Bull. Environ. Contam. Toxicol. (1997) 58:291-298

Bulletin of Environmental Contamination and Toxicology

Effects of *Bacillus thuringiensis* subsp. *israelensis (B.t.i.)* Applications on Invertebrates from Two Streams on Prince Edward Island

I. R. McCracken, S. L. Matthews

Environmental Protection Branch, Environment Canada, 45 Alderney Drive, Dartmouth, Nova Scotia, B2Y 2N6, Canada

Received: 15 June 1996 Accepted: 29 October 1996

Efforts to control black fly populations stem from their adverse effects, which include reductions in livestock productivity, pathogen transmittal, and severe restrictions in outdoor recreation and tourist activities. Although chemical larvicides such as DDT, methoxychlor and temephos were used initially (Fredeen 1975, Thompson 1975, Back *et al.* 1979), the mortality of non-target organisms (Mohsen and Mulla 1982, Fredeen 1983) and the loss of efficacy of through the development of larval resistance (Kurtak 1986) prompted the search for a more suitable agent. Several studies have confirmed the efficacy of *Bacillus thuringiensis* subsp. *israelensis (B.t.i.),* serotype H-14, against various species of blackfly larvae (Molloy *et al.* 1981, de Moor and Carr 1986, Gibbs *et al.* 1986, Merritt *et al.* 1989, Riley and Fusco 1990). *B.t.i.* is a gram-positive, spore-forming bacterium highly toxic to certain dipteran larvae upon consumption (Federici *et al.* 1990). Widespread use of *B.t.i.* has resulted in concern for the potential effects on non-target organisms, particularly chironomids which constitute an important part of the diet of young salmonids (White 1930). Several studies have reported the sensitivity of chironomids to *B.t.i.* and high concentrations of *B.t.i.* are often used to eradicate chironomids from lakes and mesocosms (Mulls *et al.* 1990, Rodcharoen *et al.* 1991). As a number of streams on Prince Edward Island are treated, with treatments frequently extending from the headwaters to the mouth of the stream, it was decided to determine the concentrations of *B.t.i.* in the water following two operational applications of *B.t.i.,* and to monitor the potential impact on aquatic invertebrates.

MATERIALS AND METHODS

Two streams were examined in 1992. Elliotts is a second order stream, ranging from 2.7 to 4.2 m wide and 15 to 23 cm deep. The substratum was comprised largely of gravel and rocky areas with flat riffle sections. Less than 50% of the stream was overstoried. Lower Tryon is a first order brook with a width of 0.9 to 1.6 m, and a depth of 22 to 36 cm. It was soft bottomed and contained two riffle areas. Overstory was less than 30%. Each stream was treated twice, and in each case the stream was sampled prior to and after treatments over a three-day period.

Treatment methods were typical of those employed in the black fly control program by Prince Edward Island applicators, so that the impacts of actual application methods were observed. The quantities of product applied varied according to the volume and current speed of the stream, as well as black fly larval density and suspended solids in the water column. The *B.t.i.* product used, Vectobac® 1200L, had an activity of 1200 International Toxic Units mg^{-1}. On May 8, 100 and 170 mL were applied to Elliotts and Lower Tryon, respectively. The product concentrate was mixed with 4 L stream water

Correspondence to: I. R. McCracken

and poured over the width of the stream during a one minute period. In a similar manner, 100 mL Vectobac® 1200L was applied to Lower Tryon on June 2 and 55 mL to Elliotts on June 11. Treatments were applied over the entire length of the stream, to prevent simulid recolonization from upstream sources, generally at 100 m intervals, one of which was selected for study in the mid to lower reaches of each stream.

