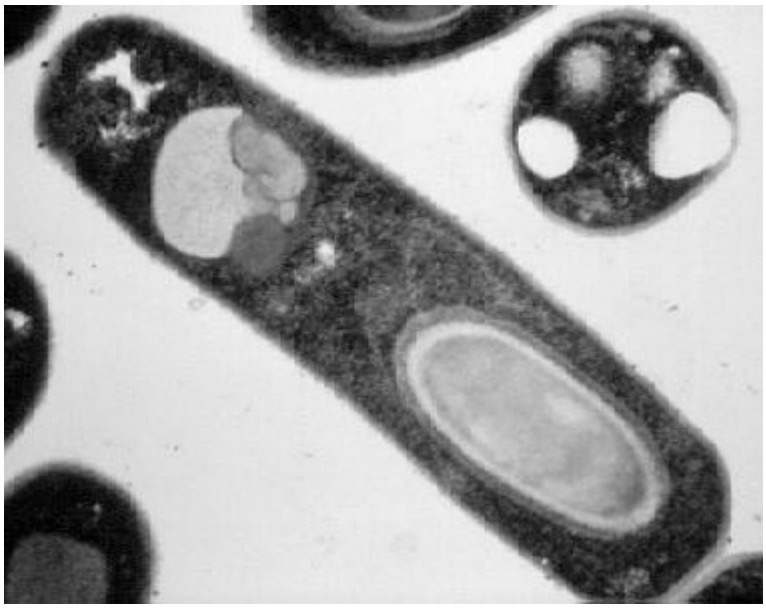
REPORT FOR THE MINISTRY OF HEALTH

Environmental and health impacts of *Bacillus thuringiensis israelensis*



Bacillus thuringiensis israelensis sporulating cell during formation of the crystal (TEM by J-P Charles, Institut Pasteur).

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Abbreviations:

Bt - Bacillus thuringiensis; Bts - Bacillus thuringiensis strains (plural); *Bti - Bacillus thuringiensis israelensis*; *Btk - Bacillus thuringiensis kurstaki*

1. Summary

- Mosquito vectors of human disease pose a constant threat to New Zealand and recent interceptions of exotic mosquitoes with the potential to vector serious mammalian diseases has highlighted the need for agents for use in control and/or eradication programmes.

- *Bacillus thuringiensis* (*Bt*) strains and varieties are pathogenic to a number of pests, including Lepidoptera and Diptera. The discovery of *B. thuringiensis israelensis* (*Bti*), a variety specific to Diptera (especially mosquitoes and blackflies) in Israel in 1978, has led to the development of many products based on this bacterium. These products have been used extensively in mosquito and biting fly control programmes, especially in Africa, USA and Germany.

- There is a well documented history of environmental safety of *Bt* strains used in pest control. The environmental safety of *Bt*, coupled with the nature of toxicity and level of specificity for target hosts, has led to the use of *Bt* in many pest control programmes in environmentally sensitive areas, including the eradication of tussock moth in New Zealand.

- Naturally occurring *Bt* strains have been isolated from the New Zealand environment, including strains similar to *Bti*. However, the aquatic environment has not been sampled for *Bt*, so the natural occurrence in New Zealand waterways is unknown.

- The mode of action of *Bti* involves the synergistic interaction of four toxic proteins. *Bti* rarely recycles in natural environments and the insect toxicity is due to crystal proteins formed during sporulation.

- Aspects of the environmental impact which need be considered for any pesticide include mammalian and non-target safety, effect on the environment, persistence and occurrence in the natural environment and possible host resistance. For microbial-based pesticides, such as *Bt*, gene transfer must also be considered.

- A review of the literature on host range and effect on non-target organisms indicates that *Bti* is relatively specific to the Nematocera suborder of Diptera, in particular filter-feeding mosquitoes (Culicidae) and blackflies (Simuliidae). It has also been shown to be pathogenic to some species of midges (Chironomidae) and Tipulidae, although usually to a lesser extent than mosquitoes and biting flies.

- *Bti* has not been reported to affect a large number of other invertebrate species including most aquatic fauna. It is not toxic to bees. Fish are not affected, either in the laboratory or after field application.

- *Bti* is considered to pose little threat to mammalian safety. *Per os* inoculations of animals and humans have not resulted in clinical symptoms. Concerns have been raised that the solubilised δ-endotoxin of *Bti* activated in the laboratory was toxic to mice when administered by injection and cytolytic to human erythrocytes. However, solubilisation occurs at high pH (such as in insect guts) and does not occur in mammalian guts.

- The close genetic relationship between *B. thuringiensis* and the occasional human pathogen, *B. cereus* has raised concerns about possible implication of *Bt* in human gastro-intestinal illnesses and other health problems caused by *B. cereus.* However, no such effect has been found after extensive field use. A specific identification system for *Bt* strains would assist monitoring of future applications.

- *Bti* does not persist in the environment after application. Generally, reports of activity after application show a decline in efficacy within days and little residual activity after several weeks. The persistence of *Bti* after application is dependent on the type of formulation/product used, with some formulations (pellets/briquettes) designed specifically to enhance residual activity.

- Some of the toxic proteins of *Bt* are encoded by genes residing on extra-chromosomal DNA (plasmids) which can be exchanged between strains and species by conjugation and/or transformation. While genetic transfer between *Bt* and other soil bacteria has been demonstrated in the laboratory (in culture, insects and sterile soils), it has not been shown in the field. No unexpected pathogenic organisms have resulted from extensive *Bt* application, suggesting that while gene transfer may have implications for genetically modified strains, it is a lesser concern for wild-type strains.

- Some insects, especially Lepidopterans, have become resistant following constant application of *Bt* strains. However, resistance has not occurred after application of *Bti*, possibly due to the complex mode of action, involving synergistic interaction between up to four proteins. Use of a single protein from *Bti* for mosquito control resulted in resistance after only a few generations in the laboratory. However, use of *Bti* for over 10 years in Africa, USA and Germany has not resulted in development of resistance.

- Over 40 tons of *Bti* were applied in west Africa alone, without any reports of safety or non-target concerns. The environmental threat posed by *Bti* would appear to be significantly less than that posed by most other forms of mosquito control which have a similar level of efficacy.

2. Introduction

Mosquito vectors of serious mammalian diseases could arrive in New Zealand at any time. The need for safe agents for their eradication and/or control is imperative. *Bacillus thuringiensis israelensis* is a subspecies of the common insecticidal bacterium; it was discovered in 1978 and has high toxicity to Diptera.

2.1. Background

Recent discovery of potential disease vectoring mosquitoes in northern New Zealand has highlighted the likelihood of serious mosquito vectored disease incursions in the near future. Past experiences with pest invasions such as fruit fly and tussock moth have demonstrated the value of preparedness. A thorough knowledge of potential control agents for mosquito vectors, including their efficacy and environmental impacts, will be essential for effective control.

Few products are registered for mosquito control in New Zealand. In general, the use of chemical insecticides is declining in New Zealand, as a result of increasing concern over negative environmental impacts such as non-target mortality and mammalian toxicity. Such concerns are exacerbated when pest control measures are required in densely populated urban environments, possibly requiring large scale aerial application. Some alternatives to chemical insecticides have been developed for control of mosquitoes. One of the most widely used is the bacterium, *Bacillus thuringiensis israelensis* (*Bti*). One product, VectoBac® 12AS (Abbott laboratories), based on *Bti*, is currently being registered in New Zealand by NuFarm NZ Ltd.

This report collates available information on environmental impacts of *Bti* use from overseas data. Such information will assist in the rapid processing of the application, and will serve as a source document for the Ministry of Health for recommendations regarding *Bti* use in mosquito control. This would include any ministerial exemption under the Biosecurity Act for use in emergency situations before full registration is approved.

2.2. Discovery of Bacillus thuringiensis israelensis

In 1975-76, a World Health Organisation sponsored project in Israel examined mosquitoes for the presence of pathogens or parasites. During this survey, a new *Bt* strain was discovered with high toxicity to mosquito larvae (Goldberg and Margalit 1977) which was later identified and designated *Bt* var. *israelensis*, serotype H14 (de Barjac 1978), since raised to subspecies status as *B. thuringiensis israelensis*. This strain was significantly more toxic to mosquitoes than other known bacterial strains at that time. It was collected from mosquitoes in the Negev desert of Israel. While dipteran active *Bt*s were known, *Bti* was found to be relatively specific to Diptera and was quickly shown to be toxic to a range of mosquito and blackfly species. Therefore, it was considered to have commercial potential as a control agent of nuisance Diptera around the world. Rapid development of *Bti* strains occurred in the early 1980s and several products were developed. The need for a more environmentally benign mosquito control agent and rising incidence of resistance to chemical pesticides provided a platform for rapid *Bti* development. Products based on *Bti* have now been used in many countries, with

extensive mosquito and blackfly control programmes based on *Bti* occurring in west Africa, USA and Europe. In particular, *Bti* has been used in areas considered environmentally sensitive (Federici 1995).

3. Characterisation of *Bacillus thuringiensis israelensis*

The classification of *Bacillus thuringiensis* is difficult because of the close genetic relationship between *B. thuringiensis, B. cereus, B. anthracis* and *B. mycoides*. The main characteristic separating *Bt* is the formation of insecticidal crystal proteins.

3.1. Bacillus thuringiensis *taxonomy*

Bacillus thuringiensis is a gram positive, rod-shaped, spore forming bacterium which often has insecticidal properties. *B. thuringiensis* belongs to the "*Bacillus cereus* complex" which also includes *B. cereus, B. anthracis* and *B. mycoides*. The taxonomic relationships between members of the *B. cereus* group are not clear (Drobniewski 1994) and the cause of some concern as the differences between *B. cereus* and *B. thuringiensis* are small and may be mainly plasmid based. DNA sequencing studies of conserved gene regions have suggested they may be strains of a single species.

During sporulation in *B. thuringiensis*, some strains produce one or more inclusions or parasporal bodies within a sporangium. The parasporal body is often toxic to specific insect groups and many different insecticidal crystal proteins (δ-endotoxins) can be found in different *B. thuringiensis* subspecies and strains.

3.2. Characterisation

The species in the complex are only differentiated from one another by a few characters, most of which are located on plasmids. Therefore, characterisation of *B. thuringiensis* has been problematic and several systems have been used. Phenotypic methods used include flagellar serotyping, description of crystal morphology, biochemical reactions and bioassays. Classification of subspecies or varieties based on serotyping using H-serovars (flagellar serotyping) resulted in identification of almost 60 varieties (Hansen *et al.* 1996). Serotype does not necessarily relate to the presence of δ-endotoxins, which determine host specificity, as flagellar genes are carried on the chromosome, while toxin genes are usually encoded on plasmids.

Characterisation methods based on phenotypic characters are insufficient when used alone in studies on the environmental ecology and fate of *B. thuringiensis*, as these methods do not provide unambiguous identification. A number of DNA-based methods have been developed for characterisation: specific primed polymerase chain reaction (PCR); Random amplified polymorphic DNA (RAPD), DNA:DNA colony hybridisation (Hansen *et al.* 1996) and rRNA-based probe (Akhurst *et al.* 1997). These methods can distinguish individual strains and isolates, allowing the tracking of the environmental fate of strains used for pest control. Such methods can also be used to identify the presence/absence of specific endotoxin genes, which mean it is possible to establish whether a particular strain has lost or acquired specific δ-endotoxin genes in the environment.

3.3. Mode of action

Bti products contain the spores and parasporal crystals of *Bti* H-14 serotype which must be ingested by the larval stage of the mosquito to cause mortality. Following ingestion, the parasporal crystals are solubilised in the alkaline larval midgut, followed by proteolytic activation of the soluble insecticidal crystal proteins. The toxin binds to a receptor on the midgut cell wall resulting in pore formation in the cell, which leads to death of the larva.

Insecticidal effect is caused by the parasporal crystal, which for *Bti* usually contains four major proteins (27, 65, 128 and 135 kDa). The crystal toxins of *Bti* are designated Cry4A, Cry4B, Cry11Aa and Cyt1Aa, according to the most recent classification (Crickmore *et al.* 1995). Cry4 protein toxin genes are Dipteran-specific, as are the Cyt genes (Tanada and Kaya 1993). The crystal is formed at the end of sporulation. All proteins are toxic to mosquitoes, however there appears to be a synergistic interaction between the Cyt1Aa protein and the Cry4 and Cry11 proteins, resulting in high toxicity to mosquito larvae (see Tanada and Kaya 1993 for expanded treatment).

