MARIANNE SHOCKLEY STEPHENS
The Effects of Algae on the Efficacy of *Bacillus thuringiensis* var. *israelensis* Against Larval Black Flies (Diptera: Simuliidae) in Laboratory Bioassays
(Under the Direction of RAY NOBLET)

The effects of algae on the efficacy of *Bacillus thuringiensis* var. *israelensis* against larval black flies (*Simulium vittatum* IS-7) were investigated in laboratory bioassays. *Microcystis,* a blue-green algae, demonstrated no statistically significant effects on the efficacy of *B.t.i*. *Navicula,* a diatom, demonstrated significant effects at cell concentrations greater than 34,000 cells/ml. *Scenedesmus,* a green algae, also demonstrated significant effects at cell concentrations greater than 7,000 cells/ml. Negative effects of algae were reversed both by increasing the concentration of *B.t.i.* and to a lesser degree by increasing exposure period. Differences attributable to introduction time of algae and *B.t.i*. into the bioassay could not be determined from our experiments. Residual toxicity determinations using the *Aedes aegypti* bioassay revealed that, if given the opportunity, black flies ingest significantly more algae and *B.t.i*. particles in an increased exposure period.

INDEX WORDS: *Aedes aegypti,* Algae, *Bacillus thuringiensis* var. *israelensis*, Bioassay, Black flies, *B.t.i*., Diptera, *Microcystis, Navicula,* Simuliidae, *Simulium vittatum* IS-7, *Scenedesmus*

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by

MARIANNE SHOCKLEY STEPHENS

B.A., Georgia College and State University, 1997

A Thesis Submitted to the Graduate Faculty

of The University of Georgia in Partial Fulfillment

of the

Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2001

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by

MARIANNE SHOCKLEY STEPHENS

Approved:

Major Professor: Ray Noblet

Committee: Darold Batzer
Beverly Sparks

Electronic Version Approved:

Gordhan L. Patel
Dean of the Graduate School
The University of Georgia
December 2001

DEDICATION

To my husband, Travis P. Stephens, and the John and Sandra Shockley family who encouraged and inspired me throughout my life and in this master's program. I also dedicate this thesis to our unborn child who will bless our family as I complete my master's degree.

ACKNOWLEDGEMENTS

I would like to thank my major advisor Dr. Ray Noblet for his mentoring, guidance and support. Dr. Noblet provided me with the opportunity to develop research and professional skills. My research and thesis could not have been accomplished without his assistance. The comments and suggestions from Dr. Batzer and Dr. Sparks as committee members have been appreciated. I also thank our laboratory: Elmer Gray, Jay Overmyer and the many student workers, who assisted with my experiments. The graduate students in the Entomology Department have also been very helpful with computer and statistical support and providing a listening ear.

Finally, I owe my success to my family, they have always supported and encouraged me in my endeavors.

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CHAPTER 1

INTRODUCTION

Black flies, commonly referred to as gnats, are severe pests of humans, birds, and other animals. In addition to being notorious blood feeders, black flies transmit protozoan and nematode parasites to domestic and wild birds as well as to mammals in North and South America and Africa (Fredeen, 1977). The small dark flies have a strongly convex thorax that gives the insect a humped appearance. Black flies cause immense agitation by their persistent swarming, crawling and biting behavior. In areas of West Africa and Central America where black flies are vectors of Onchocerciasis or River Blindness, human reactions to black fly bites can vary from disfiguring skin changes, musculoskeletal problems, and weight loss, to changes in the immune system, epilepsy, and growth arrest (Burnham, 1998). Simuliids may play some role as vectors of arbor viruses, which they may transmit from birds to humans.

Black flies are vectors of various diseases that are detrimental to human and animal health. Blood feeding by black flies also cause periodic losses in animal agriculture throughout the world. Veterinary problems associated with black flies occasionally include death, reduced production of meat, milk, and other animal products, along with reproductive dysfunction (Cupp, 1987). In North America, black flies are often a serious nuisance pest to livestock and humans in habitats supportive of black flies.

Successful completion of the life cycle of black flies requires running water. Black flies breed in rivers and streams, although the outlets of ponds and lakes can be

especially productive habitats for these filter-feeding larvae. Immature stages of *Simulium* develop in flowing water. These habitats may vary from broad rivers to small streams depending on the requirements of each individual species. Rapid-flowing water provides the oxygenation necessary for black fly development. Larval black flies are filtering/collecting organisms or suspension feeders and scrapers, feeding in the current on suspended particles in the water column. Larvae and pupae of most species develop on rocks or vegetation just below the water surface. Adult females lay their eggs on vegetation or various other substrates in the lotic system. Aquatic stages require 10-30 days to complete development, depending on stream temperature and available nutrients (Merritt and Cummins, 1996).

The adult black fly emerges from the pupal case and travels towards the surface of the water to begin its adult stage in a terrestrial environment. After adult simuliids emerge, they rest near the stream before dispersing from their breeding site. The distance of dispersal depends on the species, the density and height of ground cover and weather conditions. They may feed first on nectar of flowers for flight fuel and then mate (Davies, 1978). During adult life, usually less than a few weeks, the females of most species must mate, acquire a source of sugar for energy, a meal of blood for egg maturation, and find a suitable habitat to place eggs (Adler and McCreadie, 1997).

In an effort to reduce black fly populations in recreational areas, agricultural settings, and centers of human onchocerciasis, many streams and rivers have been treated with larvicides such as organophosphates and DDT. Although treatments of streams and rivers with these chemicals are effective the negative impacts on watersheds and non-target organisms have led to the development of environmentally safe alternatives.

In 1977, Goldberg and Margalit isolated a strain of *Bacillus thuringiensis* Berliner from the soil of a raw sewage pond in Israel (a known mosquito breeding site) that demonstrated high larvicidal activity against five species of mosquitoes. De Barjac (1978) identified this strain as a new serotype (H-14) and named the variety *israelensis.* Undeen and Nagel (1978) found *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) to be active at low concentrations against three genera of black flies.

Black fly control is achieved by targeting the larval stages of the vector through aerial or ground applications of insecticides to rivers. *Bacillus thuringiensis* var. *israelensis* (*B.t.i*) a soil bacterium, has proven to be highly effective because larval black flies are especially suspectible to *B.t.i.* (Margalit, 1990). Feeding inhibition in black fly larvae occurs as a result of the pathogenicity of *B.t.i*. (Gaugler and Molloy, 1980). The need for more control tactics that are more acceptable environmentally resulted in commercial formulations of *B.t.i.* being used extensively and effectively to regulate population sizes of larval black flies in North American streams and rivers (Molloy, 1990). *B.t.i.* is applied directly above the water surface to uniformly distribute the product to produce a wide band of *B.t.i.* drifting through that portion of the stream. The active principle of *B.t.i.* is the delta-endotoxin contained in the proteinaceous parasporal crystals produced during sporulation. After ingestion the toxin binds to specific receptors on the cell surface of the peritrophic matrix causing the midgut epithilial cells of the larvae to swell and burst.

In the late 1960's the World Health Organization (WHO) launched a campaign for the control of black flies in many of the running waterways in West Africa where the prevalence of Onchocerciasis is the highest (Hougard *et al*., 1997). Widespread efforts to

control Onchocerciasis have been directed toward suppression of its vector, the *Simulium* fly, through larvaciding of many African and South American waterways where River Blindness is a major public health problem.

Laboratory and field research with commercial and experimental formulations evaluations of *B.t.i.* have demonstrated the effectiveness of this biocontrol agent against both black flies and mosquitoes with no detrimental effects to non-target invertebrates or fish species. Molloy and Jamnback (1981), in field evaluations of *B.t.i.* as a black fly biocontrol agent, examined the effective downstream carry of the product and instar susceptibility. In addition, they monitored the effect of the treatment on non-target insect populations. In contrast to reduction in black fly larvae, increases were recorded in the numbers of various other macroinvertebrates. This increase does not necessarily reflect true population expansion and could be attributable to sampling error or disproportionate colonization within the sampled area. However, there was no evidence of any adverse effect on non-target insects following the treatment. In an investigation on the effects of *B.t.i.* on non-target benthic insects through direct and indirect exposure, no lethal or sublethal effects to the test organisms were observed (Wipfli and Merritt, 1994b). A study on direct and indirect exposure of three species of trout indicated that there was generally no measurable affect to either growth or survivorship (Wipfli *et al*., 1994).

The greatest harm of *B.t.i.* to fish and other predators may be through a food resource loss, when black fly larvae are removed from the lotic food web. Wipfli and Merritt (1994a) observed reduced feeding of predatory benthic macroinvertebrates following *B.t.i.* application. *B.t.i.* appeared to have little effect on drift and benthic densities of non-target macroinvertebrates. These results suggest that the application of

B.t.i. reduces black fly populations, but does not negatively affect most non-target macroinvertebrates (Jackson *et al*., 1994).

The black fly laboratory at the University of Georgia (UGA) maintains a colony of black flies, *Simulium vittatum IS-7* for use in laboratory bioassays (Gray, 1996). Numerous black flies are produced weekly in the artificial streams, enabling utilization of approximately 10,000 – 30,000 larvae per week for laboratory bioassays. Rearing black flies in the laboratory was investigated thoroughly by Bernado *et al.* (1986a and1986b). Modified rearing techniques are still used by black fly researchers around the world today (Brenner and Cupp, 1980).

Figure 1.1. Three stages of the black fly colony, *Simulium vittatum IS-7,* at UGA. Left: Young tank, 0-7 days old (1st-3rd instar) Back Left: Adult emergence tank, 25+ days old (pupa and emerging adults) Right Front: Middle aged tank, 7-24 days old (4th-7th instar)

Bacillus thuringiensis var. *israelensis* (*B.t.i.*) has proven to be highly effective in black fly treatment and control and has been widely adopted as a product of choice in black fly control programs around the world (Margalit, 1990, and Hougard *et al*., 1998). The susceptibility of *Simulium vittatum* larvae to *B.t.i*. in the laboratory and their distribution in water with no extensive aquatic vegetation was discussed by Fromer *et al*. (1980, 1981a, 1981b).

There have been reports of reduced efficacy of *B.t.i.* in black fly field application programs in North America and West Africa. There are several biotic and abiotic factors that have been cited as affecting the efficacy of *B.t.i.* Discharge, stream profile, turbidity, pollutants, water temperature, pH, degree of vertical mixing in the water column, and adsorption of *B.t.i.* to benthic substrates/sediments can all affect potency and stream carry of *B.t.i.* Other factors may include turbulence, density of filter feeding organisms and algae (Molloy, 1990).

Purpose of the Study

The overall focus of this thesis research project was to investigate the efficacy of *B.t.i.* against black fly larvae in the presence of algae. In order to better understand the effect algae has on the efficacy of *B.t.i.* two main objectives were developed:

1. To determine the effects of three genera of algae on the efficacy of *B.t.i.* against larval black flies in an orbital shaker bioassay system, and
2. To evaluate laboratory assessments of potential changes in black fly treatment protocols to mitigate the effects of algae of on *B.t.i*. efficacy.

Hypotheses and Experiments

Three genera of algae, *Microcystis, Navicula* and *Scenedesmus,* were cultured in the laboratory and utilized in black fly bioassays. Some of these genera are believed to reduce the efficacy of *B.t.i.* against larval black flies. The hypothesis to be tested is whether or not a particular genus of algae reduces the efficacy of *B.t.i.*

H-1, 2, & 3_0: *Microcystis, Navicula* or *Scenedesmus* does not reduce the efficacy of *B.t.i.* against black fly larvae.

H-1, 2, & 3_A: *Microcystis, Navicula* or *Scenedesmus* reduces the efficacy of *B.t.i.* against black fly larvae.

I had hoped to narrow the scope of this research to one genus of algae that demonstrated significant reductions in the efficacy of *B.t.i.* against larval black flies in the orbital shaker bioassay. Based on this, the green algae, *Scenedesmus,* was selected for the remaining experiments, and experiments 4-9 were designed to evaluate potential changes in black fly treatment protocols to mitigate the effects of algae on *B.t.i.* efficacy against black fly larvae.

Increasing the concentration of *B.t.i.* in the bioassay, while maintaining a constant concentration of algae should increase larval mortality. The simplest hypothesis to test here is whether or not higher concentrations of *B.t.i.* increase larval mortality in the presence of *Scenedesmus.*

H-4_0: Increasing the concentration of *B.t.i.* does not increase larval mortality in the presence of *Scenedesmus.*

H-4_A: Increasing the concentration of *B.t.i.* does increase larval mortality in the presence of *Scenedesmus.*

Manipulating the dosing techniques may indicate when in the assay the *B.t.i.* inhibition with *Scenedesmus* is occurring. The easiest hypothesis to test here is whether or not differences occur between the two experimental dose regimes.

H-5_0: There will not be differences observed between the two experimental dose regimes.

H-5_A: There will be differences observed between the two experimental dose regimes.

The length of the exposure period in our bioassay system may have an effect on black fly larval mortality. We want to manipulate the exposure period to determine if larval mortality will increase with increasing exposure period in the presence of *Scenedesmus.* Therefore, the simplest hypothesis to test here is whether or not differences in larval mortality were observed with increasing exposure period in the presence of *Scenedesmus.*

H-6_0: Increasing the exposure period from 10 minutes to 20 minutes will not increase black fly larval mortality in the presence of *Scenedesmus.*

H-6_A: Increasing the exposure period from 10 minutes to 20 minutes will increase black fly larval mortality in the presence of *Scenedesmus.*

If doubling the length of the exposure period in the presence of *Scenedesmus* does have an effect on black fly larval mortality, is it because the black flies are ingesting twice as much *B.t.i.* in the lengthened exposure period? The easiest hypothesis to test here is whether or not doubling the exposure period is the same as doubling the amount of *B.t.i.* available to the black fly larvae with *Scenedesmus* in the system.

