Biocontrol Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713409232

Effects of Bacillus thuringiensis var. israelensis on Target and Nontarget Organisms: A Review of Laboratory and Field Experiments

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Online publication date: 28 June 2010

To cite this Article Boisvert, Mario and Boisvert, Jacques(2000) 'Effects of Bacillus thuringiensis var. israelensis on Target and Nontarget Organisms: A Review of Laboratory and Field Experiments', Biocontrol Science and Technology, 10: 5, 517 — 561
To link to this Article: DOI: 10.1080/095831500750016361
URL: http://dx.doi.org/10.1080/095831500750016361

Biocontrol Science and Technology (2000) 10, 517–561

REVIEW ARTICLE

Effects of *Bacillus thuringiensis* var. *israelensis* on Target and Nontarget Organisms: a Review of Laboratory and Field Experiments

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(*Original manuscript received 7 December 1999; accepted in revised form 28 May 2000*)

Since the discovery of Bacillus thuringiensis *var.* israelensis (Bti) *in 1976, extensive literature has proved its efficacy to control mosquitoes and black flies, of which many species are known as important vectors of diseases or simply as pests of humans and animals. Since 1978,* Bti *has been used in many countries on all continents and numerous studies have been made on target mosquitoes and black flies, as well as nontarget organisms (NTO). This review analyses the results of 75 studies on these organisms covering approximately 125 families, 300 genera and 400 species. Different factors such as species, instar, feeding behaviour and environmental parameters (larval density, water temperature, suspended matter etc.) may drastically affect the efficacy of the* Bti *products. This is addressed in detail by reviewing the main factors affecting mosquitoes as well as black flies. The results of a wide range of laboratory and field experiments using different target and nontarget species, various preparations and formulations of* Bti *and different biotic or abiotic factors are present in the literature, making the data difficult to compare on a common basis. Our analysis shows that, under different application conditions, the effects of* Bti *on target and nontarget organisms may be hard to predict. Although* Bti *has been proclaimed to be relatively highly specific, some studies show that some NTO are affected either by single or repeated* Bti *treatments. Present use against black flies seems ecologically acceptable. High frequencies of application and/or overdosages against mosquitoes may result in some persistence of the toxin crystals and ultimately this may have adverse effects on the food web. A long-term study (published in 1998) in mosquito habitats has shown that intensive* Bti *treatments over three years did in fact produce an impact on the food web in wetlands. This raises questions, for the first time, on* Bti *environmental specificity. The importance of this impact is discussed and the alternatives for practical pest control are considered. Some modifications of* Bti *use against mosquitoes, guided by research, is probably the best of these alternatives.*

Keywords: Bacillus thuringiensis israelensis, *mosquito, black fly, nontarget organisms, laboratory experiments, field experiments, literature review*

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ISSN 0958-3157 (print)/ISSN 1360-0478 (online)/00/050517-45

INTRODUCTION

For the past five decades, humans have been almost completely dependent upon synthetic organic insecticides for agriculture, forestry and vector control purposes. However, the properties that made these chemicals useful—long residual action and toxicity for a wide spectrum of organisms—have brought about serious environmental problems and many concerns in the population. The emergence and spread of insecticide resistance in many species of vectors, the concern with environmental pollution, and the high cost of the new chemical insecticides made it apparent that insect pest control could no longer be safely dependent upon the utilization of chemicals.

Thus, increasing attention has been directed toward natural enemies such as predators, parasites and pathogens. Unfortunately, none of the predators or parasites of vectors could be easily mass-produced and stored for long periods of time. They must be reared *in vivo*, generating very expensive costs. It became evident that there was an urgent need for biological agents with the desirable properties attributed to chemical pesticides, i.e. highly toxic to the target organism, able to be mass-produced on an industrial scale, have a long shelf life and be easily transportable (Margalit & Dean, 1985). Insect pathogens were then advanced as valuable alternatives because of their relative specificity (directed to few or sometimes only a single pest species) and also because of their natural occurrence in the environment without any perceived harmful effects against nontarget organisms (Margalit, 1990).

In the mid-1970s, the World Health Organization (WHO) and other international institutions initiated studies on the development of existing and new biological control agents. In Israel, during the years 1975 and 1976, an extensive survey of mosquito breeding sites was launched to find natural pathogens and parasites of mosquitoes. As a result of this effort a new mosquito pathogen was isolated from a stagnant pond located in the Negev Desert of Israel (Goldberg & Margalit, 1977). Bioassays performed by these researchers indicated that species in four genera of mosquitoes (*Anopheles*, *Uranotaenia*, *Culex* and *Aedes*) were susceptible. This bacterium was identified as being in the genus *Bacillus* and its pathogenicity was initially (erroneously) attributed to its spores. Details of the discovery and properties of this new organism have been well documented by Margalit (1990).

Later, this pathogen was identified as a new serotype of *B. thuringiensis*, named serovariety *israelensis* or H14 after its origin, and proposed for the control of mosquito larvae (de Barjac, 1978a). At the same time, its larvicidal action on various mosquito species was confirmed, described at the cellular level (de Barjac, 1978c), and related not to spores but to a crystalline inclusion (or toxic crystal) produced during sporulation (de Barjac, 1978b,d). Since then, *Bacillus thuringiensis* subsp. *israelensis* (referred to as *Bti*) has been isolated from insects, soils or water samples from over 15 different countries (Martin & Travers, 1989; de Barjac, 1990; Bernhard *et al*., 1997). The crystal can kill mosquito larvae within minutes after ingestion.

This discovery was the first observation of a *Bacillus thuringiensis* (*Bt*) strain exhibiting a highly specific and toxic effect against certain aquatic diptera (de Barjac, 1978a). Before that discovery, only a few strains of *Bt* had been found with a low to moderate larvicidal activity against mosquitoes (Kellen & Lewallen, 1960; Reeves & Garcia, 1971; Hall *et al*., 1977). The main targets of *Bt* were principally confined to lepidopterous pests of agriculture and forestry, with *Bt* subspecies killing mostly insect larvae feeding on crops and on trees (Heimpel, 1967; Burgerjon & Martouret, 1971; Falcon, 1971).

Interest in *Bti* is increasing worldwide year by year as various commercial products are used in many countries, on all continents, for the control of mosquitoes and black flies on small to very large scales (Becker & Margalit, 1993). These authors reported that about 1000 tons of *Bti* products were being used annually in 1990. Over the years, this interest led to the production of many papers on the effect of *Bti* on target and nontarget (NTO) organisms. While reviewing the literature on these subjects, it appeared that the overall

experimental designs and methodologies used to study target and NTO were quite different, so different in fact that erroneous conclusions may have been drawn, on either the efficacy on target species or on the effects on NTO.

Some of the main problems encountered in reviewing the literature were the wide range of dosages and the types of preparations used in the numerous studies. Earlier works used spores/ml, International Toxic Units (ITU)/mg, *Aedes aegypti* units/mg, primary powder, experimental preparations, etc. Later, when more and more commercial products appeared on the market, researchers were using litres or kilograms per hectare (for mosquitoes) or mg l^{-1} (ppm) $\times$ time (min) units (for black flies) when doing field experiments. But most laboratory data were expressed in mg or units per litre. Some authors, using either commercial or experimental formulations, stated that the dosage used in their studies was higher (overdosage) than the producers' recommended dosages. Even recommended dosages have changed over the years to a point where some earlier works described using an 'overdosage', which would today be in the 'recommended dosage' category for difficult environments.

Even though *Bti* is known to be very efficient against target organisms, literature tends to demonstrate that *Bti* applied in experimental 'overdose' conditions can affect some nontarget organisms (NTO) (Sinègre *et al*., 1980a; Back *et al*., 1985; Fortin *et al*., 1986; Charbonneau *et al*., 1994; Su & Mulla, 1999). This review will focus on the effects of *Bti* on target as well as nontarget organisms. After a brief summary of the background of *Bti*, we will discuss the different factors affecting the efficacy of *Bti* on the most present main target organisms (mosquitoes and black flies). These factors can be related directly to the specific properties of mosquitoes or black flies (e.g. species, instar, feeding behaviour) or to environmental parameters (e.g. larval density, suspended matter, temperature etc.). Emphasis in the review will be placed on the effects of *Bti* on NTO, in particular how the frequency of application and the dosages applied can affect NTO and could play a role in the persistence of *Bti* crystals in the environment and the impact of *Bti* on the food web. A recent book by Glare & O'Callaghan (2000) reviews a similar theme, but concentrates more on terrestrial application of *Bt*.

BACKGROUND OF *Bt*

A member of the large family of Bacilli, *Bt* kills certain insects. It is a crystalliferous, spore-forming, aerobic bacterium closely related to *B. cereus* but differing from it by its ability to synthesize a parasporal protein crystal endotoxin generally toxic to certain insects (Krieg & Miltenburger, 1984). The presence of these crystals was first described by Berliner (1915). However, association of the crystal with the toxicity of *Bt* to insects was not established until the unique morphogenesis of sporulating cells of *Bt* was described by Hannay (1953). Hannay coined the phrase 'parasporal body' to describe the inclusion that lies alongside each spore. Today, the parasporal body is also termed parasporal inclusion, crystalline inclusion, toxic inclusion, crystal, protoxin or delta-endotoxin.

BIOLOGICALLY ACTIVE METABOLITES OF *Bti*

The toxic components of *Bti* are a range of endotoxins bound up in stable protoxin molecules in the parasporal inclusion (Larget & de Barjac, 1981; Charles & de Barjac, 1982). When sporulation is completed, the sporangium is lysed and the durable spore and the crystal are set free. The crystal and its subunits are inert protoxins and do not exhibit biological activity. The inclusion becomes active only when ingested and subsequently solubilized in the high pH of the larval midgut. Further activation may follow by proteolytic enzymes in the midgut or by proteolytic enzymes associated with the inclusion itself, releasing the protein fractions that are responsible for larvicidal activity (Chilcott *et al*.,

1983a). However, Pfannenstiel *et al*. (1984) concluded that it was unlikely that inclusion-associated proteases contributed to the activation of larvicidal toxins.

BIOSYNTHESIS AND STRUCTURE OF PARASPORAL BODY OF *Bti*

The formation of the parasporal body within the sporangium of *Bti* follows a time course similar to that of other subspecies of *Bt*. Under standard growth conditions in commonly used media, parasporal body formation is complete within 24 h, although autolysis of the sporangium usually requires another 24–48 h (Federici *et al*., 1990).

The parasporal body of *Bti* is basically spherical (oval), enveloped and averages about 1 μm in diameter, ranging from 0.7 to 1.2 μm. It contains four major proteins (27, 65, 128 and 135 kDa) assembled into three different types of inclusions (a large inclusion plus two smaller ones) bound together by a laminated net-like envelope of undetermined composition (Huber & Luthy, 1981; Charles & de Barjac, 1982; Lee *et al*., 1985; Ibarra & Federici, 1986).

The three different types of protein inclusions within the parasporal body can be ultrastructurally differentiated from one another by using a combination of size, shape and electron microscopy density. The largest inclusion makes up to 40–50% of the parasporal body and is characterized as being rounded to polyhedral and the least electron dense of the three types. This inclusion is thought to contain the 27 kDa protein based on the relative high abundance of this protein in comparison to the others present in the parasporal bodies and because it matches the solubility properties of this protein (Insell & Fitz-James, 1985; Ibarra & Federici, 1986). The second type of inclusion is often bar-shaped, of moderate electron density and constitutes approximately 15–20% of the parasporal body. This inclusion consists almost exclusively of a 65 kDa protein (Insell & Fitz-James, 1985; Lee *et al*., 1985; Ibarra & Federici, 1986). The third inclusion type is highly electron dense, hemispherical to spherical and based on its size makes up to somewhere in the range of 20–25% of the parasporal body protein composition. This inclusion type is thought to contain the proteins of 128 and 135 kDa (Mikkola *et al*., 1982).

TOXICITY AND MODE OF ACTION OF *Bti*

The toxicity of the *Bti* parasporal body varies considerably depending on whether it is intact or solubilized and how it is assayed. When ingested, either intact or solubilized, the parasporal body is toxic to mosquitoes, black flies and several other nematocerous dipterans. But, whether ingested, injected or topically applied, the intact protoxin is not active against vertebrates (de Barjac *et al*., 1980; Shadduck, 1980). Thomas and Ellar (1983a) were the first to detect the broad cytolytic activity of the solubilized parasporal body (Table 1). The solubilized parasporal body was toxic to mice upon injection and cytolytic to insect and mammalian cells *in vitro*. They attributed this activity to a 25 kDa protein cleaved from the 27 kDa protein when the parasporal body is solubilized under alkaline conditions. The intact crystals or protoxins are not toxic to mammals by ingestion because the mammalian gut conditions do not solubilize the crystals or release the haemolytic-cytolytic 25 kDa toxin and, even if they did, the mammalian gut cell membranes do not have the appropriate receptors to cause toxicity in the same way as in susceptible insects.

Thomas and Ellar (1983a), along with several other investigators, also attributed the mosquitocidal activity of *Bti* to the 27 kDa protein (Table 1). Alternatively, other investigators have reported that the 27 kDa protein is cytolytic but not mosquitocidal, with the latter activity residing in a mixture of proteins of 31–35 kDa, or the 65 kDa protein, or the 130–135 kDa proteins (Table 1). In other studies it has been suggested—and this is the current view—that the high toxicity of the parasporal body is due not to a single protein, but rather to a synergistic interaction of the 27 kDa protein with one or more of the higher molecular weight proteins (Table 1). Nevertheless, despite problems encountered in the interpretation of data, comparison of the results obtained for each parasporal body protein indicates that

TABLE 1. Toxicity (larvicidal, synergistic, cytolytic and haemolytic) produced by different proteins contained in the *Bti* parasporal body

Toxicity	Proteins	Authors	Year
Larvicidal	27 kDa	Armstrong *et al.*	1985
		Insell & Fitz-James	1985
		Sriram *et al.*	1985
		Davidson & Yamamoto	1984
		Thomas & Ellar	1983a,b
		Yamamoto *et al.*	1983
	31, 34, 35 kDa	Cheung & Hammock	1985
	65 kDa	Hurley *et al.*	1987
		Hurley *et al.*	1985
		Lee *et al.*	1985
		Ward *et al.*	1984
	130 kDa	Bourgouin *et al.*	1986
		Sekar	1986
		Visser *et al.*	1986
Synergistic	27, 65,	Crickmore *et al.*	1995
		Poncet *et al.*	1995
	128 and 130 kDa	Wu *et al.*	1994
		Chilcott & Ellar	1988
		Delecluse *et al.*	1988
		Hurley *et al.*	1987
		Ibarra & Federici	1986
		Wu & Chang	1985
Cytolytic and Haemolytic	27 kDa	Hurley *et al.*	1987
		Bourgouin *et al.*	1986
		Pfannenstiel *et al.*	1986
		Visser *et al.*	1986
		Hurley *et al.*	1985
		Lee *et al.*	1985
		Wu & Chang	1985
		Armstrong *et al.*	1985
		Davidson & Yamamoto	1984
		Thomas & Ellar	1983a,b
		Yamamoto *et al.*	1983

no single protein by itself is as toxic to mosquito larvae as the intact parasporal body (Federici *et al.*, 1990).