Bti treated mosquito larvae generally cease feeding within 1 hour, show reduced activity by two hours, extreme sluggishness by four hours and general paralysis by six hours after ingestion (Chilcott *et al.* 1990).

4. Natural occurrence and use of *Bt* in New Zealand

***Bacillus thuringiensis* occurs naturally in New Zealand, especially in soil and insects. Aquatic environments have not been extensively sampled, but strains similar to *Bti* have been found in the environment. The natural role of *Bt* is not clear and several hypotheses exist. It could be a natural insect pathogen, although it rarely recycles in insect hosts, or it could be a soil bacterium with unassociated insecticidal properties.**

4.1. Presence of Bt *in New Zealand*

Bt is ubiquitous in the New Zealand environment. Chilcott and Wigley (1993) reported isolating 6909 *Bt* isolates from various locations in New Zealand. These isolates were from soil, insect habitats and insect larvae (*Costelytra zealandica*, *Pericoptes truncatus* and *Vespula germanica*). Aquatic environments were not sampled. Some isolates were pathogenic to *Culex pervigilans* in laboratory assay. Isolates active only against Diptera contained three proteins similar to *Bt. israelensis* (28, 68 and 130 kDa), as well as a new profile for one dipteran-active strain of 40, 42 and 50 kDa.

Compared to many countries, *Bt*s have not been used extensively in New Zealand. The lepidopteran-active *Bacillus thuringiensis kurstaki* (*Btk*) has been investigated for control of kiwifruit pests, especially leafrollers (Wigley and Chilcott 1992). However, the strict quarantine and quality standards required of most export crops has limited its use in New Zealand, as *Bt* is often not as effective in horticulture crops as conventional pesticides and damage occurs before pest death. Control of blackflies (sometimes called sandflies) (Simuliidae: *Austrosimulium laticorne* and *A. multicorne*) in New Zealand was shown to be feasible by Chilcott *et al.* (1983), but no product was subsequently registered or further experimentation reported. No significant effects on population levels of non-target aquatic insects (mayflies, caddisflies, stoneflies, beetles, dixids, craneflies, snails and chironomids) were observed when *Bti* was use against simuliids in New Zealand (Chilcott *et al.* 1983).

Most current interest in use of *Bt* toxins as control agents in New Zealand is in the area of transgenic crops. Several groups are working on the expression of *Bt* toxin genes in pasture, horticultural and vegetable plants.

4.2. Occurrence and role in the environment

An understanding of the ecology of *Bti* in the environment is essential in assessment of its environmental risk. While originally recovered mainly from insects, improved isolation and identification techniques have indicated that *Bt* may be ubiquitous in soil (eg. Martin and Travers 1989). The lowest percentage recovery of *Bt* from soil reported was in the USA (60% of soils sampled) (Meadows 1993). In New Zealand, Chilcott and Wigley (1993) found that between 60-100% of soils sampled contained *Bt*, depending on source (urban, horticulture etc.). *Bt* is also indigenous in many other environments, being found in stored products, dust, on deciduous and coniferous plants and in aquatic environments. *Bt* has also been isolated from insect habitats such as rotting wood, wasp nests and stored products in New Zealand.

Bt apparently exists in the environment as spores, which have little or no metabolic processes and can survive periods of low nutrition and desiccation. There is little multiplication of the vegetative stage in the environment (see review by Meadows 1993).

There are several theories on the ecological niche filled by *Bt*. Unlike most insect pathogenic microbes, *Bt*s generally recycle poorly and rarely cause natural epizootics in insects, leading to speculation that *Bt* is essentially a soil micro-organism that possesses incidental insecticidal activity (Martin and Travers 1989). Evidence to support this view is that *Bt*s are commonly reported in the environment independent of insects and there is a lack of association between occurrence and insect activity (van Frankenhuyzen 1993). Meadows (1993) suggested four possible explanations for the presence of *Bt* in soil: 1) rarely grows in soil but is deposited there by insects; 2) may be infective to soil-dwelling insects (as yet undiscovered); 3) may grow in soil when nutrients are available; and 4) an affinity with *B. cereus*. Alternatively, Smith and Couche (1991) proposed that *Bt* is a natural component of the phylloplane microflora and has evolved in a symbiotic or mutualistic association with plants to provide protection against herbivores.

Although *Bt* is rarely found causing epizootics, the *Bti* strain was discovered causing massive death among *Culex* sp. in Israel. It is also difficult to conceive that the bacterium would use energy to produce crystals without an ecological benefit. Meadows (1993) expounds the case for *Bt* taking advantage of the insect gut pH which inhibits growth of *Bt* but liberates the toxin, allowing the *Bt* cells to multiply in the cadaver.

The production of endotoxin is the only consistent difference between *Bt* and *B. cereus*. It appears that presence of the plasmids which encode many *Bt* endotoxins is related to germination and sporulation, and may therefore make it more difficult for *Bt* strains to grow in marginal environments such as soil, compared with *B. cereus*. Loss of the endotoxin-encoding plasmids can result in *Bt* becoming, taxonomically, *B. cereus*. The significance of such loss is poorly understood. Jackson *et al.* (1995) reported the isolation of *Bt*, originally identified as *B. cereus*, from patients with gasto-enteritis (the only such report), however did not demonstrate *Bt* to be the casual organism of the symptoms.

5. Types of *Bti* formulations

There are several types of *Bti* based products available, ranging from emulsifiable liquids to pellet and granular formulations. The type of formulation influences persistence and area of effect. Pellet/briquette formulations have greater persistence and are therefore more likely to have non-target effects. At present, only a liquid formulation is under application for registration in New Zealand.

When considering the environmental impact of *Bti,* information on the range of formulations is required, as formulation can affect the persistence of toxicity, site of contamination and choice of application method, which are all factors which may impact on non-target effects.

There have been successful applications of *B. thuringiensis*-based insecticides for over forty pest species in North America, as well as elsewhere in the world (van Frankenhuyzen 1993). Therefore, the discovery of *Bti* rapidly led to development of new commercial formulations as the required technology for production and formulation already existed. Formulations based on *Bti* which had good shelf life and activity against mosquitoes and blackflies were registered with the EPA in USA as early as 1980. Commercial production commenced in USA and Europe in 1984 and *Bti* products were introduced to Australia in the same year (Clarke 1994). Early products were generally based on liquid formulation and were not stable under ambient conditions, which reduced the shelf life markedly.

TABLE 1 : Some *Bti* products for mosquito and blackfly control (possibly not all currently available) (modified from Becker and Margalit 1993).

Product	Formulation	Company
Teknar TC	Powder	Novartis (sold by Triology)
Teknar HP-D	Fluid	"
Teknar G	Granules	"
VectoBac TP	Powder	Abbott Laboratories
VectoBac 12 AS	Fluid	"
VectoBac G	Granules	"
VectoBac CG		"
Bactimos WP	Powder	"
Bactimos G	Granules	"
Bactimos	Briquettes/pellets	"
Bactimos PP		"
Cybate (Australian label)	Fluid	Cyanamid
Skeetal FC	Fluid	Entotec/Novo (purchased by Abbott?)
BMC WP	Powder	Reuter
Duplex	methoprene + *Bti*	Zoecon - PPM

While only one aqueous solution of *Bti* (VectoBac® 12AS) is currently in the process of registration in New Zealand, several other types of formulation are available (Table 1) (Becker and Margalit 1993). Highly concentrated liquid formulations are available for control of floodwater mosquitoes while formulations which float for as long as possible have been developed for use in fast-flowing or turbulent waters. Formulations which settle and persist at the bottom are required for bottom feeders. Granules which float on the surface are the most effective against surface feeders such as *Anopheles* spp. Effective formulations for control of different mosquitoes have been developed, for example briquettes for mosquitoes with continual successive generations like *Culex* spp. (Becker and Margarlit 1993). Briquettes or pellets, in particular, seem to be useful for overcoming lack of persistence, which is one of the major limitations of *Bti* formulations. A single briquette per rain barrel results in satisfactory control of *Culex pipiens* for more than one month (Becker and Margalit 1993).

In addition to the formulations in Table 1 from Europe and North America, formulations of *Bti* have also been produced in many countries such as India (eg. Deltox - Balaraman *et al.* 1981; 1987), Peru (Ventosilla *et al.* 1991), Eygpt (Morsi *et al.* 1988), Russia (eg. Bactoculicide - Cherkashin *et al.* 1987), China (eg. Chen *et al.* 1985) and Israel (Car 1984).

Other types of formulations used include culigel superabsorbent polymer controlled-release system for the slow release of *Bti* to mosquito larvae (Levy *et al.* 1990) and combinations of chemical and biological agents such as in "Duplex" (insect growth regulator s-methoprene + *Bti*) (Bassi *et al.* 1989). An experimental formulation of sprayed-dried *Bti* powder as a fizzy tablet was constructed by Skovmand and Eriksen (1993) to obtain a product for mosquito larval control that was easy to store, carry and use. They found that such a formulation may be suitable for mosquito control in breeding sites such as water reservoirs, garden pools, swimming pools and small streams. Further developments include floating bait formulations of *Bti* designed to improve the effect of bacterial toxins, especially against *Anopheles* spp. (Aly *et al.* 1987). Carrier particles such as wheat flour or products yielding surface films on water (Arosurf (alpha-isooctadecyl-omega-hydroxypoly[oxy-1,2-ethanediyl]) and Liparol (containing isoparaffins and lecithin) have been tested, although some mixtures inhibited feeding.

6. Environmental safety of formulation components

The components of any *Bti* product need to be independently assessed for environmental safety. These constituents are product specific and are closely guarded trade secrets. However, most *Bt* formulations have been cleared by the US EPA and a *Bt* formulation was cleared by the NZ Ministry of Health for use in the tussock moth eradication programme. A full list of all components of any *Bti* product to be used in New Zealand needs to be available to the licensing body.

Products based on *Bti*, such as VectoBac® contain a large percentage of bacteria and fermentation medium, however other additives are used to improve product stability and provide other desirable characteristics such as flowability. Formulations of pesticides are closely guarded trade secrets, but for registration approval, these must be provided to the appropriate government authorities. In the case of Foray 48® used in the tussock moth eradication programme, the formulation was declared to the Government, including Ministry of Health and approved for safety. According to Abbott Laboratories, there are some differences between Foray and VectoBac®, however the EPA and other agencies have approved VectoBac® formulation. Component lists need to be examined by the appropriate government agencies for safety prior to use in New Zealand.

7. Host range

***Bti* is highly pathogenic against Culicidae (mosquitoes) and Simuliidae (blackflies), and has some virulence against certain other Diptera, especially Chironomidae (midges). There are few records of susceptibility outside these dipteran groups, and most other hosts within Diptera require high doses to kill.**

Bti is generally regarded as specific to larvae of the Nematocera, which include filter-feeding mosquitoes and *Simulium* (blackflies, sandflies)(Clarke 1994). Nematocera also includes midges, craneflies and gall flies, some of which are susceptible. In the laboratory and occasionally in the field, it has been found to kill other hosts (see Table 2). However, the vast majority of susceptible hosts are recorded in nematocerous Diptera.

Among mosquitoes, different preparations of *Bti* have shown differing levels of toxicity to host species. Generally, *Culex* and *Aedes* are highly susceptible while *Anopheles* are less susceptible, but can still be killed with *Bti* (Balaraman *et al.* 1983). However, even within one genus, some species are more susceptible than others (eg. Chui *et al.* 1993).

Mosquito species which are not filter feeders do not seem susceptible. For example, against *Culicoides occidentalis* (Colwell 1982) and *Coquillettidia perturbans*, *Bti* larvicides had no effect (Walker *et al.* 1985). The mosquito predatory genus *Toxorhynchites* appears to contain both susceptible and non susceptible species (see Table 2).

Chironomidae and Tipulidae are the other groups which show some susceptibility. Chironomids are an important food source for some organisms and a pest in some areas. Chironomid larvae have been killed during field application of *Bti* against other hosts. Some species are very susceptible; for example *Chironomus kiiensis*, *C. yoshimatsui* and *Paratanytarsus* sp. where 17 µg/ml of *Bti* resulted in high (70-100%) and rapid mortality (within 2 days) (Kondo *et al.* 1995). Kondo *et al.* (1995) measured differences in susceptibility ranging from 10 to 1000 times among six species of chironomids. Other species of chironomid are not susceptible; for example, *Chironomus plumosus* (Lebrun and Vlayen 1981). Although some chironomids are susceptible to *Bti*, dose rates required for their control can be 10x the quantity required for mosquito control in the same situation (eg Becker and Margalit 1993; Mulla *et al.* 1990b). In Germany, mosquito treatment does not usually affect chironomids (Becker and Margalit 1993).