H-7_0: Doubling the exposure period is not synonymous to doubling the amount of *B.t.i.* available to black fly larvae with *Scenedesmus* in the system.

H-7_A: Doubling the exposure period is synonymous to doubling the amount of *B.t.i.* available to black fly larvae with *Scenedesmus* in the system.

The exposure period may have an effect on the efficacy of *B.t.i.* against black fly larvae with *Scenedesmus* in the system. By changing the exposure period from 10 minutes to 40 minutes, will an increase in black fly larval mortality be observed? The simplest hypothesis to test here is whether or not increasing the exposure period from 10 minutes to 40 minutes will increase larval mortality.

H-8_0: Increasing the exposure period over time from 10 minutes to 40 minutes will not increase black fly larval mortality in the presence of *Scenedesmus.*

H-8_A: Increasing the exposure period over time from 10 minutes to 40 minutes will increase black fly larval mortality in the presence of *Scenedesmus.*

When the black flies are exposed to *B.t.i.* for different periods of time it is presumed they are extracting different amounts of *B.t.i.* from the system through feeding. By using mosquito larvae in an assay following Experiment 8, we wanted to determine the level of *B.t.i.* potency remaining in the presence of *Scenedesmus.* The easiest hypothesis to test here is whether or not differences in mosquito larval mortality will be observed in response to the residual potency of *B.t.i.* in the testing medium used in the varying exposure periods.

H-9_0: Differences in mosquito mortality will not be observed with the varying exposure periods with *Scenedesmus* in the system.

H-9_A: Differences in mosquito mortality will be observed with the varying exposure periods with *Scenedesmus* in the system.

CHAPTER 2

REVIEW OF LITERATURE

Life History of Black Flies

Figure 2.1 Life Cycle of Black Flies

Egg

Black fly development involves metamorphosis from the egg, through the larval stage, to the immobile pupa, and finally to the adult. Black fly eggs are small, ranging in size from about 0.15 to 0.55 mm long. The eggs are asymmetrically ovoid differing from an elongated triangular form or reniform to oval (Peterson, 1981). The anterior end is narrowly rounded and the posterior end more broadly rounded. The dorsum is strongly convex whereas the ventral surface is more flattened (Merritt and Cummins, 1996). The eggs are laid in large masses placed side by side in a single layer (Puri, 1925). Black fly eggs are pale white and darken with age and embryonic development.

Larvae

Larvae hatch from eggs dropped into the water or placed in masses on trailing vegetation and other in-stream substrates. The larvae of Simuliidae are slender, cylindrical, and can vary in length from approximately 3.5 to 15.0 mm. They have an apneustic respiratory system, and differ in color from pale gray to pale yellowish brown to nearly black in color (Merritt and Cummins, 1996). Larvae share a consistent body plan featuring a well-sclerotized head capsule with a conspicuous pair of labral fans for filtering their food and an elongate, posteriorly swollen, variously pigmented body (Adler and McCreadie, 1997).

The head is prognathous, well developed, and sclerotized. There is usually a pair of dorsal labral fans used for straining food particles from flowing water that may be reduced or absent in some species. The thoracic segments are stout with a ventral eversible proleg having an apical ring of minute hooks arranged in rows. The abdomen has eight segments with the anterior segments somewhat slender compared to the swollen, enlarged posterior segments.

Black fly larvae lead a sedentary life, anchored by the hooklets of their posterior proleg to a pad of silk spun from their massive silk glands. Securely attached to a substrate within the stream, they can filter food from the current or browse it from the substrate. When disturbed or when the silken pad begins to deteriorate, a larva can relocate by looping over the substrate or by releasing its hooklets from the silken pad and drifting downstream, often on a line of silk (Crosskey, 1990). Simuliidae pass through a modal number of seven instars and, within one week to half a year after hatching, pupate in a silken cocoon fastened to the substrate (Adler and McCreadie, 1997).

Larvae are most commonly used in laboratory bioassays because this is the stage that is targeted in black fly control programs. Using black fly larvae in research gives us an idea not only about treatment and control, but feeding habits, toxicity to insecticides and pesticides, gut passage, and general biology and behavior.

Feeding

All larvae of Simuliidae are aquatic and most species require flowing water. With few exceptions simuliid larvae feed using two complex cephalic fans to filter particulate matter from flowing water. Abundance of simuliid larvae in riffle areas has often been attributed to respiratory requirements. Oxygen is not a limiting factor in larval simuliid distribution, but specific water velocities are required (Craig and Chance 1982, Chance and Craig 1986).

Simuliid larvae generally possess complex cephalic or labral fans for filtering food particles from water. Not all simuliid larvae possess well-developed fans and in some they are completely absent (Craig, 1974). Cephalic fans are used by larvae to filter-feed predominately on detritus and microorganisms. Many studies have been done on the structure, function and fine particle capture of the cephalic fans and associated mouthparts (Ross and Craig, 1980).

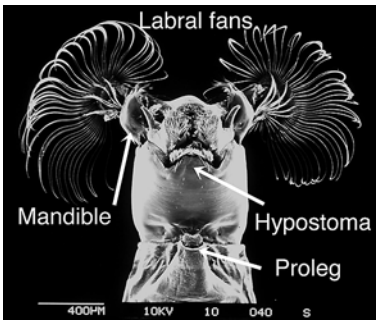

Figure 2.2 Cephalic/labral fans and mouthparts

Chance (1970) has reviewed much of the work concerning the cephalic fans and particle capture. Craig (1974) has presented detailed studies of the morphology, development and function of the mouthparts of Simuliidae.

The feeding mechanism of simuliid larvae is related to the nature and speed of cephalic fan and mouthpart movements, and water flow around larvae. It is also thought that endogenous glyconjugates are utilized in larval black fly feeding (Fry and Craig, 1995). The head and body of *Simulium vittatum* Zett. larvae are highly streamlined and water flow is laminar over the larva. Cephalic fans open and close possibly depending on filter-feeding efficiency. Ingestion rates vary among filter-feeding larval black flies (Hart and Latta, 1986). Laminar flow through fan rays produce optimal flow for filtration. Behavior, body shape, and structure of simuliid larvae are highly adapted for optimal water flow over body and fans (Craig and Chance, 1982).

Several authors have investigated the size and type of particles ingested by black fly larvae in nature (Kurtak, 1978, Fredeen 1960,1964, Wotton, 1976). The sizes reported range from 10 to 30 μm. However, materials as small as bacteria (Fredeen 1960, 1964) or colloids (Wotton, 1976), and material as large as filamentous algae (Davies and Syme, 1958; Burton, 1973) can be ingested. The larvae ingest all the particle types among the suspended material in the stream unselectively (Anderson and Dicke, 1960). Miller *et al.* (1998), Mulla and Lacey (1976) all discussed the feeding behavior of black fly larvae and retention of fine particulate organic matter. Ciborowski *et al.* (1997) considered the role of dissolved organic matter as food for black fly larvae. The effects of the food supply on the life history of Simuliidae were of interest to Colbo and Porter (1979) and described and detail.

Pupae

The black fly pupa is obtect with the head flexed beneath the thorax. The thorax is enlarged and strongly arched with one to five pairs of specialized setae. The wing and leg sheaths are distinct. The pupa is characterized by a pair of thoracic gills that are designed to function in or out of water. The pupal stage is thermally dependent, lasting from two days to a few weeks. By expelling air from its respiratory system, the pharate adult splits the pupal cuticle along the eclosion line. A respiratory organ, gill, is present at each corner of the thorax, varying in shape. Some gills may have one to many slender filaments or an enlarged antler-like structure and possibly combinations of these forms.

The pupae are covered by cocoons, which are attached to the substrate. Pupal cocoons can be a shapeless sleevelike structure or highly specific in shape and texture (Merritt and Cummins, 1996). The cocoon of pupa is varying in form, and in density and texture of weave. The cocoon can be an irregular sleeve covering part or all of pupa or a well-formed pocket-shaped structure tapering posteriorly from a large open anterior opening raised above substrate. Cocoons can also be circular and flattened. The strands composing the cocoon are coarse and loosely woven, or finer and tightly woven, incorporating small pieces of debris (Peterson, 1981).

The pupal head is small, somewhat folded on the thorax and has a pair of pigment spots on each side. The pigment spots are the remains of the larval eyes, which probably persist beneath the eyes of the imago. The thorax is very convex. Dorsally the head and thorax are covered with many minute disc-like tubercules darker than the cuticle of the body. These tubercules, which are solid chitin, are of different sizes in the various species. The abdomen has nine clearly-marked segments. (Puri, 1925).

The pupal cuticle is smooth with a faint reticulate or densely wrinkled pattern. In some cases the pupal cuticle has a series or pattern of flattened to raised granules of variable size and shape. The abdominal segments have a series of setae and small hooks, and the last segment often bears a dorsal pair of short to long terminal spines.

Adult

Black fly adults are small, stout flies that range from 1 to 6 mm in length and are usually dark brown to black but may be reddish brown, gray, orange, or yellow in color. The adult black fly has a moderately large, rounded head. The females have separated eyes that have facets unequal in size, whereas in males, the eyes usually touch along the midline above the antennae. There are no ocelli. The antennae are short, stout, and erect. The proboscis is short and thick. The mouthparts of the black fly female are usually adapted for cutting skin and sucking blood. In the male of all species and in females of some species, the mouthparts are weak and suitable only for taking in liquids such as water and nectar (Merritt and Cummins, 1996).

The legs are short and stout with elongated basal tarsomeres. The fore tibia has one apical spur; mid and hind tibiae with two apical spurs. The wings are broad, and have strong anterior and weak posterior veins. The thorax is usually high and strongly arched dorsally especially in the male (Peterson, 1981).

Emergence patterns of adult aquatic insects should reflect the underlying population dynamics of the preimaginal stages. When sampling is conducted over a long period of time relative to generation time of the insects, patterns can be drawn for the population. One of the most convenient and reliable methods of sampling adult aquatic

insects is with the use of emergence traps covering prescribed sections of the aquatic habitat (McCreadie *et al.,* 1994).

The number of emerging adults is linked with the population dynamics of preimaginal stages; consequently yearly, seasonal, and temperature effects on emergence are expected. Primary production in streams varies annually resulting from changes in larval production, which in turn determines the number of emerging adults. Seasonal patterns of emergence partly reflect changes in preimaginal abundance as a consequence of seasonal shifts in stream conditions. Seasonal patterns of emergence also reflect aspects of life history such as voltinism (McCreadie *et al.,* 1994).

Stream temperature plays a role in the population dynamics of simuliids through its influence on preimaginal development, survival, growth rate and female fecundity (McCreadie and Colbo, 1991). Therefore, stream temperature directly influences the number of emerging adult. The daily rhythm of emergence period also appears to be a response to water temperature (Crosskey, 1990). The onset and duration of the emergence is related to the local temperature regime, with reduced periods of emergence found in more northern locations (Adler *et al.,* 1982). Not only is actual temperature important, but the pattern of temperature change can also influence the growth and development of aquatic insects (Hynes, 1970).

Before mating, males usually form swarms over some conspicuous marker: the top of a tree or stump, the corner of a building, a dam or base of a waterfall, an open patch of ground, or roadway. Males of most species have eyes with larger dorsal facets, which may be adapted for detecting small objects moving above them. Females flying above such male swarms are chased and mated (Davies, 1978).

Black flies find a vertebrate host and complete their blood meal through several behavioral steps, which may include both olfaction and vision. Simuliid species are grouped into three physiological types based on nutritional requirements of females for egg development. Autogenous species are species whose females have reduced mouthparts and are unable to feed on blood. They use nutrients carried over from the larvae for their single gonotrophic cycle. Autogenous females with reduced mouthparts often emerge with eggs mature. Such females mate close to the stream, often while crawling, and quickly oviposit (Davies, 1978).

Species whose females are hematophagous, but usually produce the first batch of eggs without a blood meal are autogenous for their first gonotrophic cycle. However, these females require a blood meal for subsequent gonotrophic cycles. Sometimes autogeny may not be attained during the first cycle, which might occur if during larval life, food was scarce and temperature high. Females of species autogenous for the first cycle, after mating, rest in vegetation usually near the stream for 3-4 days until eggs mature, and after successful oviposition may fly off in search of a blood meal (Davies, 1978).

Species, whose females require a blood meal to produce any eggs, are known as anautogenous. In fully anautogenous species, newly emerged females may spend 1-2 days mating and feeding on nectar before seeking a host. After a blood meal oviposition and oogenesis requires 3-8 days depending on the temperature (Davies, 1978).

Treatment and control of Onchocerciasis has been an ongoing process since the 1970's. The Onchocerciasis Control Program (OCP) in West Africa began its activities in January 1974. The OCP was formed by four United Nations Agencies and eleven

countries. The objective of the OCP was to eliminate Onchocerciasis as a disease of public health importance and as a obstacle to socioeconomic development (Molyneux and Davies, 1997). Control programs are active throughout the world and are constantly being developed (Gray *et al.*, 1999) not only to treat black flies as vectors but also as a nuisance pest.

Bacillus thuringiensis* var. *israelensis

Bacillus thuringiensis var. *israelensis (B.t.i.)* was first isolated from a stagnant pond located in the Nahal Besor Desert river basin in the northwestern Negev Desert of Israel (Margalit, 1990). The *B.t.i.* parasporal body is a gut poison, and the midgut epithelium of affected larvae is considered to be its initial site of action. In *B.t.i.* treated mosquito larvae, midgut epithelial cells swell and burst, causing severe damage to the gut wall. *B.t.i.* parasporal body consists of at least four major proteins of 27 kDa, 65 kDa, and a doublet of 130kDa (Chilcott *et al.* 1990, Hofte and Whiteley, 1980).