When ingested by a larva, the parasporal body dissolves in the alkaline gut juices, and midgut proteases cleave the protoxin, yielding the active delta-endotoxin proteins (Chilcott *et al.*, 1983a). In *Bti*-treated mosquito larvae, binding of endotoxins to specific receptors, resulting in an osmotic imbalance across the midgut epithelial cell membranes, causes severe damage to the gut wall leading to rapid death. More detailed descriptions of the ultrastructural events in the pathogenesis of midgut cells after exposure to *Bti* are presented by Charles and de Barjac (1981) and Lahkim-Tsror *et al.* (1983) for mosquitoes and by Lacey and Federici (1979) for black flies.

The presence or the absence of appropriate cellular receptors seems to be the major factor in the high specificity of the crystals (limited to a small number of susceptible species). Considering that the proteins assembled into the crystalline inclusion can vary between subspecies of *Bt*, probably the intensity of the observed toxic effect is basically the result of a greater affinity or of the number of receptors present for a given species (Honée & Visser, 1993). This largely explains the different susceptibility of insects to the crystals of different

subspecies of *Bt*. For example, the crystals of *Bt* subsp. *kurstaki* are very active against lepidopterous insects but show only a moderate activity against mosquito and black fly larvae, while the crystals of *Bt* subsp. *israelensis* are very active against mosquito and black fly larvae but show little or no activity against lepidopterous larvae (Dulmage *et al*., 1990; Federici *et al*., 1990). However, experiments in the laboratory by Ignoffo *et al*. (1981) in 'overdosage' conditions showed that some species in two terrestrial Lepidopteran families were significantly affected by *Bti* crystals (Table 2).

To better understand the effects of *Bti* (formulated or not) on targets and nontargets, it is important to note that under both laboratory or field conditions, many factors are necessary to produce the toxic effect of *Bti* crystals. If the crystals are available in sufficient quantity, a larva, to suffer toxicity and die, must:

- capture and ingest the crystals;
- possess a digestive tract with a highly alkaline pH; and/or
- possess the enzymes capable of liberating the toxic proteins; and
- possess the gut membrane receptors, compatible with the solubilized toxins.

Regardless of the recommended dosages, to maintain 100% mortality for a certain time in a pond or a certain carry in a stream, the *Bti* crystals must be available in much larger quantities than the quantity needed for an initial effect.

For mosquito and black fly control, multiple treatments may be necessary in the course of a year and a site can also be treated for many consecutive years. Many studies have examined the short-term impacts after an application (only one treatment and a follow up during the next few days) but very few studies have continued over many consecutive years. Information on the short-term impacts are well documented but long-term environmental consequences and repercussions have largely still to be measured. Results of ten field trials by Molloy (1992) and a two-year study by Jackson *et al*. (1994) in the moving water (lotic) environment plus a five-year study by Hershey *et al*. (1998) in the standing water (lentic) environment are extremely important to evaluate the long-term impact of *Bti* in the environment. These long-term studies complement short-term work to date and give important information on the fate of *Bti* in the environment.

FATE OF *Bti* TOXIC ACTIVITY IN THE ENVIRONMENT

Formulations of *Bti* are used as larvicides, either in standing water or in moving water. However, depending on the type of environment, the fate of and/or behaviour of the toxic particles can be quite different.

In lentic environments, once applied to the surface of water, the *Bti* crystals: (1) can be ingested by mosquitoes and also by other nontarget insects with different feeding behaviours (e.g. browsers, filter-feeders etc.); (2) can sediment at various rates depending or not on how they are formulated; or (3) can interact with substrates like vegetation or sediments.

In fast running water, the situation is quite different because the *Bti* crystals will move downstream from an application point. During their downstream travel, apart from being captured by filter-feeders (including black fly larvae), they can adsorb onto floating vegetation or onto periphyton (algae) covering rocks. They can interact with suspended material and eventually find their way into slow moving or static pools along the treated streams and sediment. But contrary to mosquito habitats, black fly habitats are more complex because of the very large variety of surfaces of interaction in the streambed and the hyporheic zone (interstitial water between the streambed and ground water).

Many studies associated the fate of *Bti* with the persistence of the toxic activity or of *Bti* spores. Many workers have used spores, which are easy to detect, as useful indicators of the fate of crystals, because both are durable and have similar physical behaviour. Since persistence of the crystal was studied directly using target species, it meant that persistence

was based on the availability over time of the toxic *Bti* particles to the target and not necessarily that *Bti* crystals had been degraded into non-toxic entities. Interactions with sediments, vegetation or periphyton would render the crystals unavailable to target filter-feeders like mosquito and black fly larvae, and thus cause the observed rapid loss of efficacy in field conditions.

Studies performed on the persistence of *Bti* toxic activity in lentic environments (Luthy *et al*., 1980; Mulla *et al*., 1982a; Hougard *et al*., 1983; Su & Mulla, 1999) indicated a very short time of availability to filter-feeders (less than 7–17 days) when compared to chemical pesticides. But other research, looking at the whole environment with different methodologies, found activity for up to 15 weeks (Silapanuntakul *et al*., 1983). Ramoska *et al*. (1982), Margalit and Bobroglo (1984) and Sheeran and Fisher (1992) demonstrated that the toxic crystals could be adsorbed onto sediments and decrease efficacy because they were not available to filter-feeders, but Ohana *et al*. (1987) found that the adsorbed crystals remained toxic for up to 22 days. Dupont and Boisvert (1986) and Boisvert and Boisvert (1999), using diffusion chambers (in a cold-temperate still environment), found that the toxicity of the *Bti* crystals was lost at a slow rate but could persist for five months. Boisvert and Boisvert (1999) also found that *Bti* crystals could be adsorbed rapidly onto vegetation and remain very toxic for 22 weeks. Overall, these studies have shown that the efficacy of *Bti* formulations decreases rapidly in still water (laboratory and field experiments) but the *Bti* crystals can maintain their toxic potential for weeks if not for many months.

Very few studies on the fate of *Bti* crystals have been done in lotic environments, since it was generally agreed that once the crystals had passed the target sites, they would eventually be diluted out by the moving waters. To explain the short carry observed with *Bti* formulations, Undeen and Colbo (1980) suggested that the large substrate-surface-area/volume ratio inherent to small streams resulted in rapid removal of *Bti* by stream flora and fauna.

Back *et al*. (1985) found after a high-dosage treatment that periphyton-grazers (Diptera: Blephariceridae) were poisoned by *Bti* (as seen by the destruction of the midgut in dead drifting larvae). These results indicated that the *Bti* crystals had been adsorbed by the algae growing on rocks. This interaction was later confirmed by Tousignant *et al*. (1993) who found that periphyton collected after a *Bti* treatment (overdosage) was highly toxic to mosquito larvae, but they could not detect any toxic activity associated with sedimented matter.

Besides the persistence of its toxic activity, the fate of *Bti* activity has also been associated with the spores. Although *Bti* spores are not involved in the toxicity, any recycling in the environment could possibly generate new crystals, thus producing a toxic environment for target larvae. Larget (1981) was the first to demonstrate that *Bti* could recycle in mosquito cadavers and maintain toxic activity. This recycling in cadavers was later confirmed by Aly *et al*. (1985) and Khawaled *et al*. (1990). But Boisvert and Boisvert (1999) found that recycling in the absence of larval cadavers could occur in certain circumstances over a period of five months, although cells in the act of recycling, i.e. in the vegetative state were not observed.

It would appear that both *Bti* crystals and spores can persist for a long time in lentic environments and possibly in lotic environments. The persistence of the crystalline inclusions should not be surprising since they are extremely difficult to dissolve (Bulla *et al*., 1981; Insell & Fitz-James, 1985). Apart from boiling in strong detergents, only a high alkaline pH (10) or the presence of proteases with lower pH optima (e.g. 7 or less) will allow dissolution of the inclusions. In field environments, pH 7 is common, but highly alkaline conditions do not usually occur, in contrast to the possibility of adsorption onto natural substrates. According to Martin and Travers (1989) and Bernhard *et al*. (1997), *Bt* can be readily isolated from soils, leaves and in aquatic environments. In soil at pH 7 or less, subsp. *aizawai* lost activity at variable speeds measured in a few months, as demonstrated by a bioassay that mixed the treated soil into the insect's food (West *et al*., 1984a,b; West &

Burges, 1985; West *et al*., 1985a,b). Thus, it seems probable that the toxic protein of *Bti* is denatured at different speeds in natural aquatic environments.

The implications of these studies are important for many reasons. Even if *Bti* toxic crystals are not available to the target species, they could be captured and ingested by other filter-feeders, Diptera or even by grazer or browser organisms. There is a possibility that repeated *Bti* applications could lead to accumulation of the toxic crystals, creating an 'overdosage' situation that could have an impact on nontarget organisms. But this situation is most likely to appear in lentic rather than lotic environments.

EFFECTS ON TARGET ORGANISMS

Before going into the following sections, it is important to recall that the nature of preparations or formulations (experimental, formulated, primary and wettable powders, slow release granules etc.) used in the different experiments plays an important role in crystal availability (e.g. particle size, aggregation, rapid settling) either for mosquito or black fly larvae. Data interpretation for the different parameters described (species, instar, feeding behaviour) could be closely linked to the formulations used for the experiments because the availability, the dispersion or the settling of the crystals are different depending on the formulations used. As we will see later, the dosages required for the control of different mosquito or black fly species can be explained either by the species involved, the type of formulations used and/or the environmental conditions of the treated sites.

Spectrum of Activity

Since its discovery, *Bti* has been found to be toxic for practically all filter-feeding mosquito and black fly larvae tested. References have been reviewed by Lacey (1985) for mosquitoes and Molloy (1990) and MacFarlane (1992) for black flies. *Bti* proved to be effective against at least 72 species of mosquitoes from 11 different genera: *Anopheles*, *Aedes*, *Culex*, *Culiseta*, *Limatus*, *Uranotaenia*, *Psorophora*, *Mansonia*, *Armigeres*, *Trichoprospon* and *Coquillettidia*. Toxicity of *Bti* was also demonstrated for at least 22 species of black fly larvae from 7 different genera: *Simulium*, *Cnephia*, *Prosimulium*, *Austrosimulium*, *Eusimulium*, *Odogmia* and *Stegoptera* (Margalit & Dean, 1985).

Insects most susceptible to *Bti* crystals are mainly in genera within the same family presumably with a common ancestor. The spectrum of activity of *Bti* is mostly restricted to the members of Nematocera (suborder) within the order Diptera. However, the greatest degrees of susceptibility are found within a few families: the Culicidae (mosquitoes), the Simuliidae (black flies) and the Chironomidae (midges); with mosquitoes and black flies being the most susceptible.

Factors Affecting *Bti* Activity Against Mosquitoes

Mosquito parameters. (A) Species. Among mosquitoes, various genera exhibit different levels of susceptibility to the same *Bti* preparation. In general, *Culex* larvae are most susceptible; *Aedes* larvae are equally or slightly less so, but *Anopheles* larvae are relatively tolerant to primary products or currently available formulations (Mulla, 1990). Aly *et al*. (1988) showed that differences in susceptibility present among species of the same genus, could be caused by behavioural and physiological variations of the different species. The range of activity of different *Bti* preparations and formulations varies a great deal depending on the species and type of environment treated. Even against the same species, the range of effective dosages of different preparations can vary in environments possessing different biotic and abiotic characteristics (Mulla, 1990).

For *Aedes* and *Psorophora* species where larvae inhabit relatively shallow bodies of water, excellent control (90–100%) of the larvae at rates of 0.10–2.0 kg ha^{-1} has been provided by *Bti* formulations (Dame *et al*., 1981; Eldridge *et al*., 1985; Hougard *et al*., 1985; Lacey,

1985; Mulla *et al*., 1982b, 1985). In some situations, the required dosages may be below or above this range, but most species will be controlled for short periods using these dosages. Higher dosages are required where late-instar larvae are preponderant or in polluted and deeper water.

Studies on the efficacy of *Bti* against *Anopheles* species are indeed limited in the literature. Only a few species and situations have been investigated. In general, most *Anopheles* species studied to date were controlled at 0.5 to 6 kg ha^{-1} of *Bti* products although, in some cases, rates for successful control were as high as 19.1 kg ha^{-1} (Dame *et al*., 1981; Standaert, 1981; Hougard *et al*., 1983; Lacey, 1985; Lacey & Inman, 1985; Sandoski *et al*., 1985; Majori *et al*., 1987).

Field efficacy tests of *Bti* preparations and formulations have also been carried out against many species of *Culex*. In general, these species are quite susceptible in biotopes having low levels of organic matter but required higher dosages when breeding in brackish or polluted waters. *Culex* in clear-water situations have been readily controlled with *Bti* in the dosage range of 0.10 to 0.56 kg ha^{-1} of various preparations or formulations (Mulla *et al*., 1982a; Garcia *et al*., 1983; Majori & Ali, 1984; Mulla, 1985).

Other genera of mosquitoes have shown varying degrees of susceptibility. *Culiseta* species were not as susceptible as *Culex* species (Mulla *et al*., 1982a; Garcia *et al*., 1983). *Mansonia* species also showed a low level of susceptibility (Foo & Yap, 1983) and *Coquilletidia* species were not effectively controlled in the field by the recommended dosages of some commercial preparations (Sjogren *et al*., 1986).

(B) Feeding behaviour. Research on the feeding behaviour of larvae has provided some evidence of a relationship between the level of *Bti* activity and the feeding behaviour of larvae. For example, *Culex* and *Aedes* larvae feed actively up and down the whole depth of a shallow body of water. Some *Aedes* larvae can shift between different feeding modes during their normal behavioural activities (Shannon, 1931). They can split their feeding time between deep feeding (collecting-gathering, scraping or shredding) and feeding near or at the water surface (collecting-filtering). Their feeding modes can be related to their larval instar, younger larvae devoting comparatively less time to deep-water feeding or bottom-feeding than older instars (Nilsson, 1987). Some *Culex* species can also show similar feeding behaviours (Nilsson, 1987). Even if the toxic particles in most preparations and formulations settle rapidly towards the bottom, larvae of these two genera tend to ingest a lethal dose over a short period of time. On the other hand, the less susceptible *Anopheles* larvae, which primarily feed at the surface–air interface of water (Aly & Mulla, 1986; Rashed & Mulla, 1989), may not be able to ingest a lethal quantity of toxic particles in the relatively short period of time taken by particles to sink from the surface layer.

(C) Instar susceptibility. For most species tested, younger-instar larvae are more susceptible than older ones. Testing of four different *Bti* experimental preparations against larvae of *Culex quinquefasciatus* and *Anopheles quadrimaculatus* showed that second instars are 1.5 to 5-fold more susceptible than fourth instars (Mulla, 1990). Late fourth instars that have ceased feeding or feed little before pupation are much less susceptible because of lack of ingestion of a lethal dose in a short period of time. Prepupae and pupae are refractory to *Bti* because they do not feed and ingest the toxic particles. Early instars will definitely be killed by dosages and concentrations that will induce some mortality in older larvae. In asynchronous species such as *Culex*, *Anopheles* and some *Aedes*, all larval instars prevail in the breeding environments. Administration of maximum dosages geared to kill older larvae will be necessary to control these heterogeneous larval populations (Mulla, 1990).

