THE SAFETY OF BACTERIAL MICROBIAL AGENTS USED FOR BLACK FLY AND MOSQUITO CONTROL IN AQUATIC ENVIRONMENTS

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1. INTRODUCTION

Aquatic environments are important habitats for a multitude of species, complex food webs and the predominant sources of the essential requisite for all life in the biosphere — water. Insects contribute to several levels of the food web in aquatic systems and a multitude of terrestrial organisms that, in turn, depend on them. In the 1970's and 1980's insects became the dominant forms used in freshwater investigations of basic ecological inquiry (Barnes & Minshall, 1983).

Aquatic habitats also serve as breeding sites for some of the most serious blood sucking insect pests of humans and other animals. Black flies (Simuliidae) and mosquitoes (Culicidae) are responsible for transmitting numerous disease-causing agents to humans and domestic and sylvatic animals and for lowering the quality of life through their blood feeding activities. Immature stages of black flies are found only in lotic habitats (running water) that range from small trickles to raging rivers (Crosskey, 1990; Merritt & Cummins, 1996). Mosquito larvae occupy a huge variety of aquatic habitats that include: 1) the margins of small streams; 2) salt marshes; 3) temporary pools; 4) organically enriched standing water; 5) rice fields; 6) the vegetated margins of pristine lakes; 7) algal mats in eutrophic lakes; 8) tree holes; 9) artificial containers and a multitude of others (Laird, 1988; Merritt et al., 1992; Service, 1993). Rich complexes of other aquatic species including insects, other invertebrates and a variety of vertebrates also co-inhabit both lotic and lentic environments.

Because of the sensitive nature of aquatic habitats in terms of essential resources for humans and a wide variety of plants and animals, it is imperative that interventions that are used for the abatement of pest and vector species cause little or no deleterious effects within the habitats in which they are used. Broad spectrum conventional chemical larvicides used during the past century for black fly and mosquito control have been replaced in many countries by more environmentally acceptable alternatives such as insect specific microbial control agents and insect growth regulators. Bacteria have been more successfully used than any other group of entomopathogens for the control of larval mosquitoes and black flies (Lacey & Undeen, 1986; Becker & Margolit, 1993; Lacey &

Orr, 1994; Becker, 2000; Boisvert & Boisvert, 2000).

Bacillus thuringiensis var. *israelensis* (*Bti*), the most commonly employed entomopathogen for control of black flies and mosquitoes, is mainly active against certain families of Diptera in the suborder Nematocera. *Bacillus sphaericus* is active only against the family Culicidae (mosquitoes). The larvicidal activity of both bacteria is due to toxins produced at the time of sporulation which must be ingested by targeted larvae to be active. The molecular configuration and mode of action of *Bti* and *B. sphaericus* toxins have been studied in detail and are presented by several researchers (see Charles et al. 2000 for a comprehensive overview). Commercial formulations of both bacteria are available and employed for control of pest and vector species worldwide. Several large control programs, such as the Onchocerciasis Control Programme (OCP) in West Africa and the Rhine Valley Mosquito Control Program (KABS) in Germany, rely heavily on these microbial control agents. The OCP utilizes *Bti* as a major component of its control program in the dry season to help manage insecticide resistance in the targeted *Simulium damnosum* complex (Guillet et al., 1990; Kurtak, 1990). The KABS program uses both bacteria for mosquito control due to their efficacy and lack of negative impact on nontarget organisms (Becker, 1997, 2000).

2. METHODOLOGY FOR ASSESSMENT OF NEGATIVE IMPACT OF MICROBIAL CONTROL AGENTS IN AQUATIC HABITATS

The effects of microbials can be direct or indirect. Direct effects are due to the susceptibility of nontargets to the entomopathogens employed, and in some cases, due to formulation constituents. Indirect effects are complex and are mostly due to the mass removal of the target organisms, especially over a sustained time period. Broad guidelines for the evaluation of the effects of microbial control agents on nontarget organisms are presented by Hajek and Goettel (2000). There are relatively few specific protocols for the evaluation of entomopathogenic bacteria on nontarget aquatic organisms. Protocols will depend on: 1) the nontarget species being considered; 2) the habitat in which evaluation will take place; 3) the voltinism of the target species (mosquito or black fly); 4) the number of treatments required for its control and intervals between treatments; 5) other specific study objectives.