The first step in toxicity consists of binding of the toxin to specific receptors on the cell surface, but the nature of these binding sites is still uncertain. Black fly larvae must ingest *B.t.i.* for the crystal proteins to exhibit their toxic effects on the larvae. Upon ingestion crystal proteins dissolve into protoxins in the highly alkaline and reducing conditions of the larval midgut. The midgut epithelial cells swell and burst due to the influx of ions through toxin induced channels. Channel formation may occur indirectly by the interaction between the toxins and their membrane binding sites (Hofte and Whiteley, 1980).

Studies on the mechanism of action of *B.t.i.* parasporal body have been confined to the 25kDa protein. It is hypothesized that the 25kDa protein binds to a specific plasma

membrane receptor, most likely a phospholipid. The primary action of the toxin is to insert into the plasma membrane and generate small pores. The creation of these pores will lead to colloid-osmotic lysis; that is, an equilibration of ions through the pore resulting in a net inflow of ions, accompanied by influx of water, cell swelling, and eventual lysis (Chilcott *et al*., 1990).

Specificity of *B.t.i.* to Diptera of the suborder Nematocera, especially black flies and mosquitoes, can be attributed to two major factors. These include the degree of solubility of the protein crystals in the larval midgut and the susceptibility of the larvae to the toxins (Jaquet *et al.*, 1987). The degree of protein solubility is dependent upon the alkalinity and reducing conditions of the midgut as well as the activity of proteases. However, the susceptibility of larvae to the toxins is dependent on the toxin binding sites.

Algae

From tiny single-celled species one micrometer in diameter to giant seaweeds over 50 meters long, algae are abundant and ancient organisms that can be found in virtually every ecosystem. Freshwater lakes, ponds, and streams contain similar botanical gardens of planktonic microalgae and attached forms, periphyton. Freshwater phytoplankton and periphyton or benthic algae form the base of the aquatic food chain, without which freshwater fisheries could not exist. In addition to oceanic and freshwater environments, some algae have adapted to extreme habitats such as hot springs and brine lakes (Graham and Wilcox, 2000).

Algae are distinguished from other chlorophyllous plants by the phenomenon of sexual reproduction. In unicellular algae, the organisms themselves may function as gametes. In some multicellular algae, the gametes may be produced in special unicellular

containers or gametangia. In others, the gametangia are multicellular, every gametangial cell being fertile or producing a gamete. None of these characteristics occur in liverworts, mosses, and vascular plants. Instead, the multicellular sex organs of many of them are only partially fertile, being covered by sterile cells. In their asexual reproduction, many algae produce flagellate spores or nonmotile spores in unicellular sporangia, or if the latter are multicellular, every cell is fertile (Bold and Wynne, 1985).

The divisions of algae are distinguished by a variety of chemical and morphological differences. All divisions have chlorophyll *a*, but different divisions can also have either chlorophylls *b*, *c*, or *d*. Accessory pigments may color the algae red, blue, or golden-brown, unless the dominant pigment is green with chlorophyll. The different divisions also have chemically different cell walls and storage products, or they have distinctive forms of motility or numbers of flagella (Stevenson, 1996).

Conspicuous blooms of microscopic algae occur in marine and freshwaters, often in response to pollution with nutrients such as nitrogen or phosphate. Nutrient pollution can usually be traced to human activities, such as discharge of effluents containing sewage or industrial wastes, or the use of fertilizers. Water transparency may become so reduced that organisms such as corals, aquatic plants, and periphyton no longer receive sufficient light for photosynthesis. Often times marine and freshwater algal blooms produce poisons that affect neuromuscular systems which are toxic to the liver or are carcinogenic to vertebrates and can cause death or illness to fish, birds, cattle and other animals including humans (Graham and Wilcox 2000, Demott 1999).

Microcystis, a blue-green algae, is a member of the Cyanophycota division which lacks a nuclear envelope and mitochondria. Cyanobacteria are significant because they were the dominant forms of life on earth for more that 1.5 billion years. They were the most ancient oxygen-producing photosynthesizers; the first to produce chlorophylls *a* and *b* as well as a variety of accessory pigments; producers of massive carbonate formations in shallow waters during the Precambrian period; and the earliest terrestrial autotrophs. Modern cyanobacteria are recognized for their ability to occupy extreme habitats and valued for their ability to fix atmospheric nitrogen, bind and enrich soils, and produce medicinally useful compounds (Graham and Wilcox, 2000).

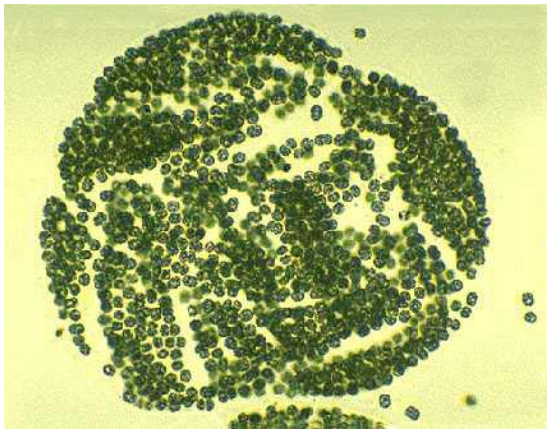

Figure 2.3 *Microcystis* colony

In *Microcystis*, the photosynthetic lamellae are single or unstacked and distributed peripherally in the cytoplasm and not within a membrane-bounded chloroplast. Morphology ranges from unicellular to filamentous; many possess extensive mucilaginous sheaths. Asexual reproduction predominates, involving binary fission, multiple fission resulting in endospores. The colonies of *Microcystis* are spherical or irregular and the cells are evenly distributed throughout the colonial matrix. *Microcystis*

is strictly planktonic and may be a common cause of water blooms (South and Whittick, 1987).

Diatoms are unicellular, although chains of cells and colonial aggregations may occur (Bold and Wynne, 1985). In terms of diversification, the diatoms have been tremendously successful. Though occurring only as single cells or chains of cells diatom diversity is rivaled among the algae only by the green algae. Diatoms are also extremely abundant and are probably the most numerous of eukaryotic aquatic organisms. In terms of contributions to global primary productivity, diatoms are among the most important aquatic photosynthesizers (Graham and Wilcox, 2000). Also present in diatoms are plastids within periplasmic endoplasmic reticulum, plastid girdle lamellae, lipid reserves, and tubular hairs on flagella of sperm of some diatoms (Graham and Wilcox, 2000). Accessory pigments, mainly beta-carotene, give the chloroplasts a brownish or yellowish color. *Navicula* is a member of the Chromophycota division which include all algae possessing chlorphylls *a* and *c*, and lacking chlorophyll *b*.

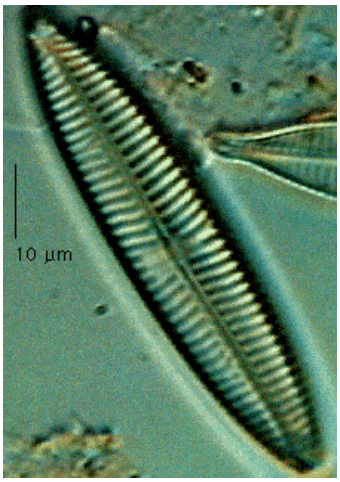

Figure 2.4 *Navicula* cell

Navicula exists as single cells or ribbons of cells. The valves are boat-shaped and each bears a raphe. *Navicula* is probably the most species-rich of all diatom genera, with

nearly 2000 widely accepted species, most being bottom-dwelling forms (Graham and Wilcox). Cells are uninucleate, and contain one or more chloroplasts. They possess a silica exoskeleton consisting of two halves each composed of a flattened plate with a connecting band attached to the edge. Certain pennate forms possess a slit-like raphe, which is associated with unique gliding motility. Asexual reproduction is normally by binary fission, the new valves and girdle formed within the parent cell (South and Whittick, 1987).

The green algae are commonly known as chlorophytes because they appear bright grass green, as do most plants. This is because the chlorophylls of green algae are usually not concealed by large amounts of accessory pigments. Green algae contain at least one plastid, and most of the green algae are considered to be autotrophic. However, the green algae exhibit a surprising level of nutritional variation some feeding on particles therefore exhibiting phagotrophy. Features that are common to nearly all of the green algae include: flagella, commonly occurring in pairs or multiples of two, that are of approximately equal length and without hairs; chloroplasts bound by a two-membrane envelope with chlorophylls *a* and *b*; and the production and storage of starch inside the chloroplasts (Graham and Wilcox, 2000).

Scenedesmus, a green algae, is a member of the Chlorophycota division, which includes all algae possessing chlorophylls *a* and *b*. *Scenedesmus* is widely distributed in fresh water and has cylindrical cells, with rounded or pointed ends. Terminal cells are often ornamented with short spines and tufts of chitinous hairs or bristles that are believed to confer buoyancy or to deter herbivores, or to space the algae for optimum light and nutrient availability (Graham and Wilcox, 2000). They are laterally joined in

groups of four, eight, or sixteen linearly arranged cells. There have even been reports of grazer-induced colony formation in *Scenedesmus* (Lurling and Van Donk, 2000). Reproduction in *Scenedesmus* is by autocolony formation, in which each parental cell forms a miniature colony that is liberated through a tear in the parental wall (Bold and Wynne, 1985).

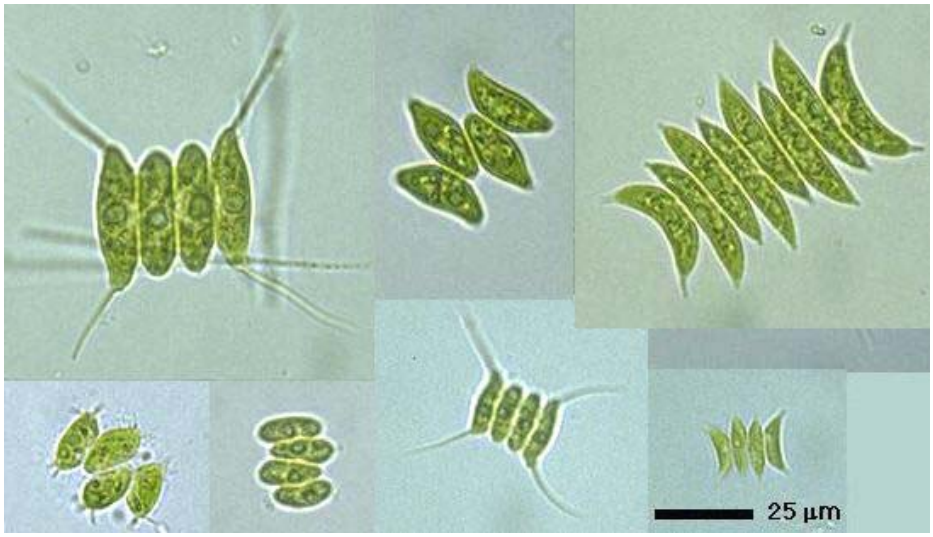

Figure 2.5 *Scenedesmus* colonies

CHAPTER 3

THE EFFECTS OF THREE GENERA OF ALGAE ON THE EFFICACY OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* AGAINST LARVAL BLACK FLIES IN AN ORBITAL SHAKER BIOASSAY SYSTEM

Introduction

Bacillus thuringiensis var. *israelensis* (De Barjac) has proven to be highly effective in black fly treatment and control programs around the world (Margalit, 1990). There are several biotic and abiotic factors that have been cited as affecting the efficacy of *B.t.i.* Discharge, stream profile, turbidity, pollutants, water temperature, pH, degree of vertical mixing in the water column, and adsorption of *B.t.i.* to benthic substrates/sediments can all affect potency and stream carry of *B.t.i.* Lacoursiere and Charpentier (1988) investigated the influence of water temperature and pH on efficacy of *B.t.i.* against black fly larvae. Other factors may include turbulence, density of filter feeding organisms and algae (Molloy, 1990).

It has been noted by workers in black fly control programs in both Pennsylvania and West Africa (unpublished) that algae are suspected to reduce the effectiveness of *B.t.i.* against black flies. Reduced mortality of black flies has been observed when *B.t.i.* is used in the presence of algae indicating that somehow the efficacy of *B.t.i.* is reduced in streams with algal populations. Various types of algae have been observed in relative abundance at treatment sites during the months when control programs are at their peak. It is during algal blooms that the field effectiveness of *B.t.i.* against black flies seems to be most negatively impacted.

The development of a standard bioassay is necessary for the evaluation of new formulation of *B.t.i.* against black fly larvae. According to Lacey *et al.* (1982) a bioassay system must allow normal feeding, produce low control mortality, should be easy to operate, economical, require little space, and produce repeatable results among different researchers. Laboratory bioassay experiments were designed and conducted to more

clearly define the relationship between algae and *B.t.i.* applied to streams for black fly control. The objective of the following experiments was to determine the effects of algae on the efficacy of *B.t.i.* against larval black flies in an orbital shaker bioassay system.

Figure 3.1 Orbital Shaker Bioassay System with flasks

The bioassay system was developed by Noblet and co-workers (personal communication) in the black fly biocontrol program at Clemson University. The orbital shaker bioassay satisfies the stipulated requirements of a bioassay system from evaluation of *B.t.i.* formulations (Barton *et al.* 1991). Three types of algae were utilized in this study: a blue-green algae, *Microcystis*, a diatom, *Navicula*, and a green algae, *Scenedesmus*.

