BACILLUS THURINGIENSIS VAR. *ISRAELENSIS*: THE BACTERIUM, ITS USE IN BLACK FLY CONTROL AND EFFECTS ON NONTARGET ORGANISMS

by

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Bacillus thuringiensis var. *israelensis*: The Bacterium, Its Use in Black Fly Control and Effects on Nontarget Organisms

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ABSTRACT

The importance of black flies as pests is mainly due to their involvement in the transmission of human onchocerciasis in the tropics and the nuisance they cause humans and cattle in temperate climates. Most attempts to control them have concentrated on killing the larvae, which breed in running water. The use of conventional chemical larvicides has led to concern over the development of resistance in target species and effects on nontarget organisms. Therefore, the discovery of a bacterium (*Bacillus thuringiensis* var. *israelensis*, or *B.t.i.*) which is toxic to most black fly species at practical application rates has stimulated much interest.

The main factors that influence the efficacy of *B.t.i.* treatments for black fly control in the field are: (1) potency of the formulation , (2) variability in susceptibility among target species and instars, (3) low temperatures, (4) organic content of the water and (5) carry of the inoculum down rivers and streams. The relative importance of each factor to a control program depends on the particular habitat in which the bacterium is used.

Most investigators in the laboratory and field have concluded that the effects of *B.t.i.* on nontarget species are minimal. Nevertheless, further long-term studies are required. An experiment was carried out as part of this study, using the caddisfly, *Clistoronia magnifica*, and two commercial

formulations of *B.t.i.*: Teknar® and Vectobac®. The results demonstrated that both formulations were innocuous at application rates recommended by the manufacturers, with one exception - increased pupal mortality and decreased adult emergence at 1 mg/l Teknar®. However, this may have been due to a fungal infection and not the formulation.

Based on its advantages over conventional larvicides, *B.t.i.* should be registered in Canada for black fly control (something which has already taken place in the U.S.). Registration would lead to competition between manufacturers for an increased market and thereby stimulate research into improved formulations.

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INTRODUCTION

Black flies continue to be a major problem throughout the world. In the tropics certain species vector human onchocerciasis, a debilitating disease which affects an estimated 20 million people (Harwood and James, 1979). In temperate climates, other species create a serious public nuisance and can cause significant losses to the livestock industry (Wood, 1985). Their control poses many problems. Breeding sites (rivers and streams) are extensive and adults may migrate great distances. Personnel involved in control efforts are frequently faced with pressures from two broad groups of people whose views on black fly control are often opposed. Those most severely affected by black flies, personally or financially, are usually in favour of insecticide use, while many of those less severely affected oppose their use on the grounds that their application may harm the environment. Indeed, most insecticides used to control black fly larvae have had deleterious effects on many organisms which coexist with them.

The discovery of *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) and its toxicity to black flies, therefore, was a welcome and important development. Researchers have emphasized its advantages over conventional insecticides: its narrow range of specificity and the lack of development of resistance in target insects. Within a short time following its discovery, formulations were developed for use in the field. It is now an

essential component in many mosquito control programs throughout North America and has found use in the Onchocerciasis Control Project in West Africa. It was registered for use against black flies in the United States in 1981, but has not yet been registered for such use in Canada.

The major objectives of this paper are to evaluate the factors that influence the efficacy of *B.t.i.* treatments in the field and to investigate possible effects on nontarget organisms. Background information on the bacterium, its toxin and mode of action is also provided.

CHAPTER I

A BRIEF HISTORY

Bacillus thuringiensis was first isolated by Ishiwata (1901) as the causal agent of "sotto disease" in silk worm (*Bombyx mori*) larvae. His identification was not complete, however, and the first morphologically valid description was by Berliner (1915), who isolated the bacillus from the Mediterranean flour moth (*Anagasta kuehniella*). Berliner identified his isolate as a new species, which he named *Bacillus thuringiensis*. Subsequently, Aoki and Chigasaki (1915) published a detailed description of Ishiwata's organism, for which they proposed the name *Bacillus sotto*.

In Europe it was originally hoped that the release of the bacterium in areas infested with damaging insects would bring pest populations below economic threshholds by initiating epidemics. Early trials, however, met with only sporadic success and interest diminished until Mattes (1927) reisolated the organism from the Mediterranean flour moth. This strain was eventually adopted in France for commercial production. Although the first commercial preparation (Sporeine) was produced prior to WW II, the greatest increase in the use of *B.t.* formulations came after the development of newer, more potent preparations during the early 1970's. In 1975 U.S. producers reported sales of approximately 500,000 kg in the U.S., with 400,000 additional kg exported or produced in foreign subsidary plants (Dulmage and

Aizawa, 1982).

Many *B.t.* strains are pathogenic to mosquito larvae when grown in a medium which stimulates β-exotoxin production. When the β-exotoxin is eliminated, fewer strains kill the larvae (Rogoff *et al.*, 1969). Strains in the varieties *thuringiensis*, *aizawai*, *morrisoni*, *toworthi* and others have detectable activity against mosquitoes in the genera *Aedes* and *Culex* (Hall *et al.*, 1977). However, most authors agree that the level of activity in lepidopteran strains is not sufficient to encourage their further development for mosquito control.

Another species of *Bacillus*, *B. sphaericus*, stimulated interest in the use of bacteria for the control of mosquitoes in the 1970's. The first insecticidal strain was isolated from *Culiseta incidens* in California by Kellen *et al.* in 1965. In 1973 Singer reported the isolation of a strain (SS II-1 = var. *fumiformis*) from *Culex fatigans* collected in Delhi, India which was at least 10,000 times more active than Kellen's strain. Within a few years Singer isolated several more strains from dead mosquito larvae. Initial laboratory and field studies utilized the SS II-1 strain, but undesirable commercial production characteristics led to its replacement by another strain, 1593 (Davidson, 1982). An industrially prepared powder of this strain has been used successfully in a number of field trials, and research into new strains and better formulations continues (Singer, 1985).

The major breakthrough in the use of bacteria for the control of mosquitoes came in 1977, when Goldberg and Margalit reported the isolation of mosquito larvicidal bacteria from soil samples taken at known mosquito breeding sites in Israel. Out of 1000 isolates screened, roughly 1 in 100 exhibited larvicidal activity similar to that of *B. sphaericus*, strain SS II-1. One isolate, 60A, proved to be 30-100 x more active when assayed against *Cx. pipiens*, with an LC50 of 6 x 10^6 spores/ml. De Barjac (1978) positively identified this strain as *Bacillus thuringiensis* and placed it in a new serotype (H-14) and new subspecies, var. *israelensis* (see Appendix). It has also been designated as strain CCBC 1897 by the WHO and as strain HD-567 by the USDA.

The earliest reported test of the pathogenicity of *B.t.* to black fly larvae, by Travis and Guttman (1966), did not indicate any potential as a microbial control agent. Later tests by Lacey and Mulla (1977), however, using 13 strains of *B.t.* (but not *B.t.i.*) against late instar field collected *Simulium vittatum* resulted in mortalities ranging from 64 - 88% for a 24 hr exposure period. Nevertheless, the authors concluded that the rates necessary for effective control (arbitrarily set at 90% or better) were not favourable compared to the use of conventional larvicides, such as temephos. Lacey and Federici (1979) concluded that the results still justified continued exploration for more toxic strains. This proved true, when further laboratory tests using the strain isolated by Goldberg and

Margalit, later to be named *B.t.i.*, demonstrated promise for practical application in the control of black fly larvae in the genera *Prosimulium, Simulium and Cnephia* (Undeen and Nagel, 1978). These tests used shorter exposure times (30 minutes and less) and indicated an even higher level of activity against blackflies (LC50 = 4.4 x 10^2 to 5.3 x 10^3 cells/ml) than had been shown against mosquito larvae. Subsequently, the toxicity of *B.t.i.* to larval *Simulium damnosum*, the African vector of onchocerciasis, was shown by Undeen and Berl (1979).

CHAPTER II

THE BACTERIUM, ITS TOXIN AND MODE OF ACTION

The Bacterium

Bacillus thuringiensis var. *israelensis* belongs to the family Bacillacea, a group of bacteria which includes several entomopathogenic species such as *B. popillae*, *B. lentimorbus*, *B. sphaericus* and *B. moritae* (Abe *et al.*, 1983). The vegetative cells of *B.t.* are peritrichously flagellated, average 3-5 by 1.2-1.5 μm and frequently occur as filaments of 4 or more cells (Luthy *et al.*, 1982). At the end of vegetative growth, these cells produce dormant spores which are extremely resistant and are able to survive unfavourable conditions for long periods of time. *B.t.* has been described as "crystalliferous" because it produces a discrete, characteristic parasporal crystalline inclusion body within the sporulating cell at the time of sporulation. This crystal is formed outside the exosporium during stages III - IV (Bechtel and Bulla, Jr., 1976). The amount of crystal formed is related to the medium and culture conditions under which the bacteria develop (Sherer *et al.*, 1973).

