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Interactions and Persistence of the Mosquitocidal Bacteria

*Bacillus sphaericus* and *Bacillus thuringiensis* var.

*israelensis*

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Abstract approved:_____

William P. Stephen

Interactions between *Bacillus sphaericus* and *Bacillus
thuringiensis israelensis* (Bti), persistence of insecticidal
activity in the mosquito habitat, and suitability of enzyme-
linked immunoabsorbent assay (ELISA) for detecting and
quantifying bacterial endotoxin in the mosquito habitat were
investigated. Interactions between the two bacterial
species were examined by laboratory and field bioassays with
larvae of *Culex pipiens*. In laboratory bioassays, *B.
sphaericus* and Bti acted independently. Antagonistic
interactions occurred with combinations of a low
concentration of one bacterium with a high concentration of
the second, however. In field bioassays, all interactions
between Bti and *B. sphaericus* were antagonistic. Field
bioassays with *C. pipiens* indicated no persistence of

insecticidal activity of either Bti or *B. sphaericus* after
two treatments and persistence of only *B. sphaericus* after 3
and 4 treatments. Organic matter and high temperatures
adversely affected the accuracy of ELISA for detecting and
quantifying bacteria in the mosquito habitat and may play a
major role in detoxification of bacterial endotoxin in
nature.

Interactions and Persistence of
the Mosquitocidal Bacteria
*Bacillus sphaericus* and
*Bacillus thuringiensis* var. *israelensis*

by

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Redacted for Privacy
_____
Professor of Entomology in charge of major

Redacted for Privacy
_____
Head of department of Entomology

Redacted for Privacy
_____
Dean of Graduate School

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INTERACTIONS AND PERSISTENCE
OF THE MOSQUITOCIDAL BACTERIA *Bacillus sphaericus*
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INTRODUCTION AND LITERATURE REVIEW


Because mosquitoes are the most important vectors of
medically significant diseases, and major pests, successful
mosquito control is critical. Major obstacles to effective
control have been the high incidence of resistance in
mosquitoes to insecticides and adverse effects of the
insecticides on the environment. Consequently, use of
biological control agents, including microorganisms, has
increased. Currently, microbial control of mosquitoes
includes the commercial or experimental application of
protozoa, fungi, viruses, nematodes and bacteria.

Since the 1950's commercially produced formulations of
the bacterium *Bacillus thuringiensis* Berliner have been used
in the control of lepidopterous pests (Lacey, 1985). In
1973 two groups of bacteria were isolated from mosquito
larvae. One group belonged to the *Bacillus alvei-B. brevis-
B. circulans* complex while the second group of bacteria was
identified as *B. sphaericus*. Since that time approximately
30 strains of *B. sphaericus* have been isolated, all of which
have shown toxicity only to mosquito larvae (Singer, 1985).
Strains 1593, 2362 and 2297 have demonstrated the greatest
insecticidal activity and are the most likely candidates for
commercial production (Singer, 1985).

In 1976 a serotype of *B. thuringiensis* was isolated from *Culex pipiens* larvae in Israel. This serotype, designated *Bacillus thuringiensis* var. *israelensis* (serovar H-14), displayed excellent activity against mosquito larvae (Lacey, 1985). The insecticidal activity of *Bacillus thuringiensis var. israelensis* (Bti) appears limited to certain families of nematocerous Diptera; the Simuliidae, Chironomidae, and Culicidae (Mulla et al., 1984). Bti displays good efficacy, specificity, biodegradability and shelf-life. Three major companies currently produce Bti formulations for the control of black flies and mosquitoes. While other species of *Bacillus* are used for the control of lepidopterous and coleopterous pests, i.e. *B. thuringiensis*, *B. popilliae,* and *B. lentimorbus*, Bti and *B. sphaericus* are the only bacteria currently used for the control of mosquitoes.

Both Bti and *B. sphaericus* are ubiquitous saprophytes found in water and soil habitats. The insecticidal activity of both bacteria derives from a delta-endotoxin located in the parasporal crystalline inclusion, formed simultaneously with the spore during sporulation. After ingestion by the mosquito larva the high pH of the larval midgut causes the breakdown of the parasporal crystal into several toxic proteins (Singh, 1986; Davidson, 1981). An alkaline environment, such as the larval midgut (pH 10), is required for breakdown of the Bti and *B. sphaericus* protoxins into the insecticial components.

The combination of different bacterial species for insect control has been investigated by several authors. Kudler and Lysenko (1964) found that combinations of *B. thuringiensis* with species of *Pseudomonas, Serratia, Enterobacter* or *Flavobacterium* are no more effective against lepidopteran larvae than an equivalent amount of Bt used alone .

Competitive interaction, where only one bacterium of a mix achieves infection and is responsible for insect mortality, has been suggested. Stephens (1959) fed different proportions of the bacteria *Pseudomonas aeruginosa* and *Serratia marcescens* to the grasshopper *Melanoplus bivittatus*. Examination of the insects' blood, and culturing of dead insects, revealed that death resulted from infection produced by only one of the bacteria, that bacterium fed in the greatest numbers. When a one-to-one proportion of the two bacteria was used all insect death was attributed to *S. marcescens*. Beard (1946) injected *B. popilliae* and *B. lentimorbus* into the body cavity of Japanese beetle larvae but never observed simultaneous infection of the beetle larvae by the two bacteria . Beard suggested that antibiotic activity of the two bacteria might account for their mutually exclusive development in the beetle larvae. Krieg (1971b) noted that bacteriocins were responsible for antagonism among strains of *B. thuringiensis*. Bacteriocins are typically proteins of high molecular weight, produced by many bacteria, that inhibit or

kill closely related species and differ from antibiotics in
having a narrower spectrum of activity (Brock et al., 1970).
Krieg found that bacteriocins are liberated by the *B.
thuringiensis* strains through autolysis of cells during the
logarithmic phase of growth.  Vankova (1965) observed in
vitro production of bacteriocins in several varieties of *B.
thuringiensis* effective against other gram positive
bacteria.  Pendleton (1969), studying infections in
lepidopteran larvae from a mixture of two strains of Bt,
demonstrated that bacteriocin production, rather than
pathogenicity, determines strain dominance.  In nature,
mixed bacterial infections of insects are rare, possibly a
result of bacteriocin antagonisms between bacterial species
(Krieg, 1971a).  Bacteriocin production by Bti and *B.
sphaericus* has not been reported but may prove to be an
important component of their interaction.

Steinhaus (1959) noted both antagonism and synergism in
his work on sequential infection of *Galleria* larvae with *S.
marcescens* and Bt.  *S. marcescens* inhibited the development
of Bt, possibly due to antibiotic action of *S. marcescens*,
whereas Bt enhanced the development of *S. marcescens*.

Zurabova (1968) reported synergism among three
serotypes of Bt:  mixtures of the three strains were more
potent than equivalent quantities of any one serotype.
Isakova (1964)  observed synergism of Bt and
Enterobacteriaceae gut bacteria in larvae of *Pieris rapae*.
Isakova proposed that synergism results from Bt interference

with the normal functioning and defense mechanisms of the insect gut, causing a subsequent increase in the insects' susceptibility to the Enterobacteriaceae bacteria.

Both Bti and *B. sphaericus* persist and recycle in the environment (Lacey, 1985 and Singer, 1985). This persistence of both bacteria is significant because of the implications for their combined use. If their interaction is antagonistic or synergistic, sequential use might be timed to avoid antagonism or enhance synergism. Larval assays and bacterial plate counts have been used as means of detecting the bacteria and determining their persistence. Both methods possess several weaknesses as indicators of bacterial presence and persistence.

There is considerable variation in the susceptibility of mosquito larvae, both within and between species, used in larval assays (Davidson, 1984). Wraight et al. (1982) demonstrated that even larval rearing conditions prior to testing can have a significant impact upon determination of lethal doses. There is additional oppportunity for inaccuracy in larval assays because the actual amount of toxin ingested by each larva is not known, only the environmental dosage to which all larvae are exposed. Thus, not only will larval variation among species be a factor in larval susceptibility to the bacteria but larval feeding behavior and feeding rate will also be significant. Attempts have been made to standardize the use of larval assays for determination of Bti and *B. sphaericus* toxicity

(Mclaughlin et al., 1984) but the protocol relies on the use of only one instar of only one mosquito species. Reseachers working with other species of mosquito may find using the standard species inconvenient and the results inapplicable.

The bacterial count plating method for identifying bacteria and establishing environmental persistence also has several limitations. First, because the pathogenicity of Bti and *B. sphaericus* is not fully understood, the relationship between the plate count and the larvicidal activity can not be defined (Singh et al., 1986; Davidson, 1981). Davidson (1984) states that plating may give misleading counts because of spore clumping and that nonviable spores, which can contribute to larvicidal activity, are not represented. Second, the objective of bacterial counts is to establish insecticidal activity found within the mosquito habitat from which the samples were obtained; plating on artificial lab media may not accurately represent the activity of the bacteria in the habitat. For example, lab media may induce bacterial toxin production which is not actually occurring in the sample's habitat. Both Bti and *B. sphaericus* are naturally occurring soil microorganisms which develop full toxicity only during sporulation and complete sporulation requires high oxygen concentrations (Davidson, 1981). A mosquito environment such as a pond lacks high oxygen levels, yet samples taken from such habitats are plated onto artificial media and typically kept in an aerobic environment. Consequently, the

plating results can not be assumed to accurately reflect the habitat of the sample source.

Enzyme linked immunoabsorbent assay (ELISA) is being explored as an alternative method to larval assays and bacterial plating. Armstrong et al. (1985) used ELISA in their procedure for purification of the Bti endotoxin. Wie et al. (1984), using a polyclonal antibody to Bti, revealed that the antibody did not cross react with other *Bacillus* spp. toxins. They also found a good correlation between LC$_{50}$ values obtained with larval assays and toxin levels detected by ELISA. Once refined, ELISA may provide an accurate and convenient procedure for detection and quantification of Bti and *B. sphaericus* endotoxins.

Several studies suggest that *B. sphaericus* remains in the environment and displays insecticidal activity longer than Bti (Silapanuntakal et al., 1983; Mulligan et al., 1980; Prasertphon et al., 1975; Ramoska et al., 1981). Factors influencing the environmental persistence and residual insecticidal activity of Bti and *B. sphaericus* in mosquito habitats are ultraviolet light, temperature, pH, organic matter content, settling of formulation, and ability of the bacteria to recycle in the environment.