Duplicate water samples were collected in autoclaved Nalgene bottles, 10 m below the application point, immediately prior to the addition of *B.t.i.*, and at 1, 15, 30, 60 and 180 minutes thereafter. Samples were frozen until analyzed. Drift nets, 1 m in length with a mesh size of 500 µm and a mouth area of 0.09 m, were positioned at 10, 30 and 100 m below the application sites. Drift samples were collected by lowering drift nets into the water for 15 minutes during 5 sampling pericds before treatment and 8 sampling periods after treatment. The organisms trapped in the nets were washed into 250 mL glass bottles and preserved with isopropanol. Five benthic samples were taken 30 to 50 m below the application point using a Surber sampler, immediately before the addition of *B.t.i* . and 24 h later to detect any changes in invertebrate abundance. Invertebrates obtained from drift and benthic samples were identified to the family, genus or species level of taxonomic classification (Pennak 1978, Merritt and Cummins 1984).

A serial dilution and plating technique was carried out on the product concentrate, Vectobac® 1200L, to determine the number of colony forming units (CFU) mL^{-1} and to characterize the appearance of the colonies. Because bacterial colonies can arise from a single spore or a mass of spores, the results are expressed as CFU mL^{-1}, rather than the number of spores mL^{-1}. The product (1 mL) was aseptically added to sterile 0.1% peptone (Difco) water (99 mL), creating a 100-fold dilution. After inverting the dilution bottle five times, an aliquot (10 mL) was aseptically transferred to another dilution bottle containing sterile 0.1% peptone water (90 mL), producing a 10^{-3} dilution. This serial dilution technique was repeated until a 10^{-9} dilution was attained, whereupon every dilution bottle was inverted five times and two aliquots (10 mL) from each were transferred to individual sterile vacuum filtration units fitted with 47 mm, 0.45 µm sterile filters (Gelman Scientific). The 0.45 µm filters are known to effectively trap all of the *B.t.i.* spores (Cabana, J., 1992, pers. comm.). Following filtration (Cardinal 1986), each filter was aseptically transferred to *Bacillus cereus* Selective Agar Base Medium (Oxoid Canada Inc.) in 9 x 50 mm sterile petri dishes, which were then incubated at 37°C for 72 h. The B.cereus selective medium, also known as PEMBA (polymixin pyruvate egg yolk mannitol bromothymol blue agar), is suitable for *B.t.i.* since both bacteria have the same biochemical requirements (Buchanan and Gibbons 1974). Although these bacteria cannot be distinguished on the basis of colonial appearance on PEMBA medium, they can be differentiated using a modification (Cardinal 1986) of a staining methcd (Smirnoff 1962) used to detect the presence of parasporal inclusions or crystals, which are present in sporulating *B.t.i.* but absent in *B.cereus* (Buchanan and Gibbons 1974).

After 72 h incubation, plates containing 30 to 300 bacterial colonies were selected and slide smears were prepared from colonies which exhibited appearances consistent with those of *B.cereus* and *B.t.i.*, cremate to slightly rhizoid, turquoise to peacock blue in colour and surrounded by an egg yolk precipitate of the same colour. The smears were heat fixed and stained with Admidoschwartz Stain for 10 min, prior to being rinsed with tap water and blotted dry with bibulous paper. The slides were then examined microscopically under oil immersion (1000 x magnification) for crystals, which stained black. The same procedure was followed for the water samples. Dilutions were prepared from 10^{-1} to 10^{-4} and plated out as described above. Randomly selected slides were sent to Abbott Laboratories to confirm the initial identifications of *B.t.i.*

RESULTS AND DISCUSSION

The water temperature ranged from 4 to 6 °C over the experimental period and dissolved oxygen levels were at or near saturation (Table 1). The treated streams were alkaline, with an average pH of 8.2, possibly due to their proximity to agricultural land. Hardness values ranged from 46 to 93 mg L^{-1} $CaC0_3$, while suspended solids were <= 12 mg L^{-1}, except on June 2 following a precipitation event, wkere they ranged from 35 to 50 mg L^{-1}. Metals such as arsenic, cadmium, chromium, copper, lead, zinc, nickel, and tin were at or below the limits of detection.