2.1 Laboratory studies

The simplest assessments of direct impact of entomopathogens on nontarget organisms have been conducted under laboratory conditions. Because the toxins of *Bti* and *B. sphaericus* must be ingested to be active, it is essential that laboratory bioassays permit feeding by test organisms that is as close to normal as possible (Lacey, 1997). For species from lentic habitats, the bioassay can be as simple as exposing nontarget larvae to suspensions of bacteria in small containers. Bioassay methods used for mosquito larvae, such as those described by Lacey (1997) and Skovmand and Becker (2000), may be suitable for use with several other aquatic Nematocera (e.g. certain species of Ceratopogonidae, Chaoboridae, Chironomidae, Psychodidae, Dixidae, etc.). Several factors may influence the degree of mortality observed (temperature, water volume and depth, number of larvae per container, etc.). For example, the provision of food increased tolerance of chironomid larvae to *Bti* in bioassays conducted by Ali et al. (1981) and Ali (1981), and it has been shown that decreased efficacy of *Bti* at low temperatures may occur

because of a decrease in larval feeding rate (Walker, 1995).

Some aquatic species will require specific conditions to feed normally in addition to being placed in a container of water. These conditions will require elucidation on a case by case basis. For example, larvae of predatory species such as beneficial mosquitoes in the genus *Toxorhynchites* do not filter particulates from water, *i.e.* would not ingest bacterial toxins in an aqueous suspension. However, they are susceptible to bacterial toxins when ingested in prey (*e.g. Aedes aegypti* larvae) that have been fed suspensions of the pathogens (Lacey & Dame, 1982; Larget & Charles, 1982; Lacey, 1983).

Methods used for laboratory bioassays of *Bti* against black fly larvae require a current that enables attachment of the larvae to the surface of the container and filter feeding (Lacey, 1997). The relatively simple methods employed for bioassays with black fly larvae will only be appropriate for a small number of aquatic species that attach to smooth surfaces in a current and feed normally. Laboratory bioassays against most aquatic nontarget insects will require a greater understanding of the environmental requisites of the species being tested and the more complex simulated field conditions that will enable normal feeding. Additionally, predator species will require provision of prey that have fed on *Bti* to determine any possible direct detrimental effects of the bacterium.

2.2 Field studies.

Although laboratory-derived nontarget susceptibility data can be useful in determining potential risk for a given species, follow up studies must be conducted under natural conditions to know the actual impact a microbial control agent will have. This will be essential for any studies on both the direct and indirect effects of the pathogens in a given organism or community. For example, Charbonneau et al. (1994) observed mortality of a nontarget chironomid in laboratory assays, but not in the field.

Censusing of insect populations before and at intervals following and sometimes during treatment usually provides the information required to determine impact of microbial control in the short and long term. Also, sampling from nearby untreated populations over the same time interval is essential to determine fluctuations in natural populations that have nothing to do with microbial treatments. A huge variety of methods are available depending on the habitat and species or community of interest. Many of the methods used for field evaluation of bacteria and other pathogens against mosquito and black fly larvae (Undeen & Lacey, 1982; Skovmand et al., 2000) will be suitable for evaluation of microbial treatments on certain nontarget species. However, for most nontargets, more extensive methods and equipment used for sampling aquatic insects in the various microhabitats (in sediments, under stones, within the water column, etc.) will be required. An overview of aquatic sampling methods and equipment is provided by Southwood (1978), Service (1993), Merritt et al. (1996) and others. Artificial substrates that are readily colonized by aquatic insects (Southwood, 1978; Merritt et al., 1996) also have been employed for the evaluation of treatment effects. In addition to population censusing, dissection of field collected nontarget organisms including fish and insects can be performed for analysis of gut contents.

Some alternatives to conducting assessments exclusively *in situ* include field exposure and laboratory incubation of the test subjects and the use of sentinel organisms placed in retrievable cages *in situ* (Lacey & Undeen, 1984; Skovmand et al., 2000). Laboratory incubation of field-exposed insects in lotic habitats provides the advantage of being able to utilize several segments of a single stream for replicated tests over a short time period. This

is accomplished by starting tests in downstream segments and successively working upstream for subsequent tests after post-treatment collections have been made (Lacey & Undeen, 1984; Wipfli & Merritt, 1994a). Trough systems or mesocosms placed in rivers or used along side of rivers and streams as a means of assessing the impact of microbials and conventional larvicides also have been used (Lacey et al., 1982; Dejoux et al., 1985; Guillet et al., 1985; Wipfli & Merritt, 1994b). The troughs offer several advantages in terms of small size, rapid set up and repeatability.