Ignoffo et al. (1981) and Davidson et al. (1984) demonstrated that both artificially induced ultraviolet light in the lab and naturally occurring ultraviolet light in the field had an adverse effect on insecticidal activity of Bti and *B. sphaericus*, respectively.

As temperature determines larval feeding rates and metabolism of bacterial toxin, it is a significant factor in the efficacy of Bti and *B. sphaericus*. Sinegre et al. (1981) showed a positive correlation of Bti activity with temperatures below 19°C and above 33°C, and no correlation within the 19-33°C range. Wraight et al. (1982) found that the efficacy of *B. sphaericus* declined more rapidly with a decrease in temperature than did that of Bti.

Mulligan et al. (1980) demonstrated no loss of Bti insecticidal activity from pH treatments of 4.3, 5.6, 8.3, 8.5 and 10.0. In contrast, *B. sphaericus*, subjected to the same pH treatments, retained only partial toxicity after 48 hours at pH 4.3 and displayed no activity after 48 hours at pH 10.

It has been shown that as the quantity of soil suspended in the mosquito habitat increases there is an accompanying decrease in insecticidal activity of Bti and *B. sphaericus* (Mulligan et al., 1980; Van Essen and Hembree, 1982; Ramoska et al., 1981; Hornby et al., 1981). The inactivation process is believed to be related to the particle charge on the bacterial crystal which acts amphoterically and adsorbs to the clay, sand and/or organic matter of the soil. The adsorption of the crystal apparently prevents it from dissolving in the larval gut and hence renders it inactive (Ramoska et al., 1981). Ramoska et al. (1981) demonstrated that silts and clays exhibit the greatest affinity to bacteria and can cause up to a 50%

reduction in Bti efficacy. Based on average LC$_{50}$ values, Ignoffo et al. (1981) found that Bti is 85 times more active against mosquito larvae in distilled water than in pond water. Mulligan et al. (1980) reported 98% mortality of *Culex quinquefasciatus* larvae in *B. sphaericus* distilled water treatments but only 15% mortality when treated with *B. sphaericus* in sewage effluent . Silapanuntakul et al. (1983) found that both Bti and *B. sphaericus* provide greater persistence, against *Aedes aegypti* and *Culex pipiens* respectively, in tap than polluted water. Van Essen and Hembree (1982) noted that smaller soil particles produce a more deleterious effect on larvicidal activity than larger particles and postulated that smaller particles have more surface area at a given concentration and thus greater opportunity for adsorption. Ramoska et al. (1981) suggested that sand traps the Bti and *B. sphaericus* crystals mechanically and causes them to settle more rapidly out of the larval feeding zone than would occur normally. In contrast to its effect on insecticidal activity, a high concentration of organic matter in water may have a beneficial effect on spore persistence. Hornby et al. (1981) found that *B. sphaericus* spores remain viable a maximum of 50 days in fresh water and 90 days in sewage water. They suggested that the organic matter in sewage water provides *B. sphaericus* spores with greater protection from ultraviolet light, and decreased exposure to particle feeding organisms.

Bti and *B. sphaericus* may retain insecticidal activity for prolonged periods; however, if they are not in the larval feeding zone they are ineffective control agents. Generalizations about the settling of the two bacteria are not possible because the type of formulation determines their settling rate. Furthermore, different species of mosquitoes display different feeding behavior, i.e. *Anopheles* spp. are surface feeders whereas *Culex* spp. are bottom feeders (Bates, 1949). Hence the site of the larval feeding zone must be defined for each mosquito species tested. Guillet et al. (1980) compared settling rates of primary powder, water-based concentrate and water-dispersed formulations of Bti and found that the sedimentation of the primary powder and water-dispersed formulations were identical; settling was noticeable at 30 minutes and after 24 hours virtually all material had settled to the bottom. In contrast, no sedimentation of the water-based concentrate was detectable after 24 hours. Davidson et al. (1984) observed that the spores of *B. sphaericus* rapidly settle to pond bottoms during field testing .

Both Bti and *B. sphaericus* are facultative rather than obligate pathogens and have the potential to recycle both in the mosquito host and in the mosquito habitat. Hertlein et al. (1979) retrieved viable and infective *B. sphaericus* from a roadside ditch where it had been applied 9 months earlier. Singer (1980) demonstrated that detritus removed from tree holes treated with *B. sphaericus* nine months earlier

displayed insecticidal activity. Bti has not demonstated this same potential for significant recycling in the environment. One study on a serotype of Bt indicated that in nature Bt only achieves significant multiplication in hosts, or in soil artificially enriched with protein, but not in unammended soil (Aly et al., 1985).

Both Bti and *B. sphaericus* have been shown to recycle in dead mosquito larvae in the lab and in the field (Ignoffo et al., 1981; Davidson et al., 1984; Aly, 1985; Aly et al., 1985). In lab and field experiments recycling of *B. sphaericus* in dead larvae resulted in increases of 100 to 1000 fold in spore numbers three days after treatment (Davidson, 1984) Aly et al. (1985) observed a thirty fold increase in Bti spore count in the gut of *Aedes* larvae seventy-two hours after treatment. They proposed a theory to account for the apparent incongruity of Bti and *B. sphaericus* recycling in larvae and in the environment but failing to cause epizootic outbreaks of disease . Toxin production in Bti and *B. sphaericus* is restricted to the sporulation phase of reproduction and sporulation typically does not occur within the first day after the death of the host larvae. Scavengers and detritivores in the habitat probably disrupt the larval cadavers and remove or disperse the substrates necessary for bacterial growth. After disintegration of the larvae, bacteria sink to the bottom of the habitat, exposing them to microbial degradation in the detritus. Competition from other microorganisms which

colonize larval cadavers may also be significant in preventing Bti and *B. sphaericus* population buildups and resulting epizootic outbreaks.

The interactions that occur with combined use of Bti and *B. sphaericus* for mosquito control should be studied for several reasons. First, insecticide interactions within insects may occur at ingestion, distribution, biotransformation reactions, target receptor sites or excretion (Wilkinson, 1976). In respect to biotransformation reactions, one compound may inhibit or stimulate the activity of the enzyme(s) responsible for the metabolism of the other compound. The result of this interaction may be synergistic or antagonisitic depending on whether the enzyme(s) affected activates or inactivates the second compound. Direct interaction at the target receptor site is rare and is usually antagonistic (Wilkinson, 1976). The overall effect of insecticide interaction results from a dominant primary mechanism combined with several secondary events which occur in direct response to the primary interaction. With the combined use of Bti and *B. sphaericus* there is the potential for interactions among spores and endotoxins. Second, because the bacteria display different host ranges their simultaneous use in a habitat containing several mosquito species may be required. In general, *B. sphaericus* is effective against *Culex* and *Anopheles* spp. while it is relatively ineffective against *Aedes* spp., and Bti controls *Aedes* and *Culex* spp. but not *Anopheles* spp.

(Mulla et al., 1984; Silapanuntakal et al., 1983; Singer, 1985; Lacey, 1985). Third, research has indicated that lab colonies can develop resistance to Bti. Georghiou (1983) applied intensive selection pressure with Bti to larvae of *Culex quinquefasciatus* for twenty-eight generations and demonstrated a slow but significant decrease in susceptibility to Bti. Georghiou examined these same mosquitoes for cross resistance to three isolates of *B. sphaericus* and observed no resistance to one isolate, and very low resistance to the other two. The toxicity of the two bacteria apparently involves different modes of action (Davidson, 1981; Lacey, 1985; Singer, 1985). Both bacteria cause loss of integrity of the gut epithelium, cessation of feeding, general body paralysis and eventual death (Lacey, 1985; Singer, 1985). The biochemical pathways on which they act appear to differ, however (Davidson, 1981). Consequently, alternating the use of the two bacteria may prove advantageous in resistance-management programs. Finally, because of differences of the two bacteria as mosquito control agents, Bti and *B. sphaericus* might be used in combination with Bti providing initial knockdown of the mosquito population and *B. sphaericus* supplying long-term control.

The primary considerations regarding persistence and residual insecticidal activity of Bti and *B. sphaericus* are whether bacterial growth is possible in the specific environment, if growth includes production of insecticidal

products, and whether insecticidal products reach and remain in the larval feeding zone.

In this study, interactions between Bti and *B. sphaericus* were investigated through laboratory and field bioassays with larvae of *Culex pipiens*. The objective of the bioassays was to establish if combined use of Bti and *B. sphaericus* has favorable or adverse effects on the efficacy of each bacterium. The residual insecticidal activity of both bacteria was determined with field bioassays and, for Bti only, with ELISA. The purpose of examining residual insecticidal activity was to establish and compare the persistence of each bacterium and to evaluate ELISA as an experimental tool in persistence studies.

MATERIALS AND METHODS

LABORATORY BIOASSAYS:

Bacterial insecticide formulations were provided by
Abbott Laboratories: Vectobac, an aqueous Bti formulation,
lot #75-018-BA, and *B. sphaericus*, an experimental powder,
strain #2362. The Vectobac has been assigned a toxicity of
600 international toxic units (ITU's) by Abbott
Laboratories. The potency of a Bti preparation is expressed
in international toxic units relative to a standard
preparation and determined by the formula: ITU/mg sample =
($LC_{50}$ standard/$LC_{50}$ sample) x potency of the standard in
ITU/mg. The toxicity of the *B. sphaericus* was unknown, and
no protocol existed for determining its ITU equivalent.
Thus, laboratory bioassays were run based on the standard
method (McGlaughlin et al., 1984) for assigning ITU values
to Bti formulations. Because *B. sphaericus* requires 48
hours to reach maximum mortality (Mulla et al., 1984), the
bioassays were run for 48 hours rather than the 24 hours
specified for Bti, and third instars were used in place of
fourth instars. Third instars were used so that pupation
would not occur during the assay period of 48 hours. Late
fourth instars and pupae do not feed and hence would not
ingest the bacteria. The changes made from the original
protocol were not considered important because the intent of
the research was not to establish an absolute ITU value for

the *B. sphaericus* formulation but rather to determine appropriate laboratory and field dosages. Probit analysis (probit 1.0 Version, G. Clinkerbeard, OSU) was performed on the data to obtain lethal dose (LD) values. Analysis indicated the toxicity of the *B. sphaericus* formulation was approximately 8.5 times greater, on a weight basis, than that of the Bti formulation. The mosquito larvae were from a colony of an anautogenous strain of *C. pipiens* collected in 1978 from a log pond in Philomath, Oregon, and reared at 26 to 28°C, with a 16:8 photoperiod. The adults received a 10% sugar solution and blood meals from japanese quail. The larval diet consisted of lab chow, liver extract, yeast and vitamin C.

