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**MIXTURE OF BACILLUS THURINGIENSIS SUBSPECIES
ISRAELENSIS AND BACILLUS SPHAERICUS FOR MANAGEMENT
OF RESISTANCE TO MOSQUITO LARVICIDES**

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Field of the Invention

The invention is directed to a method for controlling Dipteran larvae or a method for inhibiting larvicidal resistance in Diptera by introducing a larvicidally-effective amount of a combination of a strain of *Bacillus*
10 *thuringiensis* subspecies *israelensis* and a strain of *Bacillus sphaericus* into an environment containing Dipteran larvae; and a composition of the combination. Preferably, both strains are non-genetically modified.

Background of the Invention

15 Mosquitoes and black flies are representative of the order Diptera which are pests that have plagued humans and animals for generations. Mosquitoes are the major vectors for a number of human and animal diseases, including malaria, yellow fever, viral encephalitis, dengue fever and filariasis.

20 Various chemical pesticides have been developed with the goal of controlling Diptera. For example, treatment of a water source with a water-soluble alcohol in water-miscible form for mosquito abatement is disclosed in U.S. Patent No. 6,077,521. However, more recent emphasis has been placed on the use of biopesticides. For example,
25 controlled-release formulations of at least one biological pesticidal ingredient are disclosed in U.S. Patent No. 4,865,842; control of mosquito larvae with a spore-forming *Bacillus* ONR-60A is disclosed in U.S. Patent No. 4,166,112; novel *Bacillus thuringiensis* isolates with activity against dipteran insect pests are disclosed in U.S. Patent Nos.
30 5,275,815 and 5,847,079; a biologically pure culture of a *Bacillus thuringiensis* strain with activity against insect pests of the order Diptera is disclosed in U.S. Patent No. 5,912,162 and a recombinantly derived biopesticide active against Diptera including cyanobacteria transformed with a plasmid containing a *B. thuringiensis* subsp. *israelensis*



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dipteracidal protein translationally fused to a strong, highly active native cyanobacteria's regulatory gene sequence is disclosed in U.S. Patent No. 5,518,897.

Yet even these biopesticides have drawbacks; so the search for new
5 biopesticides continues. One drawback of certain biopesticides is the potential build-up of pesticidal resistance.

Resistance is defined by differences in susceptibility that arise among populations of the same species exposed to a pesticide continuously over a period of time. These differences are identified by
10 observing a statistical shift in the lethal dose (LD) either to kill 50% or 95% of the population (LD₅₀ or LD₉₅ respectively). Individual differences in susceptibility exist within each species, and pests that are substantially less susceptible may be present, generally at low frequencies, in at least some of the wild populations. In the presence of
15 the pesticide, it is these substantially less susceptible pests that survive and reproduce. Since their ability to survive is a result of their genetic makeup, their resistant genetic makeup is then passed on to their offspring, resulting in shifts in the populations' susceptibility via pesticide-induced selection. Resistance to larvicides has been encountered
20 among certain Dipteran species.

Specifically, the development of resistance in *Culex quinquefasciatus* to *Bacillus sphaericus* (B.s.) is noted by Rodcharoen *et al.*, Journal of Economic Entomology, Vol. 87, No. 5, 1994, pp. 1133-1140. A method for overcoming this resistance, by combining B.s. with
25 purified Cyt1A crystals isolated from *Bacillus thuringiensis* subsp. *israelensis* or by combining a recombinant *B.t.i.* with B.s., is disclosed by Wirth *et al.*, Journal of Medical Entomology, Vol. 37, No. 3, 2000, pp. 401-407. However, improved but naturally derived or occurring biological larvicides and compositions to overcome *Culex* mosquito
30 resistance to B.s. applications would be desirable.

Brief Summary of the Invention

The invention is directed to a composition comprising: a combination of a strain of *Bacillus thuringiensis* subspecies *israelensis* and a strain of *Bacillus sphaericus*. The strain of *Bacillus thuringiensis* subspecies *israelensis* may be non-genetically modified, or the strain of *Bacillus sphaericus* may be non-genetically modified, although a presently preferred combination includes a non-genetically modified strain of *Bacillus thuringiensis* subspecies *israelensis* and a non-genetically modified strain of *Bacillus sphaericus*.

The combination may have from about 1:10 to about 10:1 weight ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*; preferably from about 1:3 to about 3:1 weight ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*; more preferably from about 1:2 to about 2:1 weight ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*; and most preferably a 1:1 ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*.

Additional components such as surface active agents, inert carriers, preservatives, humectants, feeding stimulants, attractants, encapsulating agents, binders, emulsifiers, dyes, U.V. protectants, buffers, drift control agents, spray deposition aids, free-flow agents or combinations thereof may also be utilized in conjunction with the combination in a larvicidal composition.

The invention is also directed to a method of controlling Dipteran larvae comprising the step of introducing a larvicidally-effective amount of a combination of a strain of *Bacillus thuringiensis* subspecies *israelensis* and a strain of *Bacillus sphaericus* into an environment containing Dipteran larvae. In this method, Dipteran may be a mosquito such as *Culex pipiens*, *Culex quinquefasciatus*, *Aedes aegypti*, *Culex tarsalis*, *Culiseta incidens*, *Anopheles freeborni* or a combination thereof.

The invention is additionally directed to a method for inhibiting larvicidal resistance in Diptera comprising the step of introducing a larvicidally-effective amount of a combination of a strain of *Bacillus*

thuringiensis subspecies *israelensis* and a strain of *Bacillus sphaericus* into an environment containing Dipteran larvae. Preferably, the Diptera is *Culex* and larvicidal resistance is developed against *Bacillus sphaericus*.

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Detailed Description of the Invention

The invention is directed to a method for controlling Dipteran larvae or a method for inhibiting larvicidal resistance in Diptera by introducing a larvicidally-effective amount of a combination of a strain of *Bacillus thuringiensis* subspecies *israelensis* and a strain of *Bacillus sphaericus* into an environment containing Dipteran larvae; and a composition of the combination. Preferably both strains are non-genetically modified. A detailed discussion of the composition, and the methods utilizing the composition follows.

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The Larvicidal Compositions

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Biopesticides are a class of naturally occurring pesticides frequently derived from unicellular or multicellular organisms which have developed natural defenses against other organisms. The group of microorganisms pathogenic to insects is varied and diverse. The gram-positive soil bacterium *Bacillus thuringiensis* subsp. *israelensis* is one of many *B. thuringiensis* strains able to produce insecticidal proteins. These proteins, expressed during the sporulation cycle of the bacterium, assemble into parasporal crystalline inclusion bodies. The parasporal crystal produced by *B. thuringiensis* subsp. *israelensis* is toxic when ingested by the larvae of Diptera, including mosquitoes and black flies. Upon ingestion, crystal proteins are solubilized in the larval midgut and disrupt the epithelium of the larval midgut region. Swelling and/or lysis of the epithelial cells is followed by larval death from starvation.

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Bacillus thuringiensis subspecies *israelensis* (*B.t.i.*) has been used successfully in mosquito and blackfly control programs for many years. *B.t.i.* is utilized in clean to moderately clean organic breeding habitats, and is most effective on *Aedes* species. A commercial formulation of *B.t.i.* is known by the trademark VECTOBAC, available from Valent BioSciences Corp. Specific commercial formulations available from the

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same supplier are VECTOBAC G, VECTOBAC CG, VECTOBAC 12AS and VECTOBAC WDG. *B.t.i.* is effective against a broad range of mosquito species, offers low mammalian toxicity and is easy to apply. *B.t.i.* also has a very low susceptibility to the development of resistance, because its larvicidal activity is based on multiple toxins. The chances that individual mosquitoes within a treated population will not be susceptible to all toxins is extremely small.