A variety of biotic and abiotic factors can influence the activity of *Bti* and *B. sphaericus* against susceptible target and nontarget organisms. These include the age and species of the organism, its role in the food web and feeding behavior, environmental factors (temperature, turbidity, organic pollution, etc.), formulation constituents, concentration of bacteria, and frequency of treatments. The degree of risk for susceptible nontarget species is also a function of the persistence of the bacterial toxins. The toxins of both bacteria are degraded in organically enriched habitats, may settle from the feeding zone of the nontarget, or be diluted by particulates or addition of water, especially in lotic habitats (Gelernter, 2001). The above factors should be documented as thoroughly as possible when determining the risk of *Bti* and *B. sphaericus* for nontarget organisms.

3. DIRECT EFFECTS OF *BACILLUS SPHAERICUS* ON NONTARGET ORGANISMS

The host spectrum of *B. sphaericus* serotypes that are used for mosquito control is quite reduced relative to *Bti*, with only mosquito species being affected. Even within the Culicidae, the susceptibility of mosquito species is variable. For example, *Ae. aegypti* and certain other species demonstrate little or no susceptibility to *B. sphaericus* toxins. One important group of potentially susceptible nontargets are mosquitoes in the genus *Toxorhynchites*. Larvae of this species are voracious predators of mosquito larvae that breed in treeholes and other small collections of water. Adult *Toxorhynchites* do not take blood meals. Laboratory tests revealed that in the presence of prey (*Ae. aegypti* larvae) and 0.1 to 1.0 mg/l of a lyophilized spore preparation of *B. sphaericus* (2013 isolate; serotype 5a,5b), 24 and 48 hour old *Toxorhynchites rutilus* larvae responded with 48-97% and 37-76% mortality, respectively (Lacey, 1983). Larvae of *Tx. theobaldi, Tx. amboinensis* and *Tx. brevipalpis* were not affected by the same isolate and range of concentrations. Bioassays conducted with the 1593 isolate (serotype 5a,5b) and *Tx. rutilus* second instar larvae produced LC_{50} and LC_{95} values of 0.27 and 4.72 mg/l, respectively (Lacey et al., 1988). Not all serotypes of *B. sphaericus* are active against *Tx. rutilus*. Second instar larvae of this species were not susceptible to 10 mg/l of the 2297 isolate (serotype 25) in the presence of prey (Lacey et al., 1988).

Tests with *B. sphaericus* against several other invertebrate species in laboratory and field conditions confirms their specificity for mosquitoes and lack of effect on nontarget insects including a variety of mosquito predators and chironomids and other species of Nematocera (Mathavan & Velpandi, 1984; Mulla et al., 1984; Ali & Nayar, 1986; Aly & Mulla, 1987; Karch et al., 1990; Lacey & Mulla, 1990; Mulla, 1990; Rodcharoen et al., 1991; Walton & Mulla 1991; Yousten et al., 1991, 1992; Becker 1997; Merritt, Berg, and Ali, unpublished data).

Very little information is available on the direct effect of *B. sphaericus* on fish. Unpublished studies on the safety of *B. sphaericus* for its registration in the United States included testing against fish with no deleterious effects reported (Lacey & Siegel, 2000).

Walton & Mulla (1991) documented the combined use of the mosquitofish, *Gambusia affinis* (1 or 4 kg/ha), and *B. sphaericus* (0.11 kg/ha) for the integrated control of larvae of the mosquito *Culex tarsalis*. The combination of the two agents resulted in greater suppression of mosquito larvae than either treatment alone with no apparent deleterious effects on the mosquitofish. A multitude of laboratory tests have confirmed the safety of *B. sphaericus* for other vertebrates including mammals (summarized by Saik et al., 1990; Siegel & Shadduck, 1990a, 1990c; Lacey & Siegel, 2000).