TABLE 2: Hosts susceptible to *Bacillus thuringiensis israelensis*

(Generally no indication of relative susceptibility is given and some reports may represent field reports of treatments of mixed populations. References are representative, not necessarily the only references to a particular host. Compiled mainly through CAB abstracts).

Diptera: Culicidae	**Representative references**
Aedes aegypti.	Foo and Yap 1982; Lee and Cheong 1985; Larget *et al.* 1981
Ae. albopictus.	Lee and Cheong 1985; 1988; Huang *et al.* 1993
Ae. atlanticus.	Palmisano 1987
Ae. atropalpus.	Tousignant *et al.* 1992
Ae. campestris.	Spackman 1986
Ae. canadensis.	Knepper and Walker 1989
Ae. cantans.	Schnetter *et al.* 1983; Rettich 1983
Ae. cantator.	Christie 1990
Ae. caspius.	Sinegre 1979; Zohdy and Matter 1982; Chabanenko *et al.* 1992
Ae. cataphylla.	Luthy *et al.* 1980
Ae. cinereus.	Rettich 1983
Ae. communis.	Luthy *et al.* 1980; Rettich 1983
Ae. detritus.	Sinegre 1979; Sinegre *et al.* 1980; Merdan *et al.* 1986
Ae. dorsalis.	Garcia *et al.* 1980; Spackman 1986
Ae. dupreei	Beck 1982
Ae. fitchii.	Knepper and Walker 1989
Ae. flavopictus.	Xu *et al.* 1986
Ae. hexodontus.	Eldridge *et al.* 1985
Ae. implicatus.	Spackman 1986
Ae. melanimon.	Mulla *et al.* 1986
Ae. melanion.	Spackman 1986
Ae. mercurator.	Spackman 1986
Ae. nigromaculis.	Mulla *et al.* 1982; 1986
Ae. polynesiensis.	Barjac *et al.* 1979; Goettel *et al.* 1982
Ae. pseudoscutellaris.	Goettel *et al.* 1982
Ae. pullatus.	Spackman 1986
Ae. punctor.	Rettich 1983; Snow 1984
Ae. sierrensis	Beck 1982
Ae. sollicitan.	Christie 1990; Ward and Redovan 1983; Lake *et al.* 1982
Ae. spencerii (& varieties).	Spackman 1986
Ae. squamiger.	Webb and Dhillon 1984
Ae. sticticus.	Schnetter *et al.* 1983; Rettich 1983
Ae. stimulans.	Knepper and Walker 1989
Ae. taeniorhynchus.	Purcell 1981; Mulla *et al.* 1980; Ward and Redovan 1983
Ae. togoi.	Lee and Cheong 1985; Huang *et al.* 1993
Ae. tomentor	Beck 1982
Ae. triseriatus.	Lacey and Singer 1982; Sutherland *et al.* 1982
Ae. rusticus.	Schnetter *et al.* 1983
Ae. vexans.	Luthy *et al.* 1980; Schnetter *et al.* 1981; Gharib and Hilsenhoff 1988
Ae. ventrovittis.	Eldridge *et al.* 1985
Ae. vigilax.	Mottram *et al.* 1989; Goettel *et al.* 1982
Anopheles albimanus.	Lacey and Singer 1982; Mulla *et al.* 1986; Swezey and Salamanca 1988
An. annulipes.	Davidson *et al.* 1981
An. anthropophagus [=*An. lesteri anthropophagus*]	Xu *et al.* 1986
An. arabiensis.	Romi *et al.* 1993; Seyoum and Abate 1997; Nugud and White 1982
An. atroparvus.	Nicolescu 1982
An. balabacensis.	Lee and Cheong 1985; Foo and Yap 1982
An. culicifacies.	Dua *et al.* 1993; Bhateshwar and Mandal 1991
An. crucians.	McLaughlin *et al.* 1982

An. dthali.	Ladoni *et al.* 1986
An. franciscanus [=*An. pseudopunctipennis franciscanus*].	Kramer 1989; Garcia *et al.* 1982
An. freeborni.	Kimball *et al.* 1986
An. gambiae complex.	Hougard *et al.* 1983
An. hyrcanus.	Dubitskii *et al.* 1981
An. karwari.	Lee *et al.* 1990
An. maculatus.	Lee and Cheong 1988; Lee *et al.* 1990
An. maculipennis.	Ouzounis *et al.* 1993; Stus and Mikherskaya 1986
An. multicolor	Zohdy and Matter 1982
An. nigerrimus.	Kramer 1984
An. pharoensis.	Bekheit and Sadek-Bekheit 1984
An. pulcherrimus.	Chabanenko *et al.* 1992
An. quadrimaculatus.	Mulla *et al.* 1980; Lacey and Singer 1982; Stark and Meisch 1983
An. sacharovi.	Volzhinskii *et al.* 1984; Ceber 1992
An. sinensis.	Xu *et al.* 1986; Yu *et al.* 1982
An. stephensi.	Larget *et al.* 1981; Dua *et al.* 1993; Balaraman *et al.* 1983
An. subpictus.	Dua *et al.* 1993; Balaraman *et al.* 1983; Manonmani and Hoti 1995
An. sundaicus.	Schaefer and Kirnowardoyo 1984
An. superpictus.	Ladoni *et al.* 1986
An. vagus.	Kramer 1984
Armigeres durhami.	Lee and Cheong 1985
Ar. kesseli.	Lee and Cheong 1988
Ar. subalbatus.	Huang *et al.* 1993; Kang and Chen 1986.
Culex annulirostris.	Davidson *et al.* 1981
Cx. antennatus.	Merdan *et al.* 1986; Bekheit and Sadek-Bekheit 1984
Cx. declarator.	Habib 1983
Cx. erraticus	Beck 1982
Cx. fuscanus.	Kramer 1984
Cx. laticinctus.	Alten and Bosgelmez 1990
Cx. nigripalpu	Beck 1982
Cx. orientalis.	Yu *et al.* 1982
Cx. peus.	Mulla *et al.* 1980; Eldridge and Callicrate 1982
Cx. pipiens complex.	Farghal 1982; Zohdy and Matter 1982; Schnetter *et al.* 1983
Cx. pseudovishnui.	Lee and Seleena 1990
Cx. quinquefasciatus.	Mulla *et al.* 1980; Davidson *et al.* 1981; Foo and Yap 1982
Cx. restuans	Wraight *et al.* 1987
Cx. salinarius.	Holck and Meek 1987
Cx. sitiens.	Goettel *et al.* 1982; Balaraman *et al.* 1983; Mottram *et al.* 1989;
Cx. tarsalis.	Mulla *et al.* 1980; Garcia *et al.* 1980; Garcia *et al.* 1982
Cx. theileri.	Chabanenko *et al.* 1992
Cx. tritaeniorhynchus.	Balaraman *et al.* 1983
Cx. vishnui.	Kramer 1984
Culiseta alaskaensis.	Dubitskii *et al.* 1981
Cs. incidens	Beck 1982
Cs. inornata.	Mulla *et al.* 1980; Garcia *et al.* 1982; Spackman 1986
Cs. longiareolata .	Zohdy and Matter 1982; Merdan *et al.* 1986; Farghal 1982
Cs. melanura	Wraight *et al.* 1987
Cs. nigripalpus.	Ali *et al.* 1989
Limatus durhamii.	Lacey and Lacey 1981
L. flavisetosus.	Lacey and Lacey 1981
Mansonia spp.	Chang-Moh *et al.* 1990

Ma. bonneae.	Chang *et al.* 1990; Chang-Moh *et al.* 1990
Ma. dyari.	Lord and Fukuda 1990
Ma. indiana.	Foo and Yap 1982
Ma. titillans.	Lord and Fukuda 1990
Psorophora columbiae.	Mulla *et al.* 1980; Holck and Meek 1987; McLaughlin and Vidrine 1984
P. ferox.	Palmisano 1987
P. varipes	Palmisano 1987
Toxorhynchites splendens.	Lee and Cheong 1988
Tx. amboinensis[1].	Larget and Charles 1982; Misch *et al.* 1987
Trichoprosopon digitatum.	Lacey and Lacey 1981
Tripteroides aranoides.	Huang-Rong *et al.* 1993
Uranotaenia lowii	Beck 1982
Diptera: Anisopodidae	
Sylvicola fenestralis.	Houston *et al.* 1989; Coombs *et al.* 1991
Diptera: Chironomidae	
Chironomus attenuatus.	Garcia *et al.* 1982
C. crassicaudatus.	Ali *et al.* 1981
C. decorus.	Mulla *et al.* 1990; Rodcharoen *et al.* 1991
C. fulvipilus.	Rodcharoen *et al.* 1991
C. kiiensis.	Kondo *et al.* 1995
C. riparius.	Cilek and Knapp 1992
C. tepperi.	Treverrow 1985
C. yoshimatsui.	Kondo *et al.* 1992; 1995
Dicrotendipes pelochloris.	Kondo *et al.* 1995
Dicrotendipes sp.	Rodcharoen *et al.* 1991
Glyptotendipes paripes.	Ali *et al.* 1981
G. tokunagai.	Kondo *et al.* 1995
Hydrobaenus kondoi.	Kondo *et al.* 1992
Limnophyes minimus[1].	Houston *et al.* 1989
Metriocnemus hygropetricus	Houston *et al.* 1989
Orthocladius fuscimanus.	Houston *et al.* 1989
Paralauterborniella elachista.	Rodcharoen *et al.* 1991
Paratanytarsus sp.	Kondo *et al.* 1995
P. grimmii.	Kondo *et al.* 1995
Pentapedilum tigrinum.	Kondo *et al.* 1995
Rheotanytarsus spp.	Molloy 1992; Merritt *et al.* 1989
Rheotanytarsus fuscus.	Palmer 1993
Stictochironomus akizukii.	Kondo *et al.* 1995
Tanytarsus spp.	Ali *et al.* 1981
Tokunagayusurika akamusi[1].	Kondo *et al.* 1992
Diptera: Glossinidae	
Glossina pallidipes[1]	van-der Geest *et al.* 1982
Diptera: Muscidae	
Haematobia irritans.	Temeyer 1984
Diptera: Phlebotominae	
Phlebotomus papatasi.	Barjac *et al.* 1981; Yuval and Warburg 1989
P. argentipes.	Yuval and Warburg 1989
P. perniciosus.	Yuval and Warburg 1989

Diptera: Phoridae

Megaselia halterata.	Kiel 1991

Diptera: Psychodidae

Lutzomyia longipalpis.	Barjac *et al.* 1981; Yuval and Warburg 1989
Psychoda alternata.	Houston *et al.* 1989
P. severini.	Houston *et al.* 1989

Diptera: Sciaridae

Bradysia coprophila.	Osborne *et al.* 1985
Bradysia spp.	Harris 1993
Lycoriella mali.	Cantwell and Cantelo 1984; Kiel 1991

Diptera: Simuliidae

Austrosimulium laticorne.	Chilcott *et al.* 1983
A. multicorne.	Chilcott *et al.* 1983
Cleitosimulium argenteostriatum [=*S. argenteostriatum*].	Car and Kutzer 1988
Cnephia pecuarum.	Atwood *et al.* 1992
Cnetha verna [=*Simulium vernum*].	Olejnicek, 1986
Eusimulium vernum [=*Simulium vernum*].	Colbo and O'Brien 1984
Odagmia ornata [=*Simulium ornatum*].	Olejnicek 1986; Deschle *et al.* 1988
Prosimulium mixtum.	Colbo and O'Brien 1984
Prosimulium tomosvaryi.	Olejnicek 1986
Simulium aokii.	Nakamura *et al.* 1985
S. aureum.	Barton *et al.* 1991
S. chutteri.	Palmer 1993; Palmer *et al.* 1996
S. damnosum.	Guillet *et al.* 1982; Palmer 1993
S. gariepense.	Palmer 1993
S. goeldii.	Habib 1983
S. japonicum.	Nakamura *et al.* 1985
S. jenningsi.	Merritt *et al.* 1989
S. mcmahoni.	Palmer 1993
S. monticola.	Car and Kutzer 1988
S. noelleri.	Car and Kutzer 1988
S. notiale.	Barton *et al.* 1991
S. ochraceum.	Undeen *et al.* 1981
S. pertinax.	Araujo *et al.* 1990; Andrade *et al.* 1991; Castello-Branco *et al.* 1992
S. posticatum.	Welton and Ladle 1993
S. pugetense.	Molloy and Jamnback 1981
S. reptans.	Car and Kutzer 1988; Coupland 1993
S. rorotaense.	Habib 1983
S. tuberosum complex.	Molloy and Jamnback 1981; Car and Kutzer 1988; Merritt *et al.* 1989
S. uchidai	Nakamura *et al.* 1985
S. *variegatum*	Car and Kutzer 1988
S. variegatum group (*S. variegatum/S. argyreatum*).	Coupland 1993
S. venustum/*S. verecundum* complex.	Molloy and Jamnback 1981; Barton *et al.* 1991
S. vernum.	Riley and Fusco 1990
S. vittatum.	Frommer *et al.* 1980; Merritt *et al.* 1989
Stegopterna mutata.	Colbo and O'Brien 1984