Bacillus sphaericus (*B.s.*) is a rod-shaped, aerobic, spore-forming bacterium found commonly in soil and other substrates. To date, at least 16 strains have been found to show mosquitocidal properties of various degrees. Several strains such as 1593M, 2362 and 2297 exhibit high toxicity to mosquito larvae. *B.s.* strain 2362, (VECTOLEX, available from Valent BioSciences Corp.) has been utilized in many countries successfully. Specific commercial formulations of *B.s.* available from the same source are VECTOLEX WDG, SPHERIMOS AS and VECTOLEX CG. Moreover, this strain was found to perform well in controlling mosquitoes breeding in various habitats, especially ones with polluted water.

B. s. is most effective on *Culex* species. The activity of *B.s.* is due to a binary toxin, and repeated use can lead to development of resistance.

However, various levels of resistance to *B.s.* by mosquito larvae have been observed in *Culex pipiens* and *Culex quinquefasciatus*.

We have now found that a combination of *B.t.i.* and *B.s.* is an effective larvicidal formulation. Non-genetically modified components are utilized, which are desirable if the larvicide is to be utilized in an environment connected with production or harvesting of food sources such as crops, cattle or swine. Non-genetically modified *B.t.i.* or *B.s.* may be defined as strains which occur naturally, and are not strains resulting from recombinant DNA techniques.

B.t.i. and *B.s.* may be combined by mixing the powdered forms of each of the individual strains, or by mixing the slurries of the fermentation broths of each strain, in the desired ratio, as illustrated by Examples 1-6 which follow. The ratio of *B.t.i.* to *B.s.* may be from about 10:1 to about

1:10; preferably from about 3:1 to about 1:3, more preferably from about 2:1 to about 1:2 and most preferably about 1:1.

The compositions disclosed above may also include additional components such as a surface active agent, an inert carrier, a preservative, a humectant, a feeding stimulant, an attractant, a drift control agent, a spray deposition aid, an encapsulating agent, a binder, an emulsifier, a dye, a U.V. protectant, a buffer, a free-flow agent, or any other component which stabilizes the active ingredient, facilitates product handling and application for the particular target pests, Diptera.

Suitable surface-active agents include anionic compounds such as a carboxylate, for example, a metal carboxylate of a long chain fatty acid; a N-acylsarcosinate; mono or di-esters of phosphoric acid with fatty alcohol ethoxylates or salts of such esters; fatty alcohol sulphate such as sodium dodecyl sulphate, sodium octadecyl sulphate or sodium acetyl sulphate; ethoxylated fatty alcohol sulphates; ethoxylated alkylphenol sulphates; lignin sulphonates; petroleum sulphonates; alkyl aryl sulphonates such as alkyl-benzene sulphonates or lower alkylnaphthalene sulphonates, *e.g.*, butyl-naphthalene sulphonate; salts or sulphonated naphthalene-formaldehyde condensates; salts of sulphonated phenol-formaldehyde condensates; or more complex sulphonates such as the amide sulphonates, *e.g.*, the sulphonated condensation product of oleic acid and N-methyltaurine or the dialkyl sulphosuccinates, *e.g.*, the sodium sulphonate or dioctyl succinate.

Non-ionic agents include condensation products of fatty acid esters, fatty alcohols, fatty acid amides or fatty-alkyl- or alkenyl-substituted phenols with ethylene oxide, fatty esters of polyhydric alcohol ethers, *e.g.*, sorbitan fatty acid esters, condensation products of such esters with ethylene oxide, *e.g.*, polyoxyethylene sorbitan fatty acids esters, block copolymers of ethylene oxide and propylene oxide, acetylenic glycols such as 2,4,7,9-tetraethyl-5-decyn-4,7 diol, or ethoxylated acetylenic glycols.

Examples of a cationic surface-active agent include, for instance, an aliphatic mono-, di-, or polyamide as an acetate, naphthenate or oleate;

an oxygen-containing amine such as an amine oxide of polyoxethylene alkylamine; an amid-linked amine prepared by the condensation of a carboxylic acid with a di- or polyamine; or a quaternary ammonium salt.

5 Examples of inert materials include inorganic minerals such as kaolin, mica, gypsum, fertilizer, sand, phyllosilicates, carbonates, sulphate, or phosphates; organic materials such as sugars, starches, or cyclodextrins; or botanical materials such as wood products, cork, powdered corncobs, rice hulls, peanut hulls, and walnut shells

10 The formulation may also contain added drift control agents or spray deposition aids to control droplet size and to facilitate aerial application. Examples of suitable compounds for these purposes include polyvinylalcohol polymer solutions, polyamide copolymer solutions, polymerized acrylic acid derivatives and blends thereof, vegetable oils and blends thereof, petroleum oils and blends thereof, as well as natural
15 and synthetic polymers.

In the formulations, more than one of the additional components described above may advantageously be utilized.

The compositions of the present invention can be applied as a liquid, an aqueous suspension, an emulsifiable suspension, or a solid by
20 conventional application techniques for each. Solid formulations are presently preferred. In general, the application rate of the larvicidally-effective combination of the present invention will deliver a quantity of pesticide sufficient to control the population of a target pest.

25 Solid compositions may be formed by spray drying the *B.t.i.* and *B.s* slurries separately and combining the powder or by combining the slurries and spray drying the combined slurry to form a powder.

The composition of the present invention can be in a suitable form for direct application or as a concentrate or primary composition which requires dilution with a suitable quantity of water or other diluent before
30 application. The pesticidal concentration will vary depending upon the nature of the particular formulation, specifically, whether it is a concentrate or to be used directly. The composition may contain from about 1 to 98% by weight of a solid or liquid inert carrier, and 0.1 to 50%

by weight of a surfactant. These compositions will be administered at the rate of about 50 mg (liquid or dry) to 20 kg or more per hectare.

The Methods

5 The combination of the present invention can be treated prior to formulation to prolong the pesticidal activity when applied to the environment of a target pest as long as the pretreatment is not deleterious to the combination. Such treatment can be by chemical and/or physical means as long as the treatment does not deleteriously
10 affect the properties of the composition(s). Examples of chemical reagents include, but are not limited to, halogenating agents; aldehydes such as formaldehyde and glutaraldehyde; anti-infectives, such as zephiran chloride; alcohols, such as isopropanol and ethanol; and histological fixatives, such as Bouin's fixative and Helly's fixatives.

15 The compositions of the invention can be applied directly to the environment to be treated. Ponds, lakes, streams, rivers, still water, and other areas subject to infestation by dipteran pests are examples of environments needing such treatment. The composition can be applied by spraying, dusting, sprinkling, and broadcasting, among others.

20 The compositions of the present invention may be effective against insect pests of the order Diptera, e.g., *Aedes* sp., *Andes vittatus*, *Anastrepha ludens*, *Anastrepha suspensa*, *Anopheles* sp., *Armigeres subalbatus*, *Calliphora stygian*, *Calliphora vicina*, *Ceratitis capitata*, *Chironomus tentans*, *Chrysomya rufifacies*, *Cochliomyia macellaria*, *Culex*
25 sp., *Culiseta* sp., *Coquillettidia* sp., *Deino cerities* sp., *Dacus oleae*, *Delia antiqua*, *Delia platura*, *Delia radicum*, *Drosophila melanogaster*, *Eupeodes corollas*, *Glossina austeni*, *Glossina brevipalpis*, *Glossina fuscipes*, *Glossina moristans centralis*, *Glossina morsitans morsitans*, *Glossina morsitans submorsitans*, *Glossina pallidipes*, *Glossina palpalis*
30 *gambiensis*, *Glossina palpalis palpalis*, *Glossina tachinoides*, *Haemagogus equines*, *Haematobia irritans*, *Hypoderma bovis*, *Hypoderma lineatum*, *Leucopis ninae*, *Lucilia cuprina*, *Lucilia sericata*, *Lutzomyia longlpaipis*, *Lutzomyia shannoni*, *Lycoriella mali*, *Mansonias* sp., *Mayetiola*