Algae Dilutions

Pure cultures of each genus of algae (*Microcystis*, *Navicula*, and *Scenedesmus*) were grown in a freshwater media (Wards 88W 3251 Basic Culture Solution 1 liter) in the laboratory from cultures obtained from both Carolina Biological Supply and Wards Supply. Daily cell counts of the stock culture were taken on the morning of each bioassay with a hemacytometer cell counter. By taking the mean of four hemacytometer

fields we calculated cells per ml of the stock culture. Test dilutions were then prepared from the stock algae by using a concentration times volume formula.

$C_1 V_1 = C_2 V_2$ or Concentration1 x Volume1 = Concentration2 x Volume2

This formula is interpreted as:

(concentration *x*)(volume of *dose*) = (concentration of *algae*)(volume *total*) where *x* = concentration of cells/ml required, *dose* = volume of algae dilution added to each flask in order to reach the overall desired volume, *algae* = desired concentration of algae or # algae cells/ml and *total* = total volume of each flask. Solve for *x*. Use the same equation to determine how many cells/ml or what volume of stock algae must be added in order to make the desired concentration of algae. In the second equation the unknown variable is volume.

$C_1 V_1 = C_2 V_2$ or Concentration1 x Volume1 = Concentration2 x Volume2

(concentration of *stock algae*)(volume *x*) = (concentration of *cells/ml required*)(volume of *dilution*) where *stock algae* = cells/ml in stock culture of algae, *x* = volume of stock algae necessary to make the dilution, *cells/ml required* = *x* from the first equation and *dilution* = volume of dilution being prepared. Solve for *x.* Several factors are known in this example. The desired concentration of algae in the test flask, which has a total volume of 200 ml, is 10,000 cells/ml. A 25 ml dose of the algae dilution will be added to each flask. The stock culture of algae has a cell count of 216, 250 cells/ml. In order to dose 25 flasks with 25 ml of the algae dilution I will need a minimum of 625 ml of the algae dilution, therefore, 1000 ml of the algae dilution are required. The formula described earlier is used to calculate the volume of algae needed from the stock culture to make an algae dilution, of which a 25 ml dose is added to the flasks for a concentration of

10,000 algae cells/ml and a total volume of 200 ml per flask. Taking these known variables they are inserted into the equation:

x(25ml dose) = 10,000 cells/ml(200 ml)

x = 80,000 cells/ml

216,250 cells/ml in stock culture(x) = 80,000 cells/ml(1000 ml)

x = 369.9 ml of stock algae added to 630.1 ml distilled water

170 ml bioassay water + 25 ml algae + 5 ml *B.t.i.* or control water = 200 ml/flask with 10,000 cells/ml algae

The range of dilutions used varied with the test algae, each test consisting of generally five to six algal dilutions. Data recorded for each algal dilution included turbidity, pH, dissolved oxygen, conductivity, specific conductance, water and air temperatures.

Test Material

The *B.t.i.* formulation used in all trials was a Valent Laboratories (N. Chicago, IL) liquid formulation (Lot # 65-841-BA) kept refrigerated at 4°C in the laboratory. A stock solution of *B.t.i.* was made at the beginning of each experiment by combining 3 grams of the liquid formulation with 100ml of distilled water. All dilutions used on the day of the experiment were taken from this original stock. The stock solution was shaken with a wrist-action shaker before use to ensure that the stock solution was homogenous. Parts per million (ppm) concentration dilutions for each trial were made by serial dilutions based on 5 ml of *B.t.i.* added to the test container with a given volume of bioassay water. The standard dose (0.5 ppm for experiments 1-3, and 0.75 ppm for experiments 4-9) for these experiments was determined by baseline LC 50's obtained from laboratory

bioassays testing various formulations and potency of *B.t.i.* and compared to the standard during the course of this research.

Test Container and Test Organism

Round flat-bottomed, 250ml extraction flasks were the standard test vessels used in the bioassay. Each orbital shaker will hold 40 such flasks. The volume of bioassay water used for each test varied depending on the protocol of the individual trial; however, the total volume of water used was 200ml. The bioassay water contains distilled water and black fly food slurry (1:1 homogenized rabbit chow and soybean meal sieved through a 53 μm sieve) with an overall turbidity of 4.5 - 5.5 NTU's. Sixth to seventh instar *Simulium vittatum IS-7* larvae, from a large colony maintained from egg to adult in the laboratory at UGA (Gray, 1996) were carefully transferred to each flask with soft-tipped forceps. In an effort to minimize variation in the uniformity of test organisms three individuals loaded the flasks with each person transferring ten uniform sized larvae per flask for a total of thirty larvae.

A 25 ml dose of algae from the algae range dilutions was then added to the experimental flasks. Water and air temperature was recorded during each assay. The flasks were placed on the orbital shaker and acclimated for one hour starting at 100 rpms and gradually increasing to 150 rpms. The orbital shaker bioassay system creates a desired current in which the filter-feeding black fly larvae readily feed and ingest the algae and *B.t.i* particles. During the acclimation period, the test concentrations of *B.t.i.* were prepared and syringes filled with 5 ml of *B.t.i.* or control water. Once the *B.t.i.* or control water has been added, the overall total volume is 200 ml per flask. Syringes with

B.t.i. or water controls are inserted in a dosing board that is supported above each shaker by an aluminum rod frame.

The dosing board is designed to have a hole centered over the opening of each flask so the syringe will not hit the wall of the flask during the normal rotation of the orbital shaker. Immediately following dosing, one flask from each algae dilution level was removed from the shaker for additional cell counts to determine the number of algal cells/ml present in each flask at the time of dosing. Range tests were conducted with *Microcystis*, *Navicula*, and *Scenedesmus* to obtain baseline data on the concentrations of algal cells required to impact *B.t.i.* efficacy on black fly larvae.

Figure 3.2 The dosing board with filled syringes

Treatments

Each test consisted of controls (bioassay water + water dose), controls with algae (bioassay water + algae + water dose), a standard (bioassay water + *B.t.i.* dose), as well as an algae treatment (bioassay water + algae + *B.t.i.* dose). After the one hour acclimation

period, the flasks were dosed in unison by using a flat board to depress the syringe plungers. After a ten minute exposure period the shaker was turned off, the flasks held at room temperature for five hours. Mortality data was obtained by pouring all of the test larvae into white enamel pans, carefully determining overall percent mortality and adjusting for natural mortality with Abbott's formula (Abbott, 1925).

Data Analysis

Each trial compared the percent mortality of a standard treatment to the percent mortality of an algae treatment. Since 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 entered into the JMP program for statistical analysis. If the data were normal and passed both the goodness of fit Shapiro-Wilk W Test and Bartlett's test that the variances are equal, a one-way ANOVA was performed. If both of these tests were not passed, a nonparametric statistical analysis was conducted. SAS statistical analysis was also utilized for comparing means from the Tukey's Studentized Range Test (HSD).

Materials and Methods

Experiment 1,2, and 3: *Microcystis, Navicula and Scenedesmus* Range Trials

The first experiments were designed to determine the impacts of the various genera of algae on *B.t.i.* efficacy against black fly larvae. A constant concentration (0.75 parts per million, ppm) of *B.t.i.* was tested in the orbital shaker bioassay system with varying ranges of each type of algae using 6-7th instar larvae. The blue-green algae, *Microcystis*, were tested at a range from 10,000 to 1,000,000 cells/ml with six concentrations. The diatom, *Navicula*, was tested at a range from 3,000 to 70,000

cells/ml with five concentrations. The green algae, *Scenedesmus*, were tested at a range of 650 to 17,000 cells/ml with five concentrations. These ranges were selected based on the cell counts of the stock cultures. The stock culture of the three genera of algae were as follows: *Microcystis*, approximately 2,000,000 cells/ml, *Navicula*, approximately 200,000 cells/ml and *Scenedesmus,* approximately 100,000 cells/ml.

Results and Discussion

Experiment 1: *Microcystis* Range Trial

Microcystis is blue-green algae with a mucilaginous sheath surrounding each spherical cell. *Microcystis* exists in colonies with millions of cells/ml in the stock culture. Each individual cell has a length of approximately 5 μm, however due their existence in colonies the colony length is approximately 50 μm. We had hypothesized that this sheath, not only surrounding each individual cell but each colony, could physically bind the *B.t.i.*, therefore causing a reduction in its efficacy. This, however, was not observed in these trials.

There were no differences observed in the *Microcystis* range trial (One-way ANOVA, P=0.7607) between the standard and the algae treatments. This was the first alga tested so a large range was utilized. Overall there were seven treatments with four replicates. Treatment concentrations were as follows: treatment 0 – standard (no algae present), treatment 1 – 10,000 cells/ml, treatment 2 – 50,000 cells/ml, treatment 3 – 100,000 cells/ml, treatment 4 – 250,000 cells/ml, treatment 5 – 500,000 cells/ml, and treatment 6 – 1,000,000 cells/ml.

The *Microcystis* trials, which consisted of six concentrations from 10,000 to 1,000,000 cells/ml, demonstrated similar effects amongst each of the six concentration

levels of algae and the standard (Figure 3.3). After completing the four replications of this experiment, it was concluded that *Microcystis* demonstrated no negative effects on the efficacy of *B.t.i.* against larval black flies in the orbital shaker bioassay system.

Experiment 2: *Navicula* Range Trial

Navicula exists in aggregates and the stock culture in the laboratory generally occupied hundreds of thousands of cells/ml with a sheet like appearance. The length of a *Navicula* cell is approximately 45 μm. A blue-green algae had previously shown no effect on the efficacy of *B.t.i.* and we wanted to try a different type of algae rather than simply a different species. Due to the aggregates of cells and its common occurrence in rivers and streams, we felt that *Navicula* was a representative diatom and a good test organism. Overall there were six treatments with five replicates. Treatment concentrations were as follows: treatment 0 - standard (no algae present), treatment 1 – 3,000 cells/ml, treatment 2 – 8,000 cells/ml, treatment 3 – 14,000 cells/ml, treatment 4 – 34,000 cells/ml and treatment 5 – 70,000 cells/ml.

There appeared to be some differences among the treatments ranging from 3,000 to 70,000 cells/ml and the standard. Significant overall differences between the standard and the test containers with algae (Wilcoxon/Kruskal-Wallis Tests, $p>x^2$ 0.0037) indicate that for the diatom *Navicula,* a concentration greater than 34,000 cells/ml causes a noticable reduction in the efficacy of *B.t.i.* However, at cell concentrations below 34,000 cells/ml there were no differences in mortality compared to the standard (Figure 3.4). The consistent aggregations of this diatom might indicate physical binding of the *B.t.i.* to these colonies, making *B.t.i.* unavailable or not as readily available to the larvae at the higher concentrations during the exposure period.

Experiment 3: *Scenedesmus* Range Trial

Scenedesmus exists in cylindrical colonies consisting of four 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. The stock culture in the laboratory was generally 100,000 + cells/ml. In comparison to the blue-green algae and the diatom, the stock culture remained at a much lower concentration. This is presumably due to the larger size of the colonies and individual cells of *Scenedesmus* in comparison to *Microcystis* or *Navicula.* There were six treatments in this trial with five replicates. Treatment concentrations were as follows: treatment 0 – Standard (no algae present), treatment 1 – 650 cells/ml, treatment 2 – 1,500 cells/ml, treatment 3 – 3,000 cells/ml, treatment 4 – 7,000 cells/ml and treatment 5 – 17,000 cells/ml.

The results of the *Scenedesmus* range trials clearly indicated that when green algae are present in the system, the efficacy of *B.t.i.* was reduced. There were significant differences (One-way ANOVA, P=0.0004) observed between the standard and the treatments when there were more than 7,000 cells/ml present. However, there were also differences among the treatments. As the concentration of algae is doubled in subsequent treatments the reduction in mortality that is observed follows a distinctive negative trend (Figure 3.5). The mechanism that causes the reduction in larval mortality in the presence of *Scenedesmus* is difficult to identify. Due to the comparative size of the cell colonies with the terminal spines it is possible that the *B.t.i.* particles may be physically

binding to the *Scenedesmus* and altering its ability to bind to receptors in the midgut of the larvae after ingestion.