Two types of laboratory bioassays were performed; one to compare the effects of single and combined treatments of Bti and *B. sphaericus*, and one to determine if a time trend in mortality of the combined treatments could be detected and correlated with the activity of one of the bacteria. Both assays were performed identically except that in the first type one mortality count was taken at 48 hours on thirty replications (one per day for a month), whereas in the second mortality counts were taken at 12, 24, 36, and 48 hours on ten replications (one per day for ten days).

Laboratory bioassays tested twenty-six treatments; four concentrations of Bti, four concentrations of *B. sphaericus*, sixteen combinations of these concentrations, and two controls. Solutions of Bti and *B.sphaericus* were prepared

with dechlorinated tap water and mixed for five minutes with a magnetic stirrer. Each treatment was prepared by adding 100 ml of dechlorinated tap water to a 5-oz disposable cup and 0.1 ml of the appropriate concentration of Bti, *B. sphaericus*, or both. Dosages were: 1.0, 0.1, 0.01 and 0.001 ITU Bti/ml, 0.1, 0.01, 0.001,and 0.0001 microgram *B. sphaericus*/ml, and combined dosages of Bti ITU per ml/ *B. sphaericus* microgram per ml of 1.0/0.1, 1.0/0.01,1.0/0.001, 1.0/0.0001, 0.1/0.1, 0.1/0.01, 0.1/0.001, 0.1/0.0001, 0.01/0.1, 0.01/0.01, 0.01/0.001, 0.01/0.0001, 0.001/0.1, 0.001/0.01, 0.001/0.001, and 0.01/0.0001. Two controls, consisting of dechlorinated tap water only, were run at each assay. Twenty third-stage *C. pipiens* were transferred into each cup by pipette. The assay cups were placed in an incubator set at 28°C, with a photoperiod of 16:8, for 48 hours. The larvae were not fed during the assay. Statistical analysis of the bioassays with only a 48-hour mortality count was based on the use of isoboles for graphic representation of the effect of two pesticides applied jointly (Tammes, 1964). The data from the bioassays with mortality counts every twelve hours was examined for time trends in mortality with the Mantel-Haenszel statistical test (Mantel, 1966).


FIELD BIOASSAYS:

Field bioassays examined the residual activity, and compared the effects of single and combined use, of Bti and *B. sphaericus*. Field bioassays tested eight treatments; two concentrations of *B. sphaericus*, two concentrations of Bti, the four combinations of the Bti and *B. sphaericus* concentrations, and two controls. Ten experimental ponds on the OSU campus, earthen bottomed, containing cattails and sedges, square shaped, and ranging in size from 23 to 29.7 square meters, were used. The ponds were dug in 1976 but not used until the summers of 1984 and 1985 when testing of experimental formulations of Bti and *B. sphaericus* produced by Microbial Resources and Abbott Laboratories were conducted. Prior to the start of the field bioassays all ponds contained some rain water and each was filled to a depth of .457 meter with creek water. Cages measured .457 meter by .457 meter feet, were framed with PVC pipe, covered with fine mesh and open at both ends. One cage was placed in each pond and secured to the substrate by nails placed through holes drilled in the bottom PVC frame. Temperature was monitored throughout the experiment using a two probe Datapod temperature recorder placed in one of the ponds, with 1 probe placed 3 cm below the water surface and the other probe placed 3 cm above the pond bottom.

The hatch from eight egg rafts was placed into each cage when larvae became early third instars (approximately 800 larvae/cage). The larvae were climatized by removal from the rearing room into an unheated room two days prior

to being placed in the ponds. Twenty-four hours after the larvae were placed in the cages, a pre-treatment sample was taken and the number of live and dead larvae was recorded. The pre-treatment sample consisted of four dips per cage, one dip per corner, taken with a plastic 350 ml mosquito dipper.

Immediately after the pre-treatment sampling the ponds were sprayed with Bti or *B. sphaericus*, or both. Pond dosages were based on the recommended field dose application rate for Vectobac of 75 ITU/m². Two dosages were used for Bti and *B. sphaericus*, designated low and mid (1/5 and 1/3 of the recommended field dose) and calculated based on the surface areas of the ponds. Pond treatments were:

| Pond | ml Bti | Dosage | mg *B. sphaericus* | Dosage |
|------|--------|--------|--------------------|--------|
| 1 | 0.65 | low | – | – |
| 3 | 1.24 | mid | – | – |
| 4 | – | – | 74.73 | low |
| 6 | 1.08 | mid | 131.76 | mid |
| 7 | 0.96 | mid | 70.15 | low |
| 8 | – | – | – | – |
| 10 | – | – | 150.98 | mid |
| 11 | 0.72 | low | 87.29 | low |
| 13 | 0.70 | low | 141.72 | mid |
| 14 | – | – | – | – |

Because ponds had been used in 1984 and 1985 for testing experimental formulations of Bti and *B. sphaericus*, treatments to ponds were assigned such to minimize possible residual effects from 1984 and 1985 tests. Bti treatments were not assigned to ponds sprayed with *B. sphaericus*, *B. sphaericus* treatments were not assigned to ponds sprayed with high Bti dosages, and the two controls were assigned to ponds used only as controls. Treatments were not randomized

for each trial; all ponds received the same treatment
throughout the experiment so that residual activity of the
treatments could be tested.

Materials were prepared in the lab by suspending Bti
and/or *B. sphaericus* formulations in 100 ml of dechlorinated
tap water and mixing on a magnetic stirrer for five minutes.
Each 100 ml solution was transferred to a hand-powered Solo[R]
15-liter backpack sprayer and mixed with tap water to a
final volume of 10 liters. Three different sprayers, one
for each bacterial treatment, and one for combined
treatments, were used to avoid the possibility of cross
contamination. The surface of each pond was uniformly
covered with 10 liters of spray. Forty-eight hours later
each of the cages were sampled using the four dip method
described above. The percent reduction in number of living
larvae (expressed as mortality) was calculated.

Ponds were left undisturbed for five days after
sampling (seven days after spraying) at which time an assay
of the residual effect of the treatments was initiated. The
assay consisted of placing 800 laboratory-reared third
instars in the cages, pre-sampling after one hour, and post-
sampling after forty-eight hours, by the standard sampling
method. Percent reduction in live larvae was then
calculated. All larvae remaining from the one week post-
spray treatment residual effect were removed with a fish
net. One day later, ten days after spraying, the next group
of assays were initiated. In all, four trials were run and

ponds received a total of four spray treatments. Four pre-spray samples and four forty-eight hour post-spray samples were done for the combined use assay, and four one week post-spray samples and four nine day post-spray samples were performed for the residual effect assay.

POND SAMPLING:

Concurrent with the field bioassays, sampling was done to investigate possible changes in the background levels of Bti and *B. sphaericus* endotoxin levels in the ponds during the course of the experiment. Two water and two soil samples were collected from each pond one week after each spray treatment. One sample of each was taken from within the larval cages, and the other two samples from the opposite side of the pond. The water samples were taken with a 250 ml plastic container placed into the water unopened, situated in the larval feeding zone (7 cm above the pond bottom), the cap removed and allowed to fill. The cap was replaced and sample container removed. Two 175 cc soil samples were collected with a clam digger to a depth of 5 cm. Both water and soil samples were stored at -20°C until processed.

POND SAMPLE PROCESSING:

Samples were processed to extract the endotoxin for detection by ELISA. Both water and soil samples were concentrated by evaporation because preliminary testing indicated that the concentrations of endotoxin in the samples were too low to detect with ELISA. Initial testing also demonstated that cytolysis of Bti and *B. sphaericus* is required to free the endotoxins for detection by ELISA. Three treatments to release the endotoxin were investigated; 1) 30 min. in .05 M NaOH at pH 11, 2) sonication in water at maximum setting for 2 minutes, and 3) procedures 1 and 2 combined. Results indicated that treatment 2 was ineffective and treatments 1 and 3 gave effective and equal results. Therefore, treatment 1, 30 minute alkaline extraction, was used for all pond samples.

The 2-250 ml water samples from each pond for each sampling period were combined for a total sample volume of 500 ml. Each pond water sample was evaporated to 1 ml in a hood at room temperature over a two day period. After evaporation, approximately 20 microliters of 5N NaOH was added to each sample to bring the pH to 11. After 30 minutes the pH of each sample was adusted with 4N HCL to a pH of 9.6, the optimal binding state for ELISA. The processed water samples were stored at -20°C until analyzed, approximately one week later.

For processing of soil samples, the two samples collected from each pond were placed in a 1000 ml beaker with 50 ml of distilled water to aid in mixing. Twenty to

forty ml of 5 N NaOH was added to each sample to bring the pH to 11. After 30 minutes, each sample was diluted with distilled water. The water volume added was 1.5 times the sample volume, minus the 50 mls added to stir the soil and the number of mls of NaOH added to adjust the pH. The samples were throughly mixed and allowed to sit at room temperature for 24 hours. The liquid was removed, and the volume recorded. This liquid was evaporated to 1 ml, adjusted to a pH of 9.6 with 4 N HCL, and frozen at -20°C until ELISA analysis approximately one week later.

To evaluate the precision and recovery of the water and soil processing techniques, quantities of Bti (0.5, 0.05, 0.005 and 0.0005 microliters, quantities approximating the expected range in the pond samples) were added to water and soil samples taken from an untreated pond. These samples, plus control samples with no Bti added, were processed in the same manner as the pond water and sediment samples.

ELISA:

Monoclonal antibody to the Bti endotoxin was supplied by G. Rohrman, Oregon State University, Corvallis. Antibody to the *B. sphaericus* endotoxin was provided by E. Davidson, Arizona State University, Tempe. Unfortunately, the *B. sphaericus* antibody proved ineffective because of having been lyopholized for storage and shipping. As a result, ELISA's were run for detection of the Bti endotoxin only.