destructor, *Musca autumnalis*, *Musca domestica*, *Neobellieria* sp.,
Nephrotoma suturalis, *Ochlerotatus* sp., *Ophyra aenescens*,
Orthopodomys sp., *Phaenicia sericata*, *Phlebotomus* sp., *Phormia regina*,
Psorophora sp., *Sabethes cyaneus*, *Sarcophaga bullata*, *Scatophaga*
5 *stercoraria*, *Stomoxys calcitrans*, *Toxorhynchites amboinensis*,
Tripteroides bambusa, *Uranotaneia* sp. and *Wyeomyia* sp. However, the
composition of the invention may also be effective against insect pests of
the order Lepidoptera, e.g., *Achroia grisella*, *Acleris gloverana*, *Acleris*
variana, *Adoxophyes orana*, *Agrotis ipsilon*, *Alabama argillacea*, *Alsophila*
10 *pometaria*, *Amyelois transitella*, *Anagasta kuehniella*, *Anarsia lineatella*,
Anisota senatoria, *Antheraea pernyi*, *Anticarsia gemmatilis*, *Archips* sp.,
Argyrotaenia sp., *Athetis mindara*, *Bombyx mori*, *Bucculatrix thurberiella*,
Cadra cautella, *Choristoneura* sp., *Cochylis hospes*, *Colias eurytheme*,
Corcyra cephalonica, *Cydia latiferreanus*, *Cydia pomonella*, *Datana*
15 *integerrima*, *Dendrolimus sibericus*, *Desmia funeralis*, *Diaphania hyalinata*,
Diaphania nitidalis, *Diatraea grandiosella*, *Diatraea saccharalis*, *Ennomos*
subsignaria, *Eoreuma loftini*, *Ephestia elutella*, *Erannis tiliaria*, *Estigmene*
acrea, *Eulia salubricola*, *Eupoecilia ambiguella*, *Euproctis chrysorrhoea*,
Euxoa messoria, *Galleria mellonella*, *Grapholita molesta*, *Harrisinia*
20 *americana*, *Helicoverpa subflexa*, *Helicoverpa zea*, *Heliothis virescens*,
Hemileuca oliviae, *Homoeosoma electellum*, *Hyphantria cunea*, *Keiferia*
lycopersicella, *Lambdina fiscellaria fiscellaria*, *Lambdina fiscellaria*
lugubrosa, *Leucoma salicis*, *Lobesia botrana*, *Loxostege sticticalis*,
Lymantria dispar, *Macalla thyrisalis*, *Malacosoma* sp., *Mamestra*
25 *brassicae*, *Mamestra configurata*, *Manduca quinquemaculata*, *Manduca*
sexta, *Maruca testulalis*, *Melanchra picta*, *Operophtera brumata*, *Orgyia*
sp., *Ostrinia nubilalis*, *Paleacritia vernata*, *Papilio cresphontes*,
Pectinophora gossypiella, *Phryganidia californica*, *Phyllonorycter*
blancardella, *Pieris napi*, *Pieris rapae*, *Plathypena scabra*, *Platynota*
30 *flouendana*, *Platynota sultana*, *Platyptilia carduidactyla*, *Plodia*
interpunctella, *Plutella xylostella*, *Pontia protodice*, *Pseudaletia unipuncta*,
Pseudoplusia includens, *Sabulodes aegrotata*, *Schizura concinna*,
Sitotroga cerealella, *Spilonota ocellana*, *Spodoptera* sp., *Thaurnstopoea*

pityocampa, *Tineloia bisselliella*, *Trichoplusia ni*, *Udea rubigalis*,
Xylomyges curialis, *Yponomeuta padella*; Coleoptera, e.g., *Leptinotarsa*
sp., *Acanthoscelides obtectus*, *Callosobruchus chinensis*, *Epilachna*
varivestis, *Pyrrhalta luteola*, *Cylas formicarius elegantulus*, *Listronotus*
5 *oregonensis*, *Sitophilus sp.*, *Cyclocephala borealis*, *Cyclocephala*
immaculata, *Macroductylus subspinosus*, *Popillia japonica*, *Rhizotrogus*
majalis, *Alphitobius diaperinus*, *Palorus ratzeburgi*, *Tenebrio molitor*,
Tenebrio obscurus, *Tribolium castaneum*, *Tribolium confusum*, *Tribolium*
destructum; Acari, e.g., *Oligonychus pratensis*, *Panonychus ulmi*,
10 *Tetranychus urticae*; Hymenoptera, e.g., *Iridomyrmex humilis*, *Solenopsis*
invicta; Isoptera, e.g., *Reticulitermes hesperus*, *Reticulitermes flavipes*,
Coptotermes formosanus, *Zootermopsis angusticollis*, *Neotermes*
connexus, *Incisitermes minor*, *Incisitermes immigrans*; Siphonaptera, e.g.,
Ceratophyllus gallinae, *niger*, *Nosopsylla fasciatus*, *Leptopsylla segnis*,
15 *Ctenocephalides canis*, *Ctenocephalides felis*, *Echicnophaga gallinacea*,
Pulex irritans, *Xenopsylla cheopis*, *Xenopsylla vexabilis*, *Tunga penetrans*;
and Tylenchida, e.g., *Meloidogyne incognita*, *Pratylenchus penetrans*.

In a specific embodiment, the compositions of the invention are active
 against insect pests of the sub order *Nematocera* of the order *Diptera*.
20 *Nematocera* include the families *Culicidae*, *Simuliidae*, *Chironomidae*,
Psychodidae, *Sciaridae*, *Phoridae* and *Mycetophilidae*.

The ability of combination of the present invention to inhibit larvicidal
 resistance is described in detail hereinafter in the Examples. These
25 Examples are presented to describe preferred embodiments and utilities of
 the invention and are not meant to limit the invention unless otherwise
 stated in the claims appended hereto.

Example 1

30 The combination larvicide of the present invention was formulated
 as a mix of two commercially available strains: VECTOBAC CG, a
 commercial granular formulation of *B.t.i.* with a label potency of 200
 ITU/mg, and VECTOLEX CG, a commercial granular formulation of *B.s.*

with a label potency of 50 *B.s.* ITU/mg. Typically, the spray-dried technical concentrate of each strain is first incorporated into a known amount of vegetable oil binder. The amount of vegetable oil binder in the formula will depend upon the amount of *B.t.i.* or *B.s.* spray technical concentrate in the formula. The typical range may vary between 1% to 15% wt/wt. depending upon the amount of *B.t.i.* or *B.s.* spray dried technical concentrate, and the type, size and absorptive property of granular carrier utilized in the formula. In this example, corn cob granules of the size classification 10/14 Mesh were used. However, other size ranges such as 5/8 Mesh, 10/20 Mesh, 10/40 Mesh are also suitable. The slurry mixture was sprayed onto the granular carrier while mixing in a suitable blender and further blended until homogenous product was obtained.

Example 2

A *B.t.i.* and *B.s.* granular formulation for enhanced and broad-spectrum activity was prepared. Pre-formulated granular products of *B.t.i.* and *B.s.*, (VECTOBAC CG and VECTOLEX CG), were utilized in developing the combination formulation. The resultant combination granular product was targeted to contain *B.t.i.* at 100 ITU/mg and *B.s.* at 25 *B.s.* ITU/mg. The carrier utilized was 10/14 Mesh corn cob.

To prepare the formulation, 5 kg each of VECTOLEX CG and VECTOBAC CG were charged into a blender and blended. The product mix was then recovered.

Representative samples were taken for bioassay. Table 1 shows the amount of the raw materials which were used to form the product combination

Table 1

Raw Material	% wt/wt	kg/batch
VECTOBAC CG*	50.00	5.00
VECTOLEX CG**	50.00	5.00
Total	100.0	10.00

* Commercial *B.t.i.* granular formulation with a label potency of 200 ITU/mg

** Commercial *B.s.* granular formulation with a label potency of 50 *B.s.* ITU/mg

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Bioassay results for the *B.t.i.* and *B.s.* combination samples when tested against fourth instar *Aedes aegypti* and third instar *Culex quinquefasciatus* are shown in Table 2 below. ITU stands for international toxicity units, which are based on a reference material of known specific *B.t.i.* potency.