The Toxin

A number of toxins have been described for different strains of *B.t.* (Faust and Bulla, Jr., 1982). The major 2 are β-exotoxin and δ-endotoxin [1]. Only the δ-endotoxin has been isolated from *B.t.i.* (de Barjac, 1978). This toxin is contained in the crystal produced by the bacteria. The biochemical character of the δ-endotoxin of *B.t.i.* differs considerably from that of the δ-endotoxins of other *B.t.* strains, whose characters are quite similar. This dissimilarity undoubtably contributes to the narrow host range of *B.t.i.* and its lack of toxicity towards Lepidoptera.

Unlike most other subspecies of *B.t.*, which can be broadly characterized as containing a typical number and type of crystal (Fast,1985), *israelensis* produces multiple crystals of different shape and size (Yamamoto *et al.*, 1983). These crystals are composed of proteins. Although carbohydrates are often associated with the proteins, they may be removed by repeated washing. It is assumed, therefore, that their presence is due to either cultural conditions, preparation techniques or both, and that they are not involved in the mode of action of the toxin (Huber *et al.*, 1981). The crystals resist dissociation in

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[1]note: Use of the term endotoxin with respect to *B.t.* differs from its general use in biochemistry, where it is used to describe a heptenic (i.e. non-protein) portion of a cell wall. Its use to describe a toxin produced by *B.t.* was proposed by Heimpel in 1967. Since then it has often been used interchangably with the terms crystal and crystal protein. In this paper δ-endotoxin refers to the single toxic entity which initiates pathogenesis in the insect.

standard protein solvents, but will dissociate in the presence of thiol agents, indicating the presence of disulfide bonds. In the laboratory solubilization procedures are similar to those used for keratin, whose dissolution requires the scission of disulfide bonds in an alkaline medium.

Huber *et al.* (1981) determined the molecular weights for the crystals proteins of 9 *B.t.* serotypes, including *B.t.i.* They used the denaturing agent dithiothreitol and a carbonate buffer of pH 9.5. By polyacrylamide electrophoresis, they determined that the molecular weights of the constituent proteins for all the strains except *B.t.i.* were approximately the same (230,000, S.D.=3,000). Further dissolution of these proteins with another denaturing agent (sodium dodectyl sulphate or SDS) resulted in the isolation of proteins with molecular weights ranging from 80,00 to 150,000. Conversely, proteins with approximately identical molecular weights (25,000 and 24,000) were obtained from *B.t.i.* crystals with or without SDS.

Yamamoto *et al.* (1983) also determined the molecular weights for the crystals of *B.t.i.* using electrophoresis. Using SDS followed by (*Staphylococcus cuneus*) V8 proteinase, they found that the crystals were composed of proteins with molecular weights of 28,000, 42,000, 55,000, 67,000 and 120,000 (although they admitted that these proteins may have been derived from larger precursors). This complex of proteins corresponded with the variety of crystal types found in *B.t.i.* Interestingly, their study associated larvicidal activity with the 28 kdal

protein and its 25 kdal portion.

Armstrong *et al.* (1985) have described the purification of a single protein which they believe to be responsible for the toxic properties of *B.t.i.* They describe 2 forms, each containing an identical sequence of 10 amino acids but differing in the presence of arginine and valine residues at the amino terminus of one of the molecules. Their report indicates that these are the cleavage products of larger proteins whose molecular weights (26,000-28,000) correspond roughly to the crystal proteins described for *B.t.i.* by Huber *et al.* (1981) and Yamamoto *et al.* (1983).

The amino acid composition and sequence of the δ-endotoxins of *B.t.* vary from serovar to serovar (Fast, 1985). There is evidence that plasmids are involved in the synthesis of parasporal crystals in several subspecies pathogenic to Lepidoptera (Gonzalez *et al.*, 1981). Faust *et al.* (1983) have shown that, based on electrophoresis profiles, different strains of *B.t.i.* contain varying numbers of plasmids. Further studies have demonstrated that one of these (mw = 75 Mda) is involved in crystal production (Gonzalez and Carlton, 1984). Transfer of this plasmid to an acrystalliferous strain converted the recipient to crystal production.

The Mode of Action

Heimpel and Angus (1959) classified insects susceptible to *B.t.* into 3 types:

Type I- those which exhibit a general paralysis on ingesting crystals (e.g. *Bombyx mori*)

Type II- insects which cease feeding after ingesting the crystals and die 2-4 days later (e.g. *Malacosoma disstria*)

Type III- insects which are not killed by the toxin alone, but also require the presence of spores (e.g. *Anagasta kuehniella*)

According to this system of classification mosquitoes and black flies are type I insects in respect to their response to *B.t.i.*, since death can result without bacteraemia (Lacey and Federici, 1979; Lahkim-Tsror *et al.*, 1983). However, this does not mean that bacteraemia does not occur. Aly (1985) has reported that ingested spores germinate in living as well as moribund and dead *Aedes aegypti* and *Ae. vexans* larvae.

Huber *et al.* (1981) have put forward a model, based upon their laboratory investigations, which would explain the dissolution of *B.t.* crystals in the insect gut. They propose that in the intact crystal structure the disulfide bonds which hold the crystal proteins together are not accessible to reducing agents without the loosening of non-covalent

intermolecular forces. This is possible either under alkaline conditions or with denaturing agents at neutral pH. The fact that insects susceptible to *B.t.* are reported to have high midgut pH's (Undeen, 1979) lends support to this model. The midguts of black flies, for example, have reported pH's in excess of pH 10 (Lacey *et al.*, 1978). Such an alkaline environment could bring about the loosening of non-covalent forces necessary to allow reducing agents (gut enzymes) access to the disulfide bonds holding the crystal proteins in their place. The enzymes involved in the dissolution of the proteins are apparently gut proteases of the serine type: *in vitro* activation is possible with trypsin (Luthy *et al.*, 1982).

The only published histopathological study on the effects of *B.t.* on black flies is by Lacey and Federici (1979) using late instar *S. vittatum* and the *B.t.* strain HD-261. Other investigators have reported on the histopathological effects of *B.t.i.* on mosquito larvae (Lahkim-Tsror *et al.*, 1983; Habib, 1983). In all 3 studies absorption of the δ-endotoxin occurred in the gastric caecae and posterior stomach. This is in contrast to the effects of *B.t.* on Lepidoptera, in which changes are seen in the anterior stomach. In mosquitoes this can be explained by the greater abundance of microvilli in the posterior compared to anterior midgut. Absorption in *Ae. aegypti* larvae leads to marked cell hypertrophy and the formation of vacuoles, followed by cell lysis and sloughing (Lahkim-Tsror *et al.*, 1983). Two hypotheses have been postulated to explain these effects.

Schnell and Nickerson (1983) have suggested that *B.t.i.* δ-endotoxin acts as an ionophore, affecting anion transport. Thomas and Ellar (1983) have suggested that the δ-endotoxin interacts with plasma membrane lipids, leading to their rearrangement and disruption of the membrane's integrity.

Histopathological studies by Habib (1983) on the midgut, nerve ganglia and skeletal muscle of *Culex declarator* have shown that the δ-endotoxin liberated in the midgut proceeds to affect the ventral nervous system and consequently the skeletal muscle, provoking the disfunction of the latter and bringing about paralysis. This is supported by the work of Chilcott *et al.* (1984) who established that a protein isolated from *B.t.i.* inhibits nerve conduction in the 6th abdominal ganglion of the American cockroach, *Periplaneta americana*. The mechanism of this action is not known. On the basis of histopathological observations and the rapid onset of death of the larvae (2-3 hours), Habib has suggested that the cause of death differs from that of Lepidoptera infected by other strains of *B.t.* Mosquito larvae apparently die due to asphyxiation rather than starvation.

Whether death of black fly larvae following ingestion of *B.t.i.* crystals is brought about by the action of the toxin on the midgut cells or by its effects on the nervous system may depend on the concentration of the toxin in the midgut (Chilcott *et al.*, 1984). While Charles and de Barjac (1981) observed that black fly larvae fed high concentrations of *B.t.i.* crystals

showed no paralysis of the mouthparts or midgut and died within 30 minutes (presumably due to destruction of the midgut epithelium), Goldberg *et al.* (1982) reported that larvae fed low amounts of crystals died prior to cell damage, which occurred 60 minutes after cell death. It appears, then, that rapid progress of toxicity to the midgut epithelium may overshadow the neurological action of the *B.t.i.* δ-endotoxin.

CHAPTER III

THE USE OF *B.T.I.* FOR BLACK FLY CONTROL

Until the appearance of DDT in the 1940's, methods proposed and tested for use in controlling black flies were generally ineffective, uneconomical or involved the application of materials highly injurious to nontarget fauna (Jamnback, 1981). However, heavy agricultural use of DDT lead to adverse long term effects on the environment and it was banned for use in black fly control in most parts of the world by the 1970's. Research since then has concentrated on the identification of a substitute which is both nonpersistent and effective. Two of the most widely used and studied have been the organophosphate temephos and the organochlorine methoxychlor. Fear of black flies developing resistance to them and their effects on nontarget organisms, however, have stimulated research into possible alternatives, including other synthetic chemicals, different formulations, insect growth regulators and biological control agents.