A standard curve for ELISA absorbance of Bti endotoxin
concentration was obtained using 2-fold serial dilutions,
from .000305 microliter/ml to 2.5 microliter/ml, of
Vectobac. To release the endotoxin .05 ml of Vectobac was
alkaline extracted in 9.95 ml .05 N NaOH at a pH of 11 for
30 minutes. Two-fold serial dilutions were made into
carbonate buffer. The same dilution series was repeated
four times to establish consistency of the ELISA results.
An indirect ELISA method was used, based on Voller et al.
(1976), with several modifications. Disposable rigid
styrene microtitration plates purchased from Dynatech
Laboratories, Inc. were rinsed three times with distilled
water and then once with carbonate buffer (Fichter, 1984).
One hundred microliters of each dilution of the Vectobac in
carbonate buffer was placed in each well, with two
replications for each dilution, and kept at 4°C overnight.
The plate was washed three times with PBS/Tween and had 150
microliters of a one percent protein-carbonate buffer
solution added for 30 minutes at room temperature (Fichter,
1984). The purpose of the protein coat was to cover any
sites in the well not bound with the Bti endotoxin to
prevent direct binding of the Bti antibody or IgG conjugate,
thus avoiding false positives. A protein coat of BSA in
carbonate, as specified in Voller's paper, was tested
against a coat of rabbit mite antibody in carbonate buffer
and no coat. The results indicated that the BSA coat
allowed non-specific binding and consequent false positives

whereas no coat or the rabbit mite antibody coat did not. Therefore, a one percent solution of the rabbit mite antibody in carbonate buffer was used as the protein coat.

After three washes with PBS/Tween to remove any unbound protein coat, 100 microliters of the Bti antibody, diluted 1:500 in PBS/Tween, was added to each well and let stand for 30 minutes at room temperature. Following three washes with PBS/Tween, each well received 100 microliters of Sigma anti-mouse IgG conjugated with alkaline phosphatase, diluted 1:1000 in PBS/Tween. After 30 minutes at room temperature, unbound IgG conjugate was removed with three washes of PBS/Tween, and 200 microliters of the enzyme substrate was added to each well. The enzyme substrate consisted of a 1 mg to 1 ml ratio of p-nitrophenyl phosphate in a 10% diethanolamine buffer (Voller et al., 1976). The plate was placed in the dark for 45 minutes after which the reaction was stopped with the addition of 50 microliters of 3N NaOH to each well. The plate was read at a wavelength of 405 nm on a Biotek Instruments Microplate Autoreader.

In each ELISA run, one positive and three negative controls were included. When ELISA's for the absorbance curve of Vectobac were performed, purified Bti endotoxin, provided by G. Rohrman, was used as a positive control. In ELISA performed on the pond samples, Vectobac, 0.5 microliters per ml, served as a positive control. The three negative controls used for each ELISA were: 1) substitution of *B. sphaericus*, .05 mg/ml with a 30 minute alkaline

extraction, for the Bti in the antigen binding step; 2) complete elimination of the antigen binding step; and 3) wells containing substrate only to give a background absorbance reading. These negative controls were used to verify that any antibody and conjugate binding were specific to the Bti endotoxin. The highest absorbance value received from these negative controls was used as the cut-off value for significant values for wells containing Bti, i.e. absorbance values for wells with Bti lower than the absorbance values for the negative controls were not considered to represent specific binding.

ELISA tests on all pond samples were run at the same time, with two replications of each sample and two replications of each sample diluted 2-fold. Two fold dilutions were included to determine which side of the standard curve the absorbance value corresponded to. The following positive and negative controls, were included in each ELISA: 2-fold dilutions, 0.000305 microliter/ml to 0.3125 microliter/ml, of alkaline extracted Vectobac; twelve wells with 0.05 mg/ml of alkaline extracted Bs substituted for the Bti; two wells without any antigen; and two wells with substrate only.

Because unusual absorbance values were obtained for ELISA of the pond water and soil samples, additional assays were performed. These assays examined the effects of bentonite, pond soil, and temperature on Bti endotoxin and its expression in ELISA absorbance values.

All assays included duplicate 10 ml samples containing
0.5 microliters Bti/ml. After bentonite, soil or
temperature treatment, all samples underwent 30 minute
alkaline extraction to a pH of 11 and then a 10-fold
dilution into carbonate buffer to pH 9.6, to give a final
concentration of .05 microliters Bti/ml for use in the
ELISA. Bentonite treatments were 0.01, 0.1 and 1.0 %
solutions prepared with pharmaceutical grade bentonite.
Soil treatments were 10, 20, 30, 40, and 50 % solutions
prepared from samples taken from an untreated area of the
ponds used in the field bioassays. Bentonite and soil
treatments were kept in the dark at 16°C for one week. One-
week and three-week exposures to temperatures of 4, 8, 12,
16, 20, 25, and 30° C were used for the temperature
treatments. ELISA of the soil, bentonite and temperature
treatments used the standard protocol described previously.

RESULTS AND DISCUSSION

LABORATORY BIOASSAYS:

Results of the laboratory testing on combinations of Bti and *B. sphaericus* are presented in Tables 1, 2, and 3, and Figures 1 and 3.

The effects of the addition of Bti on *B. sphaericus* LD values are given in Table 1. All LD values in Table 1, except those that are starred, indicate an independent effect; as an increasing amount of Bti was added, a decreasing quantity of *B. sphaericus* was required to obtain the same LD value. All treatments with 0.1 or 1.0 ITU/ml Bti added to the range of *B. sphaericus* concentrations showed an independent effect. An independent effect was also observed when 0.01 ITU/ml Bti was added, except for at *B. sphaericus* $LD_{99}$.

Starred entries in Table 1 are LD values from *B. sphaericus* bioassays with Bti added which are greater than LD values for identical *B. sphaericus* bioassays with no Bti added and suggest an antagonistic interaction. All starred values occurred in treatments that combined a medium to high concentration of *B. sphaericus* with 0.001 or 0.01 ITU/ml Bti; *B. sphaericus* $LD_{60}$ to $LD_{99}$ values in treatments containing 0.001 ITU/ml Bti, and *B. sphaericus* $LD_{99}$ in treatments with 0.01 ITU/ml Bti.

The confidence intervals for the LD values, given in parenthesis in Table 1, do not overlap and indicate the effects are statistically significant.

Figure 1 illustrates the *B. sphaericus* LD values from Table 1 plotted as probit lines. The difference in the slopes of the probit lines for *B. sphaericus* only bioassays, from the slopes of the probit lines of *B. sphaericus* bioassays where .001 or .01 ITU/ml Bti was included, suggests a change in the mode of action of *B. sphaericus* when used in combination with Bti. Additionally, the crossing of these probit lines indicates an antagonistic interaction between Bti and *B. sphaericus* at these concentrations.

Table 2 shows the effects of *B. sphaericus* on the LD values of the Bti bioassays. The addition of 0.1, 0.01, or 0.001 microgram/ml *B. sphaericus* to the range of Bti concentrations produced an independent effect; all LD values are less than those for bioassays with *B. sphaericus* only. An independent effect was also observed in Bti $LD_{40}$ to $LD_{60}$ values in treatments containing 0.0001 microgram/ml *B. sphaericus*. An antagonistic interaction between Bti and *B. sphaericus* is indicated in Bti $LD_{70}$ to $LD_{99}$ values in treatments with 0.0001 microgram/ml *B. sphaericus* added; less Bti was required when used alone, than when combined with *B. sphaericus*, to produce the same LD values.

The absence of overlap of the confidence intervals for the LD values, given in parenthesis in Table 2, indicates the effects are statistically significant.

The probit lines, in Figure 1, graphed from the Bti LD values in Table 1, changed in slope with the addition of *B. sphaericus*, suggesting a difference in the mode of action of Bti when *B. sphaericus* was added. The crossing of the probit lines reflects an antagonistic interaction at some combinations.

Figure 2 shows the LD values from Tables 1 and 2 plotted against Bti and *B. sphaericus* concentrations. The shape of the isobole in this type of graph (Tammes, 1965) indicates the nature of the interaction of two compounds. Figure 2 is a reference graph from Tammes (1965) illustrating the different isoboles for synergistic, additive, independent or antagonistic interactions. The isoboles for the lab data (Figure 2) reveal an independent effect between Bti and *B. sphaericus* at $LD_{40}$ to $LD_{90}$ values and an antagonistic interaction between the two bacteria at $LD_{95}$ and $LD_{99}$ values.

The data indicate that antagonism occurred only when a low dosage of one bacterium was added to a medium or high dosage of the second. The low dosage of the added bacterium may have been sub-lethal and activated defense mechanisms of the mosquito larva so that it was better able to respond to the lethal dosage of the other bacterium. This activation parallels Wilkinson's theory (1976) on insecticide

interaction in which one compound may inhibit or stimulate the activity of the enzyme(s) involved in metabolism of the other compound. The result is synergistic or antagonistic, depending on whether the enzyme(s) affected activates or inactivates the second compound. This theory fits nicely with the combination of a low dosage of Bti with a medium or high dosage of *B. sphaericus* as research indicates Bti is faster acting than *B. sphaericus* (Davidson, 1984a). Consequently, the sub-lethal dosage of Bti could have had an effect before the slower acting *B. sphaericus* and activated some enzymatic system in the mosquito larva resulting in a decrease in its susceptibility to *B. sphaericus*. A major problem with this theory is that the antagonistic interaction was also observed when a low dosage of *B. sphaericus* was combined with a medium or high dosage of Bti. Because Bti is faster acting, a sub-lethal dosage of *B. sphaericus* is unlikely to have an opportunity to stimulate a detoxification system in the mosquito larvae before a lethal dosage of Bti caused mortality. In addition, no evidence currently exists for the presence of a detoxification system in mosquitoes effective against bacterial toxins, although defense reactions to *B. thuringiensis* have been demonstrated in other insect species (Chiang et al., 1986).

The antagonistic interaction witnessed with some of the dosage combinations may have occurred in all combination treatments but was masked. In the treatments displaying antagonism, such a low dosage of the added bacterium was

used that its contribution to total mortality would have been minimal. At treatments where a greater dosage of the added bacterium was used, however, the mortality it contributed may have masked the decrease in mortality of the second bacterium. Analysis of the data indicated that antagonism was not extreme in any of the treatments and hence masking in some of the treatments is conceivable.