4. DIRECT EFFECTS OF *BTI* ON NONTARGET ORGANISMS

Investigations on the susceptibility of non-target organisms have been conducted in laboratory bioassays, simulated habitats, or mesocosms that enabled focus on the susceptibility of several individual species. A multitude of studies conducted in lentic and lotic habitats reveal little or no direct effect of *Bti* on most nontarget organisms. Earlier literature covering these studies has been summarized and reviewed by Merritt et al. (1989), Dejoux & Elouard (1990), Lacey & Mulla (1990), Molloy (1990), and Mulla (1990). More recent literature reviews on the effect of *Bti* on nontarget organisms and one on the safety and ecotoxicology of entomopathogenic bacteria in general were presented by Boisvert & Boisvert (2000) and Lacey & Siegel (2000), respectively. The paper by Boisvert & Boisvert (2000) analyzed the results of *Bti* treatment for mosquito and black fly control on nontargets in 75 studies covering approximately 125 families, 300 genera and 400 species.

Several researchers have reported on the susceptibility of certain nontarget Nematocera, but usually at concentrations of *Bti* that were several fold higher than that required for abatement of targeted species (e.g., Wipfli & Merritt, 1994b). The largest family of susceptible nontarget organisms is the Chironomidae (Diptera: Nematocera), although several chironomid species are not susceptible to even high concentrations of *Bti* (Tables 1 and 2). Ali et al. (1981) reported relatively high LC_{90}s (in mg/l in parentheses following each species) in laboratory bioassays of formulated *Bti* against third instars of four chironomid species: *Glyptotendipes paripes* (9.8-32.4), *Chironomus decorus* (4.6-30.8), *Chironomus crassicaudatus* (4.9-47.0), and *Tanytarsus spp.* (6.3-22.6). Mortalities of 50-100% were reported by Schnetter et al. (1981) and Yiallouros et al. (1999) for laboratory bioassays against *Chironomus thummi* exposed to 0.8-1.8 mg/l. Similarly, Charbonneau et al. (1994) observed 50% mortality in the laboratory in *Psectrocladius psilopterus* exposed to 1.2 mg/l. By contrast, the LC_{95} for fourth instar mosquito larvae (*Aedes vexans*) is 0.1-0.18 mg/l (Schnetter et al., 1981).

Field-derived data on the susceptibility of chironomids from lentic habitats are presented in Table 1 and from lotic habitats in Table 2. Reduced or negligible mortality has been reported in chironomid larvae treated with *Bti* concentrations used for operational control of black fly and mosquito larvae (Miura et al. 1980, 1982; Ali, 1981; Molloy & Jamnback, 1981; Merritt et al., 1989; Liber et al., 1998; Pont et al., 1999). In an artificial marsh, Pont et al. (1999) found significant differences between treated and controls with respect to chironomid emergence at a high level of *Bti* treatment (6.7 mg/l), but no significant differences between the total number of midges emerging in the 1.6 and 3.3 mg/l treatments and controls. One midge species in this same marsh, *Polypedilum nubifer*,

Table 1. Selected References[1] on the Direct Effects of Bacillus thuringiensis var. israelensis on

Larval Chironomidae in Lentic Habitats.

Taxon/habitat	dosage (mg/l)	effect - % mortality	reference
Golf course ponds, Florida	0.5	27-65 overall	Ali 1981
Chironomus spp.		30-67	
Tanypodinae		nearly unaffected	
Experimental ponds, Florida	2.5	53-88 overall	Ali 1981
Chironomini		54-92	
Tanypodini		39-88	
Exp. ponds, California	0.25-1 kg/ha		Miura et al. 1980
Chironomus stigmaterus		slightly affected	
Goeldichironomus holoprasinus		" "	
Experimental ponds, S. California			
Chironomus spp.	5.6 kg/ha	100	Mulla & Chaney, 1987 (in Lacey & Mulla, 1990)
Chironomini	11.2 kg/ha	94	Rodcharoen et al. 1991
Residential lakes, S. California			
Chironomus decorus	4.5-6.7 kg/ha	90+	Mulla et al. 1990
Chironomus decorus	2.8-6.7 kg/ha	87-100	Rodcharoen et al. 1991
Procladius spp.	"	unaffected	
Tanypus spp.	"	"	
Wetland pond, Maui, Hawaii			
Polypedilum nubifer	15.6 kg/ha	no sig. effect	Merritt, Berg & Ali
Chironomus hawaiiensis	"	" " "	unpublished data
Wetland Ponds			
Paratanytarsus	90 kg/ha[a]	91	Liber et al. 1998
Tanytarsini	45 "	83	
Chironominae	90 "	73	
Chironomini	45-90 "	75-89	
Orthocladiinae	45-90 "	71-82	
Tanypodinae	90 "	unaffected	

[1] Observations of several researchers on the lack of adverse effect of *Bti* on chironomids are summarized by Boisvert & Boisvert (2000)

was not affected by *Bti* and this was attributed to a higher resistance to stress by this species. However, it was not determined whether the toxin was able to reach and be ingested by this species of collector-gatherer midge which burrows in the sediments.

