6th Pacific Rim Conference on the Biotechnology of Bacillus thuringiensis *and its Environmental Impact,* Victoria BC, 2005
Côté, J.-C., Otvos, I.S, Schwartz, J.-L. and Vincent, C. (eds)

Utilization of *Bacillus thuringiensis* var. *israelensis* (*Bti*)-Based Formulations for the Biological Control of Mosquitoes in Canada

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Keywords: *Bacillus thuringiensis israelensis*, efficacy, mode of action, mosquitoes, safety, vectors.

Since the discovery of *Bacillus thuringiensis* var. *israelensis* (*Bti*) in 1976, extensive literature has proven its efficacy to control mosquitoes, of which many species are known as important vectors of diseases or simply as pests of humans and animals. In an outbreak of illness, caused for example by West Nile virus, a mosquito-borne virus, the utilization of *Bti*-based products is one of the safest and most efficient method to control larval mosquito populations. The very specific and target-oriented mode of action of *Bti* makes it very safe for human health and non-target organisms. *Bti*-based products, distributed in different types of formulations, are utilized in many countries. Liquid and granular formulations are used in Canada for more than 20 years to control mosquitoes in pest control programs (either in cold or warm water conditions) and since 2003 in West Nile virus control programs. Although recognized for its high efficacy, *Bti*-based formulations may be affected by «mosquito» and «environmental» parameters. However, considering the high efficacy, specificity, long shelf life of *Bti*-based products, these should be considered first when prophylactic or regular mosquito control programs would be required.

Introduction

Since the discovery of *Bacillus thuringiensis* var. *israelensis* (*Bti*) in 1976, extensive literature has proven 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. So, considering that the *Bti* high efficacy has already been proven by many papers in the last 20 years, this paper will put more emphasis (like a review article) on topics like the mode of action, elements (mosquito and environmental parameters) affecting its efficacy, fate in the environment, innocuousness and environmental safety for a better understanding of this microbial insecticide. But in a first time, a brief presentation of the mosquitoes which are one of main target of the *Bti* will be made.

Mosquitoes

Mosquitoes are vectors of many diseases around the world like malaria, dengue fever, yellow fever and many types of encephalitis. Since 1999 in United States and 2001 in Canada, the West Nile virus is now present in both countries and has infected thousands of people in the last six years with unfortunately many fatalities.

In United States and many provinces in Canada, adulticides are used to control mosquito populations, but larvicides like *Bti* are also commonly used. In the province of Québec (Canada), almost 100 % of the products used in the last 20 years to control mosquitoes and black flies contained *Bti*. This type of insecticide is called «larvicide» and acts only on the larval stages of target organisms.

Figure 1 represents the life cycle of mosquitoes. Depending of the species, the adult female will lay eggs either on the water surface (egg rafts or single eggs) or on damp soil (single eggs). In favourable conditions, eggs will hatch and give birth to larvae. There are four different larval instars. The last aquatic stage is the pupa from which will emerge the adult. *Bti* is active only against the larvae (see mode of action) and there is no toxic activity neither against the eggs or the pupae.

Mosquitoes can breed in many types of habitats. One can find them in roadside and irrigation ditches, pastures, woodland pools, tidal waters, salt marshes, polluted waters (with organic and/or inorganic matter), small containers, tires and tree holes. They are also present in your own backyard in abandoned pools, bird baths, roofs, clogged gutters, etc. Almost everywhere where stagnant waters are present, you get high probabilities to find mosquito larvae. Different formulations of *Bti* can be used to control larvae in these various breeding sites. The type of formulations and dosages must be adjusted to the types of sites encountered. Factors affecting the efficacy of *Bti* in these environments will be discussed later.

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Introduction to *Bti*

For the past six 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.

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 ; 1978d). 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* (*B.t.*) strain exhibiting a highly specific and toxic effect against certain aquatic diptera (de Barjac, 1978a). Before this discovery, only a few strains of *B.t.* 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 *B.t.* were principally confined to lepidopterous pests of agriculture and forestry with *B.t.* subspecies killing mostly insect larvae feeding on crops and on trees (Heimpel, 1967 ; Burgerjon & Martouret, 1971 ; Falcon, 1971)

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 (Chilcott *et al*., 1983).

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 and contains four major proteins (27, 65, 128 and 135 kDa) (Huber & Luthy, 1981 ; Charles & de Barjac, 1982).

Mode of action of *Bti*

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*., 1983). In *Bti*-treated mosquito larvae, the binding of endotoxins to specific receptors results in an osmotic imbalance across the midgut epithelial cell membranes, causing severe damage to the gut wall and leading to rapid death.

To better understand the effect 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 (Figure 2). 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 (> 10);
- possess the enzymes capable of liberating the toxic proteins ;

- possess the gut membrane receptors, compatible with the solubilized toxins.

Fate of *Bti* toxic activity in the environment.

Formulations of *Bti* are used as larvicides, either in standing waters or in flowing waters. Spores, because of their ability to survive in harsh environments, may persist at gradually decreasing concentrations for weeks, but crystals will eventually degrade and their constituents will be recycled in the ecosystem. However, depending on the type of environment, the fate of and/or behaviour of the toxic particles can be quite different. The toxicity of *Bti* is mainly and strongly correlated to the availability of the crystals.

In lentic environments (standing waters), once applied to the surface of water, the *Bti* crystals 1) can be ingested by mosquitoes and also by other non-target 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.