Environmental parameters affecting field activity. (A) Larval density. Another important biological factor that influences larvicidal efficacy of *Bti* is the ratio of the quantity of toxic particles administered in the water versus the number (density) of larvae (Farghal *et al*., 1983; Aly *et al*., 1988; Vorgetts *et al*., 1988; Becker *et al*., 1992; Nayar *et al*., 1999). In field experiments, a given dosage of *Bti* that will control 95–100% of larvae prevailing at low

density will not produce the same results when larval density is materially increased. In conditions where high-density populations are met, higher concentrations or dosages will be required to produce mortalities equal to those of low-density populations. In general, denser populations of larvae (50–100 larvae per dip) will require 1.5–2 times more material than the low-density populations (5–20 larvae per dip) to yield equal mortalities (Mulla *et al*., 1982b). Organic pollution and decaying vegetation are other factors that result in denser larval populations and require higher dosages (see below). Larval density is an important factor and must be considered; but in practice, dosages are generally calculated on the basis of surface area of water to be treated and not on the larval density.

(B) Suspended organic matter. The activity patterns of *Bti* can be influenced by environmental factors such as organic pollution and the presence of colloidal particles, including food particles (Margalit & Bobroglo, 1984; Margalit *et al*., 1985). There seems to be a direct correlation between the extent and magnitude of organic pollution and the dosage of bacterial toxin required to obtain a given level of mortality. Apparently, in the presence of organic and inorganic particles and/or floating materials, fewer toxin particles are ingested per unit of time than in the absence of extraneous materials. Moreover, the availability of crystals is decreased by their adsorption onto suspended particles followed by a slow sedimentation. It is also known that denser populations of mosquito larvae are produced in the presence of high organic pollution. In both cases (high density and pollution), higher rates of application will be necessary to control mosquito larvae (Mulla, 1990).

(C) Water temperature. Water temperature is a very important factor that needs to be taken into consideration. Sinègre *et al*. (1980b) showed a decreased activity of the endotoxin against *A. aegypti* in the laboratory at low temperatures (from 17 to 7°C). Although *Bti* has been found to be active at low temperatures, its effectiveness may be reduced in cold water due to a cessation or a low rate of feeding of some species of larvae, larval diapause and a decrease in metabolic rate (Sinègre *et al*., 1980b). Becker *et al*. (1992) showed that bioassays conducted in the laboratory with second instar larvae of *Aedes vexans* at a low temperature (5°C) yielded 10-fold higher LC_{50} and LC_{90} values compared with those conducted at a higher temperature (25°C). Nayar *et al*. (1999) performed bioassays on third instar larvae of *Culex nigripalpus* and *A. taeniorhyncus* in the laboratory and found that LC_{50}s of both species were 1.4 to 3.0-fold lower at 35°C than at 15°C. Walker (1995) also demonstrated in the laboratory that low water temperatures had an effect on the LC_{50} of the test species, *A. stimulans*. The LC_{50} of *Bti* at 0°C (0.9 ppm) was 9-fold higher than that at 22°C (0.1 ppm), and 4-fold higher than that at only 4°C (0.2 ppm). The relationship of LC_{50} to temperature is also affected by the natural temperature range of the insect species, because species living in cold climates are physiologically adapted to live actively at lower temperatures than tropical species. Probably the gut enzymes are geared to have lower temperature optima. For example, *Bti* is relatively active at 5°C in snow melt mosquito species.

Other factors commonly encountered in nature like slow flowing water (rice fields), intensive vegetative cover and increased water depth are also important considerations that decrease the efficacy of *Bti* formulations against mosquitoes (Mulla, 1990) but will not be discussed here in detail.

As we have seen, many factors can influence the efficacy of *Bti* formulations against mosquitoes. In addition to these factors, the design of formulations used in all the studies is still one of the most important things to consider when comparing results and data. As already mentioned, the availability of the crystals is essential to larvae as the first step for a lethal action to occur. Thus, it is not surprising that most commercial formulations contain a substantial amount of 'inert ingredients'. Some of the inert ingredients are intended to facilitate the dispersion of the crystals, maintain the proper particle size, prevent clumping during storage and adsorption onto particulate material, etc., described in detail by Burges and Jones (1998). Overall, they should play a role in maintaining the toxic crystals in a stage where availability can be sustained for maximum efficacy of a given formulation. This should also hold true for some NTO, but unfortunately, some authors have suggested that

the inert ingredients themselves (when present) could be directly involved in the observed toxic effect on NTO of some formulations (Pistrang & Burger, 1984; Fortin *et al*., 1986; Holck & Meek, 1987; Snarski, 1990; Wipfli *et al*., 1994).

Factors Affecting *Bti* Activity Against Black Flies

Black fly parameters. (A) Species. For mosquitoes, where different species can be found according to the type of breeding habitats encountered (snow pools, ponds, marshes, tree holes, tyres etc.) and also because of the facility to rear different species in the laboratory, many studies have been done on species susceptibility to *Bti* formulations. However, for black fly larvae living in a more standard habitat (flowing waters) where many different species are present together, added to the fact that they are very difficult to rear or maintain in laboratory, species susceptibilities are not as well documented as for mosquitoes. Contrary to mosquitoes, most data were obtained in semi-field (gutter system) or field conditions.

Despite these qualifications, studies showed that all simuliid species tested so far have proven to be susceptible to *Bti* (Margalit & Dean, 1985). For example, different *Simulium* species such as *Simulium corbis*, *S. venustum*, *S. verecundum*, *S. vittatum*, and *S. tuberosum* etc., showed various levels of susceptibility to the toxic crystals (Lacoursière & Charpentier, 1988; Molloy, 1990) but in general larger larvae are less susceptible to *Bti* than smaller larvae. Molloy *et al*. (1981) and Habib (1993) reported differences in species susceptibility among *Simulium* species. While Habib (1993) did not provide body-size data to explain differential susceptibility, Molloy *et al*. (1981) showed that last-instar *S. vittatum* were three times less susceptible than last-instar *S. verecundum*. They mentioned that differences in body-size (larger larvae are generally less susceptible) could explain the differences between the two species; *S. vittatum* being about three times the size of *S. verecundum*.

(B) Feeding behaviour. In contrast to mosquito larvae that feed at the water surface or throughout the entire water depth, black fly larvae have different feeding strategies. Simuliid larvae are particularly well adapted for filter-feeding, since most possess a pair of labral fans. Filtration (collector-filterers), through the large paired cephalic fans, is passive and energy-saving because the water current does the work by delivering the food to the trapping mechanism (Currie & Craig, 1987). Although the majority of simuliid larvae are filter-feeders, many are known to be capable of scraping (scrapers–grazers) (Chance, 1970; Craig, 1977) and can forage over submerged substrates for algae and associated material (periphyton) (Cummins, 1973). They can also be collector-gatherers (Cummins, 1973) and feed upon decomposing, fine particulate organic material deposited as a loose surface film. Hart and Latta (1986) have shown that larvae can change their territorial behaviour and filter-feeding behaviour in response to food concentration.

Black fly larval feeding behaviour can play a significant role in determining the efficacy of field applications if we consider that the toxicity of *Bti* is solely due to its role as a stomach poison. Studies presented by different authors indicate that the following factors may reduce *Bti* efficacy by interfering with, or otherwise causing the cessation of normal feeding: lack of feeding during moulting (Back *et al*., 1985), feeding inhibition due to excess particulate load (Gaugler & Molloy, 1980) or formulation additives (Molloy *et al*., 1981); reduced or intermittent feeding at very cold temperatures (Colbo & O'Brien, 1984; Olejnicek *et al*., 1985).

(C) Instar susceptibility. As with mosquitoes, *Bti* is effective against all larval instars and early instars are considerably more susceptible. This correlation between susceptibility and larval age was first noted by Guillet and Escaffre (1979) and reaffirmed since then by other authors (Gaugler *et al*., 1983; Olejnicek, 1986; Morin *et al*., 1988b).

Environmental parameters affecting field activity. (A) Discharge. Black fly larvae inhabit lotic environments where conditions of slow and fast flowing water can be encountered. Many factors like the profile, the depth and the width of a stream will influence its discharge.

Discharge is well known to be a principal factor determining the carry of black fly larvicides (i.e., the distance downstream that an application of a larvicide produces high mortality). According to some authors, there appears to be a positive correlation between downstream carry and stream flow rate, with carry being greatest when discharge is high (Undeen & Colbo, 1980; Palmer *et al*., 1996). However, stream discharge is only useful as a crude estimator of surface area and hence carry. A fast, shallow stream and a slow, deep stream may have identical discharges, but vastly different substrate surface areas. For example, the poor carry (about 200 metres) of *Bti* in low-discharge streams (1 m^3 min^{-1}) (Riley & Fusco, 1990) contrasted sharply with the carry (about 5000 metres) achieved in a large river trial (2480 m^3 min^{-1}) (de Moor & Car, 1986).

(B) Stream profile. Undeen and Colbo (1980) and Undeen *et al*. (1984) have shown that carry can be correlated with stream profile, especially with the depth-to-width ratio of a stream. Moreover, Undeen *et al*. (1984) observed that because of increased contact and possible adherence of *Bti* particles to stream substrates, decreased carry has been noted in streams with a high ratio of surface area to water volume.

In a study performed by Tousignant *et al*. (1993) where two streams were treated with *Bti*, experiments were conducted to monitor the transfer of *Bti* toxicity from the open-channel water to the underlying hyporheic zone. Results showed that *Bti* toxic crystals were found as deep as 65 cm under the streambed, thus confirming the exchange of particulate material between the open-channel water and the hyporheic zone, another factor explaining the loss of *Bti* in open-channel water.

The interruption of flow caused by a low or negative relief (i.e. gullies, pools) is another important element limiting downstream carry of *Bti* formulations. In slow-moving pools, the particles have a tendency to settle out or bind onto larger particles, which subsequently will accelerate settling to the bottom, thus reducing activity downstream from the pool and contributing to a decreased carry (Molloy & Jamnback, 1981; Colbo & O'Brien, 1984).

(C) Turbidity. According to Guillet *et al*. (1985), the turbidity of water under natural conditions affected the efficacy of formulations in which spores and crystals were clumped as large particles. When the *Bti* clumps originating from large-particle formulations and large, naturally-occurring particles increase in the water, the probability of ingestion of *Bti* clumps is lowered because of the 'competition' between the two types of particles present. That competition in turbid water could explain the low efficacy of large-particle formulations rather than a feeding inhibition of the larvae. However, turbidity did not affect the efficacy of formulations with individual free spores and crystals. In their study, Morin *et al*. (1988b) indicated that twice the amount of *Bti* formulation should be applied in a stream containing 20 mg l^{-1} of seston (organic matter composed of living organisms and non-living particles) compared to 10 mg l^{-1}, in order to obtain the same mortality of black fly larvae.

(D) Water temperature. Colbo and O'Brien (1984) suggested that the lack of continuous feeding by the larvae in very cold temperatures (0–7°C) could explain the reduced efficacy of *Bti* in these conditions. Thus, they recommended a longer application time to increase the probability that all larvae would ingest some *Bti* during its passage downstream. In laboratory experiments, Lacoursière and Charpentier (1988) mentioned that changes in larval feeding behaviour and physiology could result in the reduced efficacy of *Bti* observed at lower temperatures. Their observations on the physiological behaviour of *Simulium decorum* and *Prosimulium mixtum/fuscum* larvae confirmed earlier results obtained by Olejnicek *et al*. (1985) on *Odogmia ornata* larvae who reported much lower efficacy at 0–3°C than at 17–19°C, suggesting that the efficacy of the toxic crystals in cold water was inhibited not only by reduced ingestion but also by slower digestion of the toxic crystals. Morin *et al*. (1988b) showed that ingestion rates of the *S. venustum* and *S. verecundum* larvae rose exponentially with increasing temperature between 9 and 19°C. They mentioned that increase in feeding with increasing temperature implied that for the same amount of *Bti*, mortality would be higher at warmer temperatures, even without a change in the specific activity of the insecticide. To thwart this reduced efficacy, treatments have to be modified by

increasing the exposure time and/or concentration in cold water conditions. In either case, more *Bti* is required per application.

(E) Interaction of *Bti* with benthic substrates/sediments. The short carry generally observed in streams with a high ratio of substrate surface area to water volume suggested that *Bti* was being removed from stream water as a result of the direct contact of *Bti* particles with benthic substrates or sediments (Undeen *et al.*, 1984). Back *et al.* (1985) showed that two chironomid genera (Diptera) that normally feed on organic debris, and blephariceridlarvae (Diptera) which feed by scraping periphyton growing on rocks, were adversely affected after a *Bti* treatment thereby suggesting that *Bti* toxic crystals were attached to these substrates. Tousignant *et al.* (1993) found that, after treating a small stream with *Bti*, high percentages of mortality were obtained when periphyton samples were tested against mosquito larvae, indicating that *Bti* toxic particles were associated with this substrate.

The short carry observed with *Bti* formulations has also been attributed in part to interactions with particulate matter suspended in the water, leading to a rapid sedimentation of *Bti* crystals in streams or rivers. Jackson *et al.* (1994), using an operational dosage to treat a large river, could not detect *Bti* spores in sediments collected from pools and depositional zones of the river. Tousignant *et al.* (1993) treated a small stream with a high dosage of a *Bti* formulation and they found that sediments collected behind rocks did not show any toxic activity. These results indicate that in flowing waters, *Bti* attachment to suspended matter is very low in contrast with attachment to static material covering the stream substrate.

Other environmental parameters are also important and can affect *Bti* efficacy, e.g. vegetation, pH, degree of vertical water mixing, pollutants, turbulence, density of other aquatic filter-feeding organisms, reduced water velocity due to negative relief, also formulation parameters (particle size, powders, liquid formulations, formulations additives) and treatment parameters (concentration, duration of application). These parameters are reviewed in Molloy (1990) and will not be discussed in this paper.

As we have seen, the final toxic activity of *Bti* crystals in either mosquitoes or black flies is influenced by numerous factors, some biotic others abiotic. Most of these factors can be condensed into three tiers: (a) the availability (fate) of a sufficient amount of toxic particles; (b) their ingestion (feeding behaviour); and (c) the subsequent release of enough specific toxic proteins in the insect gut to induce lethal damage in larvae. Unfortunately, we will see in the next pages that these three tiers can also occur in certain insects other than mosquitoes and black flies, i.e. in the NTO.

EFFECTS ON NONTARGET ORGANISMS (NTO)

Regardless of all the situations mentioned before, it appears that in some conditions users of *Bti* will apply the highest recommended dosages in order to achieve a control with sufficient economic value. Because of the extreme variations in biotic (e.g. species) and abiotic (e.g. temperature) factors encountered within a treated area, whether it is on a small or large scale control program, users will have the tendency to use a single application to avoid repeating treatments. Thus, it is likely that high dosages will be used most of the time subject to the limitations of cost. For example, temperature is a very important factor when considering the dosages to be applied. In both lentic or lotic environments, water temperature can vary a lot depending on the time of the day. A dosage selected for an early morning treatment could become an 'overdosage' during an afternoon treatment. Despite treatments at recommended dosages or higher 'overdosage' conditions, some adverse effects (some people prefer 'no catastrophic effects') have been demonstrated among major groups of aquatic invertebrates living in the same aquatic environments as mosquito and black fly larvae.