Table 2. Selected References[1] on the Direct Effects of Bacillus thuringiensis var. israelensis on Larval Chironomidae in Lotic Habitats

Taxon/location	dosage (mg/l)	effect - % mortality	reference
Chironomidae/ New Zealand	0.2-2/15 min	no effect	Chilcott et al. 1983
Chironomini/Ivory Coast	0.2/10 min	increased drift	Dejoux et al. 1985

Chironomidae/S. Africa	1.6/10 min	increase	Car 1984
Tanytarsini/S. Africa	2.3/7min	some reduction	Car & de Moor 1984
Tanytarsini/S. Africa	1.6/10 min	some reduction	de Moor & Car, 1986
Tanypodinae/Canada	10^5 CFU/ml 1 min	no effect	Colbo & Undeen, 1980
Eukiefferella/*Polypedilum* Canada	5.6/15 min	26-39	Back et al. 1985
Rheotanytarsus spp./USA	5-50/1 min	15-32	Molloy 1992
Chironomidae/USA	10/1 min	"some mortality"	Pistrang & Burger, 1984
Chironomidae/USA	0.5/15 min	slight increase	Molloy & Jamnback, 1981
Rheotanytarsus sp./USA	1.0/13 min	no effect	Jackson et al. 1994
Rheotanytarsus sp./USA	22.5/1 min	0-27	Merritt et al. 1989
Rheotanytarsus fuscus/ S. Africa	1.2/10 min	83	Palmer 1993
R. Fuscus/S. Africa	20/10 min	47-73	" "
Cardiocladius sp./ S. Africa	20/10 min	68	" "

[1] Observations of several researchers on the lack of adverse effect of *Bti* on chironomids are summarized by Boisvert & Boisvert (2000)

Bti has been proposed for control of nuisance chironomid midges, but the concentrations required are several fold higher than that required for mosquito control (Ali et al., 1981; Lacey & Mulla, 1990; Mulla et al. 1990; Rodcharoen et al., 1991; Ali, 1996).

Beneficial mosquitoes in the genus *Toxorhynchites* constitute another taxon of nontarget Nematocera susceptible to *Bti*. Because they do not filter feed, they are susceptible to *Bti* only when it is consumed inside of prey. Lacey & Dame (1982), Larget & Charles (1982) and Lacey (1983) observed mortality in larvae of *Tx. amboinensis, Tx. brevipalpis and Tx. rutilus* that were exposed to *Ae. aegypti* larvae that had fed upon concentrations of *Bti* ranging from 0.05 to 10 mg/l. Mortality was positively correlated with *Bti* concentration and negatively correlated with larval age.

In lotic habitats, the most commonly affected group of nontarget organisms are chironomid midges (Table 2), most notably species in the genus *Rheotanytarsis*. Larvae in this genus filter fine particulate organic matter, including *Bti* toxins from the current using mucous webs. Either their filtering efficiency or their susceptibility is not equal to that of black fly larvae. Several researchers report reduced mortality of *Rheotanytarsis* larvae relative to black fly larvae from the same streams treated with *Bti* (Table 2).

Another noteworthy example of mortality and sustained drift in larvae of a nontarget nematoceran was reported by Back et al. (1985). A field population of *Blepharicera* (Blephariceridae) responded with approximately 30% mortality and pronounced drift, up to 50 times that of pretreatment drift, when treated with 5.28 mg/L/15 min (= 79.2 mg/L/min) of the Teknar formulation of *Bti.* Drift remained high for 3 days, longer than that of the intended targets, a mixture of *Simulium venustum, S. tuberosum, Prosimulium mixtum* and *Stegopterna mutata* (Back et al., 1985). The concentration used in their study was somewhat high considering that the most commonly used range of *Bti* concentrations for control of black fly larvae is 10-20 mg/L for a one minute application (1-2 mg/L/10 min.).