At several points some interaction may have occurred between the two bacteria. Bacterial protoxins in the water might have had an effect on the other species. After the protoxins were ingested and subjected to proteases and the high pH of the mosquito larval midgut, the released endotoxins might have interacted. Differential binding of the endotoxins to the peritrophic membrane of the mosquito larval midgut, the general target site for both Bti and *B. sphaericus*, is also possible. Unfortunately, the modes of action and receptor sites for the two bacteria have not been defined. Consequently, it is not known if competition for a receptor site could occur with their combined use, and what biochemical steps are involved in each bacterium's toxic action that might be affected by the other bacterium or its metabolic by-products.

Commercial formulations, rather than pure bacterial cultures, were used in this research. As a result, possible interactions discussed may have occurred not only between bacterial metabolites but also between bacteria and carriers in the commercial formulations. The carriers in

one bacterial formulation may have had an adverse effect on the bacterium in the other.

Mantel-Haenszel analysis of the lab bioassays, examined for mortality every 12 hours, is given in Table 3. Treatments designated statistically significant are those where the time trend of mortality of the combined bacterial treatment differed significantly from the time trend of mortality of the single bacterium treatment. As expected, the greatest effect occurred when a high concentration of one bacterium was added to a low concentration of the second bacterium.

Mortality was recorded at 12 hour intervals to determine 1) if there was a significant difference in the time trend of mortality between the two bacteria; and, 2) if the mortality of the combined bacteria treatments could be attributed primarily to one of the species. It was hoped that if antagonism was observed this information would be useful in identifying the species adversely effected. Unfortunately, the time trend of mortality for either of the two bacteria did not closely parallel that for the combined treatments and thus the source of reduced mortality with combined use of Bti and *B. sphaericus* could not be determined.

FIELD BIOASSAYS:

Results of the field tests on combined use and persistence of Bti and *B. sphaericus* are presented in Table 4 and Figures 4 through 8.

In Field Trial 1 ponds treated with only one bacterial species had higher larval mortality than ponds receiving combined bacteria treatments (Figure 4). For example, a mid-dosage of Bti produced 100% larval mortality, and a mid-dosage of *B. sphaericus* provided 98% mortality, yet the treatment combining these same dosages resulted in only 7% mortality. Although not as pronounced, this decrease in insecticidal activity with the combined use of Bti and *B. sphaericus* was also evident in Field Trial 1 for all other dosage combinations; low Bti (31%) with low *B. sphaericus* (72%) gave only 43%, low Bti (31%) with mid *B. sphaericus* (98%) gave only 65%, and mid Bti (100%) with low *B. sphaericus* (72%) gave only 69% larval mortality. Increase in concentration of all single bacterium treatments produced increased larval mortality but this trend was not evident in the combined treatments; the treatment made up of the lowest concentration of each bacterium produced 43% larval reduction compared to only 7% mortality in the treatment combining the mid concentration of each bacterium.

Results of Field Trial 2 parallel those of Trial 1; the most antagonistic interaction occurred in the mid Bti-mid *B. sphaericus* treatment (Figure 5). Larval mortality in the mid Bti treatment was 97%, and 80% in the mid *B. sphaericus* treatment, but only 38% for the two combined. Similarly,

mortality in the other combined treatments was substantially lower than total mortality of the respective single treatments. In contrast to the results of Trial 1, both single and combined treatments in Trial 2 displayed an increased mortality with an increased concentration.

In Field Trial 3 differences between mortality in single and combined treatments was less than in Trials 1 and 2 and was most extreme in the low-low, rather than in the mid-mid, combination of Bti and *B. sphaericus* (Figure 6). The low dosage treatments of Bti and *B. sphaericus* provided 83% and 82% mortality, respectively, whereas the combined treatments produced only 62% mortality. Difference in larval mortality of the other treatments was small: 100% and 99% for mid *B. sphaericus* and Bti treatments, respectively, and 96% for their combined use; 83% and 99% for low *B. sphaericus* and mid Bti, respectively, and 92% for their combined use; and 100% and 82% for mid *B. sphaericus* and low Bti, respectively, and 85% for their combined use. These results suggest an independent, rather than an antagonistic, effect. In Trial 3 larval mortality in both single and combined treatments increased as concentration increased.

In Field Trial 4 mortality in all treatments was 97% or higher and differences between single and combined treatments was 3% or less (Figure 7). The effect of the combined use of Bti and *B. sphaericus* appears independent but is difficult to analyze because of high mortality in all treatments.

Although the results of the laboratory bioassays suggested antagonism at specific dosage combinations of Bti and *B. sphaericus*, the results of the field bioassays indicate a more extensive and extreme antagonism. In the field bioassays pond treatments were not randomly assigned, nor were they randomized from trial to trial (see discussion in Materials and Methods) thus, some or all of the recorded antagonism may be attributable to differences among ponds. The ponds were similar in size and location, but other differences, (i.e. pH, soil type, flora and fauna ...), that may cause variations in the efficacy of Bti and *B. sphaericus*, were not evaluated. Consequently, the observed antagonisim may result from pond assignment rather than from an antagonistic interaction between the two bacteria.

Results of bioassays on persistence of Bti and *B. sphaericus* are presented in Table 4 and Figure 8. Cumulative residual activity of Bti and *B. sphaericus* was detectable in only one treatment in Trials 1 and 2 (Figure 8). All treatments in Trial 1, except one, produced mortality of 9% or less. A background mortality of 10% or less is considered normal (Davidson, personal correspondence). The residue of the low-low treatment in Trial 1 produced 35% mortality. No residual insecticidal activity was noted in Trial 2, all yielded mortality of 8% or less.

In Trials 3 and 4, residual activity was observed in all *B. sphaericus*, and *B. sphaericus* combined with Bti,

treatments. Treatments of Bti only showed 8% or less
mortality in both Trials 3 and 4. In Trial 3 the order of
residual activity was mid *B. sphaericus* (44%), low *B.
sphaericus* (36%), low-low (32%), mid-mid (31%), mid-low
(28%), and low-mid (20%). Comparable residual activity was
observed in Trial 4; mid *B. sphaericus* (43%), low *B.
sphaericus* (33%), mid-low (31%), mid-mid (30%), low-low
(23%), and low-mid (19%).

Residual mosquitocidal effect was observed both for the
sampling at one week, discussed above, and for the sampling
at 48 hours after treatment. Data for both sampling periods
were analyzed statistically with a sign test (Table 4)
(Snedecor and Cochran, 1980). The sign test was based on
the null hypothesis that mortality of mosquito larvae in
each treatment did not significantly differ between trials.

Plots in Figure 8, and sign test values in Table 4, for
the one week bioassay, indicate a residual effect in several
treatments in Trials 3 and 4. Trial 3 differs in mortality
from Trial 1 at significance level 0.05 and from Trial 2 at
significance level 0.01. The mortality in Trial 4
treatments differs statistically at a level of 0.01 from
that in the same treatments in Trials 1 and 2.

The purpose of the 48 hour post-treatment sampling was
to evaluate combined use of Bti and *B. sphaericus*. The
mortality values of the 48 hour samples reflect the same
pattern of residual activity as observed in the one week
samples, however. Figures 4, 5, 6, and 7 show results of

the 48 hour bioassays which differed only in when they were performed. A residual effect in some treatments was first apparent in Trial 3. For example, the mid-mid treatment gave 7% mortality in Trial 1, 38% in Trial 2, 96% in Trial 3 and 100% in Trial 4. The sign test (Table 4) indicates significant larval reduction resulting from insecticidal persistence in Trial 3; Trial 3 differs from Trial 1 at a 0.05 significance level and from Trial 2 at a 0.01 significance level. Trial 4 differs in its residual activity from all three earlier trials at a 0.01 significance level. Sign tests on the 48 hour samples were comparable to those on the one week samples except for the statistically significant difference between percent reduction of the treatments in Trial 4 from those in Trial 3 in the former. A major difference between results of the 48 hour and the one week bioassays was residual activity of Bti; 1 week sampling showed no significant Bti residual activity in all 4 trials (Figure 8) whereas mortality in 48 hour sampling increased from 31% in Trial 1 to 82% in Trial 3 and 98% in Trial 4 (Figures 4, 6, and 7).

There is disagreement as to the insecticidal persistence of Bti and *B. sphaericus*, with some claims of no persistence and others of persistence up to several months (Mulligan et al., 1980; Hornby et al., 1981; Hertlein et al., 1979). In this research insecticidal residual activity is documented and the residues appear to be cumulative, requiring three applications to reach significant larval

mortality. In Trials 1 and 2 there may have been persistence of the bacteria but the spore and endotoxin concentrations were apparently sub-lethal. Treatments were only one-fifth and one-third of the recommended field dosages so that residues of the first two applications may have been quite low. By Trials 3 and 4 the cumulative amount of bacteria and bacterial metabolites in the ponds had increased to lethal levels. Another posible explanation for the difference in residual activity between Trials 1 and 2, and Trials 3 and 4, may have been weather conditions as efficacy of both Bti and *B. sphaericus* is temperature dependent (Wraight et al., 1982; Lacey, 1985). Average water temperature in the ponds was 10°C at the beginning of Trial 1 and increased to 30°C by the end of Trial 4. The low temperatures in the early trials may have inhibited larval feeding and ingestion of toxin.

The results indicate that larvicidal persistence is more pronounced in *B. sphaericus* than in Bti treatments; mortality was significantly higher in treatments with only *B. sphaericus* or with *B. sphaericus* combined with Bti, than in treatments of Bti only. The same objection raised to the validity of the results of the combined use assays, however, also applies to the persistence assays; pond treatments were not randomized and hence results may be due in part, or in whole, to pond differences.

ELISA:

Enzyme-linked immunoabsorbent assay (ELISA) values were used to indicate the concentration of Bti in the pond sediment and water samples; Bti concentrations were extrapolated from the standard curve of known Bti concentrations plotted against absorbance.