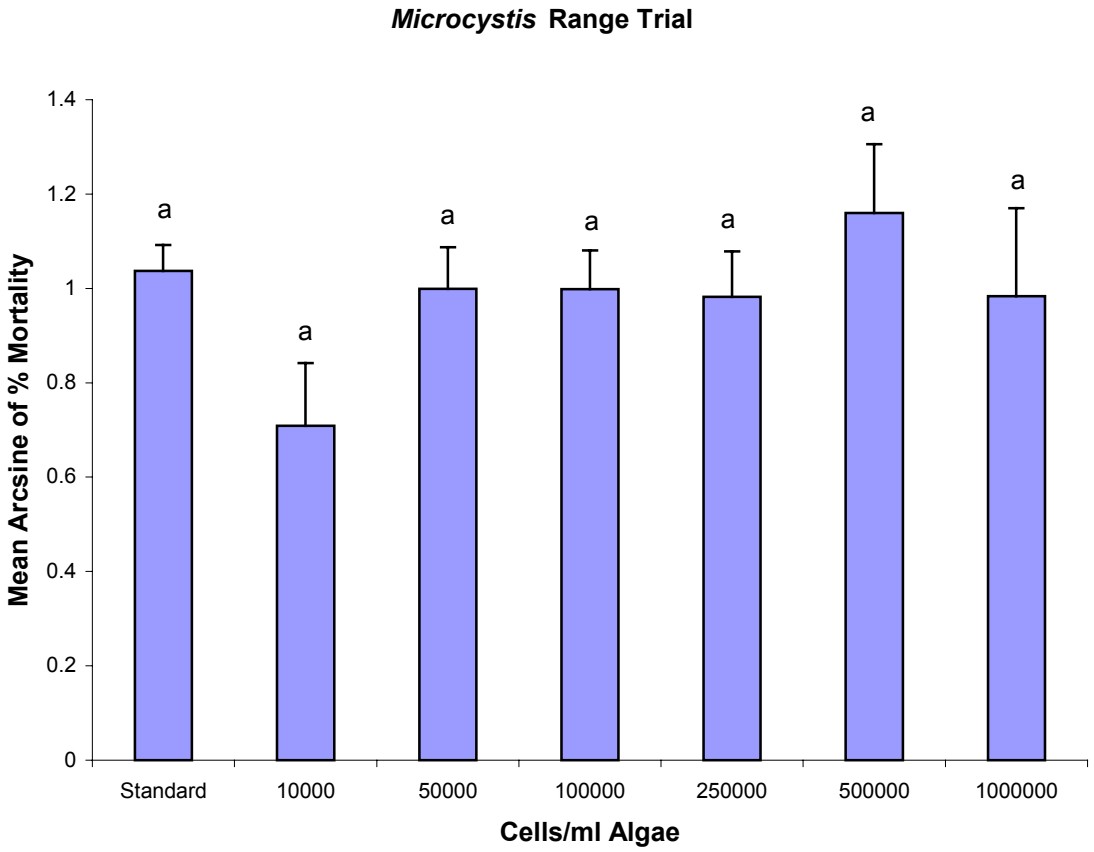

Figure 3.3 Percent larval mortality observed with varying concentrations of *Microcystis*. All treatments were dosed with 0.75 ppm *B.t.i.* and compared to the standard (no algae present). Means with the same letter are not significantly different.

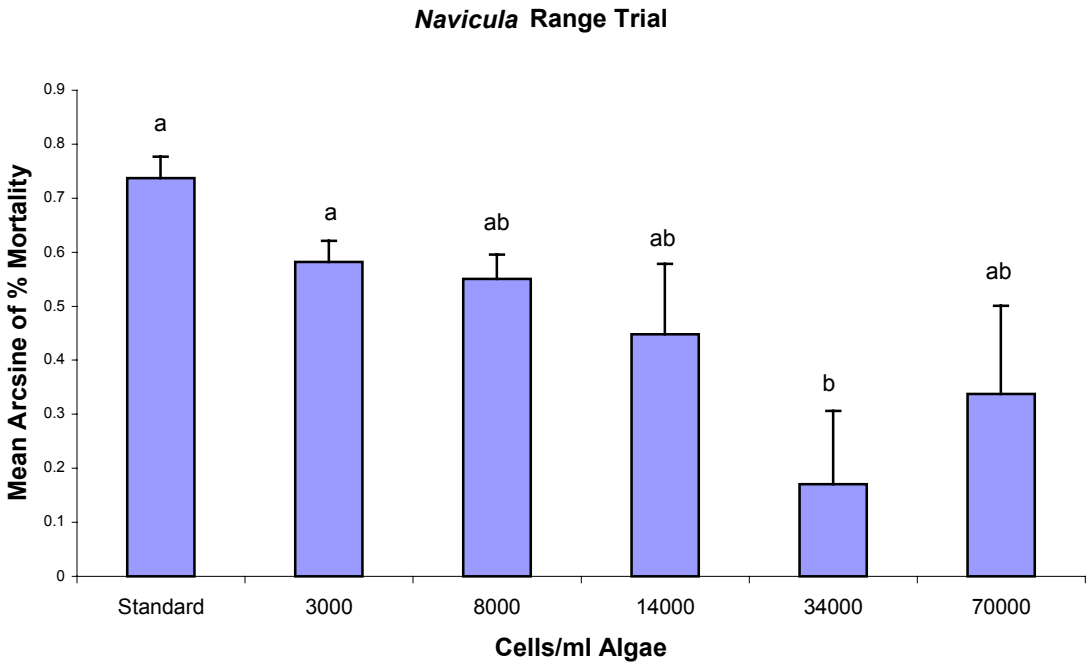

Figure 3.4 Percent larval mortality observed with varying concentrations of *Navicula*. All treatments were dosed with 0.75 ppm *B.t.i.* and compared to the standard (no algae present). Means with the same letter are not significantly different.

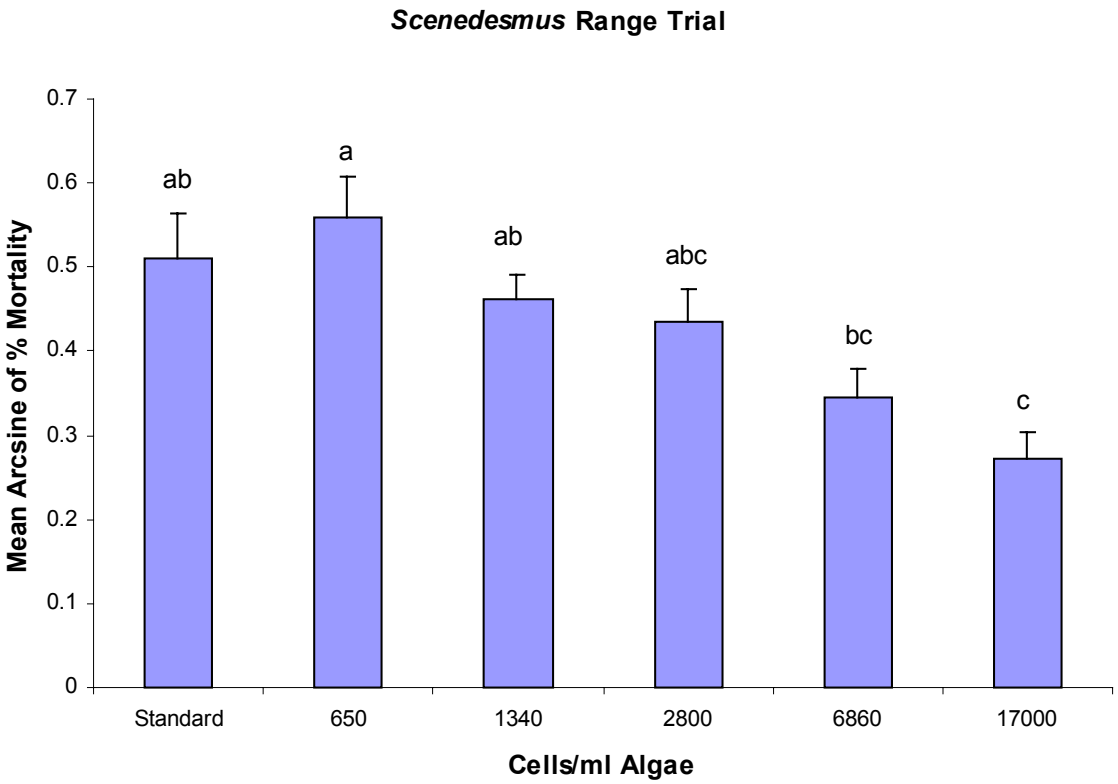

Figure 3.5 Percent larval mortality observed with varying concentrations of *Scenedesmus*. All treatments were dosed with 0.75 ppm *B.t.i.* and compared to the standard (no algae present). Means with the same letter are not significantly different.

Conclusions

Several conclusions can be drawn from the algae range trials. First, not all types of algae cause a reduction in the efficacy of *B.t.i.* against larval black flies in the orbital shaker bioassay system. Based on the results of these experiments, the blue-green algae, *Microcystis,* showed no statistically significant effects on larval mortality when present in the system as compared to a standard. The diatom *Navicula,* however, demonstrated statistically significant effects on larval mortality at concentrations greater than 34,000 cells/ml, which indicates that a reduction in the efficacy of *B.t.i*. occurs in the presence of this diatom. In the presence of *Scenedesmus,* there were significant reductions in larval mortality as the concentration of *Scenedesmus* cells increased. *B.t.i.* did not work as effectively and larval mortality was significantly reduced when greater than 7,000 cells/ml of green algae were present in the system.

Black fly control efforts and treatment programs around the world are working diligently to reduce the populations of this important disease vector and nuisance pest. The evidence suggests that in the presence of some types of algae, alternative methods of control or treatment modifications whether it is more material, longer exposure, or simply not treating until natural algal populations have declined, will have to be considered to ensure effective levels of control.

CHAPTER 4

LABORATORY ASSESSMENT OF POTENTIAL CHANGES IN BLACK FLY TREATMENT PROTOCOLS TO MITIGATE THE EFFECTS OF ALGAE ON THE EFFICACY OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* AGAINST BLACK FLY LARVAE

Introduction

Black flies are viewed as a threat to the comfort and health of both man and animal throughout many regions of the world because of their blood feeding and ability to transmit causal agents of disease. Therefore attempting to mitigate the effects of algae in *Bacillus thuringiensis* var. *israelensis* (De Barjac) based control programs is essential. Black flies are not only significant impediments to tourism and recreation, but transmit disease agents such as River Blindness (Onchocerciasis).

Based on the results of the algae range trials, the focus of these experiments was the green alga, *Scenedesmus* because it demonstrated significant reduction in black fly mortalities in our laboratory bioassays with *B.t.i*. With *Scenedesmus,* we were able to perform additional bioassays investigating various *B.t.i*. exposure manipulations that could be validated through field studies and incorporated into control programs. Some of the proposed changes in application procedures result in increased cost in control programs. Other variations that might be incorporated into control programs are as simple as increasing the exposure period by prolonging the application period.

In large control programs (such as the Black Fly Control Program of the Pennsylvania Department of Environmental Protection) increasing exposure period could be accomplished by flying the *B.t.i.* dispersal helicopter over a river multiple times instead of once. With this technique, the same amount of product could be used while increasing the drift period and availability of *B.t.i.* through the river over a given period of time. In smaller control programs where the application occurs by pouring the *B.t.i.* product directly into the river or stream the exposure period could, likewise, be increased by increasing the application period.

From the algae range trials it was determined that *Scenedesmus*, the green algae, demonstrated a clear reduction in *B.t.i.* efficacy against black fly larvae as was described by black fly control personnel in Pennsylvania and West Africa (unpublished). We wanted to determine whether or no an increased concentration of *B.t.i.* in the assay would result in increase larval mortality. The algae range trials all utilized 0.75ppm *B.t.i.* and the variable was the concentration of algae per treatment. In this *B.t.i.* range trial, we varied the concentration of *B.t.i* and a held the algae concentration constant.

Materials and Methods

Experiment 4: *B.t.i.* Range Trial

From algae range trials with *Scenedesmus* we determined that in experiments with algal concentrations of 8,000 – 10,000 cells/ml, a reduction in black fly larval mortality and *B.t.i.* efficacy, due to the presence of algae, would occur. Therefore, a constant concentration of 8,000 cells/ml of *Scenedesmus* was used in the *B.t.i.* range trial with six dilutions of *B.t.i.* ranging from 0.75 – 2.00 ppm. There were a total of thirty larvae per flask, a one hour acclimation period, a 10 minute exposure period, a five hour hold and then mortality evaluations were conducted. There were seven treatments in this trial with four replicates. Treatment concentrations were as follows: treatment 0 – standard, 0.75 ppm (no algae present), treatment 0.75 ppm, treatment 1.00 ppm, treatment 1.25 ppm, treatment 1.50 ppm, treatment 1.75 ppm and treatment 2.00 ppm. Treatments 0.75 – 2.00 ppm all consisted of 8,000 – 10,000 cells/ml *Scenedesmus*. The standard was dosed with 0.75 ppm, but had no algae present.

Experiment 5: *Scenedesmus* with Two Treatment Regimes

We wanted to determine if we could differentiate whether the inhibition of *B.t.i.,* due to the presence of *Scenedesmus,* was occurring in the test vessels prior to ingestion or in the guts of the black flies where the *B.t.i.* toxin must bind to exhibit its lethal effect. Therefore, an experiment using *Scenedesmus* with two treatment regimes was designed to demonstrate if there were differences attributable to the time of introduction of the algae (*Scenedesmus* at 10,000 cells/ml) into the flasks. There were a total of thirty larvae per flask, a one hour acclimation period, a 10 minute exposure period, a five hour hold and then mortality evaluations were conducted.

There were two treatment regimes in this experiment. The first treatment regime was "previous treatment." In this treatment the algae were added to the flasks with the black fly larvae prior to the flasks being placed on the shaker for the acclimation period. The larvae feed during the acclimation period prior to *B.t.i.* dosing and continue to feed until the orbital shaker is turned off at the end of the exposure period. In this case, the black flies' entire digestive tracts would be full of algae prior to the *B.t.i.* being introduced to the system.

The second treatment regime was "simultaneous treatment." In this treatment the algae was added to the dosing syringes with the *B.t.i.* The larvae were acclimated with bioassay water only. After the acclimation period the flasks were dosed with syringes containing both algae and *B.t.i.* In this treatment the larvae were not feeding on algae during the acclimation period, but only during the exposure period. Also, the algae and *B.t.i.* had an opportunity to interact before being introduced to the flasks with the black fly larvae. In the second dose regime treatment the black fly larvae had opportunity to

ingest both algae and *B.t.i*. at the same time because they were introduced to the flasks simultaneously. There were three treatments in this trial with five replicates. Treatments were defined as follows: treatment 0 – standard (no algae present), treatment 1 – previous treatment and treatment 2 – simultaneous treatment.