Variable mortality has been reported in other families of Nematocera exposed to *Bti* in laboratory and field studies. For example, at recommended field application rates, Merritt

et al. (1989) and Wipfli & Merritt (1994b) found no significant effects of *Bti* on crane fly larvae (Diptera: Tipulidae); however, some mortality was observed when immatures were exposed at dosages considerably higher (>50X) than recommended field rates (Wipfli & Merritt, 1994b). Direct adverse effects of *Bti* have also been observed in Dixidae, Ceratopogonidae, and Psychodidae (Garcia et al. 1980, 1981; Becker & Margolit, 1993; Boisvert & Boisvert, 2000) exposed to variable *Bti* concentrations.

Increased drift has also been reported for certain non-nematoceran lotic species following application of *Bti*. In some cases drift has been attributed to formulation components or increased particulate load. Temporary increases in drift of Ephemeroptera, Trichoptera, and Plecoptera due to these factors have been reported (Pistrang & Burger, 1984; Dejoux & Elouard, 1990, Lacey & Mulla, 1990; Wipfli & Merritt, 1994b; Boisvert & Boisvert, 2000). More rarely, reductions in some non-nematoceran species have also been reported, but they have not been specifically attributed to the direct effects *Bti* toxins. Filter feeding species are the most likely to capture and concentrate the parasporal crystals of *Bti* and formulation components that may be harmful to species that are not susceptible to *Bti* toxins. Palmer (1993) reported a significant reduction in the filter-feeding mayfly, *Tricorythus discolor*, after moderately high dosage treatment (20 mg/l for 10 min). Wipfli & Merritt (1994b) produced mortality in the filter-feeding mayfly, *Arthroplea bipunctata*, at 500 times the concentration required for black fly control. Dejoux et al. (1985) and Dejoux & Elouard (1990) reported a reduction in certain families of insects living under stone in streams treated on a weekly basis over a 9 week period. The Tipulidae (Diptera), Hydropsychidae (Trichoptera), and Baetidae (Ephemeroptera) were the most affected insects. Palmer & Palmer (1995) reported one of the few cases where a non-insect, the snail *Burnupia* sp., declined in numbers following application of *Bti* for black fly control.

With the exception of predatory Nematocera, the majority of insect predators of mosquitoes and black flies are not susceptible to *Bti*. Populations of predators that specialize on the target organisms might be affected if the reduction of the black fly or mosquito is severe and sustained (Wipfli & Merritt, 1994a). Generalist predators on the other hand have demonstrated remarkable adaptation to other prey. However, the switch to other species may in turn negatively impact other prey taxa. The effects of host removal on the survival of predators will depend on the specificity of the predator, and the availability of other prey. Studies conducted in lotic habitats demonstrate some changes in feeding habits by two species of Plecoptera. *Acroneuria lycorias* (Plecoptera: Perlidae), a general predator, preferred live larvae, but after *Bti* treatment of prey populations (simuliids) it fed on dead larvae and exploited other food sources (Merritt et al. 1991). A detrivore, *Prostoia completa* (Plecoptera: Nemouridae), prefers dead larvae and was not affected by *Bti* treatments (Merritt et al. 1991). In another series of field experiments assessing the effects of *Bti* on black flies and on nontarget organisms through direct and indirect exposure, Wipfli & Merritt (1994a, 1994b) found that stonefly predators consumed equal numbers of *Bti*-killed and live black flies with no ill effects, and one stonefly species switched to alternative prey (i.e. hydropsychid caddisflies) after larval black flies were removed. Larvae of a shredder-detrivore mayfly (Siphlonuridae) switched to consuming *Bti*-killed black fly larvae and attained a greater body mass over 7 days than individuals that were not provisioned with dead black fly larvae.

Predatory insects in lentic habitats are also for the most part unaffected by concentrations of *Bti* used to control mosquito larvae (Miura et al., 1980; Schnetter et al., 1981; Mulla et al., 1982; Mulligan et al., 1982; Aly & Mulla, 1987; Lacey & Mulla, 1990;

Boisvert & Boisvert, 2000). However, densities of predator species can be influenced by the abundance, or lack thereof, of prey mosquito larvae. Purcell (1981) reported on the effect of treating salt marsh habitats for control of *Aedes taeniorhynchus* on 39 species of associated nontarget insects, including several species of predators. Only *Notonecta indica*, an effective predator of mosquitoes, significantly declined. This species is known to abandon habitats in search of a better food supply (Purcell, 1981). Aly & Mulla (1987) observed no adverse effects on *Notonecta undulata* fed prey mosquito larvae that had fed upon *Bti.* Neri-Barbosa et al. (1997) combined *Bti* with *Notonecta irrorata* and observed optimal reduction of mosquito larvae with no deleterious effects on the predator.