Gaugler and Finney (1982) have prepared a table which summarizes field trials using *B.t.i.* against black flies. An updated version is presented in table 1. Trials have been carried out in several countries under many varied conditions. Factors which have influenced the perceived success or failure of these trials may influence the success or failure of operational treatments. These include: application methods,

Table 1 - A Summmary of Field Trials using *B.t.i.* against Black Flies

reference	location	product	dosage: conc, duration	% mortality, distance downstream	stream temp, discharge	principal species
Guillet and de Barjac, 1979	Ivory Coast	Biochem 1° powder	0.2 ppm, 10 min	100% (24 hr), 0m	*,*	*Simulium damnosum s.l.*
Undeen and Colbo, 1980	Nfld.,Canada	water suspension	6.6×10^4 viable cells/ml	80-100% (48 hr), 0-50m	19°C, 600 l/min	*S. verecundum*
			7.7×10^4 viable cells/ml	10-70% (48 hr), 0-180m	3°C, 3400 l/min	mixed genera and species
			1.5×10^5 viable cells/ml	48-100% (48 hr), 0-180m	15°C, 2000 l/min	*Simulium spp.*
			2.2×10^5 viable cells/ml	60-100% (48 hr), 0-100m	22°C, 200 l min	*Simulium spp.*
Molloy and Jamnback, 1981a	N.Y.,USA	Biochem 1° powder	0.5 ppm, 15 min	11-96% (5 days), 20-705m	13°C, 1770 l/min	*Simulium spp.*
Molloy and Jamnback, 1981b	N.Y.,USA	Bactimos® wettable powder	3.7 ppm, 15 min	76-100%, 400-2000m	12 °C, 8600 l/min	*Simulium spp.*
			13.4 ppm, 15 min	100%, 400-1000m	17°C, 7456 l/min	
Frommer *et al.*, 1981a	Tennessee,USA	Abbott 1° powder	3.1 ppm, 35 min	0-40% (24 hr), 37-312m	16-19°C, 18269 l/min	*S. vittatum*
			1.55 ppm, 70 min	50-70% (24 hr), 37-312m		
Frommer *et al.*, 1981b	Tennessee,USA	Abbott 1° powder	3.1 ppm, 35 min	27-89% (24 hr)	*, 23900 l/min	*S. vittatum*

* no data available

Table 1 - continued

reference	location	product	dosage: conc, duration	% mortality, distance downstream	stream temp, discharge	principal species
Undeen *et al.*, 1981	Guatemala	water suspension	2 x 10^5 spores/ml, 1 min	92% (48 hr), 0-25m	19°C, 10 l/min	*S. ochraceum*
			1 x 10^5 spores/ml, 1 min	21-82% (24 hr), 25-100m	210-840 l/min	
		Sandoz 402 wettable powder	1 x 10^5 spores/ml, 10 min	0-100% (24 hr), 0-75m	22°C, 156 l/min	
Lacey *et al.*, 1982a	Ivory Coast	Teknar® wettable powder	1.5 ppm, 10 min	100% (6 hr), 0-19km	26.8°C, 2742000 l/min	*S. damnosum*
Stoltz, 1982	Idaho,USA	*	*, 15 min	50-100% (7 days), 10-1610m	6792 & 27168 l/min	*
Gaugler *et al.*, 1983	Mexico	Teknar® wettable powder	45 ppm, 0.5 min	29.3-100% (24 hr), 10-100m	19.4°C, 1020 l/min	*S. ochraceum, S. metallicum, S. callidum*
			7.5 ppm, 1 min	24.4-100% (24 hr), 10-100m	18.5°C, 1080 l/min	
Chilcott *et al.*, 1983	N.Z.	Sandoz 402 wettable powder	2 ppm, 15 min	90-100% (24 hr), 0-577m	19°C, 3372 l/min	*Austrosimulium laticorne, A. multicorne*
		Biochem 1° powder	0.2 ppm, 15 min	65-100% (24 hr), 0-580m	10.5°C, 1680 l/min	

* no data available

Table 1 - continued

reference	location	product	dosage: conc, duration	% mortality, distance downstream	stream temp, discharge	principal species
Lacey and Undeen, 1984	Tennessee,USA	Teknar® wettable powder	10 ppm, 1 min	84.5% (24 hr), 0-400m	23°C, 25000-27000 l/min	*S. vittatum*
		Vectobac® wettable powder	10 ppm, 1 min	79.8% (24 hr), 0-400m	11.3-23°C, 25000-31400 l/min	
			20 ppm, 1 min	86% (24 hr), 0-400m	20°C, 21400 l/min	
		Bactimos® wettable powder	10 ppm, 1 min	60.9 % (24 hr), 0-400m	20°, 17200-21400 l/min	
			30 ppm, 1 min	87% (24 hr), 0-400m	11.3°C, 31400 l/min	
Pistrang and Burger, 1984	New Hampshire,USA	Sandoz flowable concentrate	10 ppm, 1 min + 10 ppm, 5 min	100% (24 hr), 0-150m; 0%, 800-1300m	8.7-16.4°C, 13500 l/m	*S. venustum*
Colbo and O'Brien, 1984	Nfld.,Canada	Teknar® flowable concentrate	10 ppm, 1 min	23-100% (5 days), 10-750m	0°C, 39900 l/min	mixed genera and species
				100% (5 days), 25 & 160m	7°C, 23500 l/min	
				65-95% (5 days), 10-350m	7-19°C, 2500-25000 l/min	
Car, 1984	South Africa	1° powder	3 ppm, 10 min	54% decrease-371% increase (24 hr), 20-200m	*, 2040 l/min	*S. adersi*, *S. hargreavesi*
		Teknar® flowable concentrate	1.6 ppm, 10 min	85% decrease-312% increase (24 hr), 20-200m	*, 3060 l/min	

* no data available

concentration, potency and formulation, species and stage of target and stream parameters.

Application Methods

In the majority of trials the material has been applied to the stream in liquid form using a sprinkling can, calibrated to deliver the correct application rate. In one trial the method was described simply as "pouring" (Molloy and Jamnback, 1981a). More sophisticated methods have been employed. Frommer *et al.* (1981a) used a rotometer to calibrate output and a T-shaped dispenser to deliver the material. In the treatment of rivers in the Ivory Coast, *B.t.i.* was applied in transverse bands from barrels attached to small boats (Lacey *et al.*, 1982a).

The choice of method will be influenced by the size of the stream and its accessability. Of main importance is that the material be applied across the entire width of the stream and that suspension and dilution of the active ingredient ensure adequate dispersal.

Concentration, Potency and Formulation

Before applying *B.t.i.* the dispenser must choose the appropriate formulation, potency, initial concentration, rate and duration of application. *B.t.i.* is available from at least four companies in the U.S., one in the U.K. and one in France

(Table 2). Formulations include primary powders, wettable powders (wp or wdc), emulsifiable concentrates (ec), flowable concentrates (fc or as) and granules. All five have been used in field trials against black flies.

Formulation has a marked influence on the efficacy of *B.t.i.* through at least 3 mechanisms: (1)direct effects of diluents and processing on the toxin , (2)the effect of particle size on black fly filter feeding and (3)the effect of the particle's physical characteristics on settling (Lacey and Undeen, 1984). Larger particles apparently have the greatest lethal effect. Guillet and Escaffre (1979), in tests using *S. damnosum s.l.* and Bellon Laboratory primary powder, wettable powder, emulsion and aqueous suspension formulations, found a direct correlation between efficacy and particle size, with the primary powder having the largest aggregates (mean = 29 μm; range = 6-100 μm). Presumably smaller particles are more likely to pass between the fan rays of the black fly's mouth parts and therefore not be ingested. Chance (1970) has reported that the particles ingested by all instars of *Cnephia dacotensis*, *S. decorum* and *S. venustum* ranged from 0.8-300 μ long and 10-60 μm wide. Kurtak (1978) examined the ingestion of of synthetic particles by nine black fly species in the laboratory and concluded the "ideal" particle would be 100-150 μm in diameter.

Since *B.t.i.* is only active in the midgut of the larvae, any factors which influence feeding can affect its effectiveness. There is a limited time during short exposures for the larvae to

Table 2 - Manufacturers of *B.t.i.*

Company	Name of Product
Abbott Laboratories (U.S.)	Vectobac®
Bellon Laboratories (France)	-
Biochem Laboratories (U.S.)	Bactimos®
Reuter Laboratories (U.S.)	BMC® (Biological Mosquito Control)
Tate and Lyle Industries, Ltd. (U.K.)	Skeetal®
Zoecon Corporation, Sandoz Incorporated(U.S.)	Teknar®

ingest sufficient toxic material for mortality to occur. If feeding is inhibited by some component of the formulation, the dose might possibly pass by before feeding is resumed. Gaugler and Molloy (1980) reported that the addition of an emulsion of *B.t.i.* in tests carried out under simulated stream conditions resulted in abnormal clumping of cephalic fan rays and inhibition of feeding at concentrations of 20-50 ppm.