Experiment 6: Exposure Trial

The variable in this experiment was exposure time. A black fly bioassay was conducted using *Scenedesmus* (10,000 cells/ml) with two varying exposure treatments to determine if there were differences in mortality. Each treatment was dosed with 0.5 ppm *B.t.i.* The first treatment consisted of a 10 minute exposure period. The second treatment at 20 minutes doubled the exposure time.

Exposure is the period of time after *B.t.i.* dosing has occurred before the orbital shaker is turned off. Once the orbital shaker is turned off after the exposure period, black fly feeding ceased because water current is necessary for the black flies to extract algae and *B.t.i.* particles from the water. There were four treatments in this trial with three replicates. Treatments were defined as follows: treatment 1 – standard (no algae present) and a 10 minute exposure period, treatment 2 – standard (no algae present) and a 20 minute exposure period, treatment 10 – 10 minute exposure period and treatment 20 – 20 minute exposure period. All treatments were dosed with 0.5 ppm *B.t.i*. and algae treatments had 10,000 cells/ml *Scenedesmus*. There were a total of thirty larvae per flask, a one hour acclimation period, a 10 and 20 minute exposure period, a five hour hold and then mortality evaluations were conducted.

Experiment 7: Exposure Trial II

We wanted to determine if doubling the exposure period from 10 minutes to 20 minutes was synonymous to doubling the amount of *B.t.i.* available to the black fly larvae. In Experiment 6, 0.5ppm *B.t.i.* was introduced to the black flies at the 10 and 20 minute exposure periods. The 10 minute exposure period was dosed with 0.5ppm *B.t.i.,* whereas the 20 minute exposure period was dosed with 0.25ppm *B.t.i.* or half the dose and twice the exposure of the first treatment. If, in fact doubling the exposure period is the same as doubling the amount of *B.t.i.* available to the black flies, mortalities in the treatments should be similar between the 10 and 20 minute exposure periods. There were four treatments in this trial with four replicates. There were a total of thirty larvae per flask, a one hour acclimation period, a 10 and 20 minute exposure period, a five hour hold and then mortality evaluations were conducted. Treatments were defined as follows: treatment 1 – standard (no algae present) and a 10 minute exposure period dosed with 0.5 ppm *B.t.i.,* treatment 2 – standard (no algae present) and a 20 minute exposure period dose with 0.25 ppm *B.t.i,* treatment 10 – 10 minute exposure period dosed with 0.5 ppm *B.t.i* and treatment 20 – 20 minute exposure period dosed with 0.25 ppm *B.t.i.* Algae treatments consisted of 10,000 cells/ml *Scenedesmus.*

Experiment 8: Series Exposure Trial

In Experiment 8 we increased the number of exposure treatments while maintaining a constant 0.5 ppm *B.t.i.* per treatment. Hence, *Scenedesmus* (10,000 cells/ml) was tested in four treatments at 0.5ppm *B.t.i.*: 10, 20, 30, and 40 minute exposure periods. There were a total of thirty larvae per flask, a one hour acclimation period, a 10, 20, 30 and 40 minute exposure period, a five hour hold and then mortality

evaluations were conducted. The exposure periods were increased to determine whether or not the effects of algae could be counteracted and the reduction in mortality reversed. There were eight treatments in this trial with three replicates. Treatments were defined as follows: treatment 1 – standard (no algae present) and a10 minute exposure, treatment 2 – standard (no algae present) and a 20 minute exposure, treatment 3 - standard (no algae present) and a 30 minute exposure, treatment 4 - standard (no algae present) and a 40 minute exposure, treatment 10 - 10 minute exposure, treatment 20 - 20 minute exposure, treatment 30 - 30 minute exposure and treatment 40 - 40 minute exposure. All treatments were dosed with 0.5 ppm *B.t.i.* and algae treatments had 10,000 cells/ml *Scenedesmus.*

Experiment 9: Residual Toxicity Assay

The *B.t.i.* toxicity remaining after the completion of the treatments was of interest in Experiment 9. We wanted to determine how much *B.t.i.* is left in the system after each of the four exposure periods, 10, 20, 30, and 40 minutes for the black fly bioassay. With a longer exposure period we expected to see higher mortality rates. If so, this would suggest that given a longer period of exposure the black flies have more opportunity or time to ingest a larger number of the *B.t.i.* particles in the presence of *Scenedesmus*. Black flies can only feed on the algae and *B.t.i.* particles during the exposure time because once the orbital shaker is turned off the water current required for filter feeding is eliminated.

To achieve this goal of determining residual toxicity following the black fly series exposure test, a mosquito assay was conducted following the Valent Laboratories Standard Mosquito Bioassay Protocol. Half of the flasks from the series exposure test for each of the eight treatments were removed from the orbital shaker after the exposure

period. The black flies were removed from the flasks by straining through nylon screening and 100 ml of the bioassay water was placed in five ounce wax paper cups. Third-instar *Aedes aegypti* mosquito larvae obtained from the molecular entomology lab at UGA were the test organisms. Twenty mosquito larvae were used in each wax paper cup for the assay. The residual toxicity mosquito assay ran for a total of 16 hours with 8:8 light:dark regime implemented at a constant temperature of 27-29°C.

Mosquito larvae were utilized in the treatments with four exposure periods, 10,20, 30 and 40 minutes, as follows: *B.t.i.* + algae, *B.t.i.*, water control + algae, water control, *B.t.i.* with no black flies (mosquito toxicity) and mosquitoes in rearing water (mosquito control). All treatments were dosed with 0.5 ppm *B.t.i*. and algae treatments had 10,000 cells/ml *Scenedesmus*. In each case black fly larvae were removed from the flasks after the designated exposure period following the series exposure assay and mosquito larvae were added. The mosquito assay ran for 16-18 hours before mortality evaluations were conducted. There were eight treatments in this trial with three replicates each trial utilizing. Treatments were defined as follows: treatment 1 – standard (no algae present) and a 10 minute exposure, treatment 2 – standard (no algae present) and a 20 minute exposure, treatment 3 - standard (no algae present) and a 30 minute exposure, treatment 4 - standard (no algae present) and a 40 minute exposure, treatment 10 - 10 minute exposure, treatment 20 - 20 minute exposure, treatment 30 - 30 minute exposure and treatment 40 - 40 minute exposure.

Results and Discussion

Experiment 4: *B.t.i.* Range Trial

The results of the *B.t.i.* range trial suggested several practical applications. There were significant differences between the treatments and the standard (One-way ANOVA, P< .0001). By increasing the concentration of *B.t.i.* we were able to bring larval mortality back up to and beyond the standard (Figure 4.1). It appeared that as the concentration of *B.t.i.* was increased and the concentration of algae remained constant, the reduced efficacy of *B.t.i.* could be reversed.

Larval mortality was reduced at concentrations of both 0.75 and 1.00 ppm *B.t.i.* However, at 1.00 ppm and 1.25 ppm the mortality is similar and not statistically significantly different from the standard. At concentrations above 1.5 ppm the treatments are different from the standard, which would indicate that at increasing concentrations, the efficacy of *B.t.i.* in the presence of algae is increased. For treatment and control programs these results suggest that during the presence of higher algal populations, if *B.t.i.* treatments are not performing as usual, the field operators could increase the concentration of *B.t.i.* to achieve the desired larval mortality as one option to their normal application methods. However, this technique may be more expensive because if higher concentrations of *B.t.i*. are required, more *B.t.i*. product may also be required to achieve the desired concentration.

Experiment 5: *Scenedesmus* with Two Treatment Regimes

Experiment 5 offered possible insight into mechanisms behind the interaction between algae and *B.t.i*. particles. It was demonstrated in the *Scenedesmus* range trials that in the presence of algae, the efficacy of *B.t.i.* is significantly reduced. This

experiment attempted to provide a closer look at this interaction. By utilizing the two treatments (previous and simultaneous) it was our intention to better define at what point in the assay that the inhibiting interaction between algae and *B.t.i.* particles was occurring.

There were some distinctive differences between the set up of the two treatments utilized in this assay. In the previous treatment the black fly larvae were exposed to algae prior to being dosed with *B.t.i.* This allowed for the possibility that the larval guts were completely full of algae before the *B.t.i.* was added to the system. In the simultaneous treatment the algae and the *B.t.i.* were in the same dosing syringe and introduced to the system simultaneously. This would allow for a possible interaction between the *B.t.i.* and algae to occur before they were made available to the larvae.

Although differences between the two treatments were hypothesized, this was not the case. There were differences between the treatments with algae and the standard with no algae (One-way ANOVA, P=0.0040). The reduction that had been previously documented was observed once again. However, there were no differences between the two treatments (One-way ANOVA, P=0.9394) (Figure 4.2).

We expected but did not observe marginal differences between the two treatments due to the variation in when the algae were introduced to the system. In actuality, the two treatments almost mirrored one another. By allowing the algae and *B.t.i.* particles a chance to interact outside the system in the dosing syringes we anticipated that the black flies would respond differently. This led us to believe that the interaction that was occurring and causing the *B.t.i.* inhibition was not dependent upon the guts being full or empty at the time of exposure to *B.t.i.* These results also suggested that allowing the

B.t.i. and algae interaction time before being introduced to the larvae does not increase or reduce the efficacy of *B.t.i.* in a system with algae.

Experiment 6: Exposure Trial

The exposure trial allowed us to draw inferences about field applications of *B.t.i.* in control programs when algae are present and reduction of *B.t.i.* efficacy is suspected. Adjusting application protocols to increase the exposure period would be feasible in most programs and such adjustments could be easily implemented in the field. We wanted to determine if differences would be observed by increasing the exposure time from 10 minutes to 20 minutes in our laboratory bioassays while keeping everything else constant in the two treatments.

The results indicated that increasing the exposure period has the potential to increase larval mortality in the presence of algae. Several interesting results were determined from the exposure trial. There were no differences observed between the standard of the two treatments (One-way ANOVA, P=0.8093). This suggested that most of the *B.t.i.* is extracted by the black flies, when no algae is present, in the first 10 minutes. Mortality neither increases nor decreases with increased exposure time in either standard treatment. However, this was not the case when comparing the differences between the algae treatments.

Although there were no significant differences between the 10 minute and 20 minute algae treatments (One-way ANOVA, P=0.2298), there was an observable difference in mortality between these two treatments (Figure 4.3). The mortality in the 20 minute algae treatment was higher than that of the 10 minute algae exposure treatment. This suggests a trend toward increased mortality with increased exposure.

As previously discussed, the mortality produced by the standard was similar in the two treatments, however, mortality in the algae treatments increased with an increase in exposure time. In the 10 minute treatment there was a 44.3% reduction in mortality as compared to the efficacy of the standard, whereas in the 20 minute treatment, there was an 18.5% reduction in mortality. The percent reduction was halved or mortality was nearly doubled when comparing the 10 and 20 minute exposure treatments. This proposes an interesting question that was addressed in the next experiment. Does doubling the exposure period essentially double the amount of *B.t.i.* available to the larvae; if so, does this account for the increase in mortality observed in the 20 minute treatment?

Experiment 7: Exposure Trial II

This experiment was designed to attempt to answer questions concerning the results from the exposure trial and the observed increase in mortality with the 20 minute exposure treatment. The central question was – did doubling the exposure period result in twice the amount of *B.t.i.* being available to the larvae?

As expected, there were significant differences between the standard and the algae treatment in the 10 minute exposure treatment (One-way ANOVA, P=0.0004). This further supported our earlier findings that the efficacy of *B.t.i.* at 0.5ppm was reduced in the presence of *Scenedesmus* with a 10 minute exposure period. In the exposure trial no differences were observed between the standard of the two treatments, which indicated that most of the *B.t.i.* was removed by the larvae within the first 10 minutes. However, in the second exposure trial differences were observed between the standard of the 10 and 20 minute treatments (One-way ANOVA, P=0.0015). Differences were also seen

between the algae treatments of the two trials (One-way ANOVA, P=0.0243) (Figure 4.4).

We had expected that the larval mortalities of the algal treatments would be similar if our hypothesis about doubling the amount of *B.t.i.* available to the larvae by doubling the exposure was correct. By halving the concentration of *B.t.i.* in the 20 minute exposure period, the mortality of the larvae, although exposed to half as much *B.t.i.* should be similar to that of the 10 minute treatment. The mortalities between the two standard treatments were different indicating that the lower concentration of *B.t.i.* had an effect on larval mortality.

Because differences were found between the two standard treatments in the second exposure trial, two conclusions can be drawn. First, we believe that the larvae, if no algae are present, are able to remove most of the *B.t.i.* in the first 10 minutes. The LC 50 of the *B.t.i.* formulation at 0.25 ppm was similar to what was observed in this trial even though it was exposed for 20 minutes. The second conclusion deals more with the fact that algae has an impact on the efficacy of *B.t.i.* and the ability of the larvae to ingest *B.t.i.* is hindered unless more exposure time is given. The increased mortality of the algal treatments observed in the exposure trial was not occurring because twice the amount of *B.t.i.* was made available to the larvae due to the doubled exposure period. The increased mortality observed in the exposure trial was occurring because the larvae were allowed twice as much time to ingest the *B.t.i.*

Exposure trial II answered questions concerning the effectiveness of maintaining the same concentration throughout the assay. It was demonstrated by reducing the concentration of *B.t.i.* while increasing the exposure period that the effects observed were

not anticipated. However, this further supports the idea that by maintaining a constant concentration of *B.t.i.* while increasing the exposure, effects may be observed that are dependent upon the exposure period alone.