Several studies on the effects of *Bti* have been conducted against a number of fish species in the laboratory. The results of most of these studies show no deleterious effects of formulated *Bti* on fish when tested at concentrations and exposure times similar to those used for control of black fly and mosquito larvae. For example, Lebrun & Vlayen (1981) reported no effect of *Bti* on *Tilapia* at label rates, but at 4000 mg/l, 50% of the *Tilapia* died. Similarly, Lee & Scott (1989) observed no mortality in the mummichog, *Fundulus heteroclitus*, when the fish were exposed to 22.4 mg/L, a concentration that is a hundred fold higher than that required to kill most mosquito larvae, but a concentration of 980 mg/L produced 50% mortality after 96 h. Garcia et al. (1980) observed no deleterious effect of the bacterium on mosquito fish, rain water killifish or stickle backs. Snarksi (1990) studied the effect of *Bti* on fathead minnows (*Pimephales promelas*) at spore concentrations ranging from to 2.0×10^4 to 6.5×10^6 colony forming units (CFU)/ml of water. No adverse effects at 6.4×10^5 CFU/ml and below were observed and spore counts in fish dropped rapidly upon transferring them to clean water. Mortality was observed in minnows exposed to concentrations at and above 2.0×10^6, but was attributed to severe dissolved oxygen depletion due to formulation components. Wipfli *et al*. (1994) exposed the embryos of Brook trout (*Salvelinus fontinalis*), Brown trout (*Salmo trutta*) and Steelhead trout (*Oncorhynchus mykiss*) to extremely high concentrations of *Bti*, but mortality was only observed at a concentration 70 times greater and an exposure 192 times longer than that recommended for black fly control. Mortality was attributed to formulation components and not to *Bti* toxins. Similarly, Fortin et al. (1986) only observed mortality in brook trout fry that were exposed to extremely high concentrations of a liquid formulation of *Bti*, but concluded that it was caused by formulation components.

In the United States, as part of the process necessary for registration, *Bti* products have been tested against several vertebrates. Unpublished studies for registration purposes included tests on Sheepshead minnow (*Cyprionodon variegatus*), Steelhead trout (*Oncorhynchus mykiss*) and Bluegill sunfish (*Lepomis macrochiurus*). These species were exposed to *Bti* in concentrations as high as 2.87×10^{10} CFU/l in a 30-day static renewal test; test solutions were renewed twice weekly (J. Siegel, personal communication). There was no evidence of pathogenicity or infectivity. In one study, there was significant mortality among Steelhead trout exposed to *Bti*, but this was attributed to the extreme turbidity of the water in the test group. The fish could not see their food, and in turn attacked each other (World Health Organization, personal communication to J. Siegel).

The number of studies on the effects of bacterial entomopathogens on fish conducted in the field are somewhat limited and for the most part are to short term. Merritt et al. (1989) reported no significant difference in fish numbers and species composition following application of *Bti* for black fly control in the Betsie River (Michigan, USA). No mortality of caged rock bass below the treatment point was observed nor was there significant

change in length-weight relationships of caged and free ranging rock bass before and after treatment. Jackson et al. (2001) studied the effects of single *Bti* applications for black fly control in the Susquehanna River (Pennsylvania, USA) on the abundance, feeding, condition and growth of several species of fish. Species composition and abundance of fish did not change following application of *Bti*. Although fewer numbers of black fly larvae were found in the diets of spotfin shiners, banded darters, and to a lesser extent shield darters, margined madtom and young channel catfish following Bti treatment, the total number of food items consumed did not change. Gibbs et al. (1986) reported no effect of *Bti* (10 mg/l for 1 and 5 minutes) on diet of slimy sculpin (*Cottus cognatus*) or brook trout following treatment for black fly control in the Carrabassett River (Maine, USA).

Numerous laboratory tests that were necessary for the registration of commercial products have confirmed the safety of *Bti* for other vertebrates including mammals (summarized by Saik et al., 1990; Siegel & Shadduck, 1990b, 1990c; Lacey & Siegel, 2000).