The absorbance values for the ELISA of the water and pond sediment samples taken from the ponds used in the field bioassays are shown in Tables 5 and 6. The significance level for the assay is 0.498 nm; the mean of the twelve control (*B. sphaericus*) replications plus two standard deviations. The values starred in the two tables are those which exceed the significance level and which have a reasonable relationship to the absorbance value for the two-fold dilution given in parenthesis. Absorbance values for two-fold dilutions equal to or greater than the original concentration invalidated a result.

A concurrent assay was run to determine how efficient the procedures of alkaline extraction and evaporation were in recovering the original concentration of Bti in a sample. Results are displayed at the bottom of Tables 5 and 6. A standard curve was run the same day as the pond samples from 2-fold dilutions of Bti (Figure 9).

In the ELISA for the pond water samples only pond 1 samples from trials 1 and 4 have significant absorbance values (Table 5). The significant absorbance values for the pond sediment samples are pre-trial; ponds 1,3,4 and 6,

trial 1; ponds 1,3,6,7,8,10 and 14, trial 2; ponds 4 and 10, and trial 3; ponds 3 and 6. ELISA results for both pond water and sediment samples greatly differ from what was expected; an increase in Bti levels over the trials and no detection of Bti in ponds receiving *B. sphaericus* only or in ponds used as controls (ponds 4,8,10 and 14).

The only pond water samples with significant absorbance values came from a pond which had been treated with a low dosage of Bti (pond 1). All other samples from ponds which received comparable or higher dosages of Bti produced absorbance values lower than the significance level of 0.498 nm. Calculations, based on estimating the average pond volume to be 11.50 $m^3$, indicate that with the Bti treatment range of .65 ml (lowest pond treatment) to 1.24 ml (highest pond treatment) a 250 ml sample might contain 1.3 to 2.5 microliters of Bti. This calculation assumes an equal distribution of the bacteria throughout the pond and that no settling occurred in the one week between spraying and sample collecting. The values from 2.6 to 5.0 microliters per ml (2-250 ml samples evaporated to 1 ml) fall above the linear part of the standard curve. For every ELISA performed during this research there was a decrease in absorbance value for Bti concentrations greater than 0.3125 microliters per ml. This phenomenon, of decreasing values with increasing concentrations, is not uncommon in ELISA and is thought to occur when a high protein concentration causes excessive interactions between proteins which interfere with

their binding to plate wells. If settling of Bti in the ponds was great (see introduction) the amount of Bti in the water samples may have been minimal. Consequently, Bti concentrations may have fallen below the linear area of the standard curve. Water samples were taken from the 3 cm above pond bottom not because it was considered the richest source of Bti but because *C. pipiens* are bottom feeders and the purpose of the sampling was to determine their level of exposure to Bti endotoxin.

Recovery values for the water samples indicate that the processes of alkaline extraction and evaporation did not significantly diminish the Bti concentration in a sample (Table 5). The absorbance values for the samples with 0.5, 0.005 and 0.0005 microliters Bti per ml treated by alkaline extraction and evaporation are all close to the absorbance values from the standard curve. The low absorbance value of 0.358 nm for the 0.05 microliter per ml samples is most likely due to laboratory error as all samples were prepared by serial dilution and the lower concentrations show a good relationship.

The absorbance values for the ELISA of the sediment samples are also irregular and do not follow any expected trend (Table 6). Nine significant values are found in samples which would be expected to give insignificant readings (i.e. samples from ponds which received no Bti or which were taken from ponds during the pre-trial sampling)

whereas only six significant values were obtained from samples of ponds which received Bti treatments.

Comparison of the recovery of Bti endotoxin from water and sediment samples suggests that the extraction and condensation of Bti endotoxin from sediment was not very successful (Tables 5 and 6). The absorbance for the percent recovery of 0.5 microliter/ml from the water samples was 1.40 nm whereas the same concentration of Bti in sediment gave an absorbance value of only 0.346 nm, a value substantially below the control level of 0.498 nm. The percent recovery values for the Bti concentrations of 0.05, 0.005 and 0.0005 microliters/ml are also higher for the water samples than for the sediment samples. The difference in processing of the water and sediment samples may account for the greater recovery of Bti from water samples. Whereas the entire water sample was evaporated to the 1 ml used in the ELISA, only the supernatant of the alkaline extracted sediment sample was retained for evaporation while the rest of the sample was discarded. It is at this stage that some of the Bti from a sample, located in the precipitate, could have been lost.

Calculations indicate that the area of a sediment sample represented approximately 3 percent of the total pond surface area. If all Bti settled to the pond bottom during the week prior to sampling a sediment sample might contain 19.6 to 32.9 microliters of Bti. This range of Bti concentration does not fall on the linear area of the

standard curve (Figure 9). With unequal distribution from spraying and settling, and variations in efficiency of extraction of Bti from sediment samples (due to differences in soil type) it is possible that the final 1 ml volume of some of the samples contained Bti concentrations on the linear area of the standard curve. Some of the significant sample values may reflect a specific reaction between Bti endotoxin and antibody whereas others may result from false positives.

One problem that may have occurred during processing of both water and sediment samples is inaccurate and variable pH adjustment. Each sample was titrated with HCL to give a final pH of 9.6. The pH of a sample is crucial for optimum binding of antigen to well. This is demonstated by a test in which 0.3 microliter Bti in 250 ml water was evaporated to 1 ml and alkaline extracted. Samples in which the pH was not adjusted after alkaline extraction had an average absorbance value of 0.314 nm whereas the samples with a pH adjusted to 9.6 had an average absorbance of 0.950 nm. Large numbers of pond samples were processed and possibly some of the pH adjustments were not stable.

Inconsistencies in the ELISA data prompted speculation that the bentonite and organic matter concentrations of the ponds, as well as high temperatures at the end of the trials, may all have contributed to the low absorbance values for the water samples, the variable absorbance values

of the sediment samples, and the low absorbance values for all Trial 4 samples.

In a series of lab experiments the effect of mixing Bti with different concentrations of soil on ELISA absorbance was determined (Figure 10). The erratic results and the absence of a direct relationship between the 2-fold dilution and the undiluted material suggests that soil has an effect on Bti, the endotoxin and/or ELISA not expected in this experiment. This is evident from the absorbance value (0.850) of the control sample. It is well documented that soils of smaller particle size and organic matter inactivate most toxicants through adsorption. Values in Figure 10, substantially lower than the control value, may result from binding of Bti to the soil particles, making the Bti unavailable for binding to the ELISA plate and/or monoclonal antibody. Values higher than the control value may be produced by components of the soil acting as a glue and causing non-specific binding, giving positive readings unrelated to a specific reaction between the Bti and antibody.

In lab studies the effect of bentonite on Bti, the endotoxin and/or ELISA results was dramatic. Bentonite was rototilled into the bottom of some of the ponds in 1985 to aid in water retention but not all ponds received bentonite and the ones that did were not recorded. Increasing concentrations of bentonite caused corresponding decreases in absorbance values (Figure 11). As discussed in the

introduction, Bti may be adsorbed by clay particles and suffer a decrease in insecticidal activity. The results in Figure 4 suggest that the endotoxin is inactivated, or the ELISA disrupted, by bentonite clay.

High water temperatures in the ponds are also suspected as a factor in the unexpected absorbance values. Pond water temperature was monitored hourly with a two-probe data pod for the duration of the experiment. When the first trial was begun in late April the average temperatures at the pond surface and bottom were 9.5°C and 10°C, respectively. By the end of May, when the 4th trial was run, average surface and bottom water temperatures had climbed to 31.5°C and 28.5°C, respectively. A lab experiment was performed to determine if temperatures during the course of the experiment were significant. The results of this experiment illustrate the detrimental effect a one week exposure to temperatures over 25°C may have on the immunological activity of Bti (Figure 12). In the three week temperature exposure test, all values are below that of the same concentration of Bti prepared the day of the assay (Figure 13) although all fall within the two standard deviation level of the control. Distilled water was used and the vials were wrapped in foil so that the observed decrease in activity after three weeks can not be attributed to adsorption by organic material or u.v. degradation. Apparently, three weeks in solution, regardless of

temperature, adversely effects the immunological activity of Bti endotoxin.

Further experiments, to identify the factors contributing to the high variability in the absorbance values of the soil samples, involved testing for the presence of phosphatase in the sediment. The substrate used in the ELISA is acted upon by this enzyme, and the production of this enzyme by soil microbes is common. It was hypothesized that unequal distribution of this enzyme within and between ponds might account for the erratic absorbance values. The experiments indicated that levels of phosphatase in the sediment samples were not significant. Variations in the deitritivore quantity and make-up among pond sediments, resulting in more depletion of Bti in some ponds than in others, may account for some of the unexpected absorbance values of the pond sediment samples.

There is also the possibility that there was Bti pre-existing in the ponds, residue from treatments applied during the summers of 1984 and 1985. Those ponds treated only with *B. sphaericus* and which showed significant absorbance values (ponds 4 and 10) were sprayed with Bti during 1984 and 1985. If the Bti survived in the soil over the fall and winter it might have been included in the sampling for this experiment. This is not supported in the positive results for the control ponds (ponds 8 and 14) as they had never received Bti treatments. However, Bti may have been transferred from treated ponds to untreated ponds

by insects, birds, flooding or persons spraying and
sampling.

The results suggest that ELISA holds promise as a tool
in evaluating the fate of Bti and *B. sphaericus* endotoxin in
the environment. However, there are three major
considerations for using ELISA as a means of detecting and
quantifying bacterial endotoxin in pond water and sediment
samples. First, it is necessary to work with dosages and
sample sizes that will give absorbance values on the linear
area of the standard curve. It is also important to
identify, and to control when possible, environmental
factors causing depletion of the Bti endotoxin and/or a
decrease in immunological activity of the endotoxin.
Second, lab methods used for purifying and preparing the
samples for ELISA must be consistent and produce a high
recovery of the original endotoxin concentration. Third,
the ELISA process itself must be designed to minimize the
potential for false negative and positive results. An
indirect ELISA, not possible in this research because of the
use of a monoclonal antibody, would minimize this
variability; by binding the antibody first, the antigen
(i.e. bacterial endotoxin) assayed for in the pond samples
could be selectively bound to the well and competitive
binding by other proteins would be reduced.