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In a preferred formulation, the one to one weight ratio of *B.t.i.* to *B.s.* has a potency of 100 ITU/mg for *B.t.i.* and 25 ITU/mg for *B.s.*, which is equivalent to a 4:1 ratio on a potency basis.

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Table 2

Sample	<i>B.t.i.</i> Potency	<i>B.s.</i> potency
VECTOBAC CG	185 ITU/mg	9 <i>B.s.</i> ITU/mg
VECTOLEX CG	4 ITU/mg	53 <i>B.s.</i> ITU/mg
1:1 <i>B.t.i.</i> to <i>B.s.</i>	102 ITU/mg	30 <i>B.s.</i> ITU/mg

Example 3

The larvicidal combination product may also be formulated by combining the required levels of both *B.t.i.* and *B.s.* technical powders in the same binder liquid, and then impregnating or spraying onto the granular carrier such as corn cob or any other suitable carriers.

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To prepare the formulation, both spray dried technical concentrates can be slurried in vegetable oil binder liquid and sprayed onto granular carrier in a suitable blender and mixed until a homogenous product is

obtained. The theoretical components of the combination formulation containing *B.t.i.* at 100 ITU/mg and *B.s.* at 25 *B.s.* ITU/mg are provided in Table 3 below.

Table 3

Component	% wt/wt	kg/Batch	Purpose
<i>B.t.i.</i> spray-dried technical concentrate or powder (5000 ITU/mg)	2.00	20.00	Active Ingredient
<i>B.s.</i> spray dried technical concentrate or powder (1000 <i>B.s.</i> ITU/mg)	2.50	25.00	Active Ingredient
Vegetable oil binder	10.00	100.00	Binder
Granular carrier	85.5	855.00	Carrier

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Example 4

The combined formulation may also be formed by pre-mixing fermentation beers or slurry concentrates of *Bti* and *Bs* at the desired solids or potency level and spray drying the slurry mixture to produce a combined technical spray dried powder concentrate. In such a formulation, the slurry concentrate may contain preservatives, stabilizers, surfactants, dispersants and other binders. The spray-dried technical concentrate or powder may then be utilized in formulating a granular product as in Examples 2 and 3 or as wettable powders, water dispersible granules, and aqueous or non-aqueous concentrates. These combined powder concentrates may also be utilized in pellet and/or briquette formulations. A spray drying experiment was performed combining *Bti* and *Bs* fermentation slurry concentrates at various ratios based on % solids level in each of the slurry concentrates. A *Bti* slurry concentrate was first preserved with 0.12% wt/wt of potassium sorbate and 0.06% wt/wt of methyl paraben. % solids in the preserved *Bti* slurry concentrate were 11.3% wt/wt. Similarly, a *Bs* slurry concentrate with 0.12% wt/wt of potassium sorbate and 0.06% wt/wt of methyl paraben was preserved.

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Per cent solids in the preserved *Bs* slurry concentrate had a mean % solids of 10.1% wt/wt. Slurry mixtures prepared and their ratios on solids basis are given in Table 4

5 Table 4 *Bti* and *Bs* slurry mixtures evaluated

Material\Ratio of <i>Bti</i> to <i>Bs</i> on solids basis	1:0	1:1	3:1	1:3	2:1	1:2
	A	B	C	D	E	F
<i>Bti</i> preserved slurry concentrate	8.85 Kg	4.42 Kg	6.64 Kg	2.21 Kg	5.89 Kg	2.95 Kg
<i>Bs</i> preserved slurry concentrate	----	5.10 Kg	2.55 Kg	7.65 Kg	3.41 Kg	6.80 Kg
De-ionized water	1.15 Kg	0.48 Kg	0.81 Kg	0.14 Kg	0.70 Kg	0.25 Kg
Total	10.0 Kg	10.0 Kg	10.0 Kg	10.0 Kg	10.0 Kg	10.0 Kg

The compositions as shown in Table 4 were combined and spray dried utilizing a Niro spray. Inlet temperature ranged between 180 °C and 190 °C and outlet temperature during drying ranged between 68 °C to 81 °C. The technical powders were sieved through 100-mesh standard sieve and samples were bioassayed against L4 *Aedes aegypti* and L3 *Culex quinquefasciatus*. Average potency data is represented in Table 5.

15 Table 5 Potency values of *Bti* + *Bs*. spray dried technical powders as affected by their ratios on solids basis. Mean *Bti* spray dried technical powder potency = 7474 ITU/mg. Mean *Bs* spray dried technical powder potency = 3030 *Bs*. ITU/mg (All assays are average of initial and 2 month 5 °C stored samples)

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Ratio of <i>Bti</i> to <i>Bs</i> on solids basis	Theoretical <i>Bti</i> Potency* (ITU/mg)	Actual <i>Bti</i> Potency (ITU/mg)	Actual Potency expressed as % of theoretical potency in Column 2.	Theoretical <i>Bs</i> Potency+ (<i>Bs</i> . ITU/mg)	Actual <i>Bs</i> Potency (<i>Bs</i> . ITU/mg)	Actual Potency expressed as % of theoretical potency in Column 5
1:1	3737	5174	138%	1515	1642	108%

3:1	5606	6122	109%	758	1088	144%
1:3	1869	4769	255%	2273	2655	117%
2:1	4983	5499	110%	1009	1479	147%
1:2	2489	3738	150%	2020	2503	124%
Mean	3737	5060		1515	1873	

Biopotency data presented in Table 5 reveal an interesting but very synergistic increase in actual potency of both *Bti* and *Bs* over theoretical potencies, which are, based on actual potencies of 100% of either *Bti* or *Bs* spray dried technical powders. The best combination for increased activity on both *Aedes* and *Culex* appeared to be when *Bti* and *Bs* slurry concentrates are combined at 1 part of *Bti* to 2 parts of *Bs* on solids basis. For enhancing the *Bs* potency, the best combination was when two parts of *Bti* solids was combined with 1 part of *Bs* solids. In this combination, *Bs* potency showed 47% increase over theoretical potency. By combining the *Bti* and *Bs* slurry concentrates prior to spray drying, *Bti* potency on average showed an increase of 35% over mean theoretical mean potency while *Bs* potency showed an increase of 24% over theoretical mean potency. There appeared to be significant advantage in combining the slurry concentrates prior to spray drying and further formulating these powders as granules, wettable powders, water dispersible granules, or pellet formulations. The best possible explanation for these enhanced potency values appeared to be due to the fact that each spray dried particle carries both *Bti* and *Bs* toxins and spores. In other words, these are not physical mixtures as revealed in Example 2 or 3. Thus, these novel formulation approaches are likely to not only result in broad-spectrum activity but also will minimize the potential for build up of resistance. In other words, resistance management can also be achieved yet by another novel formulation approach.

Example 5

The combination larvicidal formulation may also be prepared in liquid form by adding, at the desired level, both VECTOBAC WDG (3000 ITU/mg) and VECTOLEX WDG (650 ITU/mg) to water in the spray tank and mixing until a homogeneous dispersion is obtained. The suspension

so formed may be delivered to the target habitat by various application methods. A liquid formulation is ideal for spray operations.

Example 6

5 A liquid formulation may be prepared from liquid formulations of each individual strain. VECTOBAC 12AS and SPHERIMOS AS (aqueous suspension product forms marketed by Valent BioSciences Corp.) may also be mixed in water in the spray tank and applied by various spray application equipment. Formulating *B.t.i.* and *B.s.* as one aqueous
10 formulation with preservatives, stabilizers, surfactants, dispersants, diluents is yet another preferred method for delivering both toxins to the mosquito habitats.