Molloy *et al.* (cited in Lacey and Undeen, 1984) have presented evidence of a positive correlation between particle size and efficacy in tests conducted in short simulated streams. Although a large particle of agglomerated *B.t.i.* within the size range upon which a species feeds may provide greater quantities of toxin/unit time, it may also settle out of the water column more quickly in slower segments of the stream or become entrapped more easily in vegetation or other components of the substrate. In a field trial against *S. vittatum*, Lacey and Undeen (1984) found that wettable powder formulations caused about the same level of mortality close to the treatment point as an aqueous solution with smaller particle sizes. The larger-sized particles of the wettable powders however either settled out or were filtered from the water column more quickly, resulting in lower mortalities downstream.

B.t.i. concentration has been expressed as mg/l, ppm and viable cells or spores/ml. Mg/l and ppm are equivalent measures of weight/volume. Frommer *et al.* (1980a) have shown that observed spore counts do not follow a linear trend when

volumetric dilutions are made, but instead fit a polynomial distribution. They attribute these differences either to inconsistent or imperfect suspensions of spores when powdered formulations are mixed with water, or a normal variation in quantifying plated spore counts, or both. No published studies report the consistency of spore suspensions in flowable concentrates and while such differences may be important in the laboratory, their importance in the field when compared to other factors would most likely be minimal.

In fact, the importance of a spores/ml measure is questionable, since according to Dulmage (1971) this may not be directly and consistently correlated to *B.t.* toxicity. Evaluation of the toxicity of *B.t.* is a difficult task which is complicated by the varying susceptibilities of different target species. At present the toxicities of *B.t.i.* formulations are rated in International Toxicity Units (ITU's or IU's), which are based on the susceptiblity of 4th instar *Aedes aegypti* larvae to a standard preparation (Dulmage *et al.*, 1971). Laboratory determined ITU's for *Ae. aegypti* however do not always parallel the relative activities of formulations against mosquitoes in the field (Dame *et al.*, 1981) and could not be expected to be applicable to black fly species. In fact, a comparison of 3 formulations (Teknar® wdc, Vectobac® wp and Bactimos® wp) in small streams against *S. vittatum* revealed that the product containing the lowest ITU was more effective than the product containing the highest amount (Lacey and Undeen, 1984).

The recommended rates and durations of application are provided on the product label. These vary depending on the manufacturer and the formulation. In laboratory studies using *S. vittatum*, Frommer *et al.* (1980b) reported a sharp decrease in LC50 (0.81 to 0.32 ppm) and LC90 (1.71 to 0.86 ppm) values when exposure times were increased from 30 to 60 minutes but a minimal change when exposure times were increased beyond this. Most likely this was the result of feeding inhibition as a consequence of rapid gut filling, a phenomenom described by Gaugler and Molloy (1980). In subsequent field trials Frommer *et al.* (1981a) used treatment concentrations and exposure times selected from LC90 bioasssay values. Treatment with 3.1 ppm for 35 minutes resulted in a 25% reduction in the number of *S. vittatum* larvae attached to artificial substrates, while treatment with 1.55 ppm for 70 minutes resulted in 50-70% reduction (both measured over 312 metres after 24 hours). From these results the authors concluded that increased duration of exposure led to greater spore dispersion and increased spore contact with larvae. Lacey and Undeen (1984) have argued that better control can be obtained with several formulations using an application rate of 10 mg/l for 1 minute. Results they have published for replicated paired tests in streams against *S. vittatum*, however, do not appear to be conclusive. When applied as the 2nd treatment, the application of 0.5 mg/l for 20 minutes resulted in almost the identical mean mortality as obtained in the 1st treatment using 10 mg/l for 1 minute. This would support the conclusions reached by Jamnback and Means (1966) when they

tested conventional larvicides; that there is little difference in control for periods ranging from 5 to 25 minutes for the same quantity of larvicide.

Species and Stage of the Target

Species and instar are important factors affecting susceptibility in the laboratory. Lacey *et al.* (1978) found species in the subgenus *Psilozia* (eg. *S. vittatum*) more susceptible than species of other subgenera. The results of tests conducted by Molloy *et al.* (1981) also demonstrated differences in susceptiblity, albiet somewhat contradictory to the conclusions of Lacey *et al.* In these tests *S. verecundum*, a representative of the subgenus *Simulium*, was found to be significantly more susceptible than *S. vittatum*. Differences in susceptibility between species has also been observed in at least one field trial. Treatment of a large river in the Ivory Coast resulted in complete removal of *S. damnosum s.l.* larvae for 19 km but removed larvae of *S. shoutedeni* for only 4 km (Lacey *et al.*, 1982a).

Molloy *et al.* (1981) concluded that the small size of *S. verecundum* (ultimate instars are 1/3 the size of those of *S. vittatum*) probably contributed to their high susceptibility. It has been suggested by Gaugler and Finney (1982) that such interspecific susceptibility might be related to differences in cephalic fan ray size and spacing, since these are correlated

with feeding efficiency.

An inverse relationship between larval instar and susceptibility has been demonstrated both in the laboratory and field. In laboratory tests using *S. verecundum* and *S. vittatum*, Molloy *et al.* (1981) showed significantly greater effectiveness of *B.t.i.* in killing 2nd-5th instars than in killing 7th instars. Correspondingly, Gaugler *et al.* (1983) reported that the ratio of early:late instars was much lower posttreatment than pretreatment in a field trial using *B.t.i.* against *S. ochraceum* in Mexico.

Stream Parameters

Stream parameters of major concern include chemical content, particulate concentration, temperature, discharge, substrate surface area and vegetation. Some of these parameters may vary over the length of the stream, which complicates the evaluation of their contribution to the overall success or failure of an application.

Ignoffo *et al.* (1981) examined the effects of chemical content and particulate matter on the larvicidal activity of *B.t.i.* to *Ae. aegypti* mosquito larvae. Mortality in a natural pond was less than in water which had been acidified or alkalized (although the difference was not statistically significant). Salt concentrations within the range tolerated by the larvae had no effect. The concentration of sediment in the

water had a definite effect; increased sediment concentration gave rise to decreased activity. The authors contend that this was due to *B.t.i.* crystals binding with organic materials in the sediment rather than inactivation of the toxin *per se*.

The importance of the organic content of the water is reflected in the manufacturers recommendations on the labels of commercial *B.t.i.* preparations. Higher rates are suggested for use against mosquito larvae in polluted water. Its importance in black fly control has been shown by Car (1984) in field trials carried out in South Africa. In one trial *B.t.i.* was applied to a river heavily polluted with effluent from a nearby sewage works. The rate of application had to be increased almost double (1.6 ppm to 3 ppm) to achieve mortality comparable to that obtained at the lower rate in an unpolluted river. The water in the polluted river was also found to be high in chlorine content. This probably contributed to the lowered efficacy of the *B.t.i.* applications, since chlorine has been shown to exhibit an inhibitory effect on the δ-endotoxin in the laboratory (Sinegre *et al.*, 1980).

Apparently, low temperature can reduce the efficacy of *B.t.i.* applications. The most successful field trial to date was conducted in a river in tropical Africa where temperature of the water was 26.8°C (Lacey *et al.*, 1982a). Molloy *et al.* (1981) reported that in the laboratory mortality among *S. vittatum* treated at 20°C was almost twice that at 10°C (80.6 vs 40.4%). Temperature also affected the rate of mortality: at 20°C 95% of

the mortality (during the 4 day post-treatment period) was recorded in the first 24 hours, compared to only 56% at 10°C. A strong correlation between temperature and mortality has also been recorded in tests of another *B.t.* strain (*B.t. kenyae*) against *S. vittatum* over a temperature range from 4° to 24°C (Lacey *et al.*, 1978).

The lowest temperatures at which *B.t.i.* has been tested in the field were reported by Colbo and O'Brien (1984) in a study conducted in streams in Newfoundland. Significantly lower mortality was obtained in the first few hundred metres of a stream where the water temperature was 0°C, than later in the year when the temperature had risen to 7°C. These results and subsequent laboratory studies by J.R. Finney, J. Harding and H. Colbo (unpubl., cited in Colbo and O'Brien, 1984) have indicated that, in order to obtain a high mortality close to the treatment point, it is neccesary to extend the duration of application in cooler water: beyond 1 minute in water temperatures below 10°C and up to 5 minutes or greater in water temperatures under 6°C. The reason for reduced efficacy at lower temperatures, Colbo and O'Brien speculate, is that in water that is too cold not all the larvae feed continuously. Therefore, unless dose time is protracted, *B.t.i.* particles may pass by larvae which briefly have stopped feeding.

When applying *B.t.i.* to a moving body of water, it is important to determine the distance downstream that a lethal concentration of the bacteria will move before removal or

dilution reduces it to non-lethal levels. This is referred to as the "carry" of the material (Colbo and O'Brien, 1984) and is generally considered to be the single most important factor limiting the pathogen's efficacy (Gaugler and Finney, 1982).