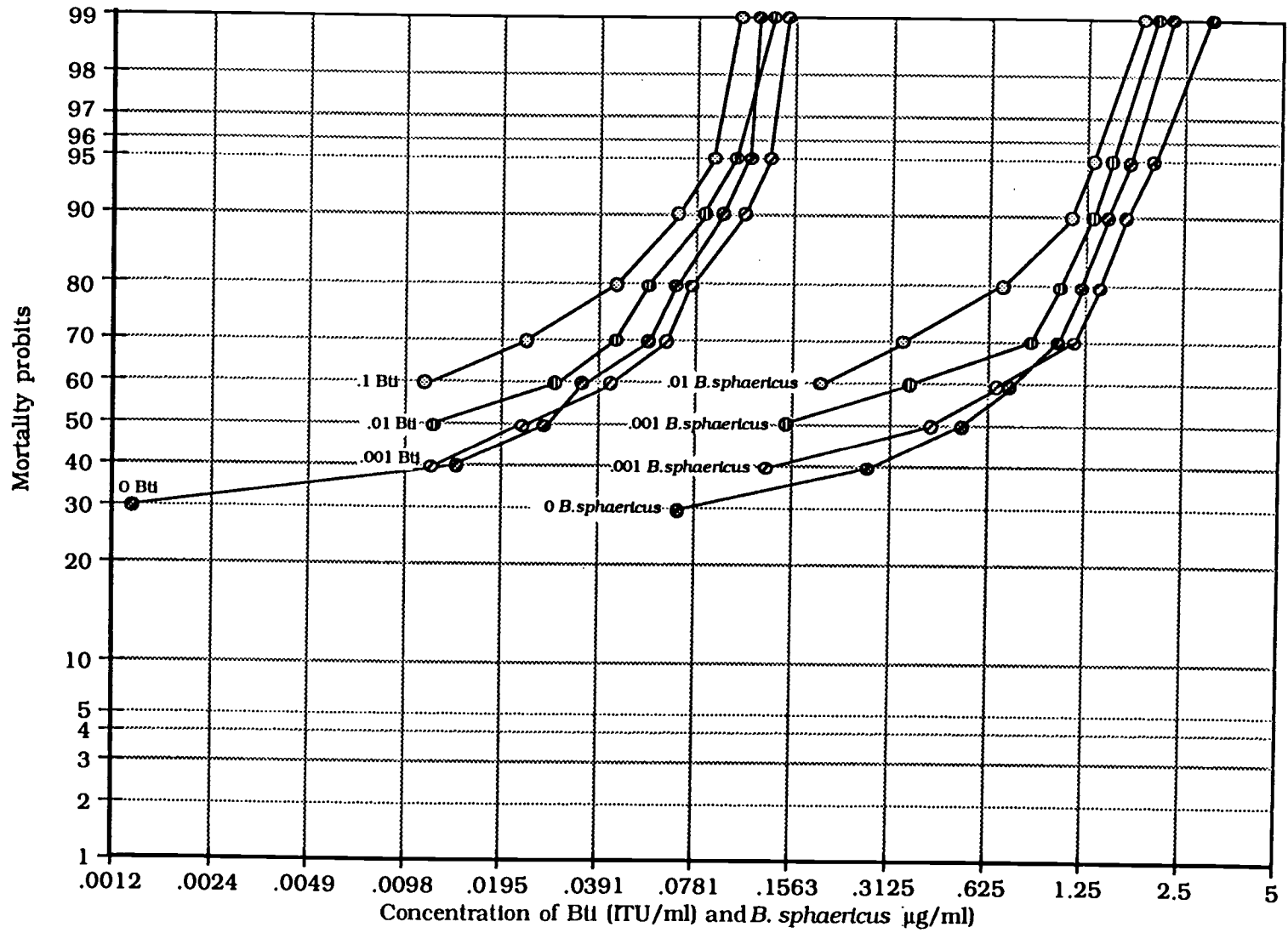Figure 1
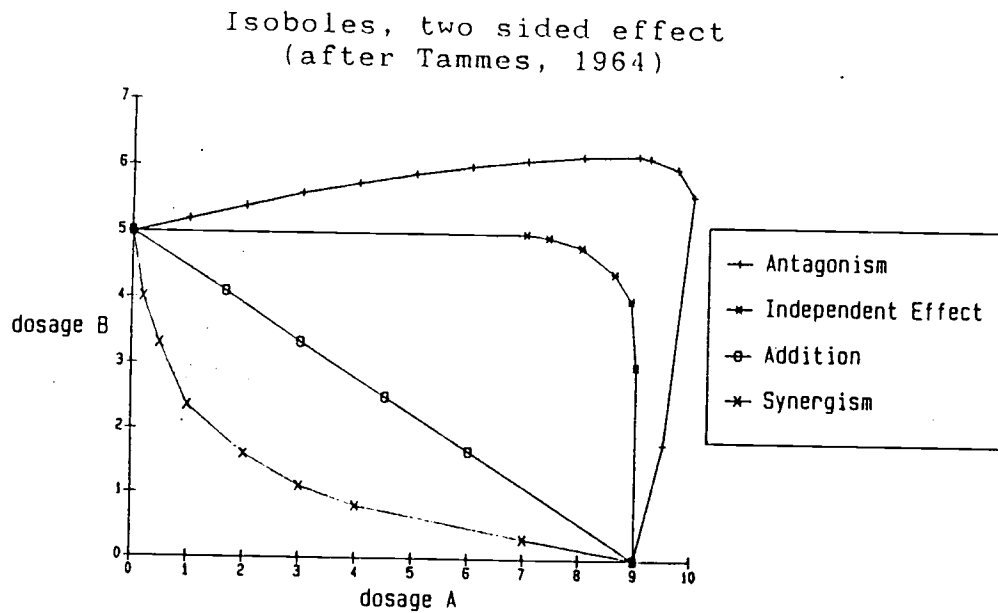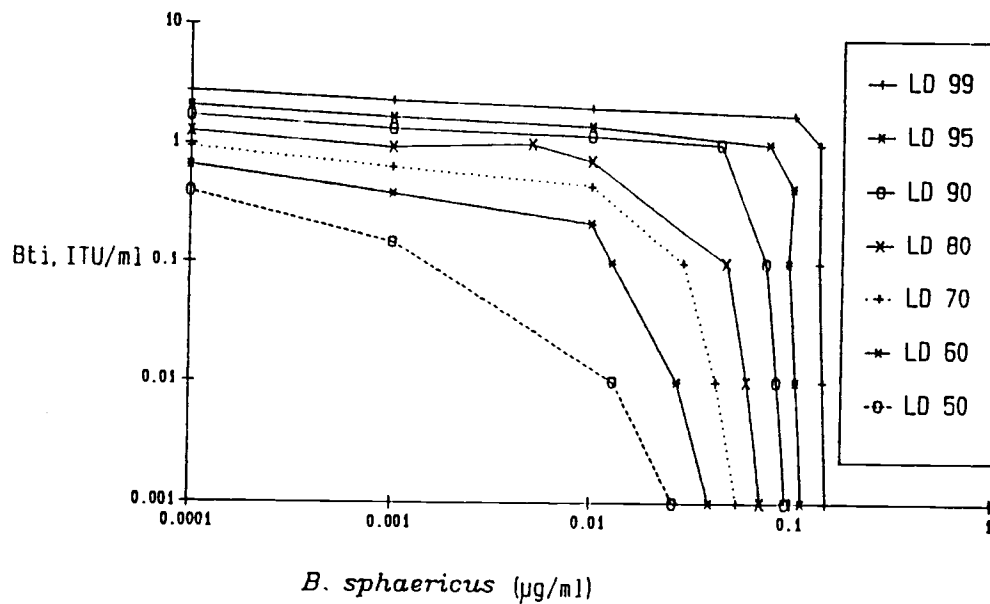Probit lines for lab bioassays

FIGURE 2

Isoboles, two sided effect
(after Tammes, 1964)



FIGURE 3

Isoboles for laboratory bioassays



*B. sphaericus* (µg/ml)

# Figure 4
## Field trial 1

Effect of *B. sphaericus* on Bti control of mosquito larvae

Effect of Bti on *B. sphaericus* control of mosquito larvae



Left chart — Y-axis: Percent Reduction in Number of Mosquito Larvae (0% to 100%), X-axis: Bti field dosage (0, low, mid)

- 0: 0%, 72%, 98%
- low: 31%, 43%, 65%
- mid: 100%, 69%, 7%

Legend:
- Bti only
- + low dosage *B. sphaericus*
- + mid dosage *B. sphaericus*

Right chart — X-axis: *B. sphaericus* field dosage (0, low, mid)