Example 7

15 The change in susceptibility of *Culex quinquefasciatus* laboratory colonies known to be *B. s.* resistant, in response to selection with a mixture of *B.t.i.* and *B.s.* was determined in the laboratory in the following manner. Selection refers to treatment at less than LC₁₀₀ level.

 A colony of *Culex quinquefasciatus* resistant to *B.s.* was started
20 from susceptible larvae collected from a waste water lagoon of a dairy in the western United States. Field collected larvae were subjected to selection at LC₉₀ every generation for forty generations. At the 40th generation, the colony showed a 54.4 and 14.2 fold resistance at LC₅₀ and LC₉₀ levels respectively. This colony was used for the subsequent tests.

25 A resistant colony is one which demonstrates a significant decrease in susceptibility to a particular pesticide over that expected for wild type insects. Generally, a five-fold or more decrease in susceptibility indicates resistance. Rodcharoen *et al.*, Journal of Economic Entomology, Vol. 87, No. 5, 1994, pp. 1133-1140 explore the concept of resistance more fully.

30 A susceptible colony is one which is effectively killed by a particular pesticide. For example, in *Culex quinquefasciatus*, if a particular pesticide, *Bacillus sphaericus*, has a measured LC₅₀ value of less than 0.1 ppm, the colony is characterized as susceptible to that pesticide.

Stock suspensions were prepared by mixing 0.2 g of *B.t.i.* or *B.s.* in 20 ml of distilled water to make a 1% suspension; mixtures were made by combining the stock suspensions in the desired ratio. Suspensions were subsequently diluted as required for test treatments.

5 The parental colony and the tenth generation were bioassayed by placing 20 late third or early fourth instar larvae in a 116 ml waxed paper cup containing 100 ml distilled water. One drop of larval diet (2 g of ground up rabbit pellets in 20 ml distilled water) was added per cup. The cups were treated with a range of concentrations of either larvicide alone,
10 as well as the mixture. Five to seven different concentrations in the range of 0.0001-0.1 ppm were utilized in each bioassay to yield mortalities. Each concentration was replicated four to five times in each test. The treated larvae were held at 82-85 °F. To determine LC₅₀ values, the number of dead larvae were counted at regular intervals from the time of
15 treatment with the test larvicide. Once all larvae died, the concentration wherein 50% had been killed could be determined.

Colonies treated individually at the LC₈₀ level with *B.s.* (*Bacillus sphaericus* strain 2362, ABG-6184, VECTOLEX, available from Valent BioSciences Corp.) or *B.t.i.* (*Bacillus thuringiensis* subsp. *israelensis*
20 (VECTOBAC available from Valent BioSciences Corp.) were compared to a colony treated with a 1:2 weight ratio of *B.s.* (VECTOLEX) to *B.t.i.* (VECTOBAC) for five generations at the LC₈₀ level, followed by treatment with a 1:1 weight ratio of *B.s.* (VECTOLEX) to *B.t.i.* (VECTOBAC) for five more generations at the LC₈₀ level.

25 Following the first five generations of these treatments, lower mean *B.s.* LC₅₀ values were obtained for the colonies subjected to selection by the 1:2 combination than for either colony selected by the individual components, indicating an increased susceptibility and consequently decreased resistance as illustrated by Table 6. Mean *B.s.* LC₅₀ values
30 continued to decline under selection with the 1:1 combination during the subsequent five generations.

Table 6

This table shows data from the original selection study through F25. One key change in the selection was that the *B.t.i.* selected colony was switched to *B.s.* selection after F10 to assess stability of susceptibility in the colony.

Table 6 Change in *B.s.* Susceptibility in ppm of a *B.s.* Resistant Colony in Response to selection with *B.t.i.*, *B.s.* and mixtures

Treatment	LC50 of Parental Colony	LC50 of 5th Generation	LC50 of 10th Generation	LC50 of 15th Generation	LC50 of 20th Generation	LC50 of 25th Generation
<i>B.t.i.</i>	0.49	0.351	0.205	*0.315	*0.366	*0.308
<i>B.s.</i>	0.49	0.452	0.42	0.42	0.554	0.464
<i>B.t.i. and B.s.</i>	0.33	0.273	0.103	0.153	0.035	0.04

*Selection was switched back to *B.s.* from generation 10 to 25.

Example 8

The bioassay procedure described in Example 7 was utilized in order to assess the susceptibility of *B.s.* resistant and susceptible *Culex quinquefasciatus* colonies to *B.t.i.*, *B.s.* a 2:1 mixture of *B.t.i.* to *B.s.*

Mean LC₅₀ values were determined for each treatment. A lower mean LC₅₀ value indicates that the particular treatment can be used effectively at a lower concentration, which indicates that the organisms are more susceptible to the treatment.

The results are provided in Table 7 below. As expected, the *B.s.* treatment gave the highest mean LC₅₀ for the *B.s.* resistant colony. However, the 2:1 ratio mixture shows an improved result over the result for *B.t.i.* and *B.s.* alone, both in susceptible and resistant colonies, indicating a higher susceptibility and possible synergism when the components are combined.

Table 7

Susceptibility in ppm of Two Laboratory *Culex quinquefasciatus* Colonies to *B.t.i.*, *B.s.* and a 2:1 Mixture of *B.t.i.* to *B.s.*

Treatment	Mean LC ₅₀ in Resistant Colony	Mean LC ₅₀ in Susceptible Colony
<i>B.t.i.</i>	0.025	0.017
<i>B.s.</i>	0.330	0.009
2:1 <i>B.t.i.</i> : <i>B.s.</i>	0.011	0.004

5 Example 9

The efficacy of a 2:1 mixture of *B.t.i.* to *B.s.* on *Culex quinquefasciatus* colonies determined to be susceptible to *B.s.* was tested as follows.

10 A mixed susceptible colony of *Culex quinquefasciatus* was established from a combination of egg rafts collected from a site in the western United States. The collected egg rafts were individually transferred to 230 ml waxed paper cups each holding 200 ml tap water and 0.5 g rabbit pellets as larval diet. The larvae were hatched out. Then pupae were removed into cups with water and placed in screen cages, where the adults emerged. The adults were provided with 10% sucrose solution, and on day five after emergence, females were allowed to feed on restrained chicks. On day 5 subsequent to this blood feeding, oviposition cups were introduced into the cages to collect eggs. To maintain the colony in the laboratory, 4-5 egg rafts were placed in an enamel pan containing 2 liters of tap water and 2 g. of rabbit pellets as larval diet.

20 The sample preparation conditions, bioassay method, and LC₅₀ determination were the same as those described in Example 7. The results are provided in Table 8 below. The results show that over time, the test colony which was *B.s.* susceptible, becomes less susceptible by the fifth generation in response to treatment with *B.s.* alone, since the LC₅₀ value increases. In contrast, the 2:1 mixture does not show the same

tendency to the same extent. This fact is indicated by the data which, while showing an increase in LC₅₀ over the parental strain, also show less of an increase than for the treatment with *B.s.* alone. Therefore, use of the 2:1 *B.t.i.* and *B.s.* mix slowed the resistance over time.

5

Table 8

Table 8 also shows data from the original selection study through F20. One key change in the selection was that the mixture-selected colony was switched from a 2:1 *B.t.i./B.s.* (VectoBac WDG/VectoLex WDG) to a 1:1 mixture selection after F5.

10

Table 8 Change in susceptibility of a *B.s.* Susceptible Colony in Response to *B.t.i.* and *B.s.* mixtures.

Treatment	LC50 of Parental Colony	LC50 of 5th Generation	LC50 of 10th Generation	LC50 of 15th Generation	LC50 of 20th Generation
<i>B.s.</i>	0.009	0.035	0.066	0.194	0.124
<i>B.t.i. And B.s.</i>	0.009	0.024	0.028	0.013	0.044

15

Example 10

The following experiment was performed to demonstrate the utility of the mixture for controlling mosquitos of varying species. In this example, the effectiveness of the mixture and each individual larvicide were determined on a mixed population of *Culex quinquefasciatus* and *Aedes aegypti*.