Diptera: Tabanidae	
Tabanus triceps.	Saraswathi and Ranganathan 1996
Diptera: Tephritidae	
Ceratitis capitata.	El-Sebae and Komeil 1990
Diptera: Tipulidae	
Tipula paludosa.	Smits and Vlug 1990
T. oleracea.	Smits *et al.* 1993; Smits and Vlug 1990
Acari: Argasidae (ticks)	
Argas persicus.	Hassanain *et al.* 1997
Acari: Ixodidae	
Hyalomma dromedarii.	Hassanain *et al.* 1997
Acari: Pyroglyphidae (mites)	
Dermatophagoides pteronyssinus.	Saleh *et al.* 1991
Mallophaga: Menoponidae	
Menopon gallinae[1]	Lonc and Lachowicz 1987
Nematoda: Meloidogynidae	
Meloidogyne incognita.	Sharma 1994

[1] Low virulence found

Lepidopterans are not generally considered susceptible to *Bti*. However, Ignoffo *et al.* (1981) found *Bti* effective against *Trichoplusia ni*, *Heliothis zea* and *H. virescens*; efficacy was comparable with previous commercial products formulated from *B. thuringiensis thuringiensis* and *B. thuringiensis galleriae*, and it was only one-third to one-tenth less active than current commercial preparations of *Bt. kurstaki*. The LC_{50}s for *Bti* against *T. ni*, *H. zea* and *H. virescens* were 109.6, 19.3 and 27.6 μg/ml, respectively. Corresponding values for *Bt. kurstaki* were 15.9, 2.0 and 7.8 μg/ml.

7.1. LC_{50}

Formulation plays a part in determining final virulence, differences in production and formulation, having up to a 10-fold increase on potency in some situations (Mulla 1990). For all the species tested, the LC_{50} fell within the range 4×10^3 to 4×10^4 viable spores/ml (Barjac *et al.* 1979). The LC_{50}s for *Culex pipiens pallens*, *Anopheles sinensis* and *Aedes albopictus* species were 0.55, 2.05 and 6.37×10^4 spores/ml, respectively (Chen *et al.* 1984). Twenty-four hour LC_{90} values for Teknar against *Limnophyes minimus*, *Metriocnemus hygropetricus*, *Psychoda severini* and *Sylvicola fenestralis* ranged from 1.1×10^5 to 5.5×10^7 spores/cm^2 (Houston *et al.* 1989). However, measurements of virulence based on spore concentrations are not always reliable, since the toxic component of *Bt* formulations is the δ-endotoxin, which does not always correlate directly with spore density in final product.

An experimental *Bti* formulation (R-153-78) gave LC_{90}s of 4.56-9.84 ppm against the midges and LC_{90}s of 0.13-0.24 ppm against mosquitoes (Ali *et al.* 1981). For comparison, against non-target hosts, the 96-h LC_{50} for Bactimos was 12.8 and 13.3 ppm for the amphipod *Elasmopus bampo* and the polychaete *Neanthes arenaceodentata*, respectively (Reish *et al.* 1985).

8. Effects on non-target organisms

Tests against invertebrates, fish and mammals have established that *Bti* has little non-target pathogenicity. Mammalian safety tests show a very low safety risk from direct exposure. One cautionary note with *Bti* is a toxicity of solubilised δ-endotoxin to mice when administered by injection; the solubilised toxin is also cytolytic to human erythrocytes. However, solubilisation occurs at high pH (such as in insect guts) and does not occur in mammalian guts. No effect was found after ingestion by mammals. The closely related *B. cereus* can cause human problems such as gastro-intestinal disorders, but *Bt* strains have not been implicated in such disorders. A specific identification method which separates *Bt* from *B. cereus* would be useful to monitor mammalian populations after *Bti* use.

8.1. Invertebrates

Bti has been extensively studied for effects on non-target organisms and environmental consequences of use with no reported adverse effects (Burges *et al.* 1981). *Bti* has no direct effect on aquatic organisms other than mosquitoes, blackflies and chironomids. Other aquatic organisms, such as shrimps, mites and oysters are generally unaffected (eg. Table 3). This large safety margin of preparations of *Bti* for non-target organisms indicates their suitability for mosquito control programmes in areas where protection of the natural ecosystem is important (Sinegre *et al.* 1980). Two extensive field programmes have failed to find any non-target organisms significantly affected by *Bti* application. In Africa, ten years of applications of *Bti* against simuliids in the *Onchocerciasis* Control Programme (OCP) found little evidence of non-target environmental effects. Also, an extensive spray programme for mosquitoes in Germany had little impact on non-target organisms (Schnetter *et al.* 1981).

Several authors have reviewed the non-target effects of *Bti* (eg. Lacey and Mulla 1990; Becker and Margalit 1993). Field applications have often been monitored for effects on non-target organisms but no significant non-target effects have been reported (eg. Ali 1981; Colbo and Undeen 1980; Molloy and Jamnback 1981; Yameogo *et al.* 1988; Burgoyne 1985; Jackson *et al.* 1994; Miura *et al.* 1980; Hershey *et al.* 1995; Mulligan and Schaefer 1981; Becker and Margalit 1993; Mulla *et al.* 1982; Gharib and Hilsenhoff 1988; Rettich 1983; Sinegre *et al.* 1980; McCracken and Matthews 1997; Merritt *et al.* 1989). Other researchers have examined susceptibility in the laboratory without effects on non-target organisms (eg. Garcia *et al.* 1980; Wipfli and Merritt 1994). Overall, the findings indicate that *Bti* is specific to Nematocera, including mosquitoes, Simuliidae and some chironomid midge larvae. Chironomid midge larvae show only low level susceptibility and in some cases, no effect on chironomid populations was observed under field conditions (Miura *et al.* 1980; Lebrun and Vlayen 1981) and in New Zealand by Chilcott *et al.* (1983). However, in other cases chironomids (Pistrang and Burger 1984; Back *et al.* 1985; de Moor and Car 1986; Sinegre *et al.* 1980; Charbonneau *et al.* 1994) and some Blephariceridae (Back *et al.* 1985) were susceptible in the field.

Many other smaller studies have examined non-target mortality without reporting concerns. Except for moderate mortality among filter-feeding chironomids, *Rheotanytarsus* spp., the results of ten field trials with *Bti* in New York State indicated a wide margin of safety to the chironomid community and other non-target stream insects (Molloy 1992). Merrit *et al.*

(1989) found no detectable non-target effects of *Bti* application against blackflies in Michigan on: (1) invertebrate macro- or micro-drift; (2) numbers of invertebrates in benthic samples; (3) mortality or feeding of drifting and non-drifting insects; (4) growth or mortality of caged *Stenonema* sp. (Ephemeroptera) larvae. There was no measured mortality of non-target organisms (insects in six orders) during tests of *Bti* for the biological control of simuliid larvae in a small stream in Newfoundland (Colbo and Undeen 1980). The effects of *Bti* and methoprene on non-target benthic invertebrates were studied in a divided pond experiment in south-central Minnesota, USA, during the spring and summer of 1989. No evidence of negative effects of larvicide treatment on density or biomass of any invertebrate group was seen, nor was there a treatment-related decrease in richness of benthic invertebrate taxa (Hershey *et al.* 1995).

Out of 39 non-target species collected before treatment in saltmarshes in Florida, only *Notonecta indica* showed a significant decrease in population when *Aedes taeniorhynchus* was treated with *Bti* (Purcell 1981). However, this decline could have been due to other causes, such as lack of food.

Inoculum densities above recommended levels can cause non-target effects among a few groups. *Chironomus plumosus* was susceptible only to doses much higher than those used against mosquito larvae (Larget *et al.* 1981). In another study, chironomid larvae were almost as susceptible to *Bti* as were culicid larvae, the LC_{50} for an exposure period of 1 day being 0.1 mg/litre (Sinegre *et al.* 1980).

Bti is non toxic to bees (Krieg *et al.* 1980). There are some reports of non-target organisms showing susceptibility in the laboratory. *Bti* has been found to affect population growth of the free-living nematode *Turbatrix aceti* (Meadows *et al.* 1990) and weakens nematode egg shells (Wharton and Bone 1989). In the host list (Table 2), some tick and mite species and horn flies have shown susceptibility when inoculated in the laboratory (Temeyer 1984).

TABLE 3: Organisms not susceptible to *Bacillus thuringiensis israelensis*

Higher order	Genus and species	Common name	Reference
ACARI	*Hydrachnella* sp.		Becker and Margalit 1993
	Hydracarina sp.	mite	Beck 1982
	Hydrachna sp.	mite	
AMPHIBIANS	*Hylo regilla*	tree frog tadpole	Abbott Laboratories; Garcia *et al.* 1980
	Bufo sp.	toad tadpole	
	Bufo bufo		Becker and Margalit 1993
	Bufo viridis		
	Bufo calamita		
	Taricha torosa	California newt	Abbott Laboratories; Garcia *et al.* 1980
	Rana temporaria		Paulov 1985a & b
	Triturus alpestris		Becker and Margalit 1993
	Triturus vulgaris		
	Triturus cristatus		
	Bombina variegata		
	Rana esculenta		
FISH	*Gambusia affinis*	mosquito fish	Abbott Laboratories; Garcia *et al.* 1980
	Lucania parva	rainwater killifish	
	Gasterosteus wheatlandi	twospine stickleback	
	Lepomis macrochirus	bluegill	Christensen 1990a
	Salvelinus fontinalis	brook trout	Wipfli *et al.* 1994
	Salmo trutta	brown trout	
	Oncorhynchus mykiss	rainbow trout	Wipfli *et al.* 1994; Christensen 1990b
	Pseudomugil signifer	Pacific blue-eye fish	Brown *et al.* 1998
	Poecilia reticulata	larvivorous fish	Mittal *et al.* 1994
	Tilapia nilotica		Lebrun and Vlayen 1981
	Esox lucius		Becker and Margalit 1993
	Cyprinus carpio		
	Perca fluviatilis		
	Ambloplites rupestris	rock bass	Merritt *et al.* 1989
	Epiplatys sp.	killifish	Beck 1982
	Cyprinoidei	goldfish	
	Cyprinodon variegatus	sheephead minnow	Christensen 1990c
CRUSTACEANS	*Orconectes limosus*	crayfish	Becker and Margalit 1993
	Amphipoda		
	Gammaridae sp.	scuds	Abbott Laboratories; Garcia *et al.* 1980
	Hyalella azetaca	sideswimmer	Abbott Laboratories; Garcia *et al.* 1980
	Gammarus duebeni	Salt-marsh	Roberts 1995
	Gammarus pulex	crustaceans	Becker and Margalit 1993
	Hyallela azteca		Gharib and Hilsenhoff 1988
	Palaemonidae		
	Leander tenuicornis		Brown *et al.* 1996
	Palaemonetes varians		Roberts 1995
	Decapoda		
	Hemigrapsus sp.	purple shore crab	Abbott Laboratories; Garcia *et al.* 1980
	Anostraca		
	Artemia salina	fairy shrimp	Abbott Laboratories; Garcia *et al.* 1980
	Conchostracans		
	Eulimnadia texana	clam shrimp	Mulla 1990
	Cladocera		
	Simocephalus vetulus	water flea	Abbott Laboratories; Garcia *et al.* 1980
	Daphnia		Ali 1981
	Daphnia magna		Lebrun and Vlayen 1981
	Daphnia pulex		Becker and Margalit 1993
	Moina rectirostris		Mulla 1990