Although a number of studies have been carried out to demonstrate the efficacy of *B.t.i.* against black fly larvae, the concentrations of *B.t.i.* present in the water were not quantified, but were reported as nominal concentrations. Those studies which attempted through microbiological plating techniques to determine the number of *B.t.i.* spores mL^{-1} of stream water did not analyze for the presence of parasporal bodies (Undeen *et al.* 1980, Frommer *et al.* 1981, Gibbs *et al.* 1986, Merritt *et al.* 1989), which is necessary to distinguish *B.t.i.* from *B.cereus.* Hence the reported concentrations of *B.t.i.* in those studies can only be viewed as rough approximations. In this study, the staining technique was used to detect parasporal bodies, thus confirming that the CFUs measured were derived from the *B.t.i.* actually present.

The Vectobac® 1200L product contained 6.2 x 10^9 CFU mL^{-1}. Based on the dimensions and flow velocity of the streams, the nominal concentrations of Vectobac® 1200L added to Lower Tryon on May 8 and June 2 were 32 and 25 mg L^{-1}, respectively. At Elliotts, the nominal concentrations were 15 mg L^{-1} for the application on May 8 and 10 mg L^{-1} on June 2. With the exception of data for Elliotts on May 8, concentrations of *B.t.i.* in the water column 10 m downstream from the application site showed a consistent temporal pattern (Figure 1). Pretreatment samples were devoid of *B.t.i.* in all cases. Peak concentrations of *B.t.i.* appeared 1 min posttreatment, ranging from 2400 to 100 000 CFU mL^{-1}. After 15 min, 40 to 150 CFU mL^{-1} were observed, followed by further declines at 30 min posttreatment to 4 to 25 CFU mL^{-1}. *B.t.i.* was not detected 60 min after treatment. A similar pattern was observed by Gibbs *et al.* (1986), with a peak concentration of 10 000 spores mL^{-1} 6 min after *B.t.i.* application at a site 150 m downstream, which rapidly fell to a concentration of only 30 spores mL^{-1} after 18 minutes, before disappearing after 20 min. On May 8, at the Elliotts stream, the concentrations gradually increased from 10 to 1400 CFU mL^{-1} after 180 min and appeared to have reached a plateau after three hours, at less than the peak concentrations measured one minute after the other applications. There are several possible explanations, including entrapment of *B.t.i.* particles by moss (Tousignant *et al.* 1993), or stagnation in slower pools with a gradual release over time.

In a total of 159 drift and 20 Surber samples taken before and after treatment, 12 orders and 32 families were found. Simulid and chironomid larvae were numerically dominant, constituting up to 95% of the drift and thus form the focus of the discussion. The remaining non-target invertebrates were present either in low numbers or sporadically in drift samples, hence changes in abundance could not be clearly correlated with *B.t.i.* applications.

Following the initial application of *B.t.i.* to Lower Tryon on May 8, there was a significant increase in drift of larval simulids (Mann-Whitney U-test, p=0.011) (Figure 2), accompanied by a decline in the number of simulid larvae in Surber samples (Mann-Whitney U-test, p = 0.072) (Figure 3). On June 2, the number of simulid larvae in the pretreatremt drift samples was lower than on May 8 (Figure 2), which suggested a decline in simulids over the season. After the June 2 treatment no simulids were observed in the drift. Data from the Surber samples (Figure 3) confirmed that simulids were present in

Table 1. Water quality charaeteristics of three streams on Prince Edward Island in 1992.

Parameter*		Lower Tryon May 8	Lower Tryon June 2	Elliotts May 8	Elliotts June 11
Hardness (mg L^{-1})		76	93	46	51
BOD (mg L^{-1})		<10	<10	<10	<10
Suspended Solids (mg L^{-1})		10	35	6	12
Magnesium (mg L^{-1})		3.3	4.0	4.8	5.2
Nitrate (mg L^{-1})		2.2	2.4	1.0	0.9
Sulphate (mg L^{-1})		4.4	4.8	4.1	4.2
Iron (mg L^{-1})		0.21	0.08	0.13	0.09
Manganese (mg L^{-1})		0.04	0.05	0.03	0.04
Chloride (mg L^{-1})		9.0	9.8	10.7	10.4
Temperature (°C)		4	-	4	6
pH		7.6	8.1	8.9	8.5
Dissolved Oxygen (mg L^{-1})		13	13	-	-
Max.Width(m)	Station 1	1.0	1.1	4.2	3.5
	Station 2	1.2	0.9	4.8	2.5
	Station 3	1.6	1.1	3.8	2.7
Max.Depth(cm)	Station 1	36	36	23	15
	Station 2	28	25	23	18
	Station 3	30	22	20	20
Velocity(cm s^{-1})	Station 1	26	20	54	67
	Station 2	48	52	103	60
	Station 3	30	36	120	44