In Table 2, studies on more than 125 families and nearly 400 different species have been listed. For this work, only Culicidae (Diptera) and Simuliidae (Diptera) are described as target organisms although sometimes other insect groups can be regarded as targets (see below). All the other organisms in the table have been identified from 75 published studies performed on target and nontarget organisms. For the purpose of this review, references have been cited and compiled to the best of our knowledge but it may be possible that the list is not fully comprehensive although every effort has been made to make it complete. Readers should notice that all the authors cited at the end of Table 2 are ordered chronologically and not alphabetically. Furthermore, each cited study is individually numbered. Some studies on NTO used increasing dosages or concentrations of *Bti* formulations. In that case, they will appear in both operational and 'overdosage' treatments (Example: Fortin *et al*. (1986), #45 in Table 2). Finally, for some authors, part of their studies have been performed in the laboratory and another part in field conditions (lotic or lentic). Their results will be presented in both laboratory or field environments (Example: Miura *et al*. (1980), #6 in Table 2).

Table 2 describes the experimental procedures used for each study, e.g. if experiments have been performed in a lotic environment (from rivulets to large rivers), lentic environment (from potholes to large ponds) or in laboratory or artificial environments (from styrofoam cups to gutter systems). Overall, of the 75 studies cited, 29% were performed in lotic environments, 32% in lentic environments, 36% in laboratory or artificial environments and 3% were done both in laboratory and field conditions. Of the studies, 33% have been done in 'overdosage' conditions, 58% were done using the recommended operational dosage while 9% of the studies were performed from operational to 'overdosage' conditions. More specifically, for each environment type, if we look at the percentage of 'overdosage' versus 'operational' dosage, we obtain the following results: (1) in lotic environments, a ratio of 25%:75%; (2) in lentic environments, a ratio of 10%:90%; and finally (3) in laboratory or artificial environments, 67%:18% (plus 15% in both conditions). Although the distribution of the different environments is quite balanced (29%:32%:36%), the experiments were performed using the recommended operational dosage is almost 60% of the examples (58%). We will see later that operational dosages can apparently affect many nontarget organisms!

For the dosage evaluations, we based our criteria according to two principles: (1) the authors explicitly expressed in their paper that they used an 'overdosage' or an operational treatment according to the producers (label or producer's recommendations); (2) if no criteria concerning the dosage used in the work were expressed, we characterized the dosage according to other similar experiments with similar dosages and formulations or according to the producer's prescription for these products. We have in our laboratory many labelled formulations of different products (since 1982) for reference. For some studies, it was difficult to characterize the dosage because of the nature of the preparation used (ex. experimental powder with units in spores ml^{-1}) or because in some laboratory studies the dosage was expressed in mg l^{-1} while field treatments used kg ha^{-1}. Although some studies were reported as using a high-dosage or an 'overdose' of the products, it was still difficult to make a decision because of the recommended dosages prescribed by the producers. Some recommended dosages (e.g. for black fly control) can vary by a factor of 750 between the lowest and the highest dosages according to the water temperature and the amount of organic pollution or suspended matter. This created anomalies where some studies labelled as high-dosage treatment, e.g. Back *et al*. (1985), would today be in the 'recommended dosage' category!

Most studies clearly indicated whether there was an effect or not on the described organisms (Table 2). Some others indicated only if there was mortality (with no explicit percentage) and others reported observations like 'drift increase' or 'density reduction'. The purpose of Table 2 is to provide general information and for more complete experimental details, it is suggested that the readers refer directly to the original papers. However, various studies (Table 2) showed that, even considering the specificity of *Bti* for mosquito and black

TABLE 2. Toxicity of *Bti* for target and nontarget organisms (organisms directly or indirectly affected by *Bti* products other than mosquitoes and black flies)

TAXA	Lot[1]	Len[1]	Lab[1]	Dose[2]	Effects[3]	References[4,5]
KINGDOM ANIMALIA						
COELENTERATA (cnidaria)						
Hydrozoa (hydra)						
Hydra sp.			×	+	No effect	59
PLATYHELMINTHS	×			+	No effect	47
Turbellaria	×			+	No effect	60
Planaridae			×	+	No effect	5
Planaridae	×			0	No effect	62
Dugesia tigrina			×	+	No effect	59
Dugesia dorotocephalis			×	+	No effect	5
Planaria sp.	×			0	No effect	30
MOLLUSCA	×			0	No effect	2
Bivalvia (Pelecypoda)			×	+	No effect	5
Bivalvia (Pelecypoda)		×		0	No effect	65
Bivalvia (Pelecypoda)	×			0	No effect	62
Corbiculidae						
Corbicula africana	×			0	No effect	60
Corbicula africana	×			+	No effect	60
Corbicula sp.	×			0	No effect	30
Lymnacidae						
Galba palustris			×	+	No effect	59
Ostreidae						
Crassostrea gigas			×	+	No effect	17
Ostrea edulis			×	+	No effect	7, 17
Sphaeridae						
Pisidium sp.			×	+	No effect	59
Sphaerium sp.	×			0	No effect	56
Gastropoda	×			0	No effect	24, 62
Gastropoda		×		0	No effect	65
Ancylidae	×			0	**Drift, +23%**	**34?**
Ancylidae	×			0	No effect	62
Burnupia capensis	×			0	No effect	60
Burnupia capensis	×			+	No effect	60
Burnupia sp.	×			0	**Density, −58%**	**30?**
Burnupia sp.	×			+	**Density red.**	**68**
Planorbidae						
Anisus leucostomus			×	+	No effect	59
Bulinus tropicus	×			+	No effect	60
Gyraulus sp.			×	+	No effect	13
Hippeutis complanatus			×	+	No effect	59
Physidae						
Aplexa hypnorum			×	+	No effect	59
Physa acuta			×	+	No effect	59
Physa sp.			×	+	No effect	3, 5, 13
Pleuroceridae	×			0	No effect	56
ANNELIDA						
Hirudinea	×			0	No effect	2, 34
Glossiphoniidae						
Helobdella sp.			×	+	No effect	3
Helobdella stagnalis			×	+	No effect	13
Helobdella stagnalis			×	+	No effect	59
Salifidae						
Salifa perspicax	×			0	No effect	60
Salifa perspicax	×			+	No effect	60

TABLE 2. *Continued*

TAXA	Lot[1]	Len[1]	Lab[1]	Dose[2]	Effects[3]	References[4,5]
Oligochaeta	×			0	No effect	30, 34, 60, 62
Oligochaeta		×		0	No effect	9, 61, 65
Oligochaeta		×		+	No effect	9
Oligochaeta		×		+	**Density, −50%**	**61?**
Naididae	×			+	No effect	60
Tubificidae						
Tubifex sp.			×	+	No effect	59
Polychaeta						
Nereidae						
Neanthes arenaceodantata			×	+	No effect	42
ARTHROPODA						
CRUSTACEA						
Branchiopoda (Phylopods)						
Anostraca		×		0	No effect	65
Chirocephalidae						
Artemia salina			×	+	No effect	5
Artemia salina			×	+	No effect	7
Chirocephalus grubei			×	+	No effect	59
Cladocera		×		0	No effect	28
Daphnidae						
Ceriodaphnia sp.			×	0	No effect	6
Daphnia pulex			×	+	No effect	59
Daphnia magna			×	+	No effect	7, 59
Daphnia magna			×	0	No effect	15
Daphnia magna			×	+	**20–80% mort.**	**15?**
Daphnia sp.		×		+	No effect	9
Simecephalus sp.			×	0	No effect	6
Simecephalus vetulus			×	+	No effect	5
Moinidae						
Moina rectirostris		×		0	No effect	39
Moina sp.			×	0	No effect	6
Conchostraca						
Caenestheriidae						
Caenestheriella sp.			×	+	No effect	13
Limnadiidae						
Eulimnadia sp.			×	0	No effect	6
Eulimnadia texana		×		0	No effect	39
Lynceidae						
Lynceus sp.			×	+	No effect	13
Copepoda			×	+	No effect	13
Copepoda		×		0	No effect	28
Cyclopoida						
Cyclopidae						
Cyclops fuscus			×	+	No effect	7
Cyclops sp.		×		0	No effect	9
Cyclops sp.		×		+	No effect	9
Cyclops strenuus			×	+	No effect	59
Cyclops vernalis			×	0	No effect	6
Cyclops viridis			×	+	**30% mort.**	**3?**
Macrocyclops sp.			×	+	No effect	5
Megacyclops sp.			×	+	**30% mort.**	**3?**
Malacostraca						
Amphipoda						
Gammaridae	×			0	No effect	56, 62

TABLE 2. *Continued*

TAXA	Lot[1]	Len[1]	Lab[1]	Dose[2]	Effects[3]	References[4,5]
Gammaridae			×	+	No effect	5
Elasmopus bampo			×	+	No effect	42
Gammarus duebeni			×	+	No effect	69
Gammarus lacustris			×	0	No effect	44
Gammarus pulex			×	+	No effect	59
Hyalellidae						
Hyalella azteca			×	+	No effect	5, 51
Hyalella azteca		×		0	No effect	9, 61
Hyalella azteca		×		+	No effect	61
Decapoda						
Cambaridae						
Orconectes limosus			×	+	No effect	59
Grapsidae						
Hemigrapsus sp.			×	+	No effect	5
Palaemonidae	×			0	No effect	56
Leander tenuicornis		×		0	No effect	74
Palaemonetes varians			×	+	No effect	69
Isopoda			×	+	No effect	5
Asellidae	×			0	No effect	56
Asellus aquaticus			×	+	No effect	59
Asellus forbesi		×		0	No effect	54
Ostracoda			×	+	No effect	13, 59
Ostracoda		×		+	No effect	9, 22
Ostracoda		×		0	No effect	9, 28
Cypridinidae						
Cypridae sp.			×	+	No effect	5
Cyprois sp.			×	0	No effect	6
ARTHROPODA						
INSECTA						
Collembola	×			0	No effect	60
Collembola	×			+	No effect	33
Collembola		×		0	No effect	65
Coleoptera (beetles) (adults)		×		+	No effect	9
Coleoptera (beetles) (larvae)		×		+	No effect	9
Chrysomelidae						
Donacia sp.		×		+	No effect	18
Dytiscidae	×			0	No effect	56
Dytiscidae		×		+	No effect	18, 22
Acilius sp.		×		+	No effect	18
Anodocheilus exiguus		×		+	No effect	18
Copelatus caelatipennis		×		+	No effect	18
Copelatus chevrolati renovatus		×		0	No effect	6
Copelatus sp.		×		0	No effect	65
Coelambus impressopunctatus			×	+	No effect	59
Dytiscus marginicollis			×	+	No effect	13
Dytiscus sp.		×		0	No effect	65
Guignotus pusillus			×	+	No effect	59
Hydaticus sp.		×		0	No effect	65
Hydroporus palustris			×	+	No effect	59
Hydroporus sp.		×		0	No effect	65
Hydroporus undulatus			×	+	No effect	51
Hydrovatus sp.		×		0	No effect	65
Hygrotus inaequalis			×	+	No effect	59
Hygrotus sp.		×		0	No effect	6, 28, 65
Hyphydrus ovatus			×	+	No effect	59

TABLE 2. *Continued*

TAXA	Lot[1]	Len[1]	Lab[1]	Dose[2]	Effects[3]	References[4,5]
Ilybius fuliginosus			×	+	No effect	59
Ilybius sp.		×		+	No effect	18
Laccophilus maculosus			×	+	No effect	51
Laccophilus maculosus decipiens		×		0	No effect	6
L. mexicanus atristernalis		×		0	No effect	6
L. mexicanus mexicanus		×		0	No effect	6
Laccophilus sp.		×		0	No effect	28, 65
Rhantus calidus		×		+	No effect	18
Rhantus consputus			×	+	No effect	59
Rhantus gutticollis		×		0	No effect	6
Rhantus pulverosus			×	+	No effect	59
Thermonectus basillaris		×		0	No effect	6
Thermonectus basillaris		×		+	No effect	18
Dytiscidae (adults)	×			0	No effect	56
Elmidae (adults)	×			0	No effect	56, 60
Elmidae (adults)	×			+	No effect	60
Elmidae (larvae)	×			+	No effect	33, 60
Elmidae (larvae)	×			0	No effect	4, 16, 30,
Elmidae (larvae)	×			0	No effect	34, 46, 56, 60
Dubiraphia sp.	×			0	No effect	62
Optioservus sp.	×			0	No effect	62
Stenelmis sp.	×			0	No effect	62
Gyrinidae			×	+	No effect	3, 5
Gyrinidae	×			+	No effect	33
Gyrinidae	×			0	No effect	56, 60
Aulonogyrus sp.	×			0	No effect	30
Orectogyrus sp.	×			+	No effect	60
Haliplidae	×			0	No effect	56
Halipus confluentus		×		+	No effect	18
Halipus immaculicollis			×	+	No effect	51
Halipus sp.		×		0	No effect	65
Halipus sp.		×		+	No effect	18
Peltodytes edentulus			×	+	No effect	51
Helodidae						
Cyphon sp.		×		0	No effect	65
Hydraenidae						
Hydraena sp.		×		0	No effect	65
Hydrophilidae		×		+	No effect	22
Hydrophilidae		×		0	No effect	9
Hydrophilidae	×			0	No effect	4, 24, 34, 56
Anacaena globulus			×	+	No effect	59
Berosus infuscatus		×		+	No effect	18
Berosus metalliceps (*adults*)		×		+	No effect	22
Berosus signaticollis			×	+	No effect	59
Berosus sp.			×	+	No effect	7
Berosus sp.		×		0	No effect	65
Berosus sp.		×		+	No effect	18
Berosus sp.	×			0	No effect	62
Berosus styliferus		×		0	No effect	6
Helophorus sp.		×		0	No effect	6, 65
Hydrobius fuscipes			×	+	No effect	59
Hydrobius sp.		×		0	No effect	65
Hydrochus sp.		×		0	No effect	65
Hydrochus sp.		×		+	No effect	18
Hydrophilus caraboides			×	+	No effect	59
Hydrophilus triangularis		×		0	No effect	6
Hydroporus sp.		×		0	No effect	65
Tropisternus lateralis		×		0	No effect	6, 28
Tropisternus lateralis nimbatus		×		+	No effect	18