	Moina macrocopa		Beck 1982
	Chirocephalus grubei	anostacan snail	Becker and Margalit 1993
	Ostracoda		
	Ostracoda		Becker and Margalit 1993; Ali 1981
	Cypridae sp.	seed shrimp	Abbott Laboratories; Garcia *et al.* 1980
	Copepoda		
	Macrocyclops sp.	copepods	Abbott Laboratories; Garcia *et al.* 1980
	Macrocyclops albidus	Larvivorous	Marten *et al.* 1993
	Mesocyclops longisetus	copepods	
	M. ruttneri		
	Acanthocyclops vernalis		
	Cyclops spp.		Ali 1981; Beck 1982
	Cyclops strenuus		Becker and Margalit 1993
	Isopoda	marine sow bug	Abbott Laboratories; Garcia *et al.* 1980; Knepper and Walker 1989
	Asellus aquaticus		Becker and Margalit 1993
INSECTS	**Ephemeroptera**		
	Baetis sp.		Ali 1981
	Callibaetis sp.	mayfly nymphs	Abbott Laboratories; Garcia *et al.* 1980
	Callibaetis pacificus		Mulla 1990
	Stenonema		Merrit *et al.* 1989
	Cloeon dipterum		Becker and Margalit 1993
	Leptoplebia sp.		Beck 1982
	Caenis lactea		
	Ephemera danica		
	Odonata		
	Ischnura sp.	damselfly nymphs	Abbott Laboratories; Garcia *et al.* 1980
	Anax sp.		
	Erythemis simplicicollis		Painter *et al.* 1996
	Tarnetrum corruptum		Aly and Mulla 1987
	Enallagma civile		
	Ischnura elegans		Becker and Margalit 1993
	Symetrium striolatum		
	Orthetrum brunneum		
	Cordulia sp.	dragonfly nymph	Beck 1982
	Hemiptera		
	Trichocorixa reticulata	water boatmen	Abbott Laboratories; Garcia *et al.*, 1980
	Hesperocorixa leavigata		
	Trichocorixa		
	Corixidae		
	Bueona scimitra	backswimmer	Abbott Laboratories; Garcia *et al.* 1980
	Notonecta kirby	backswimmer	Abbott Laboratories; Garcia *et al.* 1980
	Notonecta undulata	backswimmers	Aly and Mulla 1987
	Notonecta glauca		Beck 1982; Olejnicek and Maryskova 1986
	Buenoa antigone		Quiroz-Martinez *et al.* 1996
	Pleidae	pygmy backswimmer	Abbott Laboratories; Garcia *et al.* 1980
	Micronecta meridionalis		Becker and Margalit 1993
	Sigara striata	water bug	Beck 1982; Becker and Margalit 1993
	Sigara lateralis		
	Ranatra sp.		Beck 1982
	Ilyocoris cimicoides		Becker and Margalit 1993
	Anisops varia		
	Heteroptera		
	Plea leachi		Becker and Margalit 1993

Coleoptera		
Tropisternus salsamentus	scavenger and predaceous water beetle	Abbott Laboratories; Garcia *et al.* 1980
Peltodytes edentulus,		Gharib and Hilsenhoff 1988
Haliplus immaculicollis		
Hydroporus undulatus		
Laccophilus maculosus		
Tropisternus sp.		Abbott Laboratories; Garcia *et al.* 1980
Dytiscidae		Abbott Laboratories; Garcia *et al.* 1980
Hyphydrus ovatus		Becker and Margalit 1993
Guignotus pusillus		
Coelambus impressopunctatus		
Hygrotus inaequalis		
Hydroporus palustris		
Ilybius fuliginosus		
Rhantus pulverosus		
Rhantus consputus		
Hydrobius fuscipes		
Anacaena globulus		
Hydrophilus caraboides		
Berosus signaticollis		
Bombyx mori		Nataraju *et al.* 1993
Baeosus sp.	beetles	Beck 1982
Coelambus sp.		
Gyrinidae sp.		
Laccophilus sp.		
Trichoptera		
Mystacides alafunbriata	caddisfly larvae	Abbott Laboratories; Garcia *et al.* 1980
Several species	caddisfly nymphs	Abbott Laboratories; Garcia *et al.* 1980
Limnephilus flavicornis (F.),	caddisfly	Lebrun and Vlayen 1981
Limnophilus sp.		Becker and Margalit 1993
Phryganea sp.		
Diptera		
Ephydra riparia complex	brinefly larvae	Abbott Laboratories; Garcia *et al.* 1980
Dicraneta sp.	cranefly larvae	
Chelifera sp.	dancefly larvae	
Musca domestica	housefly	Vankova 1981; Larget *et al.* 1981
Procladius freemani	tanypodine midges	Mulla *et al.* 1990
P. sublettei		
Tanypus sp.		
Chanoborus sp.	gnat	Beck 1982
Drosophila melanogaster	fruit fly	
Erioischia brassicae	cabbage maggot	
Toxorhynchites splendens	predatory mosquito	
Culicoides sp.	predatory midge	
Chironomus plumosus	midge	
Chironomid larvae		
Lucilia cuprina		Akhurst *et al.* 1997
Plecoptera		
Several species	stonefly nymphs	Garcia *et al.* 1980; Kreig *et al.* 1980
Hymenoptera		
Apis mellifera	honey bee	Abbott Laboratories; Garcia *et al.* 1980
Trichogramma evanescens	egg parasite	Beck 1982

FLATWORMS	**Tubellaria**		
	Dugesia dorotocephala	flatworm	Abbott Laboratories; Garcia *et al.* 1980
	Dugesia tigrina		Becker and Margalit 1993
	Bothromesostoma personatum		
	Platyhelminthes		
	Dugesia tigrina	planarian flatworm	Beck 1982
EARTHWORMS	**Nadididae**	earthworms	Abbott Laboratories; Garcia *et al.* 1980
	Lumbricidae		
	Tubifex sp.		Becker and Margalit 1993
	Helobdella stagnalis	Leech	
	Oligochaeta	roundworm	Beck 1982
NEMATODA	*Neoaplectana carpocapsae*	entomopathogenic	Poinar *et al.* 1990
	Heterorhabditis heliothidis	nematodes	
MOLLUSCS	Gastropoda		Abbott Laboratories; Garcia *et al.* 1980
	Physa sp.	freshwater snail	
	Pelecypoda sp.	mussels	
	Taphius glabratus	snail	Larget *et al.* 1981
	Physa acuta		Becker and Margalit 1993
	Anisus leucostomus		
	Bathyomphalus contortus		
	Hippeutis complanatus		
	Pisidium sp.		
	Aplexa hypnorum	moss bladder snail	Beck 1982; Becker and Margalit 1993
	Galba palustris	marsh snail	
	Bithynia tentaculata	snail	Beck 1982
	Planorbis planorbis	snail	
	Radix sp.	ear pond snail	Beck 1982
	Vivparus contectus	snail	
	Ostrea edulis	oyster	
	Mytilus edulis	blue mussel	
Cnidaria			
	Hydra sp.		Becker and Margalit 1993
Rotatoria			
	Brachionus calyciflorus	zooplankton	Becker and Margalit 1993

Abbott Laboratories: from 1993 publicity on VectoBac®.

8.2. Fish and amphibians

No effect has been found on fish present during *Bti* application, nor in direct exposure experiments (see Table 3). Lee and Scott (1989) examined the acute toxicity of *Bti* to the mummichog, *Fundulus heteroclitus*, in the laboratory compared to other common mosquito larvicidal pesticides. *Bti* was the least toxic of the five preparations, with a 96h LC_{50} of 980 mg/l compared to temephos, the most toxic, at 0.04 mg/l (Table 4). Using fenoxycarb and *Bti* together was more toxic than fenoxycarb alone, suggesting mixtures of control agents should be examined carefully.

TABLE 4: Acute toxicity of mosquito larvicides to *Fundulus heteroclitus* (from Lee and Scott 1989).

Insecticide	Mean 96h LC_{50} (mg/l)	95% confidence limits (mg/l)
Temephos	0.04	0.02-0.05
Fenoxycarb	2.14	2.01-2.27
Diflubenzuron	32.99	29.01-37.52
Methoprene	124.95	90.01-171.64
VectoBac (*Bti*)	980.00	730-1330
Fenoxycarb/VectoBac	1.55	1.40-1.72

Bti application against blackflies in Michigan found no effect on mortality or weight change of caged rock bass (*Ambloplites rupestris*) or fish numbers and species composition (Merritt *et al.* 1989). Christensen (1990a, b and c) in laboratory studies found no effect of *Bti* on bluegill sunfish (*Lepomis macrochirus*), sheephead minnow (*Cyprinodon variegatus*) and rainbow trout (*Oncorhynchus mykiss*) when exposed to 1.3-1.7 x 10^{10} cfu/g of diet.

No effect of *Bti* on amphibians has been reported (Table 3). The World Health Organisation (1992) reviewed a number of laboratory and field studies that examined the impact of *Bt* on frogs, newts, salamanders and toads in which no adverse effects were recorded.

8.3. Mammalian toxicity

*Bt*s have been used for many years in agriculture and forestry situations with no reported problems. Most workers in the field of safety of *Bti* have found no reason to discontinue the use of *Bti* on grounds of risk to human health (eg Siegel and Shadduck 1990; Becker and Margalit 1993; Drobniewski 1994; World Health Organisation WHO - Becker 1992).

There have been concerns about the mammalian pathogenicity of the genus *Bacillus* because one species, *B. anthracis*, is a virulent mammalian pathogen and others, including *B. cereus*, have been isolated from wounds (see Warren *et al.* 1984). Therefore, there has been extensive safety testing of *Bt*s, including *Bti*, and very few problems have been encountered. In early studies on *Bt* safety, rats fed 2 x 10^{12} spores/kg and human volunteers fed 3 x 10^{9} spores /day for 5 days showed no ill effects (Drobniewski 1994). *Bt* has been isolated from a fatal case of bovine mastitis and a controversial case of a corneal lesion (see Siegel and Shadduck 1990). Siegel and Shadduck (1990) reported on "maximum challenge" tests for mammalian toxicity of *Bti;* a total of 6 isolates of *Bti* were administered in several ways (oral, intraperitoneal, subcutaneous, aerosol, intra-cerebral and ocular) to mice, rats and rabbits. Intraperitoneal

injection of *Bti* was the only route that led to significant mortality, but as it only occurred at high concentrations (10^7 cfu per rat), it was concluded that *Bti* was not a significant mammalian pathogen. Studies by Siegel and Shadduck (1990) included immunosuppressed mice which showed that an intact immune system was not necessary for successful clearance of *Bti* although athymic mice had higher levels of *Bti* in their spleens than euthymic mice. They further concluded that *Bti* "can safely be used in environments where human and mammalian exposure is likely to occur" (pg 215). All acute studies have been negative. Ingestion of up to 10^{12} bacteria/ animal had no effect on mice and rats. No anaphylactic shock was observed in guinea pigs. *Bti* failed to multiply in mammals and ingested cells were eliminated rapidly (Siegel and Shadduck 1990).

A number of other studies in several countries have found no effect on warm-blooded mammals (eg. Ignatiev *et al.* 1988; Halkova *et al.* 1993; Tsai-San *et al.* 1996). One concern raised about *Bti* is that the solubilized δ-endotoxin of *Bti* activated in the laboratory has been demonstrated as toxic to mice when administered by injection and cytolytic to human erythrocytes. However, the δ-endotoxin was not toxic when administered *per os* and, as the mammalian gut is not alkaline, the toxin is not activated. (Siegel and Shadduck 1990). The δ-endotoxin is only solubilised under alkaline conditions, such as an insect midgut. Other results from tests on human erythrocytes *in vitro* suggested that the *Bti* insecticidal crystal proteins are safe to humans when they are intact and not when solubilised (Rani *et al.* 1996). Extensive studies on birds and mammals with high doses of unsolubilised *Bti* spores and crystals have shown no effect (Meadows 1993). No effect has been found on birds in other laboratory studies. Lattin *et al.* (1990a and b) found on toxicity, pathogenicity or weight loss when *Bti* was administered orally in large doses (3.4-6.2 x 10^{11} cfu/kg/day for 5 days) to young brown quail (*Colinus virginanus*) and young mallards (*Anas platyrhynchus*).