*Analysis was also completed for additional parameters which were below detection limits (mg L^{-1}) as follows: As (<0.05), Cd (<0.01), Cu (<0.01), Pb (<0.02), Zn (<0.01), Ni (< 0.01), Sn (<0.05).

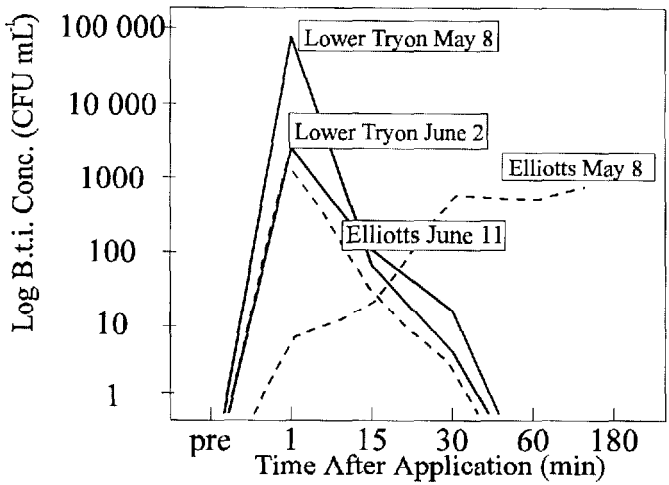

Figure 1. Concentrations of *B.t.i.* in stream water over a three hour interval after each application

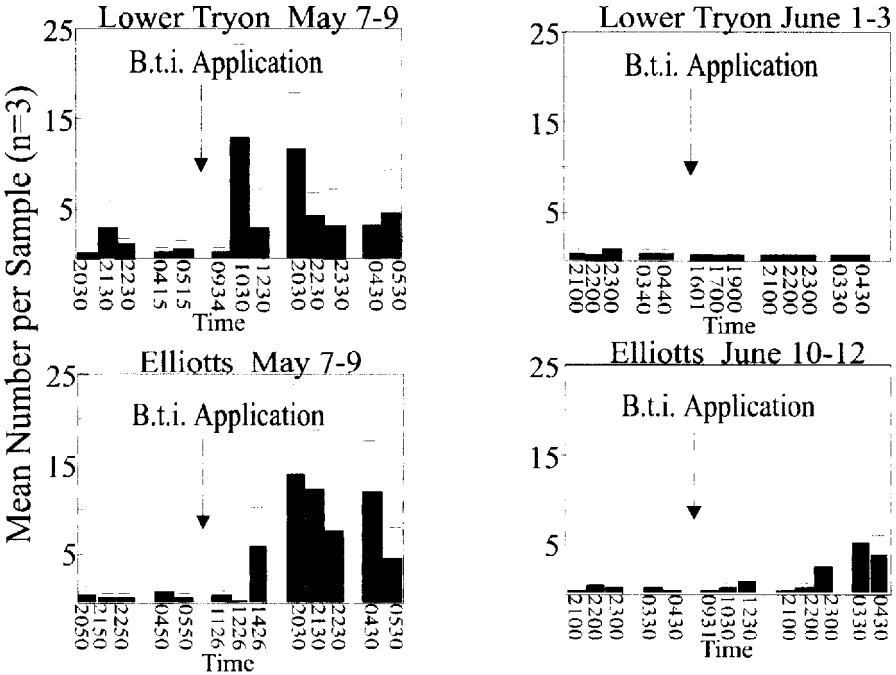

Figure 2. Mean number of larval simulids in drift samples before and after *B.t.i.* applications.