Undeen and Lacey (1982) have presented a table which relates carry to discharge (Table 3). From this table it can be noted that in the majority of cases the greater the discharge, the further the carry. The significance of this relationship is apparent when one compares the success of *B.t.i.* field trials in the control of the vectors of onchocerciasis. While Lacey *et al.* (1982a) obtained carry for at least 34 km of a large West African river where the discharge was 2.7×10^7 l/min, Undeen *et al.* (1981) in Guatemala reported carry of only 14, 30 and 30 metres in streams having discharge rates of 10, 150 and 210 l/min respectively. Smaller bodies of water with a lower discharge usually have a greater substrate area per unit volume discharge than larger bodies of water. This results in a rapid spread and loss of *B.t.i.* and therefore, shorter carry.

Gaugler *et al.* (1983) have emphasized that the interaction of discharge with other stream parameters is poorly understood and that other factors such as stream depth, turbulence, turbidity, pools, substrate and larval density should also be considered.

Frommer *et al.* (1981b) examined the effects of vegetation on the success of treatment of a stream with *B.t.i.* The test stream

Table 3- Carry of *B.t.i.* as a Function of Discharge

discharge l/min	carry[1] metres	test location	reference
10	14	Guatemala	Undeen *et al.*, 1981
156	30	Guatemala	Undeen *et al.*, 1981
210	55	Guatemala	Undeen *et al.*, 1981
600	58	Nfld.,Canada	Undeen and Colbo, 1980
200	80	Nfld.,Canada	Undeen and Colbo, 1980
1770	180	N.Y.,USA	Molloy and Jamnback, 1981
2000	200	Nfld.,Canada	Undeen and Colbo, 1980
25000	500	Nfld.,Canada	Undeen and Lacey, 1982
7500	1000	N.Y.,USA	Undeen and Lacey, 1982
8500	1500	N.Y.,USA	Undeen and Lacey, 1982
27420000	3400	Ivory Coast	Lacey *et al.*, 1982a

[1] the furthest distance downstream where mortality was recorded as ≥80%

was treated before and after the appearance of a dense growth of aquatic weeds (*Potomogeton crispus* and *P. pectinatus*). They found that the weeds had little effect on the effectiveness of *B.t.i.* against black flies. In fact, there was a greater reduction in the number of black fly larvae when the weeds were present than when they were absent. The authors observed delays in the anticipated arrival of the treatment suspension and suggested that the vegetation acted to retard the rate at which the spores moved through the stream, resulting in greater dispersion of the spores.

CHAPTER IV

THE EFFECTS OF *B.T.I.* ON NONTARGET ORGANISMS

A Review

When introduced into running water *B.t.i.* becomes part of a more or less constant flow of small particles and organisms carried by the current. Studies made with membrane filters have shown that stream waters carry 1000's of bacteria per millilitre (Beling, 1953; Rodina, 1964). Studies on bacterial counts in estuaries and lakes using nucleopore filters and flourescent microscopy have shown that these waters contain bacterial cells in the order of 10^6 cells/ml (Hobbie *et al.*, 1977), so spore counts in streams could be even higher using newer techniques. Of the wide variety of organisms - algae, aquatic plants, plankton, benthos, fish and birds - which coexist with black fly larvae in streams and rivers, those with the greatest possibility of being directly affected would be animals that ingest particles of similar size to those found in *B.t.i.* formulations. This would include flagellates, filter feeders belonging to the Chironomidae and Trichoptera, molluscs and arthropods which subsist on detritus on the bottom, and fish which feed upon the drift. Animals which in turn feed upon these organisms would consume the bacteria indirectly.

Measurement of B.t.i.'s Effects on Nontarget Organisms

The effect of *B.t.i.* on nontarget organisms has been measured at 3 levels: on cells *in vivo* and *in vitro*, on intact organisms under controlled conditions and on animals present during field trials.

Thomas and Ellar (1983) examined the effects of the δ-endotoxin of *B.t.i.* on cells *in vivo* and *in vitro*. The toxin, extracted from the crystals by incubation in an alkaline solution at a high temperature, was found to affect not only mosquito cells, but also a wide variety of of cell types outside the *in vivo* host range, including mammalian cells and erythrocytes. In contrast, a solubilized preparation of the δ-endotoxin from *B.t. kurstaki* (a subspecies pathogenic to a number of Lepidoptera) was found to exert no effect on mosquito cells, mammalian cells or those of Lepidoptera outside its natural host range. As well, the solubilized toxin from *B.t.i.* proved lethal at low dosages (15-35 μg/g body weight) when inoculated intravenously into adult mice or subcutaneously into suckling mice. No effects were observed when the toxin was ingested orally, however.

Few studies on the effects of *B.t.i.* on nontarget organisms in the laboratory or experimental plots have been published. Most have concerned animals found predominantly in mosquito breeding habitats (Sinegre *et al.*, 1979; Muira *et al.*, 1980; Garcia *et al.*, 1980; Ali, 1981), including fish, amphibians,

crustaceans, flatworms, molluscs and numerous insects. None of the tests has examined the effects of *B.t.i.* treatment for longer than 28 days, or looked at growth, weight gain or reproduction. The only organisms shown to be adversely affected have been members of the Chironomidae, Dixidae and Ceratopogonidae: Diptera which belong to the same suborder (Nematocera) as the black flies and mosquitoes.

Lethal concentrations required to kill larvae in these families appear to be higher than the concentrations required to kill Culicidae or Simuliidae in the field. Sinegre *et al.* (1980) reported a 24h LD50 of 0.1 mg/l for chironomid larvae, using Bellon Laboratories primary powder. This was within the range (0.03 to 0.1 mg/l) required to kill 50% of a culicine mosquito population in the same study. However, in a study designed to investigate the potential of *B.t.i.* treatments for the control of pest chironomids, Ali (1981) concluded that the lethal activity of Abbott wp was relatively low when compared to its effectiveness against mosquitoes.

B.t.i. has since been found to affect other Diptera as well, including one species outside the Nematocera. Cantwell and Cantelo (1984) have reported on its effectiveness in controlling a sciarid fly, *Lycoriella mali*, found in mushroom compost. Temeyer (1984) has reported larvicidal activity in the horn fly, *Haematobia irritans*.

Most frequently, the evaluation of the effects of *B.t.i.* has been carried out under natural conditions during field trials. In fact, Undeen and Lacey (1982) have recommended that "during each field test (against black flies) efforts should be made to ascertain the effects of *B.t.i.* on nontarget organisms". They have suggested that methods include the use of Surber or Hess samplers, artificial substrates and natural substrates, and that sufficiently replicated samples (≥10) be taken pretreatment and at 72 hours posttreatment to provide an index of nontarget density.

Overall, the effects of *B.t.i.* applications to nontarget organisms in running water have been reported as minimal (Table 4). The only nontarget organisms for which increased mortality has been detected have belonged to the Chironomidae (Pistrang and Burger, 1984). Increased drift in other insect families has been noted also: Dejoux (1979) and Undeen and Colbo (1980) both make mention of noticable drift in several minor groups, Pistrang and Burger (1984) report temporary increases in the drift of Ephemeroptera, Trichoptera and Plecoptera.

Interpreting the Results of Field Trial Nontarget Studies

Confidence in the conclusions reached in field trials is weakened considerably by difficulties inherent to the assessment of animal populations in running water. To quote Hynes (1970), "to assess populations of animals on river beds in terms of numbers or biomass/unit area presents formidable, perhaps

Table 4 - A Summary of the Effects of *B.t.i.* on Nontarget Organisms in Field Trials against Black Flies

reference	location	product	dosage	sampling method	major invertebrate groups	results/conclusions
Dejoux, 1979	Ivory Coast	Biochem 1° powder	0.2 ppm for 10 min	troughs set into stream	Chironomidae, Coleoptera, Ephemeroptera, Lepidoptera, Mollusca, Odonata, Trichoptera	nearly total short-term innocuity
Colbo & Undeen, 1980	Nfld.,Canada	water suspension	1 x 10^5 spores/ml for 1 min	natural substrates	Chironomidae, Elmidae, Ephemeroptera, Odonata, Plecoptera, Trichoptera	increased drift in some minor taxa
Molloy & Jamnback, 1981a	N.Y.,USA	Bellon Lab 1° powder	0.5 ppm for 15 min	Surber sampler	Chironomidae, Elmidae, Ephemeroptera, Plecoptera, Trichoptera	no adverse effect
Lacey *et al.*, 1982a	Ivory Coast	Sandoz wdc	1.6 ppm for 10 min	artificial substrates	Chironomidae, Ephemeroptera, Trichoptera	no apparent ill effects

Table 4 - continued

reference	location	product	dosage	sampling method	major invertebrate groups	results/conclusions
Chilcott *et al.*, 1983	N.Z.	Sandoz wdc/ Biochem 1° powder	2 ppm for 15 min	Surber sampler	Chironomidae, Coleoptera, Dixidae, Ephemeroptera, Gastropoda, Plecoptera, Trichoptera	adverse effects unlikely
Pistrang & Burger, 1984	New Hampshire,USA	Teknar® fc	10 ppm for 1 min	standardized kick samples and drift samples	Chironomidae, Ephemeroptera, Plecoptera, Trichoptera	mortality of Chironomidae, increased drift of Ephemeroptera, Plecoptera and Trichoptera

insurmountable difficulties". He points to the doubts many investigators have concerning the validity of quantitative sampling due to the varying selectivity of different sampling techniques and the large variances in samples obtained.