The close relationship between *Bt* and *B. cereus* led Damgaard (1995) to investigate the levels of diarrhoeal enterotoxin produced by strains isolated from various *Bt* products. He found that all strains produced diarrhoeal enterotoxin, including the *Bti* based products, Bactimos® and VectoBac®. These two products had titres of 242 and 120 respectively compared to *B. cereus* standard of 1629. The presence of diarrhoeal enterotoxin in *Bti* products is of some concern without tests to distinguish which *Bacillus* species is the cause of gastro-intestinal problems. In future, works such as Damgaard (1995), Jackson *et al.* (1995) and Warren *et al.* (1984) recommended careful screening of *B. cereus* cases for presence of paracrystals indicative of *Bt.*

There have been very few isolated cases where *Bti* has been implicated in mammalian clinical effects. In one case, a research student in England accidentally punctured his hand with a hypodermic needle containing a suspension of spores and endotoxin crystals of *Bti* together with the vegetative bacterium *Acinetobacter calcoaceticus* var. *anitratus*, the latter a normal human skin bacterium which seldom causes serious soft-tissue infection (Warren *et al.* 1984). He recovered after 5 days. Sample and Buettner (1983) reported a corneal ulcer attributed to *Bt* after accidental splashing of a commercial preparation (Dipel, *Btk*) into an eye. Treatment with gentamycin cured to ulcer. Both these cases resulted from exposure to highly concentrated inocula.

Jackson *et al.* (1995) reported the isolation of *Bt* from four patients suffering gastro-enteritis. The isolations were originally identified as *B. cereus*. It was not confirmed that the *Bt* caused the gastro-enteritis. The high genetic similarity between *B. cereus* and *B. thuringiensis* often raises alarms with those clinicians with only a passing knowledge of the group.

Green *et al.* (1990) monitored cultures obtained for routine clinical purposes from people who lived in a spray zone of *Btk* used against gypsy moth in Oregon, USA. Over a 2 year period, 55 *Bt*-positive cultures were identified (around 120,000 people lived in the sprayed area), 95% of which were thought to be probable contaminants. Of the remaining three patients, *Bt* could not be determined to be a pathogen. All had pre-existing medical problems, suggesting *Bt* may have some risk for immuno-compromised hosts. Damgaard *et al.* (1997) isolated *Bt* from infections in burn wounds, contamination which occurred from water used in the treatment of the wounds. They were non-flagellate *Bt*s (all strains used in pesticides are flagellate) and showed no activity against insects. The patients had extensive burns and were therefore immuno-suppressed. Recently, Hernandez *et al.* (1998) presented a case of a French soldier with wounds infected by *Bacillus thuringiensis konkukian* serotype H34, a new serotype from Korea. Whether this was simply a contaminant or infection by *Bt* is unclear. However, the report includes experimental evidence of the ability of serotype H34 to infect immuno-suppressed mice after cutaneous inoculation.

Proven cases of *Bt* causing clinical disease in mammals remain extremely rare and after reviewing available data, Drobniewski (1994) concluded that the risk to public health from *Bti* was extremely small. Risks to immuno-suppressed individuals may be higher than to the general public, as is the case with most bacterial contaminants. During a spray programme, monitoring which incorporates specific identification methods for the *Bacillus* spp., capable of identification to the level of isolate, would greatly assist evaluation and monitoring the effect of use. Such a monitoring programme should be conducted in conjunction with health monitoring in the spray areas to detect any possible symptoms resulting from spraying.

Similar safety data to that of Drobniewski (1994) have been presented in New Zealand for the use of *Btk* against tussock moth during the aerial application programme, "Operation Evergreen". A follow-up study on the safety of *Btk* spraying over an urban area found no increase in health problems (http://webnz.com/evergreen/Health.html & http:/www.moh.govt.nz/9605.htm). The Ministries of Forestry and Health commissioned Auckland Healthcare services to carry out a health risk assessment following "Operation Evergreen". The HRA found that, by September 1997 there were:

- no miscarriages or premature deliveries associated with *Btk* spraying.
- no allergies caused by bacteria.
- no increased attendance at Baycare health centre as a result of spraying.
- no increased incidence of measles or meningococcal disease .

The Government has commissioned a health surveillance programme to run until 1999, monitoring any health effects which might arise following tussock moth spraying.

9. Persistence and activity in the environment

Persistence of an insecticidal agent has a strong influence on the risk to non-target organisms and the environment. A persistent agent is more likely to control the target pest, but is also more likely to have unintended effects, such as with DDT. *Bti* does not usually persist very long after application. Generally, toxicity to mosquitoes persists for only days and efficacy can be reduced within 24 hours. Formulation and application techniques can extend the persistence of activity for over one month in some situations, but activity remains sensitive to factors like UV degradation.

9.1. Persistence

Bti has many properties which make it a superior mosquito control agent; for example it is environmentally benign, easily produced and host specific. One characteristic which contributes to a lack of environmental risk is lack of persistence. *Bti*, in common with other *Bt*s, lacks the ability to recycle readily in insect populations. Generally, *Bti* persists for days rather than months as reported for some of the more toxic chemicals for mosquito control. Early reports showed that a primary powder formulation of *Bti* (serotype H-14) had virtually no residual effect against mosquito larvae beyond application, although the delta-endotoxin remained chemically stable in neutral and acid waters (Sinegre *et al.* 1980). Some reports suggest that *Bti* activity reduces in effectiveness within 24 h, while others have shown effectiveness lasting at least one week.

While *Bti* gives good control initially (within days of application), it does not appear to persist in most situations. Young mosquito larvae can appear 3-4 days after treatment of habitats (Mulla 1990). In a four weekly application regime, Mulla (1985) found no residual activity after 2 weeks. Beehler *et al.* (1991) found the application of 100 ppm VectoBac® to *Ae. triseriatus* in scrap tyres caused no mortality 4 weeks after application. In pottery water jars on the Ivory Coast, treatment of mosquitoes with 1 or 5 mg/litre *Bti* lost its effectiveness within 1-4 weeks (Hougard *et al.* 1985). Laboratory studies in France with 4th-instar larvae of *Ae. aegypti* showed that suspensions prepared from 2 preparations of *Bti* retained their activity against the larvae for only 3-5 days, but this period could be extended by leaving dead larvae in the medium (Larget 1981). In contrast, the *Bti* product Acrobe® provided significant control for 47 days in tyres in Florida (Becnel *et al.* 1996).

The specificity of *Bti* reduces the possibilities for the bacterium to survive outside the aquatic environment. Studies on *Bt* growth outside the insect indicate little or no multiplication outside the host (see Meadow 1993 for review). *Bt* rarely spreads from the point of inoculation (Burges 1973), although non-target organisms, such as fish (Snarski 1990), have been shown to disseminate *Bti*.

9.2. Effect of formulation on persistence

Long lasting pesticides will have greater use in eradication campaigns than short lived products. Formulation methods are constantly being developed to overcome the short persistence of *Bti* products. Extended persistence with *Bti* is possible through use of improved formulations. Briquettes may result in more prolonged control than liquid formulations as these products have greater persistence through slow release. Bactimos (*Bti*) briquettes provided complete control of *Ae. aegypti* adult emergence in Malaysia in plastic containers of water for up to 75 days post-treatment, while Altosid (7.9% methoprene) briquettes gave 100% mortality for up to 122 days (Sulaiman *et al.* 1991). In Martinique, Bactimos briquettes controlled larvae of *Ae. aegypti* for 10 days in drums (using one-quarter briquette), and larvae of *Culex quinquefasciatus* for 10 days in a stream with stagnant water (1.5 briquettes for 11 m^2) (Yebakima 1991). Half a Bactimos briquette in 20 litres of tap water gave effective control (90-100% mortality) of 2nd instar larvae of *Ae. aegypti* in the laboratory for 29 days in the absence of soil and 16 days with soil present, but its efficacy decreased as the briquette broke up and sank (Saleh 1989). Recommended rates of some *Bti* formulations vary according to the depth of water (eg. Table 10.1 in Cowley *et al.* 1998).

Other factors affecting persistence of *Bti* include UV (eg. Bagci and Shareef 1989), agitation, sedimentation (eg. Standaert 1981), water quality and constituents such as pollutants, environmental conditions such as pH and temperature (eg. Standaert 1981; Cokmus and Elciin 1995), and target host and microbial competition. Sheeran and Fisher (1992) found agitation to be the most important factor in maintaining persistence of *Bti* cells and the bioavailability of the *Bti* toxin. In their study, sediment acted to decrease efficacy against 3rd-instar larvae of *Ae. aegypti* by increasing settling of the toxic particles, but did not decrease the persistence of the *Bti* cells themselves.

Excessive VectoBac® 12AS dosages did not significantly extend the duration of effectiveness for controlling mosquito larvae, although treated populations never recovered to the same level due to increased predation and lack of breeding sites (Mulla *et al.* 1993). A 5-fold increase above standard label dosage still failed to prevent *Culex* spp. emergence in Kenya (Logan and Linthicum 1992). While doubts exist over the effectiveness of *Bti* against *Culex* spp., it has been successfully used against some *Culex* species (Becker and Margalit 1993; Brown *et al.* 1998).

9.3. Application methods and rates

Application rates for *Bti* products vary depending on the type of formulation (liquid, briquettes, etc) and have been summarised in the National Mosquito Pest Management Strategy (Cowley *et al.* 1998). Application rates of all products depend on the surface area of water to be treated (not depth of water), larval density and stage and water quality.

Choice of application method has implications for the environmental risk as it determines the spread of *Bti*, the range of non-target organisms exposed and even the deposition of dose. Applications can be made by pouring into water, spraying (including ultra low volume), aerial techniques and by specialist formulation such as briquettes. Against Culicidae in Louisiana, roadside ditches are treated with VectoBac® 12AS every 10-12 days from a jeep equipped with a CO_2 pressurised system (Palmisano 1987). A truck-mounted forced-air generator (Scorpion 20) was evaluated in an urban residential area of Santo Domingo, Dominican Republic. This method has the potential to simultaneously control adults and larvae (Tidwell *et al.* 1994). Aerial application has been used in several control efforts (eg. Schmidt 1988; Knepper *et al.* 1991; Palmisano 1987; Yates 1985) and often ultra low volume technology is used in conjunction with aerial application. For example, low volume aerial applications of Bactimos (1.17 litres/ha) and Teknar (at 1.17 litres/ha) afforded good control of *Ae. sollicitans* larvae in Maryland (Lesser 1984).

10. Gene transfer

Most toxin encoding genes in *Bti* are based on plasmids, extra-chromosomal DNA which can be transferred from cell to cell. Studies on transfer of genetic elements in the environment are in their infancy, but it has been demonstrated in the laboratory that *Bt* strains can transfer toxin-encoding plasmids to other bacterial species. There is no evidence that such transfer has ever resulted in a dangerous new combination in nature.

A relatively new area of environmental concern regarding the use of *Bt*s is the mobility of toxin genes. The majority of *Bt* toxin genes are encoded by extra-chromosomal DNA (eg. plasmids) and such DNA is known to be exchanged between bacteria by conjugation and transformation. Therefore, several recent studies have examined the mobility of toxin-encoding plasmids between *Bt* and other bacteria.

Under laboratory conditions, plasmids can be shuttled between *B. thuringiensis*, *B. cereus* and *B. anthracis* (Battisti *et al.* 1985; Wiwat *et al.* 1990). Plasmid transfer between strains of *Bt* and other bacteria was demonstrated in infected lepidopterans at rates similar to those obtained by *in vitro* plasmid transfer events (Jarrett and Stephenson 1990). Muller-Cohn *et al.* (1994) have shown conjugation among *Bt*s in sterile nutrient amended soil and in insect larvae. Conjugation was only observed if at least one of the *Bt* strains was toxic to the insect larvae.

Jarrett and Stephenson (1990) isolated sporeforming soil bacteria and used the vegetative cells as recipients in *in vitro* conjugation experiments. Up to 3% of the transconjugants (cells receiving DNA) gained the ability to produce protein crystals.

These experiments demonstrate that there is potential for conjugative DNA transfer from *Bt* to indigenous soil bacteria. However, a prerequisite for gene transfer is the presence of metabolically active bacteria and *Bt* is primarily present in the environment as spores which cannot participate in gene transfer. Therefore, gene transfer could be expected to occur in habitats with actively growing *Bt*, primarily insect cadavers. In addition, genetic transfer is unlikely to take place at high rates in soil or water because of the low population levels of bacteria (Meadows 1993). There may also be environmental barriers between such transfer in nature.

Genetic transfer may also be mediated by transposons, DNA structures that are able to move around in the genome. While transposon structures are well recognised in *Bt* (Malvar and Baum 1994), no investigations of transposon mediated conjugal transfer of genes involving *Bt* in natural environments have been described. Transformation, where a piece of naked DNA is taken into a foreign cell and incorporated into the genome, has been demonstrated for *Bacillus* (Reanney *et al.* 1982).

Despite the widespread use of *Bt*, no examples of *Bacillus* with unexpected pathogenic properties which could be due to gene transfer have been reported (Meadows 1993).