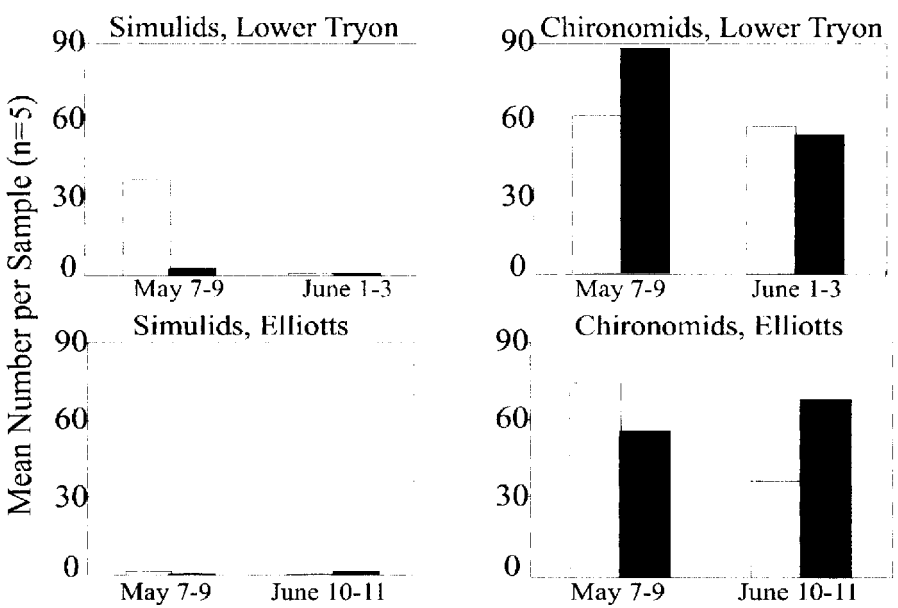

Figure 3. Mean numbers of larval simulids and chironomids in Surber samples before (□) and after (■) *B.t.i.* applications

very low numbers both before and after treatment on June 2, and that there was no significant difference between pretreatment and posttreatment abundance. A highly significant increase in simulid drift was observed after the application of *B.t.i.* on May 8 at Elliotts (Mann-Whitney U-test, $p < 0.001$) (Figure 2). The mean number of larval simulids in Surber samples was less than 5 per sample before and after both treatments. *B.t.i.* did not appear to have a significant effect on the number of larval simulids in drift samples following the June 11 application at Elliotts.

At Lower Tryon on May 8, there was a significant increase in larval chironomid drift after *B.t.i.* was added to the stream (Mann-Whitney U-test, $p < 0.05$) (Figure 4). Although the change in chironomid drift was marginally significant (Mann-Whitney U-test $p = 0.047$) after the June 2 *B.t.i.* application, the magnitude of the change was negligible. There were no trends in Surber samples from Lower Tryon consistent with an impact due to the *B.t.i.* applications (Figure 3). Neither *B.t.i.* application appeared to have significant effects on chironomid larval drift or benthic abundance at Elliotts.

Significant increases of drifting larval simulids occurred after the initial applications of *B.t.i.* on May 8 to the Lower Tryon and Elliotts streams, which suggested substantial mortality. Although mortality rates of simulid larvae following treatment were not evaluated directly in this study, it is reasonable to assume that increased drift rates were indicative of mortality, as observed in similar studies (Pistrang *et al.* 1984, Back *et al.* 1985, Merritt *et al.* 1989). The concentrations of *B.t.i.* at Elliotts may not have been high enough to affect the chironomids present. As simulids were affected by the May 8 treatment in both streams and chironomids were affected only at Lower Tryon which received a higher concentration of *B.t.i.*, there may be a concentration range of *B.t.i.* sufficient to affect simulids, yet not impact chironomids.