TABLE 2. *Continued*

TAXA	Lot[1]	Len[1]	Lab[1]	Dose[2]	Effects[3]	References[4,5]
T. salsamentus (*nymphs + adults*)			×	+	No effect	5
Tropisternus sp. (*nymphs + adults*)			×	+	No effect	5
Tropisternus sp.		×		+	No effect	18
Hydrophilidae (adults)	×			0	No effect	56
Psephenidae	×			+	No effect	33
Psephenus sp.	×			0	No effect	62
Scirtidae						
Scirtes sp.		×		+	No effect	18
Staphylinidae		×		0	No effect	65
Diptera (Nematocera)						
Athericeridae	×			0	No effect	46, 56
Atherix variegata	×			+	No effect	63
Blephariceridae	×			0	No effect	46
Blepharicerica sp.	×			+	**30% mort.**	**33**
Blepharicerica sp.	×			+	**Drift, + 50x**	**33**
Ceratopogonidae		×		+	No effect	18
Ceratopogonidae			×	+	No effect	59
Ceratopogonidae	×			+	No effect	33, 60
Ceratopogonidae	×			0	No effect	4, 62
Ceratopogonidae			×	+	**100% mort.**	**5**
Celicoides sp.		×		0	No effect	65
Palpomyia sp.			×	+	**42% mort.**	**5**
Chaoboridae			×	+	No effect	7
Chaoboridae	×			+	No effect	33
Chaoborus astictopus			×	+	No effect	13
Chaoborus crystallinus			×	+	No effect	59
Chaoborus sp.		×		0	No effect	9
Mochlonyx culicomorphis			×	+	No effect	59
Chironomidae		×		+	No effect	18, 61
Chironomidae	×			+	No effect	21, 33, 63
Chironomidae	×			0	No effect	4, 16, 24
Chironomidae	×			0	No effect	30, 46, 56
Chironomidae	×			0	**Drift increase**	**72**
Chironomidae	×			0	**Mortalities**	**32, 47**
Chironomidae			×	+	**15–100% mort.**	**5**
Chironominae	×			0	No effect	58
Chironomus crassicaudatus			×	+	**Mortality**	**10**
Chironomus decorus			×	+	No effect	13
Chironomus kiiensis			×	+	**80–100% mort.**	**67**
Chironomus maturus			×	+	**97% mort.**	**13**
Chironomus plumosus			×	0	**100% mort.**	**15**
Chironomus stigmaterus		×		0	**100% mort.**	**6**
Chironomus yoshimatsui			×	+	**70–100% mort.**	**67**
Chironomus sp.			×	+	**90% mort.**	**7, 59**
Chironomus sp.		×		+	**90% mort.**	**9, 61**
Chironomus sp.			×	+	**100% mort.**	**3**
Chironomus sp	×			0	No effect	60, 62
Chironomus sp.			×	+	**93% mort.**	**61**
Cladopelma sp.	×			0	No effect	62
Cladotanytarsus sp.	×			0	No effect	62
Cryptochironomus sp.	×			0	No effect	62
Dicrotendipes pelochloris			×	+	**10–100% mort.**	**67**
Dicrotendipes sp.	×			0	No effect	62
Dicrotendipes sp.			×	+	**93% mort.**	**61**
Glyptotendipes paripes			×	+	**Mortality**	**10**
Glyptotendipes tokunagai			×	+	**30–100% mort.**	**67**
Goeldichironomus holoprasinus		×		0	**100% mort.**	**6**

TABLE 2. *Continued*

TAXA	Lot[1]	Len[1]	Lab[1]	Dose[2]	Effects[3]	References[4,5]
Micropsecta sp.			×	+	**43–100% mort.**	**61**
Microtendipes sp.	×			0	No effect	62
Paratanytarsus grimmii			×	0	**Mortality**	**66**
Paratanytarsus sp.			×	+	**40–100% mort.**	**67**
Paratanytarsus sp.			×	+	**93% mort.**	**61**
Pentapedilum tigrinum			×	+	**90–100% mort.**	**67**
Phaenopsecta sp.	×			+	**Moribund**	**33**
Polypedilum sp.	×			+	**Density, −39%**	**33**
Polypedilum sp.	×			0	**Drift increase**	**62?**
Polypedilum sp.	×			+	No effect	60
Rheotanytarsus distinctissimus	×			0	**23% mort.**	**58**
Rheotanytarsus exiguus	×			0	**23% mort.**	**58**
Rheotanytarsus fuscus	×			+	**Density red.**	**60**
Rheotanytarsus fuscus	×			0	**Density red.**	**60**
Rheotanytarsus sp.	×			+	No effect	33
Rheotanytarsus sp.	×			0	No effect	62
Rheotanytarsus sp.	×			0	**27% mort.**	**56**
Stempellinella sp.	×			0	No effect	62
Strictochironomus akizukii			×	+	**60–100% mort.**	**67**
Tanytarsi sp.	×			0	**Density, −60%**	**30**
Tanytarsi sp.	×			0	No effect	62
Tanytarsi sp.		×		+	**88% mort.**	**9**
Tanytarsi sp.			×	+	**43–100% mort.**	**61**
Tanytarsi sp.			×	+	**Mortality**	**10**
Xenochironomus sp.	×			+	**Density red.**	**68**
Diamesinae	×			0	No effect	58
Orthocladiinae	×			0	No effect	30, 34, 58
Orthocladiinae	×			0	**Drift increase**	**53**
Bryphaenocladius sp.		×		0	No effect	65
Cardiocladius sp.	×			+	**Density red.**	**60**
Cardiocladius sp.	×			0	No effect	60, 62
Chaetocladius sp.		×		0	No effect	65
Corynoneura sp.	×			+	No effect	33
Eukiefferella sp.		×		0	No effect	65
Eukiefferella sp.	×			+	**Density, −26%**	**33**
Orthocladius sp.			×	+	No effect	59
Orthocladius sp.	×			0	No effect	62
Orthocladius sp.		×		0	No effect	65
Pseudorthocladius sp.		×		0	No effect	65
Rheocricotopus sp.	×			+	No effect	33
Smittia			×	+	No effect	59
Tvetenia sp.	×			0	No effect	62
Tanypodinae	×			0	No effect	4, 34, 58
Ablabesmyia sp.	×			0	No effect	62
Coelotanypus sp.		×		+	No effect	9
Conchapelopia sp.	×			0	No effect	60
Larsia sp.	×			+	No effect	33
Nilotanipus sp.	×			0	No effect	62
Procladius sp.		×		+	No effect	9
Procladius sp.			×	+	No effect	59
Procladius sp.	×			0	No effect	62
Tanypus spp.			×	+	No effect	59
Thienemannimya sp.	×			+	No effect	33
Thienemannimya sp.	×			0	No effect	62
Culicidae						
Aedes sp.		×		0	100% mort.	23, 35
		×		0	100% mort.	36, 37, 40
Anopheles sp.		×		0	100% mort.	12, 20, 27
		×		0	100% mort.	38, 43, 50

TABLE 2. *Continued*

TAXA	Lot[1]	Len[1]	Lab[1]	Dose[2]	Effects[3]	References[4,5]
Coquelletidia sp.		×		0	100% mort.	48
Culex sp.		×		0	100% mort.	26, 31, 37, 39
Culiseta sp.		×		0	100% mort.	26
Mansonia sp.		×		0	100% mort.	25
Psorophora sp.		×		0	100% mort.	12, 23
		×		0	100% mort.	35, 36, 40
Dixidae			×	+	No effect	5
Dixidae	×			0	No effect	24
Dixa sp.			×	+	**100% mort.**	**5, 59**
Empididae	×			+	No effect	33
Empididae	×			0	No effect	4
Ephydridae		×		+	No effect	18
Ephydra riparia			×	+	No effect	5
Muscidae						
Musca domestica			×	+	No effect	59
Phlebotomus sp.			×	0	**Mortality**	**11**
Psychoda alternata			×	+	**100% mort.**	**59**
Psychoda sp.		×		0	No effect	65
Rhagionidae	×			0	No effect	34
Scathophagidae		×		0	No effect	65
Sciaridae			×	+	**79% mort.**	**59**
Lycoriella mali			×	0	**Mortality**	**29**
Simuliidae	×			0	100% mort.	56
Austrosimulium sp.	×			0	100% mort.	24
Cnephia sp.	×			0	100% mort.	4, 8
Cnephia sp.			×	0	100% mort.	1
Eusimulium sp.			×	+	100% mort.	3
Odagmia sp.	×			0	100% mort.	41
Odagmia sp.			×	+	100% mort.	3
Prosimulium sp.	×			0	100% mort.	4, 8
Prosimulium sp.			×	0	100% mort.	1
Simulium sp.	×			0	100% mort.	4, 8, 16
Simulium sp.	×			+	100% mort.	21
Simulium sp.			×	0	100% mort.	1
Stegoptora sp.	×			0	100% mort.	4, 8
Stegoptora sp.	×			+	100% mort.	33, 60
Stegoptora sp.			×	0	100% mort.	1
Stratiomyidae		×		0	No effect	9
Odontomyia sp.		×		+	No effect	18
Syrphidae						
Helophylus pendulus			×	+	No effect	59
Tabanidae		×		0	No effect	65
Tephritidae						
Anastrepha ludens (*adults*)			×	+	**65–80% mort.**	**71**
Tipulidae (craneflies)	×			+	No effect	33
Tipulidae (craneflies)	×			0	No effect	4, 24, 56
Molophilus sp.		×		0	No effect	65
Tipula abdominalis	×			+	**36–100% mort.**	**63**
Tipula sp.			×	+	**50% mort.**	**59**
Ephemeroptera (mayflies)	×			+	No effect	21, 33, 47
Ephemeroptera (mayflies)	×			0	No effect	2, 16, 58
Ephemeroptera (mayflies) (nymphs)		×		0	No effect	28
Caenoidea						
Caenidae	×			0	No effect	34
Afrocaenis sp.	×			0	No effect	60
Austrocaenis sp.	×			0	No effect	30
Caenis amica	×			+	No effect	63
Caenis lactea			×	+	No effect	3
Caenis sp.	×			0	No effect	62

TABLE 2. *Continued*

TAXA	Lot[1]	Len[1]	Lab[1]	Dose[2]	Effects[3]	References[4,5]
Ephemerellioidea						
Ephemerellidae	×			0	No effect	46, 56
Ephemerella sp.	×			0	No effect	4
Ephemerella subvaria	×			+	No effect	63
Serratella sp.	×			0	No effect	62
Ephemeroidea						
Ephemeridae	×			0	No effect	46
Ephemera danica			×	+	No effect	3
Hexagenia sp.	×			0	No effect	62
Trycorythidae	×			0	No effect	56
Trycorythus discolor	×			+	**Density red.**	**60**
Trycorythus discolor	×			0	No effect	60
Trycorythus sp.	×			0	No effect	30, 62
Heptagenioidea						
Baetidae	×			0	No effect	30, 34, 56, 60
Baetidae	×			+	No effect	60
Baetidae		×		+	No effect	18
Baetidae	×			0	**Density red.**	**4?**
Afroptilum sp.	×			+	No effect	60
Baetis brunneicolor	×			0	**Drift, + 29x**	**32**?
Baetis flavistriga	×			+	No effect	63
Baetis glaucus	×			+	No effect	60
Baetis glaucus	×			0	No effect	30, 60
Baetis latus	×			0	No effect	60
Baetis sp.	×			0	No effect	60, 62
Baetis sp.		×		+	No effect	9
Callibaetis pacificus		×		+	No effect	22
Callibaetis pacificus		×		0	No effect	39
Callibaetis sp.			×	+	No effect	5, 13
Callibaetis sp.		×		0	No effect	6
Centroptilum excisum	×			0	No effect	60
Centroptilum excisum	×			+	No effect	60
Centroptilum medium	×			0	No effect	30
Centroptilum sp.	×			0	No effect	30, 62
Coleon dipterum			×	+	No effect	59
Heterocoleon sp.	×			0	No effect	62
Pseudocoleon maculosum	×			0	No effect	30
Pseudocoleon sp.	×			0	No effect	62
Heptageniidae	×			0	No effect	46, 56
Afronurus peringueyi	×			0	No effect	60
Afronurus peringueyi	×			+	No effect	60
Afronurus sp.	×			0	No effect	30
Arthroplea bipunctata	×			+	**24% mort.**	**63**
Epeorus fragilis	×			0	**Drift, + 14x**	**32?**
Epeorus sp.	×			0	No effect	4
Heptagenia sp	×			0	No effect	62
Leucrocuta sp.	×			0	No effect	62
Rhithrogena sp.	×			0	No effect	62
Stenacron sp.	×			0	No effect	62
Stenonema sp.	×			0	No effect	4, 56, 62
Oligoneuriidae						
Isonychia sp.	×			0	No effect	62
Siphlonuridae	×			0	No effect	46, 56
Siphlonorus rapidus	×			+	No effect	63

TABLE 2. *Continued*

TAXA	Lot[1]	Len[1]	Lab[1]	Dose[2]	Effects[3]	References[4,5]
Leptophlebioidea						
Leptophlebiidae	×			0	No effect	24, 56
Choroterpes elegans	×			0	No effect	60
Choroterpes elegans	×			+	No effect	60
Choroterpes sp.	×			0	No effect	62
Habrophlebia vibrans	×			0	No effect	4
Leptophlebia sp.			×	+	No effect	3
Paraleptophlebia adoptiva	×			+	No effect	63
Paraleptophlebia sp.	×			0	No effect	4
Potamanthidae						
Anthopotamus sp.	×			0	No effect	62
Hemiptera (water bugs)						
Belostomatidae						
Belostoma lutarium		×		+	No effect	18
Belostoma sp.		×		+	No effect	18
Belostoma testaceum		×		+	No effect	18
Corixidae		×		0	No effect	28
Corixidae	×			+	No effect	60
Corixidae	×			0	No effect	4, 62
Corisella sp.		×		0	No effect	6
Hesperocorixa laevigata			×	+	No effect	5
Micronecta meridionalis			×	+	No effect	59
Sigara lateralis			×	+	No effect	59
Sigara striata			×	+	No effect	59
Trichocorixa verticalis		×		+	No effect	18
T. reticulata (*nymphs* + *adults*)			×	+	No effect	5
Gelastocoridae						
Gelastocoris oculatus		×		+	No effect	18
Gerridae						
Limnogonus hesione		×		+	No effect	18
Hebridae						
Hebrus buenoi		×		+	No effect	18
Hebrus concinnus		×		+	No effect	18
Merragata brevis		×		+	No effect	18
Mesoveliidae						
Mesovelia amoena		×		+	No effect	18
Mesovelia mulsanti		×		+	No effect	18
Mesovelia sp.		×		+	No effect	18
Naucoridae						
Ilyocoris cimicoides			×	+	No effect	59
Notonectidae						
Anisops varia			×	+	No effect	59
Buenoa elegans		×		+	No effect	18
Buenoa scimitra (*nymphs* + *adults*)			×	+	No effect	5
Buenoa scimitra			×	+	No effect	13
Buenoa scimitra		×		0	No effect	6
Notonecta indica		×		+	**Density red.**	**18?**
Notonecta glauca			×	+	No effect	59
Notonecta kirbyi (*nymphs* + *adults*)			×	+	No effect	5
Notonecta kirbyi			×	+	No effect	13
Notonecta sp.			×	+	No effect	5
Notonecta sp.			×	+	**20% mort.**	**19?**
Notonecta undulata			×	+	No effect	49