A wide variety of sampling methods for assessing both target and nontarget population levels have been employed in *B.t.i.*/black fly field trials. Those used for assessing changes in populations of nontarget organisms have measured either drift or survival. Two investigators have questioned their own results. Molloy and Jamnback (1981a) conceded that their figures may be more a reflection of sampling error due to small sampling size and bias in sampler placement than actual population changes, while Lacey *et al.* (1982a) admitted that the numbers of Trichoptera present in their study were insufficient to enable an accurate measurement of possible effects caused by *B.t.i.*

The significance of increased drift caused by *B.t.i.* applications is unclear. The drift of organisms in running water is a well established phenomenon and commonly includes species of Chironomidae, Ephemeroptera and Plecoptera. Presumably, under normal conditions drifting invertebrates either find open niches further downstream or are eaten by predators. Increased drift can occur naturally during high water and in some species of Trichoptera, Plecoptera and Simuliidae may take place seasonally shortly before pupation or adult emergence. Assuming that invertebrates which are dislodged due to *B.t.i.* applications ultimately perish, the impact of such applications could depend

on how quickly new individuals came to replace them. If only part the river or stream is treated, then these new inhabitants could originate from the normal drift. Otherwise, recolonization by nontarget organisms could take much longer, since many mayfly and caddisfly species are poor fliers which emerge at relatively long intervals (1-2 years in temperate climates).

An Experiment to Determine the Toxicity of *B.t.i.* to a Nontarget Organism

Introduction

The purpose of the experiment was to determine the effect of *B.t.i.* on the developement of the immature stages of the caddisfly, *Clistoronia magnifica* Banks. Caddisflies were chosen because of:

1. reported effects of *B.t.i.* on caddisfly larvae in the field (Pistrang and Burger, 1984)
2. the similarity in food resource, fine particulate matter, between the larvae of many caddisfly and black fly species
3. the abundance of caddisfly larvae in the streams and rivers where black flies commonly breed, and
4. the importance of caddisfly larvae to this ecosystem.

The particular species, *Clistoronia magnifica*, was chosen because it can be reared easily in the laboratory.

To quote Wallace and Malas (1976), "They (caddisflies) have exploited a food resource, extremely fine particulate matter, that few other groups of filter-feeding insects have been able to use. Undoubtably among the exceptions are the larvae of the Simuliidae (Diptera) which have been shown to possess the capacity of feeding on minute particles". Larvae of both orders select particles of a particular size, which differs between species and instars. Many larval caddisflies in the families Pschomyiidae, Hydropsychidae, Polycentropidae and Philopotamidae

spin silken nets from salivary secretions. These larvae use their nets to trap particles flowing in the current. The size of the openings in the the mesh of the nets can be correlated to the size of the particles consumed by the larvae (Malas and Wallace, 1977). The mesh opening sizes for some species (eg. in the genera *Hydropsyche* and *Diplectrona*) are within a range which would capture particles from *B.t.i.* formulations. Caddisfly larvae also have a high midgut pH (Wigglesworth, 1972), an apparent requirement for susceptibility to *B.t.i.* (Undeen, 1979).

Trichoptera are worldwide in distribution and are usually an important element in the fauna of running waters (Hynes, 1970). Their numbers may reach levels which make them pests. In Japan they have been reported to slow the rate of water flow in conduits (Tsuda, 1961). They can also obscure the vision of motorists when attracted to automobile lights (pers. obs.). Nevertheless, there are at least 3 reasons why they should be conserved:

1. As filter feeders they play an important role in streams and rivers by concentrating drifting material which otherwise might be lost (Wallace *et al.*, 1976). The fecal pellets of caddisflies are considerably larger in size than the particles they ingest. In this way they convert smaller particles into a form that is then available to organisms which feed on larger sized material.
2. Predaceous species may help regulate natural populations of

dominant stream dwellers, including black flies (Muirhead-Thomson, 1971).

3. The larvae of caddisflies are considered to form an important food source for trout and other fish (Muirhead-Thomson, 1971).

It seems clear, therefore, that their removal from the ecosystem could cause undesired consequences, especially since the Trichoptera appear to fill a role similar to that of the Simuliidae, which are targeted to be reduced or eliminated.

Materials and Methods

Physical Parameters

Larvae were reared from egg masses (collected from local lakes) by the method described by Anderson (1977). They were placed in enamel coated metal trays 25 cm wide, 40 cm long and 5 cm deep. Fine aquarium gravel provided the substrate. Temperature was maintained at 15°C. The water in the trays was replaced every 3 to 5 days using dechlorinated tap water. The pH before and after the water was changed was approximately 6. Trays were arranged on three levels, illuminated from above with a fluorescent light (15L:9D). Each time the water was changed the trays' positioning was altered by moving each tray 2 positions to the right.

Diet

Larvae were provided with a diet of red alder (*Alnus rubra*) leaves and wheat grains. Leaves were always present in a greater amount than the larvae could consume. Wheat grains are a necessary supplement for normal caddisfly growth (Anderson, 1977). In order to avoid contamination of the water, the grains were added in amounts proportional to the number of larvae present at the time the water was changed.

Exposure of Larvae and Subsequent Observation

Sixteen trays were used. Initially 40 fifth instar larvae were placed in each tray. Following a pre-exposure period of 3 days, the water in the trays was replaced by water containing one of two commercial *B.t.i.* flowable concentrates: Teknar®, manufactured by the Zoecon Corporation and rated at 1500 ITU/mg and Vectobac®, manufactured by Abbott Laboratories and rated at 600 ITU/mg. Teknar® was applied at .1, 1, 10 and 100 mg/l. Vectobac® was applied at 1, 10 and 100 mg/l. Two replicates of each concentration were used. The water in two trays was replaced by untreated dechlorinated tap water to serve as controls. After 3 days exposure the water in the trays was changed, the substrate thoroughly washed and the food source replaced.

Viable larvae and pupae were tallied for each tray each time the water was replaced. Dead larvae commonly would separate from their cases. Sometimes empty cases could be found, the larvae

presumably consumed by their neighbours. Those which pupated were placed under gauze cones to trap emerging adults. Pupae which died could be detected by the softness of their cases and their odour when held close to the nose. The experiment was concluded 112 days after exposure of the caddisfly larvae to the *B.t.i.*, at which time the remaining pupal cases were opened to determine the viability of their occupants.

Results

Table 5 summarizes the results of the experiment. An initial survey of the data indicates that:

1. A consistent number (11-18) of larvae died in each tray, both treated and untreated, except for the trays treated with 100 mg/l Vectobac®, where the numbers of larval deaths was higher (28,30).
2. The percentage mortality calculated from the number of pupae that died to those that pupated was considerably higher in the trays treated with 1 mg/l Teknar® (75.0%,80.8%) and 100 mg/l Vectobac® (75.0%,100.0%) than in the remaining trays (4.0-46.0%). One control tray had the lowest pupal mortality(4.0%), while the other had the fifth highest (46.0%).
3. The numbers of adults which emerged from the trays treated with 1 mg/l Teknar® and 100 mg/l Vectobac® were noticably lower (5,7 and 0,2) than the numbers which emerged from the other trays (13-25).

Table 5 - The Effects of *B.t.i.* Treatments on the Survival of Immature *Clistoronia magnifica* over 112 Days

treatments:	control	Teknar®				Vectobac®		
mg/l[1]	0.0	0.1	1	10	100	1	10	100
number at	40	40	40	40	40	40	40	40
beginning:	40	40	40	40	40	40	40	40
died while	14	14	14	14	15	12	15	28
larvae:	14	14	12	13	11	12	18	30
pupated:	25	26	26	26	25	28	25	9
	26	26	28	27	29	28	22	9
died while	1 (4.0)	3(11.5)	21(80.8)	5(19.2)	3(12.0)	3(10.7)	2(8.0)	9(100.0)
pupae:[2]	12(46.0)	10(38.5)	21(75.0)	7(25.9)	6(20.7)	5(17.9)	6(27.3)	7(75.0)
emerged:	23	23	5	21	22	25	23	0
	14	15	7	19	23	23	15	2
larvae	1	0	0	0	0	0	0	3
remaining:	0	0	0	0	0	0	0	1
pupae	1	0	0	0	0	0	0	0
remaining:	0	1	0	1	0	0	1	0

[1] each treatment was applied to 2 trays; the results from each tray are recorded separately

[2] % of pupae that died/larvae that pupated in parentheses

Statistical Analysis

The results were analyzed using a single factor analysis of variance. The null hypotheses were:

1. mean larval mortalities for the 8 treatments were equal
2. mean % pupal mortalities for the 8 treatments were equal
3. mean emergences for the 8 treatments were equal

The outcome of these analyses is presented in Table 6. All 3 null hypotheses were rejected. Dunnet's (1955) test for comparing a control mean to each other group mean subsequently was used to determine which treatments resulted in significant differences. Only treatment with 100 mg/l Vectobac® brought about significantly greater larval mortality than in the control group (Table 7). Both treatment with 1 mg/l Teknar® and 100 mg/l Vectobac® resulted in significantly greater pupal mortality and significantly less adult emergence than the control group (Tables 8 and 9).