Surber sampling at the spatial scale used may not have been sufficiently sensitive to detect impacts due to *B.t.i.* treatments. A larger sample size over a longer sampling period may be necessary to relate drift abundance to Surber abundance. Different sampling methods may preferentially include or exclude certain species, and although direct numerical comparisons of the results of Surbers and drift nets are not practical, generally the data indicated that simulids may have been underrepresented in the benthic samples. The data presented suggests that high concentrations of *B.t.i.* in operational black fly control programs can adversely affect non-target chironomids if the concentration exceeds 25 mg L^{-1}, the maximum concentration on the pesticide label, as observed at Lower Tryon on May 8. A spatially and temporally more intensive sampling regime is required to determine the impacts on other non-target species over time, and to determine whether stream biomass is reduced or whether changes in diversity allow other species to serve as alternative sources of food for predators.

It has been recommended that long-term control of black fly larvae would require carefully timed repeated treatments with *B.t.i.* (Colbo and O'Brien 1984). In earlier studies of the effects of *B.t.i.* on simulid larvae and non-target invertebrates, *B.t.i.* was added to the downstream portion of a stream while the upstream part remained untreated (Molloy and Jamnback 1981, de Moor and Carr 1986, Merritt *et al.* 1989). As a result, recolonization of previously treated areas could occur within days, necessitating retreatment. However, in this study entire streams were treated from their sources to their outflows. The second *B.t.i.* applications did not appear to be warranted based on the low numbers of simulid larvae present and the overall lack of effect on drift and benthos. A standardized sampling protocol is required to determine the need for *B.t.i.* application, which would replace a subjective evaluation of the quantities of simulid larvae present on substrates. Once the need for treatment is determined, more specific criteria should be developed which would specify maximum concentrations of *B.t.i.* that can be applied based on physical factors affecting efficacy, as well as the stage of development and species of black fly present (Molloy *et al.* 1981, Khachatourians 1990). These measures will limit the amount of pesticides entering the environment.

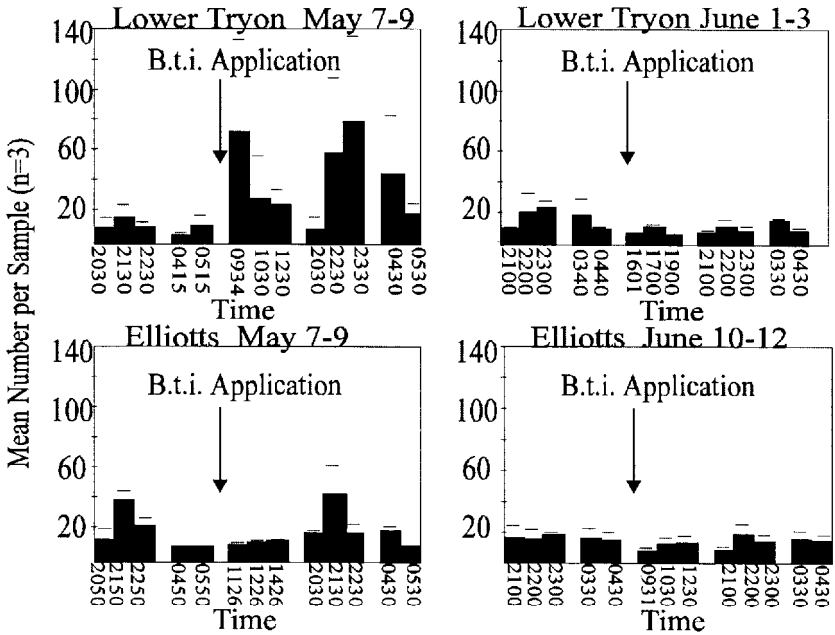

Figure 4. Mean number of larval chironomids in drift samples before and after *B.t.i.* applications.

Acknowledgments. We thank Abbott Laboratories for partially funding this study and for their help in confirming the identification of *B.t.i.* colonies. We also thank J. Cabana and C. Craig for their technical advice. We are grateful to W. Ernst, G. Julien and L. Rutherford for their help in planning and carrying out this study, and for reviewing the manuscript.

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