TABLE 2. *Continued*

TAXA	Lot[1]	Len[1]	Lab[1]	Dose[2]	Effects[3]	References[4,5]
Notonecta unifasciata		×		0	No effect	6
Pleidae	×			0	No effect	56
Pleidae			×	+	No effect	5
Plea leachi			×	+	No effect	59
Reduviidae		×		+	No effect	18
Saldidae	×			0	No effect	56
Veliidae						
Microvelia hinei		×		+	No effect	18
Microvelia sp.			×	+	No effect	13
Lepidoptera	×			0	No effect	2
Lepidoptera		×		+	No effect	18
Noctuiidae						
Heliothinae						
Heliothis viriscens			×	+	**Mortality**	**14**
Heliothis zea			×	+	**Mortality**	**14**
Plusiinae						
Trichoplusia ni			×	+	**Mortality**	**14**
Pyralidae	×			0	No effect	34, 56
Petrophila sp.	×			0	**Drift increase**	**62**
Megaloptera	×			+	No effect	33
Corydalidae						
Nigronia sp.	×			0	No effect	56
Sialidae	×			0	No effect	62
Odonata	×			0	No effect	2
Odonata	×			+	No effect	33
Anisoptera (dragonflies)		×		0	No effect	9, 28
Anisoptera (dragonflies)	×			0	No effect	56
Aeshnidae	×			0	No effect	56
Aeschna sp.	×			0	No effect	4
Anax sp.			×	+	No effect	5, 19
Anax sp.		×		+	No effect	18
Corduliidae						
Cordulia sp.			×	+	No effect	7
Gomphidae	×			0	No effect	56
Gomphus sp.			×	+	No effect	19
Libellulidae	×			0	No effect	34, 56
Libellulidae		×		+	No effect	18
Erythemis simplicicollis		×		+	No effect	22
Erythemis simplicicollis			×	+	No effect	70
Erythrodiplax sp.		×		+	No effect	18
Libellula sp.			×	+	No effect	13, 19
Orthetrum brunneum			×	+	No effect	59
Pantala sp.		×		0	No effect	6
Sympetrum striolatum			×	+	No effect	59
Tarnetrum corruptum			×	+	No effect	49
Tramea sp.		×		+	No effect	18
Zygoptera (damselflies)		×		0	No effect	9, 28
Calopterygidae	×			0	No effect	34, 56
Calopterygidae		×		+	No effect	18
Coenagrioniadae	×			0	No effect	56
Coenagrioniadae		×		+	No effect	18
Argia sp.			×	+	No effect	13
Enallagma civile			×	+	No effect	49
Enallagma sp.		×		0	No effect	6
Enallagma sp.		×		+	No effect	18

TABLE 2. *Continued*

TAXA	Lot[1]	Len[1]	Lab[1]	Dose[2]	Effects[3]	References[4,5]
Ischnura elegans			×	+	No effect	59
Ischnura sp.			×	+	No effect	5
Ischnura sp.		×		+	No effect	18
Lestidae						
Lestes stultus			×	+	No effect	13
Plecoptera (stoneflies)	×			+	No effect	33
Plecoptera (stoneflies)	×			0	No effect	16, 30
Gripopterygidae	×			0	No effect	24
Leuctridae						
Leuctra sp.	×			0	**Drift, +4x**	**32?**
Leuctra sp.	×			0	No effect	4
Nemouridae						
Amphinemura wui	×			0	**Drift, +2.4x**	**32?**
Malenka sp.			×	+	No effect	13
Nemoura cineraea			×	+	**40% mort.**	**3?**
Nemoura sp.	×			0	No effect	4
Prostoia completa	×			+	No effect	63
Perlidae	×			0	No effect	56, 62
Acroneuria lycorias	×			+	**Drift increase**	**63**
Neoperla sp.	×			0	No effect	34
Neoperla spio	×			0	No effect	60
Paragnetina media	×			+	No effect	63
Perlodidae	×			0	No effect	46
Isoperla dicala	×			+	No effect	63
Isoperia holochlora	×			0	**Drift, +3x**	**32?**
Isoperla signata	×			+	No effect	63
Isoperla sp.	×			0	No effect	4
Trichoptera (caddisflies)	×			0	No effect	2, 30
Trichoptera (caddisflies)	×			+	No effect	21, 47
Brachycentridae	×			0	No effect	56
Conoesucidae	×			0	No effect	24
Ecnomiidae						
Ecnomus sp.	×			0	No effect	60
Ecnomus sp.	×			+	No effect	60
Glossosomatidae						
Catoxyethira sp.	×			0	No effect	30
Protoptila sp.	×			0	No effect	62
Helicopsychidae	×			0	No effect	56
Hydropsychidae	×			0	No effect	30, 56
Hydropsychidae	×			0	**Drift, +62%**	**34?**
Aethaloptera maxima	×			0	No effect	60
Aethaloptera maxima	×			+	No effect	60
Amphipsyche scottae	×			0	No effect	30
Amphipsyche scottae	×			0	**Density red.**	**60?**
Amphipsyche scottae	×			+	No effect	60
Ceratopsyche sparna	×			+	No effect	63
Cheumatopscyche pettiti	×			0	No effect	4
Cheumatopscyche sp.	×			0	No effect	62
Cheumatopscyche thomasseti	×			0	No effect	30, 60
Cheumatopscyche thomasseti	×			+	No effect	60
Diplectrona modesta	×			0	No effect	4
Hydropsyche bretteni	×			0	No effect	4
Hydropsyche pellucidula			×	+	**100% mort.**	**3?**
Hydropsyche sp.	×			0	No effect	62
Hydropsyche sparna	×			0	**Density red.**	**4?**
Macrostemum sp.	×			0	No effect	62
Parapsyche apicalis	×			0	**Drift, +7.6x**	**32?**

TABLE 2. *Continued*

TAXA	Lot[1]	Len[1]	Lab[1]	Dose[2]	Effects[3]	References[4,5]
Hydroptilidae	×			0	No effect	56, 60
Catoxyethira sp.	×			0	No effect	34
Hydroptila sp.	×			0	No effect	60, 62
Hydroptila sp.	×			+	No effect	60
Orthotricia sp.	×			0	No effect	30, 60
Orthotricia sp.	×			+	No effect	60
Orthotricia sp.	×			0	**Drift increase**	**53**
Orthotricia sp.	×			0	**Drift, +82%**	**34?**
Lepidostomatidae	×			0	No effect	46
Leptoceridae	×			0	No effect	4, 56
Ceraclea sp.	×			0	No effect	34, 62
Mystacides alafimbriata			×	+	No effect	5
Oecetis sp.	×			0	**Drift, +71%**	**34?**
Limnephilidae	×			0	No effect	4, 56
Chaetopteryx sp.			×	+	No effect	3
Limnephilus flavicornis			×	+	No effect	15
Limnephilus sp.			×	+	No effect	59
Potamophylax rotundipennis			×	+	**80% mort.**	**3?**
Pycnopsyche divergens	×			0	**Drift, + 21x**	**32?**
Philipotamidae	×			0	No effect	56
Chimarra aterrima	×			+	No effect	63
Chimarra sp.	×			0	No effect	4, 34, 62
Dolophilodes sp.	×			0	No effect	4
Polycentropodidae	×			0	No effect	34, 56
Polycentropus sp.	×			0	No effect	4
Phryganeidae						
Phryganea sp.			×	+	No effect	59
Psychomyiidae	×			0	No effect	56
Rhyacophilidae	×			0	No effect	24
Rhyacophila sp.	×			0	No effect	4
CHORDATA						
VERTEBRATA						
Pisces	×			0	No effect	53
Catostomidae						
Catostomus commersoni	×			0	No effect	56
Centrarchidae						
Ambloplites rupestris	×			0	No effect	56
Lepomis gibbosus	×			0	No effect	56
Micropterus salmoides	×			0	No effect	56
Cichlidae						
Tilapia nilotica			×	0	No effect	15
Tilapia nilotica			×	+	**50–90% mort.**	**15?**
Cottidae						
Cottus cognatus	×			0	No effect	46, 56
Cyprinidae						
Cyprinus carpio			×	+	No effect	59
Clinostomus elongatus	×			0	No effect	56
Notropis cornutus	×			0	No effect	56
Notropis atherinoides	×			0	No effect	56
Pimephales promelas			×	0	No effect	57
Pimephales promelas			×	+	**100% mort.**	**57?**
Rhinichthys atratulus	×			0	No effect	56
Rhinichthys cataractae	×			0	No effect	56
Semotilus atromaculatus	×			0	No effect	56
Cyprinodontidae						
Fundulus heteroclitus			×	+	No effect	55

TABLE 2. *Continued*

TAXA	Lot[1]	Len[1]	Lab[1]	Dose[2]	Effects[3]	References[4,5]
Lucania parva			×	+	No effect	5
Esocidae						
Esox lucius			×	+	No effect	59
Gasterosteridae						
Gasterosteus wheatlandi			×	+	No effect	5
Ictaluridae						
Ictalurus natalis	×			0	No effect	56
Ictalurus nebulosus	×			0	No effect	56
Nocturus sp.	×			0	No effect	56
Percidae						
Perca flavescens	×			0	No effect	56
Perca fluviatilis			×	+	No effect	59
Ethostoma caeruleum	×			0	No effect	56
Ethostoma nigrum	×			0	No effect	56
Petromyzonidae						
Lampetra lamottei	×			0	No effect	56
Poeciliidae						
Gambusia affinis			×	+	No effect	5, 7
Gambusia affinis		×		0	No effect	28
Pseudomugilidae						
Pseudomugil signifer			×	+	**50% mort.**	**73**
Salmonidae						
Onchorynchus mykiss			×	0	No effect	64
Onchorynchus mykiss			×	+	**10–100% mort.**	**64?**
Salmo trutta	×			0	No effect	56
Salmo trutta			×	0	No effect	64
Salmo trutta			×	+	**15–100% mort.**	**64?**
Salvelinus fontinalis	×			0	No effect	46, 56
Salvelinus fontinalis			×	0	No effect	45
Salvelinus fontinalis			×	+	**20–86% mort.**	**45?**
Salvelinus fontinalis			×	0	No effect	64
Salvelinus fontinalis			×	+	**5–80% mort.**	**64?**
Umbridae						
Umbra limi	×			0	No effect	56
Amphibia						
Bombinidae						
Bombina variegata			×	+	No effect	59
Bufonidae						
Bufo americanus			×	0	No effect	52
Bufo bufo			×	+	No effect	59
Bufo calamita			×	+	No effect	59
Bufo sp.			×	+	No effect	5
Bufo viridis			×	+	No effect	59
Hylidae						
Hyla crucifer			×	0	No effect	52
Hyla regilla			×	+	No effect	5, 13
Ranidae						
Rana esculenta			×	+	No effect	59
Rana pipiens			×	0	No effect	52
Rana temporaria			×	+	No effect	59
Salamandridae						
Tarica torosa			×	+	No effect	5
Tarica torosa (*eggs* + *nymphs*)			×	+	No effect	13
Triturus alpestris			×	+	No effect	59
Triturus cristatus			×	+	No effect	59
Triturus vulgaris			×	+	No effect	59

TABLE 2. *Continued*

TAXA	Lot[1]	Len[1]	Lab[1]	Dose[2]	Effects[3]	References[4,5]
KINGDOM PROTISTA						
CHLOROPHYTA						
Chlorophyceae						
Chlorococcales						
Chlorococcaceae						
***Chlorella* sp.**			×	0	**90–99% mort.**	**75**
Desmidiales						
Desmidiaceae						
***Closterium* sp.**			×	0	**90–99% mort.**	**75**

1. Experimentation performed in: Lot: Lotic environment; Len: Lentic environment; Lab: Laboratory or artificial environment.

2. 0: Operational treatment (recommended dosages according to the labels or producers). +: Overdosing treatment according to the authors (5 to 1000 times the recommended dosage)

3. red. = reduction; mort. = mortality; when effects are mentioned without any notification, it means that the authors reported the observed effect without any specific percentages (e.g. mortality, density reduction).

4. **References in bold**: Studies where nontarget organisms are directly or indirectly affected by *Bti* formulations.

5. ?: References where the different authors suggest that the observed effects on the organisms are not necessarily caused by the *Bti* toxins but by other elements (formulation additives, methodology errors, sampling errors, high turbidity etc.).

(1) Undeen & Nagel, 1978; (2) Dejoux, 1979; (3) Weiser & Vankova, 1979; (4) Colbo & Undeen, 1980; (5) Garcia *et al*., 1980; (6) Miura *et al*., 1980; (7) Sinègre *et al*., 1980a; (8) Undeen & Colbo, 1980; (9) Ali, 1981; (10) Ali *et al*., 1981; (11) de Barjac *et al*., 1981; (12) Dame *et al*., 1981; (13) Garcia *et al*., 1981; (14) Ignoffo *et al*., 1981; (15) Lebrun & Vlayen, 1981; (16) Molloy & Jamnback, 1981; (17) Moulinier *et al*., 1981; (18) Purcell, 1981; (19) Sébastien & Brust, 1981; (20) Standaert, 1981; (21) Lacey *et al*., 1982; (22) Mulla *et al*., 1982a; (23) Mulla *et al*., 1982b; (24) Chilcott *et al*., 1983b; (25) Foo & Yap, 1983; (26) Garcia *et al*., 1983; (27) Hougard *et al*., 1983; (28) Stewart *et al*., 1983; (29) Cantwell & Cantelo, 1984; (30) Car & de Moor, 1984; (31) Majori & Ali, 1984; (32) Pistrang & Burger, 1984; (33) Back *et al*., 1985; (34) Dejoux *et al*., 1985; (35) Eldridge *et al*., 1985; (36) Hougard *et al*., 1985; (37) Lacey, 1985; (38) Lacey & Inman, 1985; (39) Mulla, 1985; (40) Mulla *et al*., 1985; (41) Olejnicek et al., 1985; (42) Reish *et al*., 1985; (43) Sandoski *et al*., 1985; (44) Brazner & Anderson, 1986; (45) Fortin *et al*., 1986; (46) Gibbs *et al*., 1986; (47) de Moor & Car, 1986; (48) Sjogren *et al*., 1986; (49) Aly & Mulla, 1987; (50) Majori *et al*., 1987; (51) Gharib & Hilsenhoff, 1988; (52) Leclair *et al*., 1988; (53) Yaméogo *et al*., 1988; (54) Knepper & Walker, 1989; (55) Lee & Scott, 1989; (56) Merritt *et al*., 1989; (57) Snarski, 1990; (58) Molloy, 1992; (59) Becker & Margalit, 1993; (60) Palmer, 1993; (61) Charbonneau *et al*., 1994; (62) Jackson *et al*., 1994; (63) Wipfli & Merritt, 1994a; (64) Wipfli *et al*., 1994; (65) Hershey *et al*., (1995); (66) Kondo *et al*., 1995a; (67) Kondo *et al*., 1995b; (68) Palmer & Palmer, 1995; (69) Roberts, 1995; (70) Painter *et al*., 1996; (71) Robacker *et al*., 1996; (72) McCracken & Matthews, 1997; (73) Brown *et al*., 1998; (74) Brown *et al*., 1999; (75) Su & Mulla, 1999.

fly larvae, some NTO can be affected to a certain degree (varying from increased drift to death) after the application of *Bti* formulations in the environments described. We must emphasize that for the purpose of this analysis, only mosquito and black fly larvae were considered as target species.

