study for *Scenedesmus*; however, increasing the concentration of *Chlorella, Dictyosphaerium,* or *Microcytis* did not result in significantly lower black fly mortality. Thus, another mechanism is likely involved.

Another plausible factor that might explain the reduced efficacy of *Bti* in the presence of algae is algal morphology. *Scenedesmus,* the only genus of algae having an effect on *Bti* efficacy, has terminal spines on the corners of its colony matrix (Bold and Wynne 1985). These spines may enable the larvae to capture cells of *Scenedesmus* more efficiently or make the cells more difficult to remove from the cephalic fan during the grooming process. This may force the larvae to spend less time collecting and more time cleaning their fans resulting in less *Bti* ingested. Algae of the genera *Chlorella, Dictyosphaerium,* and *Microcytis,* which did not reduce *Bti* efficacy, lack terminal spines, further supporting this theory.

Although we were able to show that certain types of algae can reduce the mortality of black fly larvae exposed to *Bti* under controlled laboratory conditions, the concentrations of algae that caused significant reduction in black fly mortality were higher than concentrations of algae measured in the field when efficacy was reportedly reduced. From data collected in 1997, algal concentrations in Pennsylvania rivers where larval mortality was reportedly reduced ranged from 504 to 1,032 cells/ml (Arbegast, personal communication). Examination of these data indicates that algae may be interacting with other unmeasured parameters in the water, or possibly that multiple taxa of algae combined may have a greater effect on *Bti* efficacy than a single type of alga. However, examination of data collected in 2001 showed that algal concentrations ranged from 4,100 to 11,600 cells/ml in rivers with reported reduction in larval mortality after *Bti* application (Arbegast, personal communication). The high end of this range (11,600 cells/ml) is close to the concentrations shown to significantly reduce black fly mortality in our study with *Scenedesmus* (between 8,000 and 16,000 cells/ml). Low algal concentrations reported in 1997 may have been related to unrepresentative sampling or other calculation errors. Nonetheless, concentrations of individual algal taxa did not exceed 3,200 cells/ml, indicating that mixtures of different algae may play a significant role in reducing larval mortality. The number of algal genera in rivers on treatment dates with reported reduction in efficacy ranged from 10 to 16, whereas treatment dates with reportedly good larval control had <10 genera present in the water (Arbegast, personal communication).

Another possible explanation for the higher algal concentrations needed to reduce *Bti*-induced mortality in our study was that we failed to test the type of algae causing the most detrimental effects. The genera we tested were commercially available in cultures. Culture of some of the algae present in the Pennsylvania rivers may be necessary to investigate more algal genera as well as mixtures of different algae. Further research is needed on the mechanisms responsible for algal effects on *Bti* activity in the laboratory and in field evaluations during periods when problems are observed. Such data would enable us to predict the impacts of increased algal populations on *Bti*-based black fly control programs, and to design treatment strategies to mitigate the problem.

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