20

Twenty plastic rearing tubs were placed outside in a midwestern United States location. The tubs were filled with deionized water and enriched with 2.4 g of ground guinea pig chow. The tubs were then infested with 100 third instar *Culex quinquefasciatus* and 100 third instar *Aedes aegypti*. One hour after the infestation, and just prior to treatment, tubs were sampled and the number of larvae in a test sample from each tub were counted, to obtain a control value.

25

Test larvicides included VECTOLEX CG (50 *B.s.* ITU/mg on corn cob granules), VECTOBAC CG (200 ITU/mg on corn cob granules) and a 1:1 mixture of VECTOLEX CG and VECTOBAC CG, as described in Example 2. Each tub was treated with an appropriate amount of one of the three test larvicides, equivalent to a single treatment rate of 5, 10 or 20 lbs/acre.

On the fifth and twelfth day after treatment, 100 third instar *Culex quinquefasciatus* and 100 third instar *Aedes aegypti* were added to each tub.

Larvae were sampled from the tubs on the second day, the seventh day and the fourteenth day after treatment to determine how many were still alive. The numbers were obtained and compared to the number of larvae alive prior to treatment. 100% reduction indicates that all larvae were killed. A positive number for percent reduction indicates that the larvicide does kill. The results are provided in Table 9 below, indicating that the 1:1 mixture of *B.t.i.* to *B. s.* can control *Culex quinquefasciatus* and *Aedes aegypti* larvae, even over a period of several days, at each application rate tested.

Table 9

Average Percent Reduction

Treatment	Application Rate (lb/acre)	% reduction in larvae - Day 2	% reduction in larvae - Day 7	% reduction in larvae - Day 14
1:1 <i>B.t.i.</i> : <i>B.s.</i>	5	100	55	56
<i>B. s.</i>	5	95	77	34
<i>B.t.i.</i>	5	100	84	73
1:1 <i>B.t.i.</i> : <i>B.s.</i>	10	100	98	91
<i>B. s.</i>	10	81	44	50
<i>B.t.i.</i>	10	100	92	78
1:1 <i>B.t.i.</i> : <i>B.s.</i>	20	100	97	90
<i>B. s.</i>	20	88	82	64
<i>B.t.i.</i>	20	100	100	76

Example 11

Another experiment was performed to demonstrate the utility of the mixture for killing mosquitos of varying species. In this example, the effectiveness of the mixture and each individual larvicide were determined on *Culex tarsalis*.

The test was performed on mosquitoes in a waste water pond of a waste water treatment facility in the western United States. The pond was extremely polluted, and standard tests showed the presence of *Culex tarsalis*. Tall reeds covered 80% of the water surface. The edge of the pond was divided into six plots ranging from 0.1 to 0.2 acres in size, for the purposes of the test.

Just prior to treatment, each test plot was sampled and the number of larvae in a test sample from each plot were counted, to obtain a control value.

Test larvicides were the formulations described in Example 10, and then each plot was treated with an appropriate amount of one of the three test larvicides, equivalent to a single treatment rate of 5 or 10 lbs/acre.

Larvae were sampled from the test plots on the second day, the seventh day and the fourteenth day after treatment, to determine how many were still alive. The numbers were obtained and compared to the number of larvae alive prior to treatment. 100% reduction indicates that all larvae were killed. A positive number for percent reduction indicates that the larvicide does kill. The results are provided in Table 10 below, indicating that the 1:1 mixture of *B.t.i.* to *B. s.* can control *Culex tarsalis* larvae.

Table 10
Average Percent Reduction

Treatment	Application Rate (lb/acre)	% reduction in larvae - Day 2	% reduction in larvae - Day 7	% reduction in larvae - Day 14
1:1 <i>B.t.</i> : <i>B.s.</i>	5	9	93	6
<i>B. s.</i>	5	90	100	100
<i>B.t.i.</i>	5	-25	47	28
1:1 <i>B.t.</i> : <i>B.s.</i>	10	88	96	65
<i>B. s.</i>	10	83	98	65

5 Example 12

Another experiment was performed to demonstrate the utility of the mixture for killing mosquitos of varying species. In this example, the effectiveness of the mixture and each individual larvicide were determined on *Culex pipiens* and *Culiseta incidens* outdoors in a roadside ditch.

10 Two sections of a roadside ditch separated by a driveway in the western United States were the site of the study. The ditches were vegetated with grasses and aquatic plants and received seepage runoff from a septic system as well as rainfall. A drainage swale with similar hydrology and vegetation was selected as the untreated control. Each site
15 was populated with *Culex pipiens* and *Culiseta incidens* at the time of treatment.

Just prior to treatment, each test plot was sampled and the number of larvae in a test sample from each plot were counted, to obtain a control value.

20 Test larvicides were of the formulations described in described in Example 10, and then two of the test sites were treated with an appropriate amount of one of the two test larvicides, equivalent to a single treatment rate of 20 lbs/acre. The remaining test site was untreated, to serve as the control.

Larvae were sampled from the test plots on the fourth day and the seventh day after treatment. The numbers were obtained and compared to the number of larvae prior to treatment. 100% reduction indicates that all larvae were killed. A positive number for percent reduction indicates that the larvicide does kill. The results are provided in Table 11 below, indicating that the 1:1 mixture of *B.t.i.* to *B. s.* can control *Culex pipiens* and *Culiseta incidens* larvae.

Table 11
Average Percent Reduction

Treatment	% reduction in larvae - Day 4	% reduction in larvae - Day 7
1:1 <i>B.t.i.</i> : <i>B.s.</i>	100	98
<i>B. s.</i>	100	100

Example 13

The efficacy of a 2:1 mixture *B.s.* (VECTOLEX) to *B.t.i.* (VECTOBAC) was determined in the field in the following manner.

The test field was a rice field in the western United States, measuring 156 acres. Levees were used a buffer zones between test plots. At the time of the treatment, *Anopheles freeborni* larvae were present at a density of 0.5-3.0 per dip, according to a standard dip test. Each individual larvicide, and the larvicidal combination were applied at a rate of 12 lbs/acre by airplane, which applied the granules at a speed of 85 miles per hour and with a swath width of 60 feet.

Larval counts were performed on days 2, 6 and 15 post-treatment, and measured against the larval count of an untreated control test plot.

The results are shown in Table 12 below, illustrating that a 2:1 mix effectively kills larvae, as a positive number for percent control of larvae indicates that the larvicide does kill.

Table 12

Average Percent Reduction

Treatment	% control of larvae at Day 2	% control of larvae at Day 6	% control of larvae at Day 15
2:1 <i>B.t.i.</i> to <i>B.s.</i>	50	100	0
<i>B. s.</i>	81	87	93
<i>B.t.i.</i>	100	100	90

Example 14

5 The following studies demonstrate the susceptibility of various *B.s.* susceptible and non-susceptible mosquitoes to mixtures. They support the claims of mixtures as a method of controlling mosquitoes, and controlling *B.s.* resistant mosquitoes. Three of the studies also support the method of mixing technical powders prior to formulation of granules.

10

Efficacy of a 1:1 mixture of *B.t.i.* and *B.s.* Formulations for Control of *B.s.* Resistant *Culex quinquefasciatus* Field Populations Compared to Each Formulation Separately.

Materials and Methods

15 A highly resistant population of *Culex quinquefasciatus* was identified in Wat Pikul, Bang Yai District, Nonthaburi Province, Thailand. This population was treated with various doses of VectoBac WDG (3000 Bti ITU) and VectoLex WDG (650 Bs ITU) and a 1:1 mixture of the two between January and September of 2001. Following each treatment,
20 population changes were assessed over time by dipping, and percent control of late instar larvae and pupae was calculated for post treatment days.