From the information presented in Table 2, at least 37 studies (in bold) showed that some nontarget organisms can be affected to a certain extent after a *Bti* treatment. Some species closely related to mosquitoes and black flies may have the alkaline conditions (midgut) required for the dissolution of the parasporal crystals and show high sensitivity if their midgut epithelium possesses the specific receptors on which the toxins produce their effects. Moreover, the 'formulation additives' or 'inert ingredients' (emulsifiers, dispersants, antimicrobial agents etc.) added to crystals, spores and vegetative cells to make a formulation could have a direct effect on nontarget organisms. As mentioned before, some authors facing unusual responses of NTO have suggested that 'inert ingredients' could have played a role in their observations (Pistrang & Burger, 1984; Fortin *et al*., 1986; Holck & Meek, 1987; Snarski, 1990; Wipfli *et al*., 1994) although, of course, the additives permitted in registered

products have been tested on standard experimental animals, especially those also used in the food industry.

In the course of extensive testing of aquatic nontarget species, members of the Diptera suborder Nematocera have been shown to be the most common species susceptible to *Bti* (more than 50% of the species identified in bold in Table 2). Ceratopogonids (biting midges or biting gnats) which are adversely affected at high dosage treatments (Table 2, Garcia *et al*., 1980) are blood-sucking biters of man and animal; and can vector microfilariae, protozoans and arboviruses that cause severe diseases (Rodhain & Perez, 1985). Chironomid midges, the nontarget group most frequently reported to be susceptible in mosquito habitats (40% of the species identified in bold in Table 2), are nonbiters but often occur in large swarms, creating nuisance, economic and occasionally medical problems (allergies) for humans residing or working near the midge breeding sources, eventually necessitating control measures (Ali, 1991). Many authors (Table 2) have shown mortality and/or density reduction of chironomids in mosquito habitats treated with *Bti* while in black fly habitats (where filter-feeding species are especially susceptible) the response of chironomids to *Bti* treatment was characterized by a drift increase, a density reduction but most of the time by different rates of mortality depending on the initial dosage of *Bti* (Table 2). According to those authors, who used a variety of different experimental procedures and formulations, the susceptibility of chironomid larvae to *Bti* could be between 15 to 75 times less than mosquito or black fly larvae, but the studies indicated that a high dosage of *Bti* will affect chironomid populations.

However, in Table 2, 11 out of 23 studies identified in bold (eight in lotic environments, one in lentic environments and two in the laboratory) in which chironomids were affected by *Bti* were performed using operational dosage. Nine of the 11 studies were done in field conditions. Overall, nearly 40% of the studies (9 out of 23) reporting an effect on chironomid populations were done using actual operating conditions. It would therefore appear that the feeding behaviour of certain species of chironomids could make them highly susceptible to *Bti* even in conditions where 'operational dosages' would be used for treatment of mosquito or black fly breeding sites. Recently, some liquid *Bti* formulations used for mosquitoes and black flies control have been registered for the treatment of nuisance flies (*Psychoda* sp. and *Chironomus* sp.) in sewage treatment plants using trickling filters, and for fungus gnats (*Sciara* sp.) on greenhouse ornamentals. In the UK only, over 1000 tons of *Bti* formulations are used each year for the control of chironomids in sewage treatment plant (P. van Poppelen, pers. comm.).

Apart from chironomids against which real effects have been demonstrated, many other organisms (some species in seven other Nematoceran families (Diptera), nine other invertebrate orders, four families of fish and two algal species) have either been shown to be directly or indirectly affected or have been suspected of being affected by *Bti* products (formulations) (identified in bold in Table 2). As we will see in the next paragraphs, the observed effects on certain organisms could be questionable and need to be interpreted with caution as sometimes pointed out by the authors themselves. Many factors could have an influence on the reported effects of *Bti* on the listed organisms (Table 2). Among these factors, we can mention errors in experimental procedures, methodologies not well adapted to the purpose, extensive 'overdosage' making the role of formulation additives conspicuous, lack of controls, small number of specimens collected, etc. Some results will be described but the final analysis must be left to the readers who should consult the original papers to get a better overview of the experiment, the results and the interpretation of the data.

The first exposure tests against stream nontarget organisms were conducted in small dishes at 4 and 20ºC by Weiser and Vankova (1979). Mortality was noted among two species of copepods, one species of Diptera, two species of Trichoptera and one species of Plecoptera (Table 2). However, few conclusions can be made regarding the susceptibility of these organisms to *Bti* because the tests were conducted in lentic conditions ('stagnant' water). According to these authors, the mortality in controls for Trichoptera and Plecoptera

appeared to be due to lack of oxygen while mortality in copepods resulted from the stress of the transfer during the experiment. Although the dosage and time of exposure of the test animals represented conditions which do not occur in nature during operational treatment of black fly larvae, it was nevertheless a possible early warning of the potential hazard of *Bti* for nontarget insects.

Colbo and Undeen (1980) tested the selectivity of a non-formulated aqueous suspension of *Bti* in a Newfoundland stream. Reductions were observed in populations of hydropsychid species and the mayfly family Baetidae (Table 2). For the hydropsychids, the density was reduced to a level that was statistically significant which the authors believed may have been due to pupation occurring between samples. Baetidae density showed a drop between two sampling periods that could have been due to real effects or error in the treatment of the samples. According to the authors, Baetidae were primarily in the 1 mm size range and easily overlooked in the debris making the results suspect?

In a laboratory study conducted by Lebrun and Vlayen (1981), to test the possible adverse impact of *Bti* on some nontarget organisms, the authors found different effects according to the dosages used in their experiments. On neonate larvae (0–24 h old) of *Daphnia magna* (Crustacea), no mortality was recorded up to a concentration of 1000 ppm but 54–80% mortality was recorded at concentrations between 4000 (0.4%) and 5000 ppm (0.5%) (Table 2). Similar results were obtained with 24 h old alevins of *Tilapia nilotica* (Pisces: Cichlidae). No mortality was recorded under a concentration of 1000 ppm but mortalities were observed (50–90%) at concentrations between 4000 and 5000 ppm (Table 2). Experiments were done using a primary powder and according to the authors the water was highly saturated by the powder at a concentration of 1000 ppm (0.1%). The mortality appearing on these two species was likely to be due to the plugging of the gills rather than toxic effects caused by *Bti*. Fortin *et al*. (1986) also recorded mortalities at high product concentrations when they tested a water-based formulation of Teknar® against brook trout fry (*Salvelinus fontinalis*) in the laboratory. Their results indicated 20–80% mortality after a 45 min exposure at respective concentrations of 4500 (0.45%) and 6000 (0.6%) ppm (Table 2). It appeared that the mortality was not caused by the *Bti* crystals, but by the presence of xylene (2%) in the formulation tested.

Another study was conducted in the laboratory by Snarski (1990) on 3-week-old fathead minnows (*Pimapheles promelas*) to monitor their sensitivity to different *Bti* formulations. Her results showed no mortality at low dosage, but in 'overdosing' conditions, complete mortality was observed within 24 h. Mortality was attributed to severe dissolved oxygen depletion due to formulation ingredients rather than direct toxicity from *Bti* crystals. Wipfli *et al*. (1994) also found mortalities on different species of trout, but only at very high dosages. Since generally, there was no difference in the observed mortalities between denatured (autoclaved) and non-denatured formulations, they concluded that formulation components (inert ingredients) rather than *Bti* toxin were the cause of the observed mortalities.

In a study conducted in a salt marsh, Purcell (1981) found that, after a treatment with *Bti*, the population of backswimmers, *Notonecta indica* (Hemiptera: Notonectidae), declined significantly (Table 2). Since backswimmers are well documented as efficient mosquito larvae predators (Lee, 1967; Toth & Chew, 1972), they may have died from eating larvae that had ingested *Bti*. However, other studies (Garcia *et al*., 1980; Miura *et al*., 1980; Garcia *et al*., 1981) have shown that similar exposure did not affect them (Table 2). After the death of mosquito larvae, the backswimmers more likely moved in search of a new habitat with a better food supply. Toth and Chew (1972) have reported such migrations, which could explain the observed declining populations seen in some studies. Sebastien and Brust (1981) also recorded 20% of mortality in *Notonecta* sp. in the laboratory, but since there was 10% mortality in the control group, they suggested that the mortality may have been due to confinement in the plastic containers, rather than a direct effect of *Bti*.

Pistrang and Burger (1984) treated a small stream in central New Hampshire to determine

the effects of *Bti* on an outlet-breeding black fly population and associated nontarget aquatic insects. The black fly larval population was eliminated and some chironomids were killed by *Bti* but they were not abundant enough to adequately assess impact on their populations. Temporary increases in the drift of two Ephemeroptera and two Trichoptera species were observed following treatment and a slight increase in observed drift of three Plecoptera species was also noticed (Table 2). The dominant nontarget taxa (Ephemeroptera) exhibiting elevated drift following the *Bti* treatment were not common in natural drift samples taken before treatment. However, according to the authors, in no instance could the observed elevation of drift be termed 'catastrophic' and the increase in drift may have been caused by a constituent of the formulation used, rather than the active ingredient (*Bti*).

In a West African field trial, Dejoux *et al*. (1985) concluded that a stream treatment at the recommended dosage did not adversely affect nontarget populations of invertebrates. However, some nontarget groups like Gastropoda (ancylids) and Trichoptera (hydropsychids, hydroptylids and leptocerids) may have been slightly affected by the treatment as seen by a drift increase in their populations near the application site. According to the authors, a sudden surge of turbidity caused by the application of the *Bti* formulation could have produced that drift (Table 2).

In Quebec, a high-dosage trial by Back *et al*. (1985) in a small stream demonstrated that *Bti* could kill some blephariceríd larvae (visual estimate of 30% mortality) (Table 2). They judged that the main nontarget impact of their treatment was against the chironomids (collectors-gatherers to engulfers) present on the bottom of the stream and to blephariceríds (grazers) attached to periphyton growing on rocks.

In other studies, Gastropoda (*Burnupia* sp.) were shown to be susceptible to *Bti* (Car & de Moor, 1984; Palmer & Palmer, 1995) (Table 2). *Burnupia* sp. are known to be algal browsers, not filter-feeders; but as Back *et al*. (1985) reported, blephariceríds (Diptera) that are also browsers showed decreased in density (large drift) as well as *Burnupia* sp. According to these authors, the *Bti* crystals adsorbed onto periphyton covering rocks could explain the population decrease and their susceptibility. Later, Tousignant *et al*. (1993) demonstrated that high percentages of mortality were obtained when periphyton samples collected in a stream after a high dosage treatment with a *Bti* formulation they tested against mosquito larvae, indicating that the *Bti* toxic particles had become associated with that substrate.

Merritt *et al*. (1989) examined the effect of *Bti* (using an operational dosage) on NTO but more specifically on *Rheotanytarsus* sp. (filter-feeders) because of their expected susceptibility as chironomids and the fact that their microhabitat, food and mechanism of food acquisition were similar to that of black flies (Wallace & Merritt, 1980; Coffman & Ferrington, 1984). They observed no significant effects on NTO (Table 2) although 27% of *Rheotanytarsus* sp. population was affected by *Bti*; but that level of mortality was restricted to the first sampling station (100 m) below the treatment site. According to Merritt *et al*. (1989), sampling variability, rather than *Bti*, may have been responsible for some of the earlier reported population changes observed by other authors in nontarget organisms after *Bti* treatments. Small sample sizes, choice of sampling site, emergence and pupation could have explained the observed decrease in benthic density. Later, Molloy (1992) also found 23% of mortality on *Rheotanytarsus* sp. (filter-feeders) (Table 2) after a *Bti* treatment but no effect on other chironomid species (tube-dwelling and surface-dwelling) which have another feeding behaviour. Molloy (1992) mentioned that the potential for adverse impact on filter-feeding chironomids in operational black fly programs has been clearly demonstrated and that the study also confirmed the narrow impact of *Bti* on the overall stream insect community.

Wipfli and Merritt (1994a) found some effects of *Bti* on Ephemeroptera and Plecoptera. They observed that *Arthroplea bipunctata* (Ephemeroptera: Heptageniidae) showed 24% mortality after a high dosage treatment with *Bti* (500 times the recommended dose). The filter-feeding behaviour of that species allows *Bti* direct access to the digestive tract and the high-dosage treatment may explain this rather high percentage of mortality. The high-dosage seems to be also the explanation for the drift of *Acroneuria lycorias* (Plecoptera: Perlidae) (Table 2).

Finally, in a two-year study (one application per year) on the Susquehanna River (Pennsylvania, USA), Jackson *et al.* (1994) concluded that no changes in benthic density and drift during 28 days following a *Bti* application could be interpreted as negative responses to *Bti* for nonsimuliid macroinvertebrates in the riffle (shallow areas of high water velocity and mixed gravel-cobble substrate) and depositional zone of the experiment, except for *Petrophila* (Lepidoptera: Pyralidae) and *Polypedilum* (Diptera: Chironomidae) with both of which a significant drift was observed. Several taxa exhibited significant changes (not shown in Table 2) in their field studies but according to the authors they appeared related to random error or natural processes such as variation in diurnal drift patterns or recruitment, but not to *Bti* application. In their study, Back *et al.* (1985) also observed an impact on *Polypedilum* (density reduction of 39%), but no other studies found an impact on *Petrophila*.

One of the most interesting studies on NTO was recently published by Su and Mulla (1999). These authors treated artificial ponds (basins) with two *Bti*-based granular formulations. One, a registered formulation, was used at twice the recommended dosage in polluted water while the other formulation (experimental) was used at the lowest effective dosage. With both formulations, they obtained significant control of a *Culex* species for nearly three weeks. But they also observed that the growth of two species of green algae (*Closterium* sp. and *Chlorella* sp.) was greatly reduced (90–99% reduction) during that period when compared to control basins.

Apart from Chironomidae, seven other dipteran families with different feeding behaviour were affected by *Bti*, i.e. Blephariceridae, Ceratopogonidae, Dixidae, Psychodidae, Tipulidae, Sciaridae and Tephritidae, the latter two being non-aquatic insects. During many experiments/trials using 'overdosages', some of these families showed significant mortalities (100%) (Table 2). All these families are dipteran and may possess (like the target species) in 'overdosage' conditions the capacity to capture, ingest and digest toxic crystals. In sufficient quantity, this can produce enough toxic proteins to induce cellular damage that could lead to death. It should be remembered that some terrestrial Lepidopteran species can be affected by high dosages of *Bti* (Ignoffo *et al.*, 1981).

A high-dosage treatment is often not necessary to obtain a complete control of mosquitoes and black flies during a field application, but in 'overdosage' conditions, secondary effects can be expected on nontarget organisms. Further studies on the effective dosage to apply during a field treatment (mosquitoes and black flies) can eventually avoid these problems generated by high-dosages. Since *Bti* toxic crystals have been shown to be environmentally persistent (Dupont & Boisvert, 1986; Boisvert & Boisvert, 1999), there are some concerns that intensive *Bti* applications could create a situation where accumulation of toxicity could cause long-term effects on nontarget organisms.

For many species, drift is induced after an application of *Bti* in lotic environments but no mortality is observed. Even if there is no mortality, this drift can have an impact on the food web because some predators that feed on these drifting insects could be affected. The removal of mosquito or black fly larvae by *Bti* could also disturb the food web, depending on the type of predatory behaviour (specialist or generalist) or whether the predators feed on live or dead prey.