5. INDIRECT AND LONG-TERM IMPACT OF ENTOMOPATHOGENIC BACTERIA IN AQUATIC ENVIRONMENTS.

Several studies have addressed the short term indirect effects of *Bti* and *B. sphaericus,* but only a limited number of case studies have been conducted on the long term impact of *Bti* on aquatic communities. Mosquitoes, black flies and chironomids often contribute enormously to aquatic food webs. If their populations are severely suppressed on a continuous basis and their role in the ecosystem is significant, it could result in reductions of species that depend upon them as a major food source. The more diversified the food web, the less likely that complete or partial removal of a single species will result in catastrophic consequences. The more species a given intervention affects, the greater the likelihood of altering ecosystem infrastructure. The effect of repeated applications of *Bacillus* pathogens on ecosystem structure and biodiversity warrants more attention.

Very little has been published on the effects of successive treatments with *B. sphaericus* on the abundance of species that depend upon mosquito larvae as their main source of nutrition. Indirect effects of long-term mosquito abatement with *B. sphaericus* could possibly result in the decline or emigration of predators that depend on mosquitoes as their predominant prey. However, Merritt (unpublished data for 2000-2001) reported no effect on species richness, diversity or abundance after repeated applications of *B. sphaericus* to canary grass and cattail marshes for mosquito control for two years. Similarly, Mulla et al. (1984) and Lacey & Mulla (1990) reported no noticeable adverse effects on invertebrate fauna after season long control of *Culex spp*. with *B. sphaericus*.

Mosquito control efforts in the Rhine Valley of Germany rely almost exclusively on applications of *Bti* and to a lesser extent, *B. sphaericus*. Becker (1997) reports no long-term deleterious effect on NTOs that are monitored as part of the program. In contrast, long-term monitoring of a wetland ecosystem in the United States indicated that initial regular application of *Bti* for control of mosquito larvae did not result in short term changes, but after the wetlands were treated with the bacterium for 2-3 years, species diversity and richness declined significantly (Hershey et al. 1995, 1998). However, subsequent investigations by Schmude et al. (1997) and Balcer et al. (1999) in the same habitat did not corroborate the findings of Hershey et al. (1998) and revealed no long-term effect on insect community structure due to repeated *Bti* applications.

Lotic habitats have also been periodically or regularly treated with *Bti* for control of black fly larvae in the United States and Africa. The longest ongoing use of the bacterium in rivers has been in the Onchocerciasis Control Programme in West Africa where it is used as an intervention during the dry season. Alternation with conventional chemical larvicides during the wet season precludes long term assessment of the individual impact of *Bti*. However, Dejoux & Elouard (1990) contend that there is no evidence of long term deleterious effects on ecosystems receiving weekly applications during the dry season. Yaméogo et al. (1988) assert that the substitution of *Bti* for the organophosphate, chlorphoxim, in the dry season has favored the reconstitution of the invertebrate fauna. Molloy (1992) observed very little effect on nontarget organisms after multiple applications of *Bti* in small streams in New York State. Wipfli & Merritt (1994a) observed that reduction of simuliid larvae with *Bti* indirectly and differentially affected predators. They concluded that specialist predators in black fly poor environments would be most affected, whereas generalist predators would be least affected.

6. CONCLUSION

From the above studies it was concluded that when applied at labeled rates, neither *Bti* nor *B. sphaericus* appear to pose little direct or indirect toxic threat to nontarget benthic invertebrate species or fish. The main damaging effect of *Bti* in streams and rivers may be on specialist predatory species, through loss of black fly larval and/or adult biomass (Gislason, 1992; Wipfli & Merritt, 1994b). Generalist predators, on the other hand, may be least affected by black fly prey loss if alternative prey are abundant (e.g. hydropsychid caddisflies), but in turn may negatively affect other prey taxa.

To predict potential detrimental effects over the long term, a better understanding of the basic ecological interactions between pathogen and target and nontarget organisms is warranted (Goettel & Hajek, 2001). Long-term studies of the effect of *Bti* and *B. sphaericus* use on food resource loss for predators and the effects on fisheries and other wildlife are needed to understand their impact on the overall ecology of aquatic ecosystems. The ecology of species that depend on the targeted host for food or regulation and the effect of sustained host removal on their abundance and diversity are suggested subjects for continued and future research.

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