Results

25 Doses of VectoLex WDG as high as 200 mg/m² resulted in little or no control of this population. VectoBac WDG was found to provide control

at doses as low as 20 mg/m². A 1:1 mix of the two products was found to be more effective than either product alone at a dose of 20mg/m².

Table 13

5 Percent reduction of a *B.s.* resistant *Culex quinquefasciatus* field population after treatment with *B.s.*, *B.t.i.*, and a mixture of *B.t.i* and *B.s.*

Treatment	Day 2	Day 7	Day 14
<i>B.s.</i> 650 ITU @ 200 mg/m ²	13	nd	nd
<i>B.t.i.</i> 3000 ITU @ 20 mg/m ²	87	46	-53
1:1 mix (<i>B.s.</i> 325 ITU + <i>B.t.i.</i> 1500 ITU) @ 20 mg/m ²	97	50	22

10 Efficacy of Two *B.t.i./B.s.* Combination Formulations For Control of *Culex pipiens* and *Culiseta incidens* Compared to Standard *B.s.* Formulation in Artificial Plots

Materials and Methods

Twenty eight artificial test plots were set up using wading pools in the parking area of Multnomah County Mosquito Control District's facility at 5235 N. Columbia Blvd, Portland, OR on July 27, 2001. The pools were set out in four rows with seven pools per row. Each row was designated as a test series. The pools were filled to a depth of approximately 8" with tap water from the District's water supply. This depth was maintained throughout the study. Each pool had an approximate surface area of .785 M². Each pool was enriched with straw and rabbit chow (100gr.) to provide habitat and food for the mosquito larvae.

The pools were left to season the hay/rabbit chow mix, and to allow natural populations of the local mosquitoes to become established. Artificial stocking of the test pools was done after the local mosquito populations failed to produce adequate populations in the pools for the study. After this initial stocking, populations of *Culex pipiens* and *Culiseta incidens* maintained themselves by natural re-infestation.

Three formulations, designated ABG6185, VBC60015 and VBC60019 were compared in the study. ABG-6185 consisted of *B.s.* technical powder formulated onto corncob and had a potency of 50 *B.s.*

ITU. VBC-60015 and VBC-60019 were combinations of *B.t.i.* and *B.s.* technical powders formulated onto corncob and had theoretical potencies of 200*B.t.i.*/50*B.s.* ITU and 100*B.t.i.*/50*B.s.* ITU respectively.

Formulations were tested at application rates of 2.5kg/ha and 5kg/ha compared to untreated controls. Four replications were done for each application rate and UTC in a random pattern throughout the test series.

Sampling was done by taking 5 dips per pool using standard mosquito dippers and concentrating the larval catch with fine mesh strainers. Composite samples were preserved in alcohol for counting and species identification. Larval counts were recorded as L1-L2, L3-L4 and pupae.

Pretreatment counts, and the test product applications were done on August 20, 2001. The initial post-treatment larval counts were done on August 23, 2001 approximately 64 hours after the treatment. Follow-up counts were done on August 27, August 31 and a final count on September 6, 2001.

Control success was determined by calculating mean numbers of 3rd and 4th stage larvae, and pupae in the pre and post-treatment counts from the four replicates of each test. Percent control was calculated by and applying Mulla's formula to the overall population means for each treatment.

Results

Following treatment, mean populations in all treated plots declined relative to the untreated control plots, and were significantly lower ($P=.05$ Student-Newman-Keuls) than the UTC at 7 days post treatment. There were numerical, but not statistically significant differences between individual treatments. Initial reductions (3 days post treatment) were highest overall for the combination formulations, and similar control was seen from the formulation throughout the duration of the study. Percent reductions from treatments at 2.5 kg/ha are shown in Table 14.

Table 12 Corrected percent reduction of L3-L4 *Culex* and *Culesita* larvae in artificial plots following application of treatments at 2.5 kg/ha.*

Table 14

Treatment	Day 3	Day 7	Day 11	Day 17
<i>B.s.</i> 50 ITU	33.1	55.1	71	74.6
VBC 60015 (<i>B.t.i.</i> 200 ITU/ <i>B.s.</i> 50 ITU)	65.1	97.2	97.2	81.2
VBC 60019 (<i>B.t.i.</i> 100 ITU/ <i>B.s.</i> 50 ITU)	82.5	94.1	85.8	65.7

5 *corrected against UTC based on population means from 5 replicates

Efficacy of Two *B.t.i./B.s.* Combination Formulations for control of *Culex tarsalis* and *Culex pipiens* Compared to Standard *B.s.* Formulation in Small Field Plots

10 Materials and Methods

The test site was a marsh where a small stream entered the Yakima River near Yakima, Washington. Natural populations of *Culex tarsalis* and *Culex pipiens* were present. Water was essentially stagnant. Depth was 6-12 inches and remained constant throughout the experiment. Water temperature ranged between 72 and 77 throughout the experiment. Vegetation was primarily grass with scattered broadleaf weeds covering 80% of the surface. plant height was 6-15 inches in height. Cattle occasionally grazed in the site, but were not present during the test. Organic matter was very high at the site. Predator populations were generally low in the plots.

20 The test was a randomized complete block experiment with three, 1000 square foot plots per treatment. Plots were sampled immediately prior to the application on 7-19-01 and again 48 hours, 7 and 14 days after the application. Twenty dips with a standard mosquito dipper were made in each plot. Larvae instar 1-2, larvae instar 3-4 and pupae were counted in each dip.

25 Three formulations, designated ABG6185, VBC60015 and VBC60019 were compared in the study. ABG-6185 consisted of *B.s.* technical powder formulated onto corncob and had a potency of 50 *B.s.* ITU. VBC-60015 and VBC-60019 were combinations of *B.t.i.* and *B.s.*

technical powders formulated onto corncob and had theoretical potencies of 200*B.t.i.*/50*B.s.* ITU and 100*B.t.i.*/50*B.s.* ITU respectively.

Formulations were tested at an application rate of 5 lb/acre and compared to untreated controls. Data were analyzed with Analysis of Variance.

5 Percent reductions were calculated with Mulla's formula and based on large larvae and pupae only. Overall population means for each treatment were used in this calculation.

Results

10 At two days after treatment, all three formulations provided significant although not outstanding control. The rate of 5 lbs/A may have been rather low for this site. Control at 7 and 14 days was not significant largely because the larval population in replicate two of the UTC had very low population. Nonetheless, percentage control was higher in all the
15 treated plots than in untreated plots on day 7 and 14. In comparing the formulations, there did not appear to be any difference between treatments at day 2 but both the VBC formulations were better than ABG-6185 at day 7. Corrected percent reductions calculated using overall population means of L3-pupae are shown in Table 15.

20

Table 15 Corrected percent reduction of L3-L4 *Culex* larvae and pupae in small field plots following application of treatments at 5 lb/acre.*

Table 15

Treatment	Day 2	Day 7	Day 14
<i>B.s.</i> 50 ITU	50.0	52.3	17.3
VBC 60015 (<i>B.t.i.</i> 200 ITU/ <i>B.s.</i> 50 ITU)	58.0	73.4	64.0
VBC 60019 (<i>B.t.i.</i> 100 ITU/ <i>B.s.</i> 50 ITU)	59.4	81.7	38.4

25 Efficacy of Four *B.t.i.*/*B.s.* Combination Formulations for Control of *Ochlerotatus taeniorhynchus* Compared to Standard *B.t.i.* Formulation in Artificial Plots

Materials and Methods

20 Twenty-four artificial test plots located at the John A. Mulrenan, Sr., Public Health Entomology Research and Education Center, in Panama

City, Florida were utilized in this study. The plots were filled to a depth of approximately 6" with 3-5 ppt saline water. This depth was maintained throughout the study. Each plot had an approximate surface area of 8 ft². Emergent grasses and a sandy soil substrate were present in the plots. Water temperature averaged 75 degrees Fahrenheit during the study.