FOOD WEB

Compared to black flies, very few extensive studies have been made on the effect of *Bti* on the food web in mosquito habitats. Recently, a long-term study on the effects of repeated *Bti* treatments on NTO in wetlands has been performed by Hershey *et al.* (1998) over five years in Minnesota (USA). After two years of intensive untreated control sampling, *Bti* treatments were applied during three consecutive years. Six applications were made each year between mid-April and mid-July, at rates recommended on the formulation label (considering the frequency of applications, we characterized the treatments as intensive). In general, the treat-

ments had minimal effects on NTO during the first year. However, highly significant reductions were observed in several insect groups in the second year and eventually the intensive *Bti* treatments resulted in wetland communities that were depleted of most insects during the third year. Since *Bti* was likely to be directly toxic to only nematoceran Diptera, the effects of *Bti* on other insect groups may have resulted from disruption of the invertebrate food web (Hershey *et al*., 1998). According to the authors, dramatic changes were measured in two diversity indices: richness was drastically reduced while dominance increased. In streams, *Bti* is carried by the current, and thus may not persist at a site. Long-term exposure does not occur; thus insects not intoxicated with the initial pulse will escape lethal exposure. But the Hershey *et al*. (1998) study occurred in wetlands, where *Bti* persisted in breeding sites until it degraded or became non-available. Because the application was repeated six times per season at 3-week or shorter intervals, nontarget insects were much more likely to have been exposed to the direct or indirect effects of the *Bti* products. According to these authors, the repeated treatments had a long-term adverse impact on the NTO community of the treated wetlands. Both indirect effects and direct toxicity probably contributed to the observed differences. *Bti* is likely to be directly toxic only to nematoceran Diptera; thus effects of *Bti* on the other insect groups may have resulted from disruption of the invertebrate food web (Hershey *et al*., 1998).

The recent study by Su and Mulla (1999) could possibly provide some explanations for the Hershey *et al*. (1998) results. As mentioned earlier, Su and Mulla (1999), using two *Bti* coated granules formulation (Vectobac G and Vectobac WDG) for the control of *Culex* species, found that shortly after a single treatment the growth of two species of green algae was greatly inhibited for nearly three weeks. These authors concluded that concomitant with the control of mosquito larvae, the reduction in primary productivity (algal growth) was considered as a bonus since it helps clear the water in the polluted basin. Considering the type of habitat treated and the frequency of *Bti* applications by Hershey *et al*. (1998), it is likely that primary production of algae was almost totally inhibited for three years resulting in the dramatic changes in diversity indices that they observed. It is interesting to note that the same *Bti* granular formulation (Vectobac G) was used in both studies where impacts on NTO were reported (Hershey *et al*., 1998; Su & Mulla, 1999). This formulation is made of an inert substrate (usually corn grits) coated with *Bti* with the help of a binding agent.

With regard to black flies, medium and long-term studies have been performed mainly in Africa with the WHO/OCP Program to control the onchocerciasis vector, *Simulium damnosum* s.l. After 10 consecutive years of treatments, evidence has shown that there were no long-term deleterious effects of *Bti* on the ecosystems of streams receiving weekly applications and environmental monitoring did not reveal any significant direct or indirect effects of *Bti* treatments on lotic fish populations (Yaméogo *et al*., 1988).

As mentioned earlier, Jackson *et al*. (1994) studied the effects of *Bti* on drift and benthic densities of nontarget macroinvertebrates in the large Susquehanna River (Pennsylvania, USA) during a two-year study (1989 and 1990). They focused their study on a larger lotic ecosystem because according to Hynes (1989), less than 5% of all papers published on ecological topics associated with running water address large rivers. With few exceptions in Africa (Lacey *et al*., 1982; de Moor & Car, 1986; Yaméogo *et al*., 1988), most studies were conducted in streams or small rivers. Hynes (1989) mentioned that particular care must be taken when extrapolating results from streams to large rivers, presumably because of the limited information available on large rivers and the potential population differences between streams and large rivers. According to Jackson *et al*. (1994), their results clearly support the hypothesis that *Bti* can be used to suppress black fly population without negatively affecting most nontarget macroinvertebrates in the large Susquehanna River. However, according to reports from the Academy of Natural Sciences of Philadelphia (ANSP) (1990, 1991), there was limited evidence that suggested the reduction in black fly populations in response to *Bti* treatments, may have cascaded through the food web and negatively influenced the fish assemblages in the treatment zone of the Susquehanna River.

Although many authors have stated that *Bti* appears innocuous to nearly all NTO by direct

challenge, its use to selectively remove a member of the stream community could have indirect ecological consequences in the black fly habitat. That is, the elimination of black flies from a stream removes a segment of the food web, thereby possibly reducing ecosystem diversity, abundance and stability, and potentially altering the overall community structure. Some species might increase in number to occupy the niche previously filled by black flies; species dependent upon black flies as prey could be reduced, other species groups may remain unchanged.

Information is lacking on the functional and ecological effects caused by applying *Bti* and removing an entire black fly population from the stream community food web. In a typical lake outlet system of southern Quebec (Canada), Morin *et al.* (1988a) found that black fly populations can ingest a significant portion of the seston flowing out of the lake during periods of high standing water stocks. Their results suggested that this high rate of ingestion by simuliids may help to explain the downstream decrease in abundance and growth rates of filter-feeders other than black flies in lake outlets. Any intervention that would disrupt aquatic community structure (e.g. removal of black fly populations) could affect other trophic levels. If black flies comprise a large fraction of animal standing stock in a given system, loss of black fly biomass may have negative or positive effects on other populations, particularly on black fly predators and competitors (Merritt *et al.*, 1991). Black flies are important prey for numerous predatory invertebrates and vertebrates (Davies, 1991). However, the impact on predators may be minimal, if the predators are generalists and switch to alternative prey species, assuming sufficient alternative prey are available. Specialist predators would suffer the greatest indirect consequences through a prey resource loss. Black fly competitors, on the other hand, may benefit through additional food or space resources when black flies are removed from the system.

Molloy and Daniels (1988) investigated the impact of biological control of black flies on trout and sculpin populations in a 2-km section of a creek. The population levels of slimy sculpin (*Cottus cognatus*), brook trout (*Salvelinus fontinalis*) and brown trout (*Salvelinus trutta*) were estimated, 6 months prior to the beginning of *Bti* treatments and again after 30 months of black fly removal. The frequent year-round treatments (at weekly to monthly intervals) were designed to place unusually high stress on the fish community and *Bti* was applied at about five times the normal requirement for black fly control. The authors concluded that the use of *Bti* in cold water streams for the control of black flies did not pose a major threat to slimy sculpin, brook trout and brown trout population levels. However, according to the authors, the numbers of trout observed were too low to draw any solid conclusions regarding these two species.

Merritt *et al.* (1991) investigated the changes in feeding habits of selected nontarget aquatic insects in response to live, hot water-killed, and *Bti*-killed blackfly larvae (*Prosimulium fuscum* and *P. mixtum*) in cages submerged in 4°C river water. The feeding behaviours of two blackfly predators, *Nigronia serricornis* (Megaloptera: Corydalidae) and *Acroneuria lycorias* (Plecoptera: Perlidae) and a detritivore *Prostoia completa* (Plecoptera: Nemouridae) were investigated. *Nigronia* larvae showed no significant differences in predation on larvae within the three categories. *Acroneuria* nymphs consumed more live than dead prey. Nymphs of the detritivore, *Prostoia*, showed preference for dead black fly larvae to live ones. Merritt and coworkers speculated on the consequences of the accelerated transformation of live to dead prey biomass following *Bti* application. The predators would lose an abundant food resource and the detritivores would have a new, but short-termed, resource provided. As these insects are generalists, the population effects may be of minor importance. However, the authors indicated that there still may be unfavourable consequences for predators and detritivores when a viable population of larval black flies is transformed into dead organic matter.

Wipfli and Merritt (1994b) conducted a field study to assess feeding habit changes of two predatory stoneflies following larval black fly prey loss from two streams, and to determine the relative importance of black fly larvae as prey for these and other selected predatory benthic macroinvertebrates. *Acroneuria lycorias* and *Paragnetina media* (Plecoptera: Perlidae) diets were monitored in response to local reductions in larval black fly populations caused

after *Bti* treatments in two Michigan streams (USA). Black flies were the major dietary component of both predators collected from the control sections, but the number of black flies ingested was significantly less for predators collected from *Bti*-treated habitats. The total number of prey ingested significantly decreased for *A. lycorias*, but not for *P. media*, and non-black fly prey consumption significantly increased for *P. media*, but not for *A. lycorias*. According to the authors, in blackfly-poor environments (including *Bti*-treated streams), feeding habits of specialist predators were most affected, and generalist predators least affected because the latter consumed alternative prey.

Indirect effects may be felt by other taxa at the same trophic level as black flies, through shared predators. Both positive and negative indirect interactions among non-competing prey are theoretically possible (Abrams, 1987; Holt & Kotler, 1987). Predators switching to alternative prey (following black fly biomass loss due to *Bti*) may have significant impacts on alternative prey populations, leading to a restructuring of the prey community, and potentially resulting in successional repercussions through the food web. Removing black flies should provide additional food and space and could allow other filter-feeders (e.g. hydropsychid caddisflies, midges) to colonize habitats previously occupied by black flies. Studies by McAuliffe (1984) and Hemphill and Cooper (1983) suggested that some hydropsychids can compete with black flies. Following *Bti* use, space vacated by black fly larvae may eventually be utilized by their competitors, assuming that competitors species are available for colonization.

All researchers agree that each living organism plays a role in the ecosystem but that role is rarely held, except for some exceptions, by only one species or a group of individuals. One can thus expect that the intensity of the impact (on the food web) is *inversely proportional* to the complexity of the ecosystem, i.e. the less complex the local ecosystem accommodating the treated population of mosquitoes and black flies is, the more the ecosystem can be affected by the removal of these populations.

With regard to the food web, some important comments must be made from the above studies. According to Hershey *et al.* (1998), toxicity of mosquito larvicides to nontarget invertebrate species needs to be understood in wetlands prior to implementation of large-scale mosquito control programs to prevent damage to the function of these important ecosystems. In their study of applications made at higher than normal frequencies, the 2–3 year lag time in response of NTO to larvicide treatment has demonstrated the need for long-term studies in wetland ecosystems, and the need to reconsider the conclusions based on previous short-term studies that *Bti* is environmentally safe. In addition, three years may not be adequate to evaluate fully the importance of the changes to vertebrate species because they are likely to have longer response time than shorter lived insects (Hershey *et al.*, 1998).

However, as we have seen, investigations on short-term effects of *Bti* on NTO (Table 2) have been much more numerous than those conducted on a medium or long-term basis. The reasons could perhaps be higher cost and the need for qualified personnel over a relatively long period. Also, if we consider several months to years of regular observation to constitute a long-term study, *we have to distinguish between the real impact of the tested product and the natural evolution of the population observed.*

With black flies, contrary to the Hershey *et al.* (1998) conclusion on mosquitoes so far, in a lotic environment, *Bti* has been proven safe for the ecosystem but studies have not conclusively demonstrated that there were no long-term effects on NTO and on the food web where sites have been treated with *Bti*. More long-term studies associated with *Bti* use are still needed to understand better the consequences associated with other members of the lotic food web.

CONCLUSION

Since its commercial arrival in the early 1980s, *Bti* has been considered as an environmentally safe biopesticide for the control of mosquitoes and black flies. Compared to chemicals, the

high degree of specificity, the low impact on NTO and the short persistence have meant that *Bti* formulations are now used successfully in many countries. In many of the *Bti* studies, comparisons were made with the normal conventional alternative products, e.g. chemical pesticides or oil. In general, their ecological impacts were important, often severe, even dramatic.

Many early studies have shown some effects of *Bti* treatments on NTO but at high dosages ('overdosage' conditions), thus enhancing the idea that environmental damage could not occur under normal usage. However, this review has identified that nearly 25% of the studies describing an impact on NTO (excluding chironomids) were done according to the recommended (operational) dosages. Although many studies were done in various environmental conditions, most were designed to look at the acute toxicity of *Bti* on target and nontarget organisms.

A rare long-term study in wetlands has shown significant dramatic effects on diversity indices which involve NTO but only after three years of repeated treatments at higher than normal frequency. This study combined with another one indicating that a single *Bti* treatment drastically inhibited primary production of green algae suggest that in the long-term and in certain biotic conditions, repeated *Bti* treatment could possibly induce extensive change and possibly damage in the food web. Interestingly, these two recent studies were done at operational dosage and *Bti* was formulated as coated granules. Could this mean that various formulations (*Bti* plus formulation additives) and their recommended dosages are inappropriate for certain habitats, concomitantly with abiotic parameters present at treatment time? Research is needed on alternatives, e.g. fewer, but seasonal applications and aiming at the LC_{90} level rather than total mortality, because research on some agricultural and forestry applications of *Bt* indicates that the relatively unharmed populations of predators and other natural control agents rapidly destroy the greatly reduced surviving populations of target insects.

In their studies, many authors have used the LC_{50} to compare the relative sensitivity of NTO to that of a target species (usually mosquito larvae). Even if statistically less accurate, future studies should compare the LC_{95-99} to evaluate acute toxicity since in its actual use, the goal of a treatment of the target insects is to achieve such percentages of mortality. From a practical point of view, it is unfortunate that to overcome the major influences of some biotic and abiotic parameters on the efficacy of *Bti* (thus on the dosage to be used), formulations are labelled with instructions like 'use higher dosages if ...' or 'repeat if necessary'. On the other hand, cost restrictions tend to prevent 'overdosage'.

Over nearly a quarter of a century, many studies have combined to promote a better understanding of *Bti* (biosynthesis, structure, toxicity, mode of action, fate, effects on target and nontarget organisms). Its safety and its specificity made it the ideal tool to fight many dipteran vector-borne diseases worldwide (dengue, yellow fever, malaria, onchocerciasis). However, recent studies may weaken the confidence held until now in formulated *Bti*. A long-term study has reported some negative effects on NTO, many other studies have shown effects on NTO even at the recommended dosage and, finally, a recent study described important inhibition of the primary production of green algae (the first link in the food web). These results may for the first time raise some questions on the ecological acceptability of *Bti*.

We feel that long-term and controlled field studies should be made in different biotic environments and especially in fragile ecosystems. Perhaps the use of new methods like stable isotope ratios could provide a better picture of the long-term impact of repeated large-scale *Bti* treatments on ecosystems.

The alternatives for human disease control and nuisance abatement must be faced:

(1) Do nothing to control the insects and live with the consequences. But is this ecologically sound as the mosquitoes and black flies could be regarded as ecologically out of balance?

(2) Use chemical insecticides etc. and suffer much harsher ecological consequences and probably eventual failure due to the insects becoming resistant.
(3) Use *Bti*, probably the most acceptable option, particularly if steps are immediately taken to alleviate perceived problems and if long-term research indicates improvements to these steps. Care should be taken to prevent the target insects becoming resistant to *Bti*.
(4) Sufficient control of mosquitoes and black flies would locally decimate one element near the bottom of the food chain with ecological consequences higher up. The acceptability of these consequences should be studied.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Guy Charpentier from the Université du Québec à Trois-Rivières and the referees from this journal who kindly reviewed and made valuable comments on this paper. Mario Boisvert's financial support was provided by the Natural Sciences and Engineering Research Council of Canada and the Fondation Québecoise de la Recherche sur l'Eau (Réseau Environnement).

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