Discussion and Conclusions

Insects present in fast flowing water would not be exposed to *B.t.i.* for as long a period as the caddisflies in this experiment. Three days was used as an exposure period to ensure that larval *C. magnifica*, which feed on leaves, had sufficient time to ingest the bacteria following the disturbance caused by changing the water in the trays. This could be analagous to the situation in nature where leaf-shredding caddisflies present in slower-flowing portions of a stream or river might come in

Table 6 - Results of Single Factor Anovas to Test the Effects of *B.t.i.* on the Survival of the Caddisfly, *Clistoronia magnifica* (a = 0.05)

for H_0: mean larval mortalities for the 8 treatments were equal

$P(F_{(1)7,8} \geq 29.02) < 0.0005$, therefore reject H_0

for H_0: mean % pupal mortalities[1] for the 8 treatments were equal

$0.01 < P(F_{(1)7,8} \geq 5.13) < 0.025$, therefore reject H_0

for H_0: mean emergences for the 8 treatments were equal

$0.0025 < P(F_{0.05(1)7,8} \geq 9.52) < 0.005$, therefore reject H_0

[1]transformed to approximate a normal distribution using the arcsine transformation

Table 7 - Results of Dunnet's Test for Comparing a Control Mean to Each Other Group Mean for Larval Mortality

groups (a)	1	2	3	4	5	6	7	8
treatment	Vectobac® 1 mg/l	Teknar® 1 mg/l	Teknar® 100 mg/l	Teknar® 10 mg/l	Control	Teknar® 0.1 mg/l	Vectobac® 10 mg/l	Vectobac® 100 mg/l
means (X_a)	12	13	13	13.5	14	14	16.5	29

$H_o: \mu_5 \geq \mu_a$

Comparison (5 vs a)	Difference $\lvert X_2 - X_a \rvert$	SE	q'	p	$q'_{0.05(1),8,p}$	conclusion
5 vs 1	2			5	$X_5 > X_1$	accept $H_o: \mu_5 \geq \mu_1$
5 vs 8	15	1.46	10.27	4	2.42	reject $H_o: \mu_5 \geq \mu_8$
5 vs 7	2.5	1.46	1.71	3	2.22	accept $H_o: \mu_5 \geq \mu_7$

conclusion: only treatment 8 (100 mg/l Vectobac®) resulted in greater larval mortality than the control group (group 5)

Table 8 - Results of Dunnet's Test for Comparing a Control Mean to Each Other Group Mean for % Pupal Mortality[1]

groups (a)	1	2	3	4	5	6	7	8
treatment	Vectobac® 1 mg/l	Teknar® 100 mg/l	Vectobac® 10 mg/l	Control	Teknar® 10 mg/l	Teknar® 0.1 mg/l	Teknar® 1 mg/l	Vectobac® 100 mg/l
means (X_a)	22.06	23.67	23.97	27.13	28.29	29.09	62.01	75.00

$H_0: \mu_4 \geq \mu_a$

Comparison (4 vs a)	Difference $\lvert X_4 - X_a \rvert$	SE	q'	p	$q'_{0.05(1),8,p}$	conclusion
4 vs 1	5.07			5	$X_4 > X_1$	accept $H_0: \mu_4 \geq \mu_1$
4 vs 8	47.87	12.65	3.78	5	2.55	reject $H_0: \mu_4 \geq \mu_8$
4 vs 7	34.88	12.65	2.76	4	2.42	reject $H_0: \mu_4 \geq \mu_7$
4 vs 6	1.96	12.65	0.15	3	2.22	accept $H_0: \mu_4 \geq \mu_6$

conclusion: both treatment 8 (100 mg/l Vectobac®) and treatment 7 (1 mg/l Teknar®) resulted in greater % pupal mortality than the control group (group 4)

[1]transformed to approximate a normal distribution using the arcsine transformation

Table 9 - Results of Dunnet's Test for Comparing a Control Mean to Each Other Group Mean for Adult Emergence

groups (a)	1	2	3	4	5	6	7	8
treatment	Vectobac® 1 mg/l	Teknar® 100 mg/l	Teknar® 10 mg/l	Teknar® 0.1 mg/l	Vectobac® 10 mg/l	Control	Teknar® 1 mg/l	Vectobac® 100 mg/l
means (X_a)	24	22.5	20	19	19	18.5	6	1

$H_o: \mu_6 \geq \mu_a$

Comparison (6 vs a)	Difference $\lvert X_2 - X_a \rvert$	SE	q'	p	$q'_{0.05(1),8,p}$	conclusion
6 vs 1	5.5			6	$X_6 > X_1$	accept $H_o: \mu_6 \geq \mu_1$
6 vs 8	17.5	3.76	4.65	3	2.22	reject $H_o: \mu_6 \geq \mu_8$
6 vs 7	12.5	3.76	3.32	2	1.86	reject $H_o: \mu_6 \geq \mu_7$

conclusion: both treatment 8 (100 mg/l Vectobac®) and treatment 7 (1 mg/l Teknar®) resulted in less adult emergence than the control group (group 6)

contact with bacteria particles which settle on the vegetation. The experiment is intended to give only an indication of the susceptibility of Trichoptera as a whole and the results admittedly cannot be applied directly to any other species other than that used.

All treatments were innocuous to *C. magnifica* at application rates recommended by the manufacturers (1-10 mg/l), with one exception, treatment with 1 mg/l Teknar®. Two possible reasons for this exception are:

1. At high concentrations the larvae ceased feeding until the water was replaced. Afterward they resumed feeding and went on to develop normally. At lower concentrations the amount of toxin present was insufficient to stop feeding. This explanation would require that the 1 mg/l concentration was within a range sufficient to affect the larvae but not inhibit their feeding.
2. Fungal infection from pupae which had already died spread to healthy pupae. Pupae which had died commonly were covered with white mycelium. This was true for all trays and such pupae were removed as soon as they were noticed. This also might explain the relatively high pupal mortality encountered in one control tray. Gaufin *et al.* (1965) have reported susceptibility of 2 caddisfly species, *Arctopsyche grandis* and *Hydropsyche californica*, to the fungus *Saprolegnia* when maintained in the laboratory at 18°C. The fungus may have been

introduced with the alder leaves.

The significantly greater larval mortality in the trays treated with 100 mg/l Vectobac® (10 times the highest recommended application rate) could have been due to a constituent of the formulation other than the bacteria. The water in these trays appeared "cloudy" in comparison to the other trays for the 3 days following treatment. The increased turbidity appeared to affect the larvae's respiration; two larvae constructed abnormally long cases during the treatment period. Pistrang and Burger (1984) have suggested that the increased drift of Ephemeroptera and Trichoptera which occurred following treatment in their field study may have been due to such a constituent. The formulation they used was a flowable concentrate of Teknar®.

The most important factors influencing the use of *B.t.i* for black fly control are: (1) evaluation of formulation potency, (2) variability in susceptibility between different species and instars, (3) lack of efficacy at low temperatures, (4) organic content of the water, (5) carry and (6) cost.

Recently a new standard preparation has been proposed for use in the United States in rating *B.t.i.* against mosquitoes (Dulmage *et al.*, 1985). A similar effort is required to establish the comparative toxicities of formulations against black flies, in order to provide applicators with a basis for choosing most effective product. Lacey *et al.* (1982b) have suggested that a particular strain of a black fly species be used as a standard for toxicity tests. This strain could be distributed from one laboratory, possibly as eggs. *Simulium vittatum* has been selected by many researchers in North America for laboratory bioassay and has been reared continuously in the laboratory (Edman and Simmons, 1985). An appropriate instar would also have to be selected. Lacey *et al.* (1982b) consider penultimate instars to be the most suitable because they may be easily separated from other instars and will not pupate during the test period.

The relative importance of the other factors depends, of course, upon the particular situation in which the *B.t.i.* is to be applied. The organic content of the water would be an

important consideration in the control of larvae in polluted streams and rivers. Lack of efficacy at low temperatures could reduce the usefulness of *B.t.i.* in controlling black flies early in the season in certain parts of Canada, where species of *Prosimulium* (eg. the *fuscum-mixtum* complex) overwinter in the larval stage and are ready to pupate soon after the spring thaw occurs (Wood, 1985).