11. Resistance

Resistance to *Bt* has arisen, especially in Lepidoptera. Despite many years of extensive use and several laboratory studies aimed at generating resistance to *Bti*, only low level resistance has ever been detected with this dipteran-specific subspecies. The reasons could be related to the synergistic activity of the multiple toxic proteins of *Bti*.

While resistance has arisen to some strains of *Bt*, eg. *Btk* against the lepidopteran pests *Plodia* and *Plutella* (Marrone and Macintosh 1993), the use of *Bti* has not resulted in the development of resistance in host populations. Laboratory attempts to induce resistance by continual exposure to *Bti* have generally failed to detect resistance (eg. Lee and Cheong 1985; Goldman *et al.* 1986; Gharib and Szalay-Marzso 1986) or selected for only low level resistance (Georghiou 1984; Georghiou and Wirth 1997).

Constant exposure of *Ae. vexans* in the field for 10 years in Germany resulted in no difference in the level of resistance in exposed and unexposed populations (Becker and Margalit 1993). Similar results were reported for blackflies (Kurstak *et al.* 1989). In a conflicting report by Han (1988), selection experiments generated some resistance (6-11 x) after 27 generations of *Cx. p. pallens*; however resistance was unstable and 50% of mosquito lines which developed resistance reverted after 3 generations.

The lack of resistance development to *Bti* could be due to its complex mode of action, involving synergistic interaction between up to four proteins (Becker and Margalit 1993). Use of a single protein from *Bti* for mosquito control resulted in resistance after only a few generations in the laboratory (Gill, in Becker and Margalit 1993). Georghiou and Wirth (1997) also showed that resistance could be raised in only a few generations when a single *Bti* toxin was used, and was progressively more difficult to raise in mosquitoes with combinations of two and three toxins. When all four *Bti* toxins were used, resistance incidence was "remarkably low".

12. Host and environmental factors affecting efficacy

The effect of biotic (biological) and abiotic (environmental) conditions on the efficacy of *Bti* must be considered in the context of environmental impact. As a live bacterium, *Bti* is susceptible to a number of environmental and host influences. Some of these affect persistence and therefore can result in reduced risk to non-target organisms. Others, such as distribution by non-susceptible hosts, can influence the area at risk by an application of *Bti.* Generally, *Bti* effectiveness is reduced by low temperatures but is not overly dependent on water quality. The feeding behaviour of insects greatly influences the amount of inoculum ingested, which in turn influences susceptibility. The physical factors in the area of application, such as the amount of vegetation present, can also influence efficacy.

12.1. Feeding behaviour of host

Culex usually feed throughout the water column and *Aedes* are regarded as substrate feeders, both of which allows the settling particles of *Bti* to be ingested to varying extents. *Anopheles* primarily feed at the surface where *Bti* remains for only a short time (Mulla 1990 and references therein). In addition, filtering rates vary between genera and species. For example Aly (1988) showed that in the absence of *Bti*, larvae cleared the suspensions with constant relative filtration rates of 632 (*Ae. aegypti*), 515 (*Cx. quinquefasciatus*) or 83.9 µl/larva/h (*An. albimanus*). Generally *Anopheles* spp. are less susceptible to *Bti* than *Culex* or *Aedes* (see section 7.1). Formulations have been developed which overcome this difference to some extent. Two commercially available formulations of VectoBac (not available in New Zealand yet), VectoBac® G (floating granules) and VectoBac® CG (sinking granules), have been designed to maximise contact between active *Bti* toxin and larvae in the feeding zone for different species of mosquito. Floating formulations, such as the microlipid encapsulation method, have shown similar activity against C*ulex* and *Anopheles*, while unformulated *Bti* is less active against *Anopheles* than *Culex.*

12.2. Inoculum and host density

Inoculum and host density also play a role in efficacy. As has been demonstrated with many host systems, the higher the host density, the greater the *Bti* dose required to kill (eg. Farghal *et al.* 1983; Chen *et al.* 1984; Becker *et al.* 1992). Mulla (1990) estimated that 1.5-2 x more inoculum would be required against 50-100 larvae/dip than 5-20 larvae/dip to give the same mortality. Use of VectoBac® 12AS against *Ae. albopictus* was less effective against high population densities (200-250 larvae/litre medium)(Chui *et al.* 1993) and increasing densities of 2nd-instar larvae of *Ae. aegypti* population led to a decrease in the proportion of dead individuals after treatment with *Bti* (Alekseev *et al.* 1983).

12.3. Developmental stage of larvae

For almost all species tested, increasing age of the larvae resulted in reduced susceptibility in mosquito and blackflies (eg. Mulla *et al.* 1980; Wraight *et al.* 1981; Farghal 1982; Chen *et al.* 1984). Mosquitoes go through four instars and comparative figures of mg/l of active product

can vary up to 10 fold to kill 4th instars compared to 2nd instars (Mulla 1990). Often, the final instar is feeding little or has ceased to feed, affecting the amount of inoculum ingested. Pupae and pre-pupae do not feed and are therefore not affected by *Bti*. Mortalities were consistently higher among smaller (second-fifth instar) *Simulium* larvae than seventh instar, with respective LC_{90} values at 11.5°C of 111 ppb and 615 ppb (Molloy *et al.* 1981). Even against the chironomid *Chironomus tepperi* in Australia, the activity of a wettable powder formulation of Bactimos against 4th-instar larvae had an LC_{90} of 0.79 ppm., about 4 times that for the 1st instar (Treverrow 1985).

12.4. Other organisms

Other organisms, especially filter feeders, can reduce the number of *Bti* cells available and therefore reduce efficacy. For example, competition in food intake by filter feeding *Daphnia curvirostris* resulted in lower mortality of mosquito larvae after *Bti* applications (Becker *et al.* 1992).

12.5. Water quality

Water quality, including presence of pollutants, salinity, organic and inorganic particles can all influence *Bti* efficacy. There appears to be a direct correlation between organic pollution and dosage required to kill mosquitoes. Extraneous material apparently results in less *Bti* being ingested, resulting in reduced efficacy (Bakr *et al.* 1986; Mulla *et al.* 1990; Becker and Margalit 1993). The presence of free chlorine in the water can inhibit or destroy the endotoxin and a clear inverse correlation has been observed between the amount of chlorine in the water and larval mortality by Sinegre *et al.* (1981). The presence of soil significantly reduced larval mortality, probably by assisting sedimentation and unavailability of *Bti* (Sinegre *et al.* 1981c; Essen *et al.* 1982). Ramoska *et al.* (1982) found the efficacy of spore-crystal formulations of *Bti* against larvae of *Cx. quinquefasciatus* and *Ae. aegypti* in the laboratory decreased when in aqueous environments containing a concentration of soil or clay particles greater than or equal to 0.5 mg/ml. In another study, sludge from soil decreased the effectiveness of *Bti* more than decomposing organic matter, inorganic mud or silica gel (Margalit and Bobroglo 1984). Tannic acid also reduced *Bti* efficacy at concentrations as low as 0.25 mM (425 mg/litre) (Lord and Undeen 1990).

One percent salinity did not alter the character of the correlation between dose and mortality of *Ae. aegypti* and *Cx. pipiens* when exposed to *Bti*, so the doses effective in freshwater may be used in brackish water. However, significant species-related differences were observed in the effect of salt on activity (Rasnitsyn *et al.* 1993).

12.6. Temperature

Water temperature has a marked effect on *Bti*, with decreased toxicity with reducing water temperature (eg. Wraight *et al.* 1981; Atwood *et al.* 1992; Mulla *et al.* 1980). Low water temperature reduces the amount of feeding by target larvae which is probably directly related to reduced *Bti* virulence (Walker 1995). Sinegre *et al.* (1981) found in bioassays with the larvae of *Cx. pipiens* and *Ae. aegypti* that larval mortality caused by *Bti* was not influenced by water temperatures of 19-33°C, but the mortality rate was lower at temperatures below 19°C and higher above 33°C. Low temperature (5°C) yielded 10-fold higher LC_{50} and LC_{90} values against mosquitoes in bioassays compared with those conducted at a high temperature (25°C)(Becker *et al.* 1992). Third-instar *Ae. stimulans* slowed but did not stop feeding at 0

and 4°C compared to 22°C, with comparative LC_{50} values to *Bti* of 0.1 ppm at 22°C, 0.2 ppm at 4°C and 0.9 ppm at 0°C (Walker 1995). Eldridge *et al.* (1985) found *Bti* effective against snow pool mosquitoes at temperatures as low as 5°C. Against another insect, Atwood *et al.* (1992) found that significantly lower larval mortality of the southern buffalo gnat, *Cnephia pecuarum*, occurred as water temperature decreased below 9°C.

12.7. Vegetative cover and food

Garcia *et al.* (1982) found that control of *Culex peus* in a primary oxidation pond with a dense cover of water hyacinth was unsuccessful and the presence of dense vegetation reduced the effectiveness of *Bti* against *Odagmia ornata* in the Czech Republic (Mata *et al.* 1986). Conversely, the presence of extensive aquatic vegetative growth had little effect in reducing the activity of *Bti* against larvae of *Simulium vittatum* in a stream in Tennessee (Frommer *et al.* 1981).

There was a marked difference in *Bti* susceptibility between two larval populations of a euryphagous species, *Pentapedilum tigrinum*, when reared on different foods prior to treatment; the larvae reared on aquatic plant tissue were 100 times more susceptible than the larvae fed on detritus (Kondo *et al.* 1995).

Skovmand and Eriksen (1993), using a fizzy tablet formulation of *Bti*, found that a full tablet dose per 5 m^2 pond killed 93-100% of 2nd- to 3rd and early 4th-instar *Ae. cataphylla* and *Ae. cantans* larvae in ponds without dense vegetation but only 75-82% of the same instars in ponds with dense vegetation.

12.8. pH

Bti has been found to be relatively insensitive to variations in water pH. However, in field tests against *Cx. tarsalis*, all the formulations of *Bti* were less effective in water where the pH was higher than 8 (Mulla *et al.* 1980). Sinegre *et al.* (1981) found a slight but non-significant correlation between pH from 5 and 7 and the effectiveness of the endotoxin. Floore *et al.* (1987) found no effect of pH in the range of 6.3-8.6 (27°C) on the efficacy of VectoBac® AS in the laboratory against 3rd-instar larvae of *Cx. qinquefasciatus*.

12.9. UV

Sunlight inactivates *Bti* (eg. Becker *et al.*1992). For example, the LC_{90s} for *Bti* against *Cx. pipiens* were approximately four times higher in sunlit sites than shaded sites (Becker and Margalit 1993).

12.10. Bacteriophage

Bacteriophages are viruses which attack bacteria and can therefore reduce the effectiveness of a bacterial control agent. Bacteriophages which inhibited *Bti* were found in 9 of 12 habitats surveyed in Egypt (Ali *et al.* 1993). No information is available on whether bacteriophage affect *Bti* in the field, however they are unlikely to have a direct effect as toxicity of *Bti* is dependent on the protein crystal and not the bacterial cell. Bacteriophages may have an impact on any recycling of the bacterium and can disrupt production of *Bt* preparations.

12.11. Efficacy comparisons

Not all formulations will have the same effect on target species, nor the environment. In some cases the type of formulation (ie. pellet, liquid) has a major impact on efficacy. For example, several commercial formulations of *Bti* were tested in the field in California for mosquito control by Grant *et al.* (1984). Among granular preparations formulated on maize and applied at 10 kg/ha, VectoBac® G was more effective against the larvae of *Ae. nigromaculis* than against *Cx. tarsalis*, but the reverse was true for Teknar®. Grant *et al.* (1984) showed VectoBac® sand granules were ineffective against *Cx. tarsalis* at application rates of less than 28 kg/ha, but VectoBac® aqueous suspension at 0.5 kg/ha gave 98% control.

In another trial, three commercial formulations of *Bti* were bioassayed in the laboratory in the USA to determine their effectiveness against *Simulium* spp. and *Ae. aegypti* relative to that of the international standard powder formulation IPS-78 (standard powders of *Bti* are supplied by several research institutes to standardise potency of *Bti* products). Efficacy of a formulation is generally determined by measurement against a standard formulation and expressed in terms of International Toxic Units (ITU/mg) (de Barjac 1983). Potency is calculated by:

$$\frac{LC_{50} \text{ of a standard}}{LC_{50} \text{ of a standard}} \times \text{potency of the standard} = \text{Potency of the sample (ITU/mg)}$$

The potencies of the wettable powder formulations Bactimos and VectoBac® and the water dispersible formulation Teknar were 4530, 5723 and 336 International Toxic Units (ITU)/mg, respectively, against *Simulium* spp. and 3556, 2317 and 1373 ITU/mg against *Ae. aegypti* (Molly *et al.* 1984).