Artificial infestation of the test plots was done prior to initial treatment and every other day following the treatments. Approximately 1000 third instar *Ochlerotatus taeniorhynchus* larvae were added to each plot on each infestation day.

Five formulations, designated ABG6138s, VBC60015, VBC60016, VBC60018 and VBC60019 were compared in the study. ABG-6138s consisted of *B.t.i.* technical powder formulated onto corncob and had a potency of 200 *B.t.i.* ITU. VBC-60015, VBC60016, VBC60018 and VBC-60019 were combinations of *B.t.i.* and *B.s.* technical powders formulated onto corncob and had theoretical potencies of 200*B.t.i.*/50*B.s.* ITU, 100*B.t.i.*/25*B.s.* ITU, 200*B.t.i.*/25 *B.s.* ITU, and 100*B.t.i.*/50*B.s.* ITU respectively. Formulations were tested at application rates of 2.8kg/ha compared to untreated controls. Four replications were done for each application rate and UTC in a random pattern throughout the test series.

Sampling was done by taking 8 dips per plot, using standard mosquito dippers. Numbers of larvae collected from each plot were recorded. Initial sampling was done 1 day following initial infestation and treatment, and repeated on days 2, 3, 5, 7, 10, and 13 following treatment.

Control success was determined by comparing number of larvae collected in treated plots to numbers collected in the UTC plots. Percent mortality was calculated for each treatment using the following formula.

% mortality = (# larvae in control - # of larvae in treatment)/ # larvae in control

Results

One day after treatment, populations in all treated plots were significantly lower than in the UTC plots. Mean percent reductions on day

one ranged from 79.7% for VBC60015 to 98.5 % for VBC60018. There were no significant differences between the treatments on this day. The materials continued to show efficacy through day 7 of the study, after which time percent control declined rapidly. Three of the combination treatments, VBC60015, VBC60018 and VBC60019, provided significantly higher overall percent reductions through the course of the 13-day study (LSMEANSSS multiple comparison test $p=0.05$). Percent reductions through day 7 from treatments at 2.8 kg/ha are shown in Table 16.

Table 16. Percent reductions through day 7 from treatments at 2.8 kg/ha

Table 16

Treatment	Day1	Day2	Day 3	Day 5	Day 7
<i>B.s. 50 ITU</i>	84.9	58.8	51.6	71.7	14.8
<i>VBC 60015 (B.t.i. 200 ITU/B.s. 50 ITU)</i>	79.7	71.9	76.5	67.3	43.5
<i>VBC 60016 (B.t.i. 100 ITU/B.s. 25 ITU)</i>	84.2	75	19.3	63.8	20.5
<i>VBC 60018 (B.t.i. 200 ITU/B.s. 25 ITU)</i>	98.5	65.7	64.2	70.3	38
<i>VBC 60019 (B.t.i. 100 ITU/B.s. 50 ITU)</i>	91.3	67.1	62.7	60.4	28.1

All references cited are hereby incorporated by reference.

The present invention is illustrated by way of the foregoing description and examples. The foregoing description is intended as a non-limiting illustration, since many variations will become apparent to those skilled in the art in view thereof. It is intended that all such variations within the scope and spirit of the appended claims be embraced thereby.

Changes can be made in the composition, operation and arrangement of the method of the present invention described herein without departing from the concept and scope of the invention as defined in the following claims:

Claims

We claim:

- 5 1. A method of controlling Dipteran larvae comprising the step of introducing a larvicidally-effective amount of a combination of a strain of *Bacillus thuringiensis* subspecies *israelensis* and a strain of *Bacillus sphaericus* into an environment containing Dipteran larvae.
- 10 2. The method of claim 1 wherein said strain of *Bacillus thuringiensis* subspecies *israelensis* is non-genetically modified.
3. The method of claim 1 wherein said strain of *Bacillus sphaericus* is non-genetically modified.
- 15 4. The method of claim 1 wherein said strain of *Bacillus thuringiensis* subspecies *israelensis* is non-genetically modified and said strain of *Bacillus sphaericus* is non-genetically modified.
- 20 5. The method of claim 1 wherein said combination has from about 1:10 to about 10:1 weight ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*.
6. The method of claim 1 wherein said combination has from about 1:3 to about 3:1 weight ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*.
- 25 7. The method of claim 1 wherein said combination has from about 1:2 to about 2:1 weight ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*.
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8. The method of claim 1 wherein said combination has a 1:1 ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*.

5 9. The method of claim 1 wherein said Dipteran is a mosquito.

10 10. The method of claim 9 wherein said mosquito is selected from the group consisting of *Culex pipiens*, *Culex quinquefasciatus*, *Aedes aegypti*, *Culex tarsalis*, *Culiseta incidens*, *Anopheles freeborni* and combinations thereof.

15 11. A method for inhibiting larvicidal resistance in Diptera comprising the step of introducing a larvicidally-effective amount of a combination of a strain of *Bacillus thuringiensis* subspecies *israelensis* and a strain of *Bacillus sphaericus* into an environment containing Dipteran larvae.

20 12. The method of claim 11 wherein said strain of *Bacillus thuringiensis* subspecies *israelensis* is non-genetically modified.

13. The method of claim 11 wherein said strain of *Bacillus sphaericus* is non-genetically modified.

25 14. The method of claim 11 wherein said strain of *Bacillus thuringiensis* subspecies *israelensis* is non-genetically modified and said strain of *Bacillus sphaericus* is non-genetically modified.

30 15. The method of claim 11 wherein said combination has from about 1:10 to about 10:1 weight ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*.

16. The method of claim 11 wherein said combination has from about 1:3 to about 3:1 weight ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*.

5 17. The method of claim 11 wherein said combination has from about 1:2 to about 2:1 weight ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*.

10 18. The method of claim 11 wherein said combination has a 1:1 ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*.

19. The method of claim 11 wherein said Diptera is *Culex*.

15 20. The method of claim 11 wherein larvicidal resistance is developed against *Bacillus sphaericus*.

21. The method of claim 11 wherein said Diptera is a mosquito.

20 22. The method of claim 21 wherein said mosquito is selected from the group consisting of *Culex pipiens*, *Culex quinquefasciatus*, *Aedes aegypti*, *Culex tarsalis*, *Culiseta incidens*, *Anopheles freeborni* and combinations thereof.

25 23. A composition comprising:
a combination of a strain of *Bacillus thuringiensis* subspecies *israelensis* and a strain of *Bacillus sphaericus*.

30 24. The composition of claim 23 wherein said strain of *Bacillus thuringiensis* subspecies *israelensis* is non-genetically modified.

25. The composition of claim 23 wherein said strain of *Bacillus sphaericus* is non-genetically modified.

26. The composition of claim 23 wherein said strain of *Bacillus thuringiensis* subspecies *israelensis* is non-genetically modified and said strain of *Bacillus sphaericus* is non-genetically modified.

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27. The composition of claim 23 wherein said combination has from about 1:10 to about 10:1 weight ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*.

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28. The composition of claim 23 wherein said combination has from about 1:3 to about 3:1 weight ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*.

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29. The composition of claim 23 wherein said combination has from about 1:2 to about 2:1 weight ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*.

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30. The composition of claim 23 wherein said combination has a 1:1 ratio of *Bacillus thuringiensis* subspecies *israelensis* to *Bacillus sphaericus*.

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31. The composition of claim 23 further comprising an additional component selected from the group consisting of a surface active agent, an inert carrier, a preservative, a humectant, a feeding stimulant, an attractant, an encapsulating agent, a binder, an emulsifier, a dye, a U.V. protectant, a buffer, a drift control agent, a spray deposition aid, a free-flow agent and combinations thereof.

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32. The composition of claim 23 wherein slurries of both strains are spray dried.

33. The composition of claim 32 wherein the slurries are spray dried separately.

34. The composition of claim 32 wherein the slurries are mixed together and the mixture is spray dried.

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