Variability in susceptibility between different species and instars should be examined in the laboratory, since suitable rearing methods have been developed for many species (Edman and Simmons, 1985). Lack of susceptibility in some cases may be due to the size of the larvae and their inability to ingest particles found in *B.t.i.* formulations. This might be overcome by increasing the range of particle sizes within one formulation or the development of formulations with different particle sizes. Lack of susceptibily due to physiological resistance might be overcome by selection of strains with different crystal antigens.

It appears that insufficient carry will hinder the application of *B.t.i.* in many situations. Particles precipitate out in regions of sluggish flow and the presence of such regions necessitates increased numbers of application points. This problem is common in the application of any particulate insecticide to a stream or river and is the major cost consideration of control programs. Quantities of *B.t.i* required for applications are small and costs compare favourably with

methoxychlor formulations required for treatment of the Athabasca River in Saskatchewan and temephos formulations required for the treatment of rivers in West Africa (Undeen and Colbo, 1980). The greatest costs accrue from the labour involved in application and prior survey of the river or stream system.

Manufacturers claim that *B.t.i.* is "selectively" toxic to most black fly and mosquito larvae (Anon, 1984). Although this was previously believed to be the case, it is now known that the toxicity of *B.t.i.* is not restricted to these insects or to the Suborder Nematocera, but also can extend to other members of the Order Diptera. Often literature extolling the safety of *B.t.i.* to man and other nontarget organisms refers to the good record of *B.t.* strains since their introduction as insecticides prior to W.W. II. What the authors fail to report is the uniqueness of the δ-endotoxin produced by *B.t.i.* compared to the δ-endotoxins produced by other strains. This uniqueness has been demonstrated by studies on the toxin itself (Huber *et al.*, 1981), its effects on individual cells (Thomas and Ellar, 1983) and its absence of toxicity to Lepidoptera (de Barjac, 1978).

Nevertheless, *B.t.i* is a drastic improvement over conventional insecticides. Apparently it is innocuous to practically all nontarget organisms in the field and certainly is much less harmful than other black fly larvicides such as DDT, methoxychlor and temephos, which can cause large increases in drift and mortality of nontarget species (Jamnback, 1973; Helson and West, 1978). There are also no reports of the

developement of resistance in target species, a persistent threat to the use of many pesticides. Finally, the public would presumably perceive the treatment of rivers and streams with *B.t.i.* as a safer method for black fly control than the application of conventional chemical insecticides.

In an experiment conducted as part of this paper 5th instar larvae of the caddisfly species, *Clistoronia magnifica*, were exposed to different concentrations of 2 commercial *B.t.i.* formulations: Teknar® and Vectobac®. At concentrations corresponding to the application rates recommended by the manufacturers, neither formulation brought about statistically significant differences in larval mortality when compared to controls. Teknar® applied at 1 mg/l, however, caused significantly greater pupal mortality and decreased adult emergence. This may have been due to a fungal infection rather than the *B.t.i.* treatment, so the results are not conclusive. Further laboratory studies on filter-feeding caddisfly species are recommended, since these species ingest particles of similar size to those found in *B.t.i.* formulations.

The ecosystem of running water is poorly understood, due mainly to its dynamic nature. Sampling techniques and the assessment of population changes, naturally occurring or induced by insecticides, are not reliable, which makes the assessment of insecticide impact difficult. Improved methods of sampling and analysis need to be developed and studies on the effects of *B.t.i.* treatments to nontarget organisms should accompany

applications for black fly control, wherever possible. One noticable effect of the application of *B.t.i* to streams and rivers is increased drift of some nontarget species. The significance of this effect against the background of natural drift is not known and may depend upon how much of the river or stream is treated. Undeen and Lacey (1982) have suggested that increased drift of nontarget organisms caused by *B.t.i.* applications could be measured by drift samples collected at 15 minute intervals for 1 hour after treatment and at 1 hour intervals over the next 24 hours. Background drift would have to be measured prior to treatment to establish temporal patterns, should any exist.

Application of *B.t.i* for control of black flies is at present the method which best combines efficacy and minimal effects on the environment. It can be argued that its advantages over the application of such conventional larvicides as methoxychlor and temephos justify its registration in Canada as soon as possible. Registration would significantly increase the use of commercial *B.t.i.* formulations in the field and this would result in not only the reduction of black fly populations but other benefits as well. The relative importance of such factors as instar and species, temperature and carry would become apparent and competition between manufacturers for an increased market would stimulate research into solutions. Parallel studies on population changes in black flies and nontarget organisms could also be undertaken, leading to the

refinement of sampling techniques and analysis.

APPENDIX: IDENTIFICATION AND CLASSIFICATION OF *B.T.* SEROTYPES

Several crystal forming bacteria pathogenic to insects have been described. All of these are similar to *B. cereus* Frankland and Frankland in regard to cellular and colony morphology. In 1959 Heimpel and Angus examined all crystal forming bacteria known up to that time and compared them according to a number of criteria: spore morphology, sporangium morphology, vegetative rod morphology and staining characteristics, gelatin liquefaction, colony appearance on agar and in nutrient medium, ability to hydrolyze starch, sugar fermentation, production of of acetylmethylambinol and production of phospholipase-C. On the basis of these comparisons and a review of other relevant literature the authors concluded that the isolates previously referred to as species *sotto, aleati* and *thuringiensis* should be regarded as a single species. The authors chose to call the species *Bacillus thuringiensis*, since this was the first complete species name to appear in print. The original species names were retained as varietal names to account for consistent differences, mostly minor in nature.

The generally accepted key for taxonomic division of the species today is based on the antigenic properties of the flagella. The fundamental work for this serotype classication was carried out by de Barjac and Bonnefoi in 1962. They studied 35 cultural and biochemical characters of 24 *B.t.* strains and discovered that a single test, the agglutination of flagellar

(or H) antigens, fit perfectly the biochemical classification by 10 different biochemical characters. The test is sensitive, specific, reliable and rapid. Its reliability was confirmed by Norris and Burges (1963), who studied about 50 strains and grouped them according to their esterases. The patterns they obtained coincided well with the corresponding classification by flagellar agglutination.

The steps involved in the identification of H-serotypes are described in detail by de Barjac (1981). The method relies on antisera which aggutinate the flagellae of vegetative cells. Because the motility of cells grown on conventional media is usually too poor to give strong agglutination reactions, motility has to be increased by selection. This is done by successive innoculation of the bacteria into Craigie tubes, eventually isolating those cells which migrate at the required speed. These are grown to a concentration of 10^6 - 10^9 cells/ml (checked by turbidity) and then injected into laboratory rabbits over a number of weeks. The antisera is obtained from the blood of the animals which is allowed to coagulate. Different titres of antisera are tested for their agglutination to stock cell suspensions of the unknown strain. A positive reaction is indicated by the clear contents of the test vessel and a flocular sediment on the bottom.

A cross-agglutination between 2 strains does not prove the identity of the unknown, however; it only means that the 2 share a common antigenic fraction and may differ by others. Several

B. t. strains, therefore, can agglutinate with the same H-antiserum, yet have different biochemical characters, as well as different pathogenicity spectra in host insects.

To check this possibility de Barjac uses a cross saturation technique, in which the antiserum for each strain (the unknown and the known) is saturated by a suspension of the other strain. Should the saturated anti-sera no longer react with either strain, the strains are assumed to have identical H-antigens. If not, the unknown strain is assumed to have an subfactor in its antigenic complement. These are given arbitrary letters, a, b or c.

Burges (1984) has outlined the accepted nomenclature for the species. Subgroups are designated as "varieties" according to their flagellar serotype. Sometimes the serotype number is used in place of the varietal name. Sometimes one serotype can be divided into biotypes which differ in their pathogenicity spectra. De Barjac (1981) considers these to be varieties as well. Within many varieties, further subgroups can be can be formed on the basis of crystal antigens. As of 1982, 25 H-antigen groups and 24 varieties were recognized (Table 9). These may be designated by the first 3 letters of the varietal name or the collector's code for the isolate. Further numbers may be added to denote differences in pathogenicity to a variety of host species or to identify mutations.

Table 10 - *B.t.* H-antigen Groups and Varieties

H-antigen Group	Variety	H-antigen Group	Variety
1	*thuringiensis*	8a8c	*ostriniae*
2	*finitimus*	9	*tolworthi*
3a	*alesti*	10	*darmestadiensis*
3a3b	*kurstaki*	11a11b	*toumanoff*
4a4b	*sotto*	11a11c	*kyushuensis*
4a4b	*dendrolimus*	12	*thompsoni*
4a4c	*kenyae*	13	*pakastani*
5a5c	*galleriae*	14a14b14c	*israelensis*
6	*subtoxicus*	15	*indiana*
6	*entomocidus*	15	*dakota*
7	*aizawae*	?	*wuhanensis*
8a8b	*morrisoni*	?	*fowleri*

from de Barjac, 1981; Krieg & Langenbruch, 1981; Smith, 1982

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