13. Discussion and Conclusions

It is concluded that *Bti* is one of the safer options for use against exotic mosquitoes in New Zealand. Other agents or more persistent *Bti* formulations than the VectoBac 12AS liquid formulation may be required for eradication efforts.

Consideration of the environmental safety and impacts of the use of *Bti* must be seen in the context of alternatives. There are a number of other agents (many listed by Cowley *et al.* (1998) in the draft national pest management strategy for exotic mosquitoes), which should be considered for eradication or control of exotic mosquito incursions into New Zealand. These agents include other microbial products, such as those based on *Bacillus sphaericus*, insect growth regulators, such as methoprene, and chemical insecticides. There are organophosphate chemicals, with good residual activity for use in non-sensitive regions and a number of new microbial agents which have been developed into products more recently. The insect growth regulator, methoprene, could be a useful agent, as it has low environmental impact while having a greater residual activity than *Bti*.

Selected agents then need to be considered on a number of criteria including efficacy, residual activity, environmental impact, health aspects and possibility of resistance development in the target insect.

13.1. Is Bti safe for use in New Zealand?

Bti use has been widely supported by health authorities, such as WHO, as it has demonstrated effectiveness against disease vectors with few deleterious effects on non-target organisms or the environment (Clarke 1994). The environmental safety of wild-type *Bt* products has been well documented during the 40 years or more that *Bt* products have been available (eg. Meadows 1993). In the first year of use in Africa for disease vector control (*Simulium*), 40 tons of *Bti* were applied (Meadows 1993). It has also been used extensively in the USA, Switzerland and the Rhine valley of Germany, without obvious environmental problems.

The environmental threat posed by *Bti* would appear to be significantly less than most other forms of mosquito control which have a similar level of efficacy. The environmental impact of *Bti* must be viewed in context; if exotic mosquitoes arrive in New Zealand, what will be the response? There are three response scenarios:

1. Do nothing
2. Apply control agents
3. Use environmental manipulation and/or modification techniques

Obviously, "do nothing" is an unlikely scenario, and use of management techniques alone is also unlikely to sufficiently reduce mosquito numbers. Therefore use of some agent will be necessary to suppress and possibly eradicate exotic mosquitoes. Cowley *et al.* (1998), in the draft pest management strategy for mosquitoes, put forward a number of possible agents

ranging from chemicals to insect growth regulators and *Bti*. While there are some questions over the complete safety of *Bti* in terms of relationship to *B. cereus* and effects on non-mosquito Nematocera, these are minor in comparison with concerns over chemical control agents, such as temephos, which have mammalian and non-target safety problems. Such concerns would restrict their use in many regions. In an eradication or emergency control situation, agents will need to be safe to apply in all situations as areas cannot be left untreated.

One agent which may be suitable for use in addition to *Bti* is the insect growth regulator methoprene. It has similar safety tolerances to *Bti* and greater persistence, making it more suitable for use in eradication programmes than most *Bti* products which do not persist for long periods. An environmental impact study similar to this document is required on methoprene to determine non-target impacts in the New Zealand environment.

13.2. Risks to New Zealand fauna

Bti is restricted in host range to the subclass Nematocera, with relatively few effects recorded outside this group. Non-target mortality could be expected in the Culicidae, Chironomidae and Simuliidae. New Zealand has representatives of all these groups; however, the actual susceptibility of many species can only be assessed by direct exposure experimentation. Among New Zealand's 14 known Culicidae (Laird 1995) (Table 5), *Aedes, Culiseta* and *Culex* would be expected to be susceptible while *Coquillettidia* would be unlikely to be susceptible, based on previous research (e.g. Walker *et al.* 1985). Susceptibility of the other genera, *Opifex* and *Maorigoeldia*, is unknown. In the laboratory Chilcott (pers. comm.) has found that *Bti* is highly toxic to *Ae. australis, Ae. notoscriptus* and *Cx. pervigilans.*

Some or most species of Simuliidae and Chironomidae would be susceptible, however effects would only occur at the area of application, as *Bti* does not recycle readily in populations. Simuliidae, in particular, are also a pest species in some areas of New Zealand and their control with *Bti* has already been investigated (Chilcott *et al.* 1983).

TABLE 5: Species of mosquitoes found in New Zealand (compiled from Debenham and Hicks 1989, Vol 2 pg 119-120).

Aedes antipodeus	*Maorigoeldia argyropus*
Aedes australis	*Coquillettidia tenuipalpis*
Aedes notoscriptus	*Coquillettidia iracunda*
Aedes subalbirostris	*Culiseta tonnoiri*
Culex asteliae	*Culiseta novaezealandae*
Culex pervigilans	*Opifex fuscus*
Culex quinquefasciatus	*Aedes chathamicus*[*]
Culex rotoruae	(*Aedes nocturnus*)

early record considered questionable in Debenham and Hicks (1989)

[*] Chatham Islands

13.3. Other agents

It is not the aim of this report to review the environmental impacts of all possible agents, however some mosquitocidal agents are briefly outlines below.

13.3.1. Chemicals

Cowley *et al.* (1998) in the New Zealand draft national pest management strategy for exotic mosquitoes list a number of chemicals and compounds which are used against mosquitoes overseas. For larvae, only the petroleum oils (diesel, kerosene) are currently registered for use. The insecticides alpha-cyrpermethrin (Fendona 15 SC), bendiocarb (Ficam W), betacyfluthrin (Responsar SC 125), cyfluthrin (Solfac 50EW), deltamethrin (Cislin 10), dichorvos (Nuvan 1000EC), lambacyhalotrin (Icon 10 WP) and permethrin are listed as adulticides registered for use in New Zealand. Pirimiphos methyl (Actellic), pyriproxyfen (Sumilarv) and temephos (Abate 50 SG) are larvicides used overseas for mosquito control, but not registered for use against mosquitoes in New Zealand, while bioresmethrin and malathion are adulticides not registered for use against mosquitoes in New Zealand.

One chemical that could be used in New Zealand is temephos, an organophosphate. Granules have good shelf life and persistence of over 150 days. It is relatively toxic compared to *Bti*, which has raised occupational health and safety concerns, and it is also less specific than *Bti* (e.g. Yap *et al.* 1982). The environmental concerns and the fact that there are signs of resistance in target species has lessened the attractiveness of temephos for mosquito control (Clarke 1994). However, because of the good residual activity and high toxicity to mosquitoes chemical insecticides such as temephos may have a role in treatment of specific sites, such as containers.

13.3.2. Insect growth regulators

Diflubenzuron (Dimilin) is a common insect growth regulator used in New Zealand for insect control. Cowley *et al.* (1998) note that it is not recommended for use with mosquitoes due to questions over mammalian and non-target safety, such as carcinogenic breakdown products. S-methoprene, an Insect Growth Regulator (IGR), is possibly the most attractive alternative to *Bti* currently used against mosquitoes overseas. Methoprene is the active ingredient in the larvicide Altosid. Methoprene's disruption of the mosquito growth cycle allows it to be defined as a biorational agent, rather than a conventional pesticide. It specifically targets mosquito larvae, but does not kill them until they reach their next developmental stage, the pupae. This is the key to preserving the natural food chain, since mosquito larvae can be a food source for other organisms. In addition, extensive studies have shown that methoprene breaks down quickly in the environment, spares non-target organisms and poses little hazard to humans. Methoprene is also available overseas in briquette form for slow release. There is no registration of methoprene products for mosquito control in New Zealand.

13.3.3. Microbial control agents

13.3.3.1. *Bacillus thuringiensis* strains other than *Bti*

There are several reports of mosquitocidal *Bt* strains other than *Bti*. Ragni *et al.* (1996) reported on the characterisation of six highly mosquitocidal *Bt* strains that do not belong to H-14 serotype, while Chilcott and Wigley (1993), in their survey of *Bt*s in New Zealand, found a strain virulent against a mosquito species which differed from the typical protein profile for *Bti*. Kawalek *et al.* (1995) described a new mosquito active subspecies *B. thuringiensis* subsp. *jegathesan* from Malaysia. Several patents have been applied for in the USA, based on non-*Bti* mosquitocidal *Bt*s.

13.3.3.2. *Bacillus sphaericus*

Strains of this *Bacillus* species (or collection of heterogenous strains which may be larger than a species) which are generally saprophytic but over 50 isolates are known to kill mosquitoes. Strains occur world-wide in both soil and aquatic habitats. Pathogenicity appears to be due to toxins released from both vegetative cells and parasporal crystals (Tanada and Kaya 1993). Pathogenicity is restricted to mosquitoes, which vary in their susceptibility to this agent. *Aedes* spp. are less susceptible than *Culex* spp. There is some evidence of recycling properties in polluted aquatic environments (Yap 1990); field trials assessed to date have shown some control and residual effects against mosquitoes, especially *Culex, Mansonia, Psoropora* and *Anopheles* (Yap 1990). Environmental impact is very slight. *B. sphaericus* is pathogenic to only mosquito larvae and under certain conditions, persists and recycles in nature, meaning long term control is possible. Products based on *B. sphaericus* are available, for example Vectolex-WDG (Abbott Laboratories), which has a New Zealand experimental use label (Cowley *et al.* 1998).

13.3.3.3. *Lagenidium giganteum*

Lagenidium giganteum is an Oomycete fungus which is pathogenic to a number of mosquito species, including *Aedes, Culex, Anopheles* and *Culiseta* (Tanada and Kaya 1993). It has been under development as a control agent for mosquitoes for many years and has recently been commercialised in the USA. The new product, Laginex™ AS is an aqueous formulation of *L. giganteum* and is produced by AgraQuest (Davis, California)(Jimenez *et al.* 1997). At label rates and under appropriate conditions, the formulation has been shown to kill larvae for 21 to 30 days. This is an early product and liquid flowable and dry formulations are currently under development (Jimenez *et al.* 1997).

13.3.3.4. *Romanomermis* spp.

Mermithid nematodes of the genus *Romanomermis* have been developed for mosquito control. In the case of *R. culicivorax*, field trials, including aerial application, have demonstrated efficacy against some mosquito species (eg. Galloway and Brus 1976; Brown *et al.* 1977; Levy *et al.* 1977). The nematode was commercially produced as early as 1976 and was further developed by several groups (e.g. Nickle 1980; Webster 1980). Production has since ceased (Tanada and Kaya 1993), partly due to difficulties in the production process, storage, transport and competition from *Bti*.

13.3.3.5. *Culicinomyces clavisporus*

The fungus, *C. clavisporus*, is a Deuteromycete which is unique in that it attacks mosquito larvae. Mosquito larvae die within 2 days of exposure to the conidia (spores) and cadavers support the production of more infective conidia. An Australian group attached to the army researched this agent as a mass application control in the 1980s, however no product eventuated and the project appears to be have been discontinued.

13.3.3.6. Other microbial pathogens

There are several common mosquito pathogens which are presently unsuited to mass application, but are often effective mosquitocidal agents. Fungi in the genus *Coelomomoyces* are obligate pathogens of mosquitoes and several other insects. *Coelomomyces* have involved life-cycles and cannot be easily cultured, making their use in control programmes unlikely. A *Coelomomyces* species has been described from New Zealand (e.g. Pillai 1971). Another fungus, *Tolypocladium*, is a mosquito pathogen, but no products have yet been developed based on this agent. There are also viruses and protozoa which kill mosquitoes.

13.3.3. Other biological agents

There is a large group of known predators, such as predatory mosquitoes and fish, which are effective against mosquitoes. Most, however, are generalists and specific control is not possible. The time required to demonstrate environmental safety may be prohibitive. Some agents, such as the fish *Gambusia*, are already present in New Zealand. Backswimmers in the genus *Notonecta* and copepods are also common mosquito predators.

13.4. Efficacy of agents

The efficacy of any agent used will need to be high, as the first aim of a mosquito incursion response is likely to be eradication. Not all agents are equally efficacious. For example, Becnel *et al.* (1996) evaluated the effect of 3 larvicides on the production of adult *Ae. albopictus*. The fungal pathogen *Lagenidium giganteum* was ineffective. A liquid formulation of *Bacillus thuringiensis israelensis* (Acrobe) provided significant control for 47 days, whereas a slow-release pellet formulation of the insect growth regulator methoprene (Altosid) provided almost complete control for 116 days. Such information, often available from overseas, will be required to choose appropriate agents for use in New Zealand.

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