Effects on target organims

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 the 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.

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. *Bacillus thuringiensis* var. *israelensis* 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. 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).

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. 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 that sink from the surface layer. Moreover, Mahmood (1998) in his laboratory study, where he compared the susceptibilities of *Aedes aegypti* and *Anopheles albimanus* by their feeding rates, found that the *Anopheles* larvae ingested ten times less material than *Aedes.* This laboratory result may be very important to explain and interpret the difference in susceptibilities in the field.

The physiology of the *Anopheles* can also be taken into consideration when comes the time to explain the difference in *Bti* efficacy. Figure 3a shows the physiology of *Culex* and *Aedes,* where a siphon is present at the end of the body. This siphon allows the larvae to breath at the water surface. The way the larvae stands in the water column permits a greater probability to ingest crystals when the larvae are feeding. Figure 3b, one can see that *Anopheles* larvae do not possess a siphon and must stand parallel to the water surface to breath and to feed. So, as long as the *Bti* is still in the feeding zone of the larvae (zone smaller than for *Culex* and *Aedes*), crystals are available and a toxic effect can be observed. However, as soon as the crystals are no longer in the feeding zone, they can not be captured and ingested and toxic effects are no longer possible.

Instar susceptibility For most species tested, younger-instar larvae are more susceptible than older ones. 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 do not 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.
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 that 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*., 1982).

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. In both cases (high density and pollution), higher rates of application will be necessary to control mosquito larvae (Mulla, 1990).

Water temperature Water temperature is a very important factor that needs to be taken into consideration. Sinègre and Vigo (1980) showed a decreased activity of the endotoxin against *Aedes aegypti* in the laboratory at low temperatures (from 17°C 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 & Vigo, 1980). The relationship of LC_{50} to temperature is 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 state where availability can be sustained for maximum efficacy of a given formulation.

Worldwide utilization

Interest in *Bti* is increasing worldwide year by year as various commercial products (corn cob granules, dunks, pellets, briquets, liquid) 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.

In 2005, *Bti*-based formulations have been widely tested and used worldwide in mosquito control projects. They have been used in more than 25 countries, including Canada (in all provinces). In Québec, *Bti* has been used for more than 20 years in pest control programs (nuisance) and since 2003 in the West Nile virus (WNV) control program. Figure 4 shows the evolution of quantities used in Québec since 1992. In twelve years (1992 to 2003), the quantity of *Bti* used per year increased from less than 4 tons per year to nearly 30 tons per year.

In 2003, the Québec Ministry of Health mandated the SOPFIM to treat spring species of mosquitoes (*Aedes* and *Ochlerotatus*) and summer species (*Culex*, *Coquillettidia*, *Anopheles*) to fight potential vector species of WNV. That year, 26.5 tons of *Bti* (mainly granular formulations) were added to the 27 tons used in nuisance programs, for a total of more than 50 tons of *Bti*. Contrary to other Canadian provinces and/or various states in United States, no adulticides are used in Québec to control mosquito populations. Applicators and contractors rely only on *Bti* formulations that are efficient in either cold (spring) or warm (summer) water conditions.

Two different modes of application of *Bti* can be used in control treatments : aerial and ground applications. Aircrafts and/or helicopters equipped with conventional booms and nozzles or rotary mist atomizers are commonly used to spray over large surface areas. For ground treatments, the backpack is the conventional equipment used with diluted material or granular formulations.

Environmental safety

In United States and Canada, biopesticides must be registered before their utilization. In both countries, criteria for the acceptance of the products are very stringent (infectivity, pathogenicity, toxicity, laboratory and field experiments) and when a product is finally accepted, one can be sure that the product is safe for the humans and the environment and that it has proven its efficacy toward the target insects.

In Canada, *Bti* has undergone a full health impact assessment which shows that it poses no risk to mammals, including humans. Based on the lack of human health risk and long history of safe use associated with *Bti* and other varieties of *Bt*, the Canadian Pest Management Regulatory Agency (PMRA) has no human health and safety concerns with the application of registered products containing Bt to bodies of water that will be used for human consumption (PMRA, 2001).

In 1999, conclusions and recommendations of a task group on environmental health gathering people from the United Nations Environment Programmes, the International Labour Organization and the World Health Organization stated that : «*Bti* is safe for use in aquatic environments including drinking-water reservoirs for the control of mosquito, blackfly and nuisance insect larvae». (WHO, 1999)

The United States Environmental Protection Agency (USEPA) categorizes the risk posed by *Bt* strains to non-target organisms as minimal to non-existent. So, the weight of scientific evidence indicates that *Bti* is non-infectious and non-toxic to humans and other mammals and poses little risk at dosage levels permitted in insect control programs (PMRA, 2001).

Conclusion

Since its commercial arrival in the early 1980's, *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 non-target organisms and the short persistence have meant that *Bti* formulations are now used successfully in many countries.

A promising future of this microbial control agent in mosquito control programs is ensured by its high efficacy, its specificity, its feasibility to be fermented on an industrial scale, its long shelf-life, its transportability, and finally and maybe the most important, there is actually no known field resistance documented until today.

So, compared to chemicals, use of *Bti* is probably the most acceptable option particularly if steps are immediately taken to alleviate perceived problems. *Bti* should be introduced in countries where chemical products are actually used on a large scale and abatement programs against pest insects (mosquitoes and blackflies) using that new tool should be highly considered.

Acknowledgements

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