Experiment 8: Series Exposure Trial

We have demonstrated that it is possible to increase larval mortality in the presence of algae by increasing the exposure period. We were also able to demonstrate that by doubling the exposure time the amount of *B.t.i.* available to the larvae remains the same. The difference is how available to the larvae does *B.t.i.* become with increased exposure periods. We are still unsure what the mechanism is that is causing the inhibition, but it is clear that mortality is greater if black fly larvae are given more opportunity to extract *B.t.i.* from the water. This is positive from the standpoint of developing strategies to mitigate the impacts of algae on black fly treatment and control programs.

The results from the series exposure trials support the preliminary data. There were significant differences in three out of four algal treatments (One-way ANOVA, 10 minutes P=0.0058, 20 minutes P=0.0043, 30 minutes P=0.0472, 40 minutes P=0.2018). The mortalities observed in the algal treatments of the 10, 20, and 30 minute exposure periods were different from the standard treatments. The expected reduction in *B.t.i.* efficacy occurred in these three treatments, although each time the mortality was closer to that of the standard. The mortality in the 40 minute treatment was not significantly different (Figure 4.5) from the mortality of the standard. This suggests that by increasing the exposure period the effects of the algae can be countered and mortality can be brought back up to the level of the standard. The standard treatment, however, was not

different amongst the treatments (One-way ANOVA, P= 0.9842), which indicated that the larvae were extracting most of the *B.t.i.* in the first 10 minutes. This further supports results from previous trials. There was a positive trend noted in the mortality of the four treatments.

Experiment 9: Residual Toxicity Assay

Results from the previous trials raised the question of how much *B.t.i.* the black fly larvae were removing from the system in the four exposure periods (10, 20, 30, 40 minutes). Mosquitoes are the industry test organisms for determining *B.t.i*. potency and provided us the opportunity to extrapolate residual toxicity after the black fly bioassays were completed. This also enabled us to have a better understanding of how much *B.t.i.* black flies can extract in the presence of algae in our four treatment periods.

We expected to see reversed results from those of the series exposure trial. As exposure increased, mortality in the algal treatments also increased in the series exposure trial. We expected this to reflect in the mosquito assay by decreasing mosquito toxicity as exposure increased. The thought was that as black flies are given more opportunity to extract *B.t.i.* from the system, less *B.t.i.* will be remaining for mosquito toxicity.

All of the four treatments showed significant differences between the standard and the algal treatments (One-way ANOVA, 10 minutes P=0.0035, 20 minutes P=0.0076, 30 minutes P=0.0135, 40 minutes P=0.0078). In each case, when algae was present toxicity to the mosquitoes decreased. There was a negative trend in mosquito mortality in the algal treatments (Figure 4.6). However, the standards of the four exposure periods were the same (One-way ANOVA, P=0.2481). In this assay the mortality of the mosquitoes in the standard treatment, even with four exposure periods, was similar.

Once again, this supports the finding that most of the *B.t.i.* is extracted from the system by the black flies in the first ten minutes in the orbital shaker bioassay.

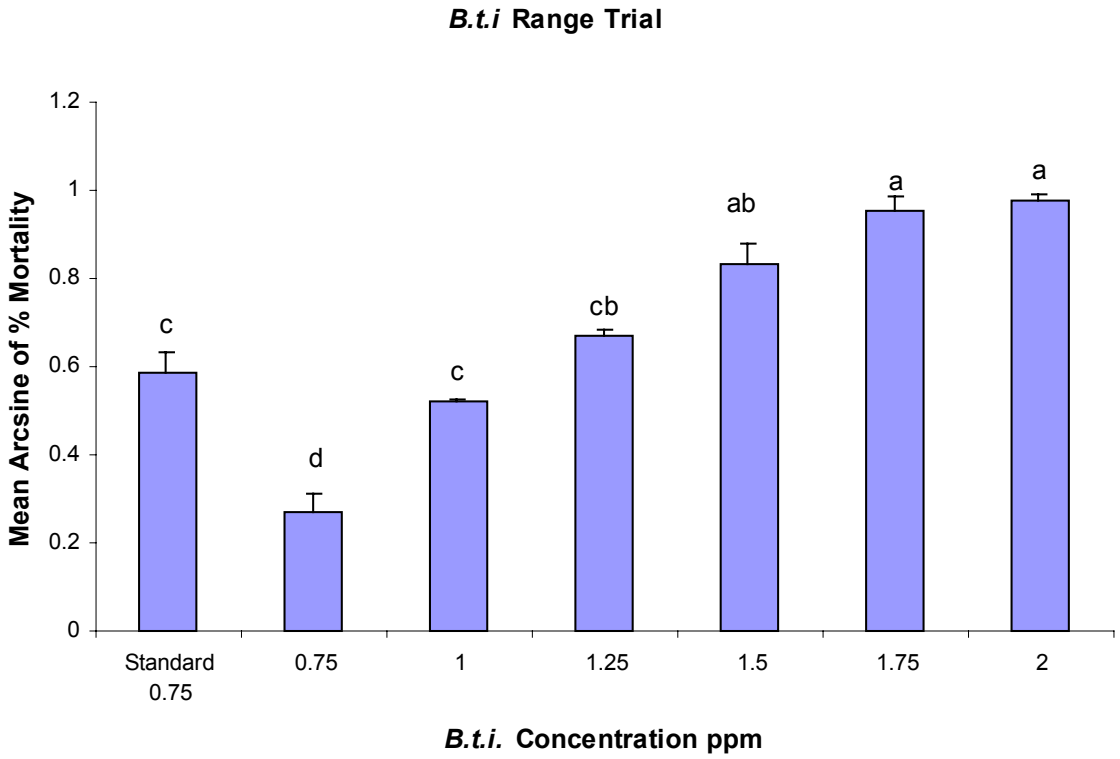

Figure 4.1 Percent larval mortality observed with varying concentrations of *B.t.i.*. All treatments contained 8,000-10,000 cells/ml *Scenedesmus* and were compared to the standard (no algae present). Means with the same letter are not significantly different

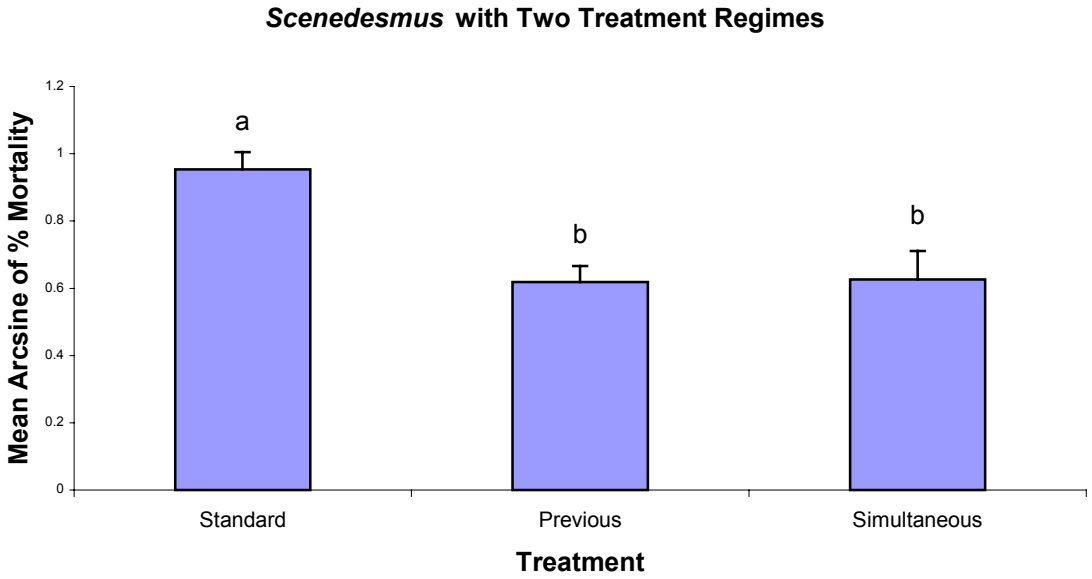

Figure 4.2 Percent larval mortality observed with two treatment regimes. The previous treatment consisted of algae added to the system prior to the *B.t.i.* The simultaneous treatment consisted of algae added to the system with the *B.t.i.* All treatments contained 8,000-10,000 cells/ml *Scenedesmus*, were dosed with 0.5 ppm *B.t.i.* and compared to the standard (no algae present). Means with the same letter are not significantly different.

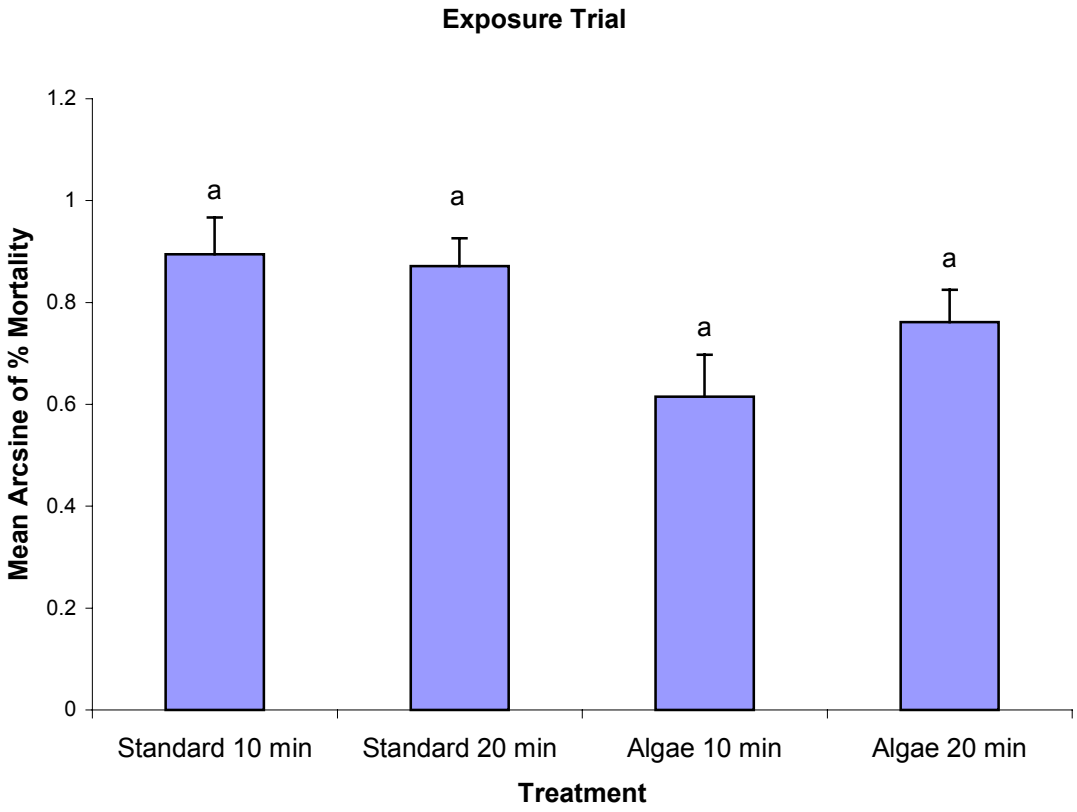

Figure 4.4 Percent larval mortality observed with two exposure periods; 10 minute and 20 minute. All treatments contained 8,000-10,000 cells/ml *Scenedesmus*, were dosed with 0.5 ppm *B.t.i*. and compared to the standard (no algae present). Means with the same letter are not significantly different.

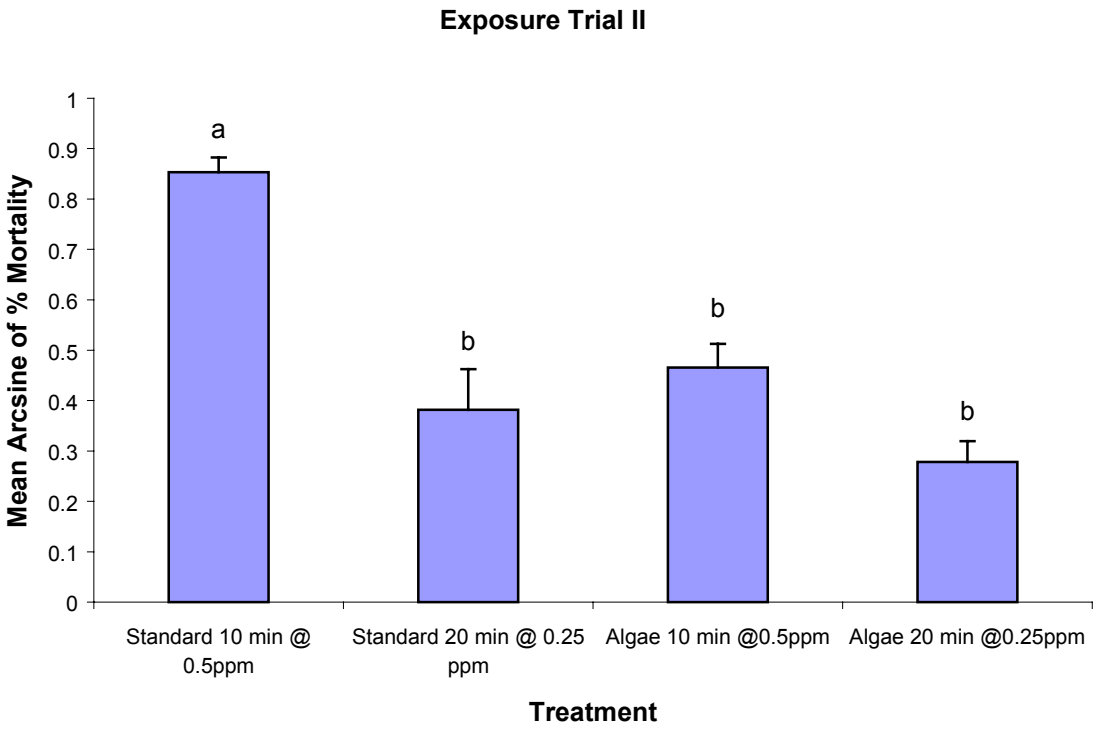

Figure 4.4 Percent larval mortality observed with two exposure periods; 10 minute and 20 minute, and two concentrations of *B.t.i.*; 0.5 ppm and 0.25 ppm. All treatments contained 8,000- 10,000 cells/ml *Scenedesmus* and compared to the standard (no algae present). Means with the same letter are not significantly different.

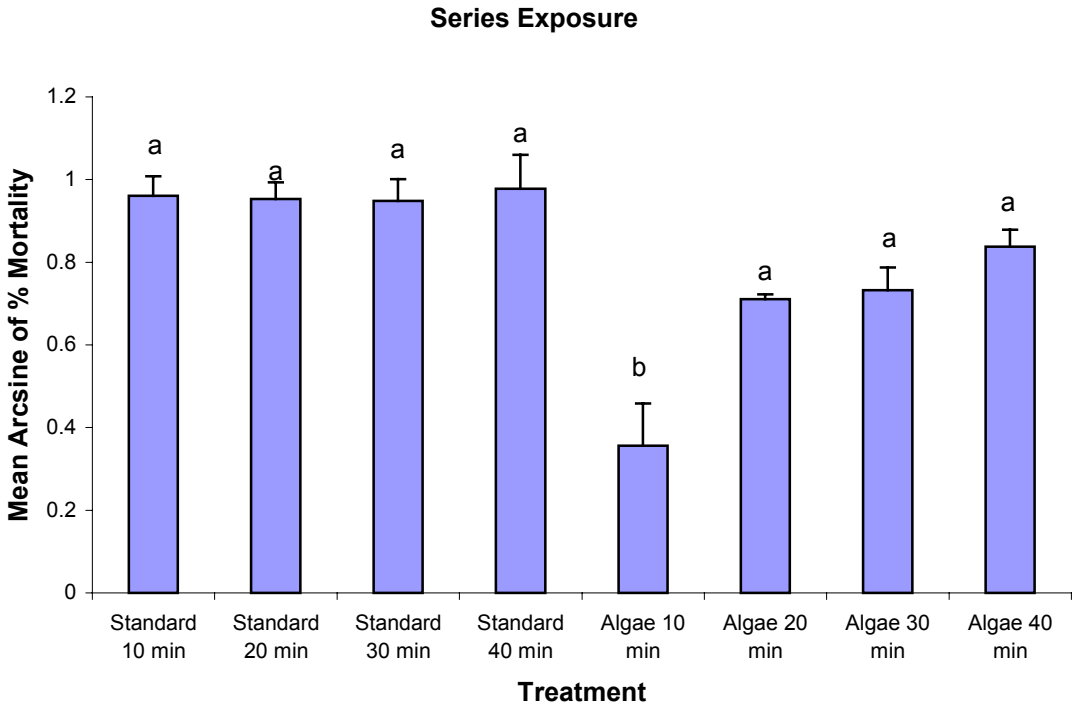

Figure 4.5 Percent larval mortality observed with four exposure periods; 10 minute, 20 minute, 30 minute, and 40 minute. All treatments contained 8,000-10,000 cells/ml *Scenedesmus*, were dosed with 0.5 ppm *B.t.i.* and compared to the standard (no algae present). Means with the same letter are not significantly different.

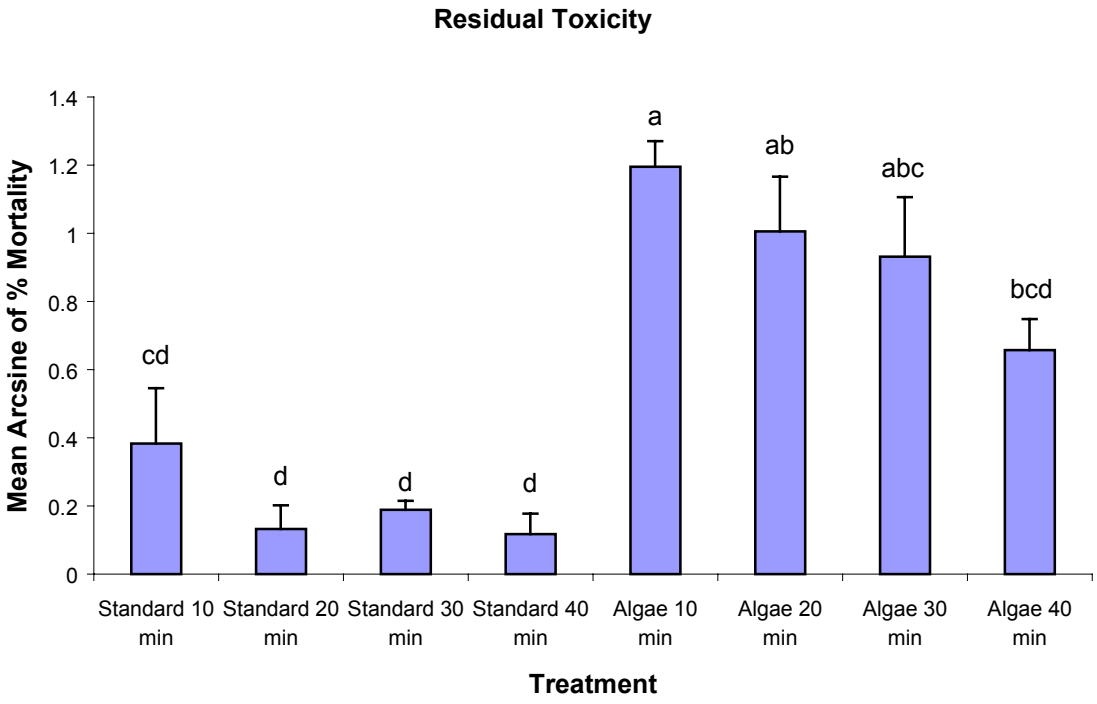

Figure 4.6 Percent mosquito mortality observed with four exposure periods; 10 minute, 20 minute, 30 minute and 40 minutes following the series exposure assay after the black fly larvae have been removed from the system. All treatments contained 8,000-10,000 cells/ml *Scenedesmus*, were dosed with 0.5 ppm *B.t.i.* and compared to the standard (no algae present). Means with the same letter are not significantly different.

Conclusions

There are several conclusions we can draw from our bioassay experiments on the evaluations of potential changes in field treatment protocols that could be used to mitigate the effects of algae on *B.t.i*. efficacy against black fly larvae. First, by increasing the concentration of *B.t.i*. we were able to reverse the negative effect of algae. Increased concentrations of *B.t.i*. can be used when algae are present in the rivers and streams to ensure acceptable levels of larval mortality.

In an effort to determine when in the assay the *B.t.i*. efficacy inhibition caused by algae was occurring, we designed an experiment to try to demonstrate differences between two experimental dosing treatments. However, there were no significant differences observed in this experiment. This indicates that whether inhibition was occurring in the 10 minute exposure period when the *B.t.i.* is introduced to the system with algae and black flies or if the algae and *B.t.i.* were combined prior to being introduced to the black flies, the inhibition still occurs.

The length of the exposure period is usually specified in treatment protocols and is thus of interest to personnel in black fly control programs. This is a modification that can be made in the field by spreading *B.t.i.* particles over a section of stream for a longer period of time without using more product. Although no statistical differences were observed in the assay, a trend towards a reversal in inhibition was noted. By increasing the exposure period from 10 minutes to 20 minutes, while maintaining a constant concentration of *B.t.i*., larval mortality was increased in the presence of Scenedesmus.

In an experiment designed to determine if increasing the exposure period produced the same results as increasing the concentration of *B.t.i.* available to the black

flies, additional exposure experiments were conducted. We concluded that increasing the exposure period was not the same as increasing the concentration of *B.t.i*.

Additional experiments were conducted to determine effects of increasing exposure time from 10, 20, 30 and 40 minutes. An initial significant reduction in the efficacy of *B.t.i.* was observed in the 10 minute exposure period. However, in subsequent treatments; 20, 30 and 40 minute exposure periods, no significant differences were observed. A positive trend in mortality indicates that with increasing exposure time an increase in larval can be detected. The standard treatments in each of the four exposure periods were not significantly different, indicating that with no algae in the system, the black flies were able to remove most of the *B.t.i.* within the first 10 minutes. We can conclude that the negative effects of *Scenedesmus* on the efficacy of *B.t.i.* can be mitigated with increased exposure times.

Lastly, we can conclude that if the black flies are given the opportunity to feed for a longer period of time during the increased exposure periods, they will extract significantly different amounts of *B.t.i*. particles from the system. This was proven in the remaining toxicity assay with the use of *Aedes aegypti* mosquitoes. When *Scenedesmus* is present in the system the black flies are unable to remove the amount of *B.t.i.* necessary to cause sufficient larval mortality, therefore a reduction in the efficacy of *B.t.i*. is noted. The reduction in *B.t.i*. efficacy was reversed if the black flies were exposed for longer periods of time in the presence of algae because the black flies were able to extract sufficient amounts of *B.t.i*. to produce a desired level of mortality. Mosquito mortality in the residual toxicity assay was highest in the 10 minute exposure period and a negative trend was observed thereafter. Interestingly, mosquito mortality of the standard

treatment, with no algae, demonstrated no significant differences. This indicated that the black flies were able to remove most of the *B.t.i.*, therefore, mosquito mortality was low throughout the standard treatments.

CHAPTER 5

CONCLUSIONS

Effects of Three Algae Genera on *Bacillus thuringiensis var. israelensis*

Presently, the effects of algae have demonstrated that two of the three genera of algae utilized in this research project have statistically significant effects on the efficacy of *B.t.i.* against larval black flies in the orbital shaker bioassay. The blue-green algae, *Microcystis*, demonstrated no negative effects on *B.t.i*. efficacy against larval black flies. At cell concentrations greater than 34,000 cells/ml *Navicula*, a diatom, demonstrated significant effects on *B.t.i.* efficacy. *Scenedesmus*, a green algae, also demonstrated significant effects on *B.t.i.* efficacy at cell concentrations greater than 7,000 cells/ml when present in the system during a black fly bioassay. Larval mortality, as compared to a standard treatment where no algae is present, is reduced. In black fly treatment and control programs around the world, during an algae bloom, a reduction in the efficacy of *B.t.i.* may be observed with select genera of algae.

Manipulations that could Mitigate the Effects of Algae on *B.t.i.* Efficacy

From manipulations in the experimental procedures to our black fly bioassay with *Scenedesmus,* we can make several conclusions. From the six experiments in Chapter 4 it can be concluded that the negative effects of algae can be reversed with a greater concentration of *B.t.i.* along with an increase in the exposure period. We can also conclude that increasing the exposure period is not the same as increasing the concentration of *B.t.i.* available to the black flies. Differences attributable to introduction time of algae and *B.t.i*. into the bioassay could not be determined from these experiments.

Residual toxicity determinations using the *Aedes aegypti* bioassay revealed that, if given the opportunity, black flies will ingest significantly more algae and *B.t.i.* particles in an increased exposure period.

Future Studies and Applications to Black Fly Treatment and Control Programs

This research demonstrates baseline data concerning the effects of algae on the efficacy of *B.t.i.* against larval black flies in the orbital shaker bioassay system. The algae genera chosen for these experiments were all present in black fly field control programs in Pennsylvania and West Africa. They represent three algal types; blue-green, diatoms and green algae respectively. However, for a more complete understanding, additional genera of algae should be tested based on differences in morphology, pigmentation and overall relative abundance in treatment areas where reduction in *B.t.i.* efficacy has been reported during an algal bloom.

Although we were able to provide suggestions for mitigating the negative effects of algae on *B.t.i.* efficacy, increasing *B.t.i.* concentration and increasing exposure period, we did not determine the mode of action behind the inhibition. Whether the inhibition of *B.t.i.* is occurring as a result to a biological, chemical or morphological change has yet to be determined.

Future studies could be focused to explore biochemical changes occurring in the black fly larval midgut due to the presence of *B.t.i.* and algae. It is suspected that the alkalinity of the black fly midgut changes when the gut is full of algae. It is also possible that the *B.t.i.* binding sites are impeded by algae or possibly that the *B.t.i.* is unable to bind to the sites in the gut when algae are present.

Field experiments would also be useful for some application methods. Our suggestions for black fly treatment and control programs, increasing *B.t.i.* concentration and increasing exposure time, have not been verified in the field during an algal bloom when *B.t.i*. efficacy could be reduced. Information gathered from field trials would be applicable wherever black fly control is necessary.

Future research is essential to better understand the interaction between algae and *B.t.i*., both in the laboratory and in the field. Hopefully, our findings will be useful to field personnel applying *B.t.i.* for black fly control in rivers and streams with high population levels of algae.

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BIOGRAPHY

Marianne Shockley Stephens was born in Cairo, Georgia to Drs. John and Sandra Shockley. Marianne graduated from Morgan County High in Madison, Georgia in 1993. She went on to complete a degree from Georgia College and State University in 1997 with a Bachelor of Art in Biology. Marianne married Travis P. Stephens in November of 1997 and moved to Charleston, South Carolina where her husband, Travis, completed the Advanced Standing Program of Culinary Arts at Johnson and Wales Culinary Institute. Marianne began a graduate program at the University of Georgia where she conducted her master's research under the guidance of Dr. Ray Noblet. After completion of her master's degree, Marianne plans to work for the state or federal government utilizing the knowledge and skills gained as a graduate student.