- 0: 0%, 31%, 100%
- low: 72%, 43%, 69%
- mid: 98%, 65%, 7%

Legend:
- *B. sphaericus* only
- + low dosage Bti
- + mid dosage Bti

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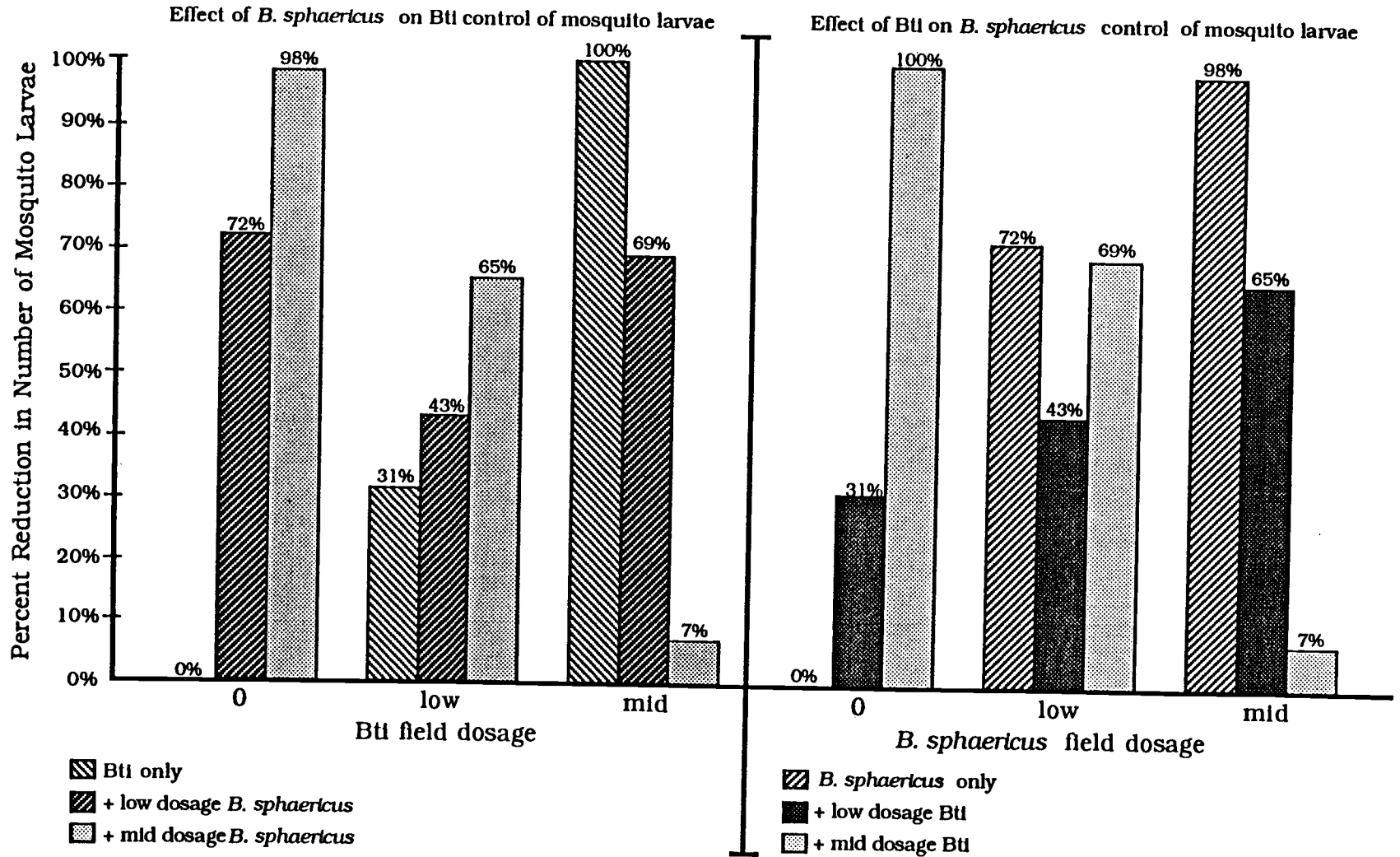
Figure 5
Field trial 2

Figure 6
Field trial 3

# Figure 7
## Field trial 4

**Effect of *B. sphaericus* on Btl control of mosquito larvae**

**Effect of Btl on *B. sphaericus* control of mosquito larvae**



Percent Reduction in Number of Mosquito Larvae

Btl field dosage

*B. sphaericus* field dosage

Btl only
+ low dosage *B. sphaericus*
+ mid dosage *B. sphaericus*

*B. sphaericus* only
+ low dosage Btl
+ mid dosage Btl
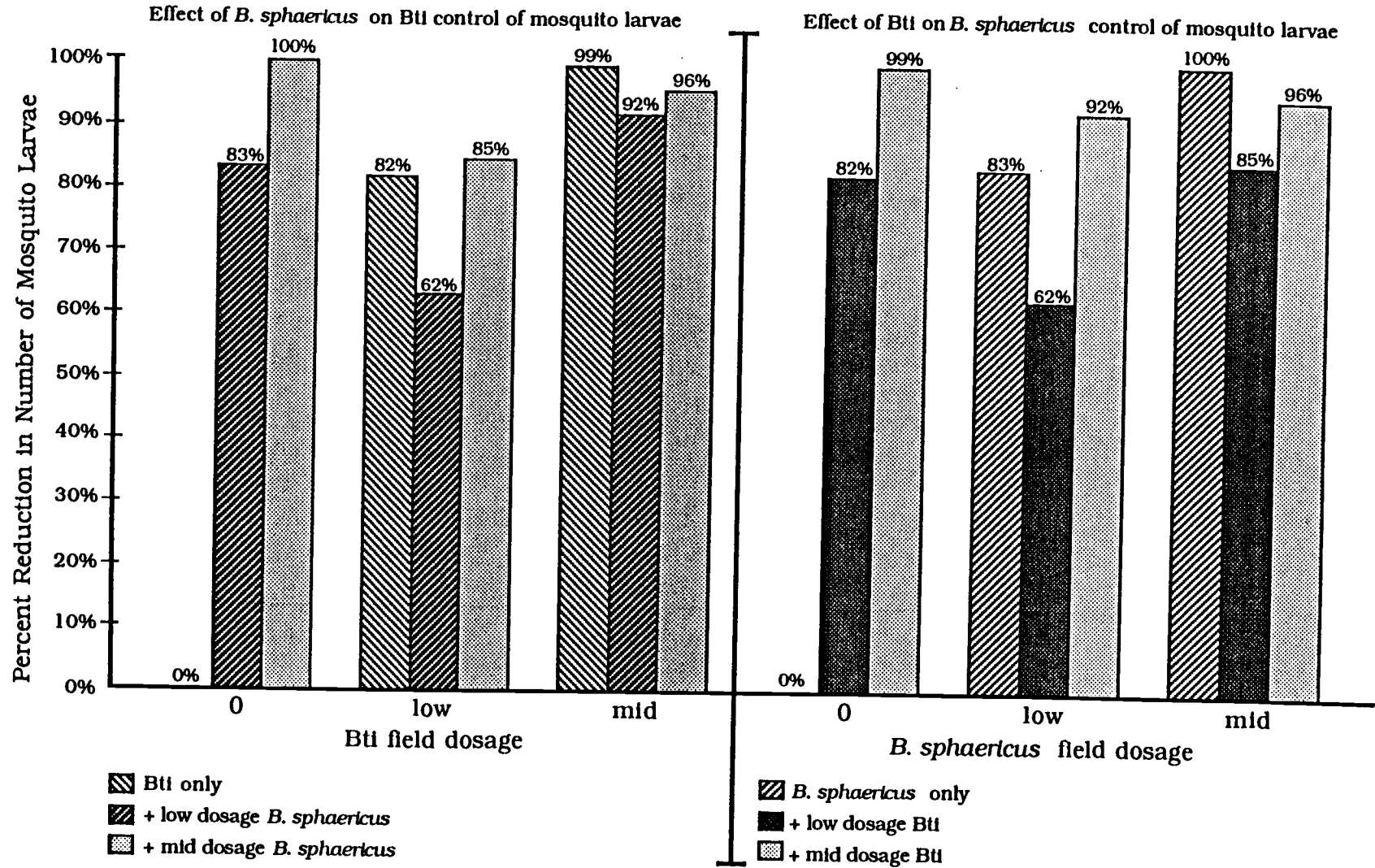
Figure 8

Cumulative residual activity of Bti and *B. sphaericus*

Legend:
- ● low Bti-low *B. sphaericus*
- ◒ low *B. sphaericus*
- ⊗ low Bti-mid *B. sphaericus*
- ⊕ mid *B. sphaericus*
- ⊘ Mid Bti
- ⊘ Mid Bti-Mid *B. sphaericus*
- ⊜ mid Bti-low *B. sphaericus*
- ⦿ low Bti

Y-axis: Percent reduction in number of larvae

X-axis: Trial 1, Trial 2, Trial 3, Trial 4
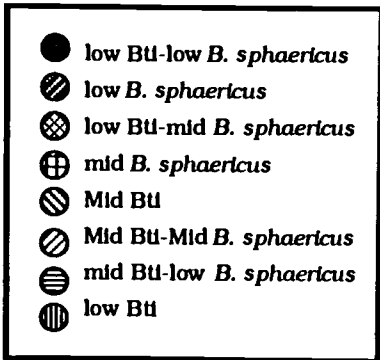
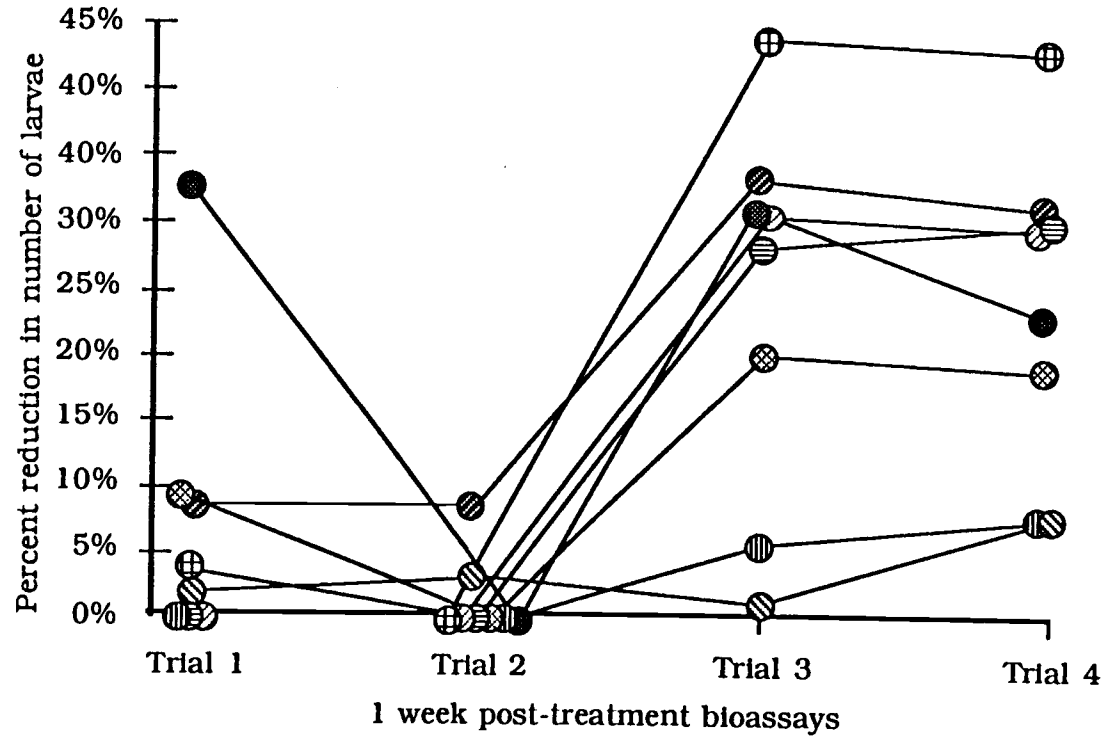1 week post-treatment bioassays

Figure 9

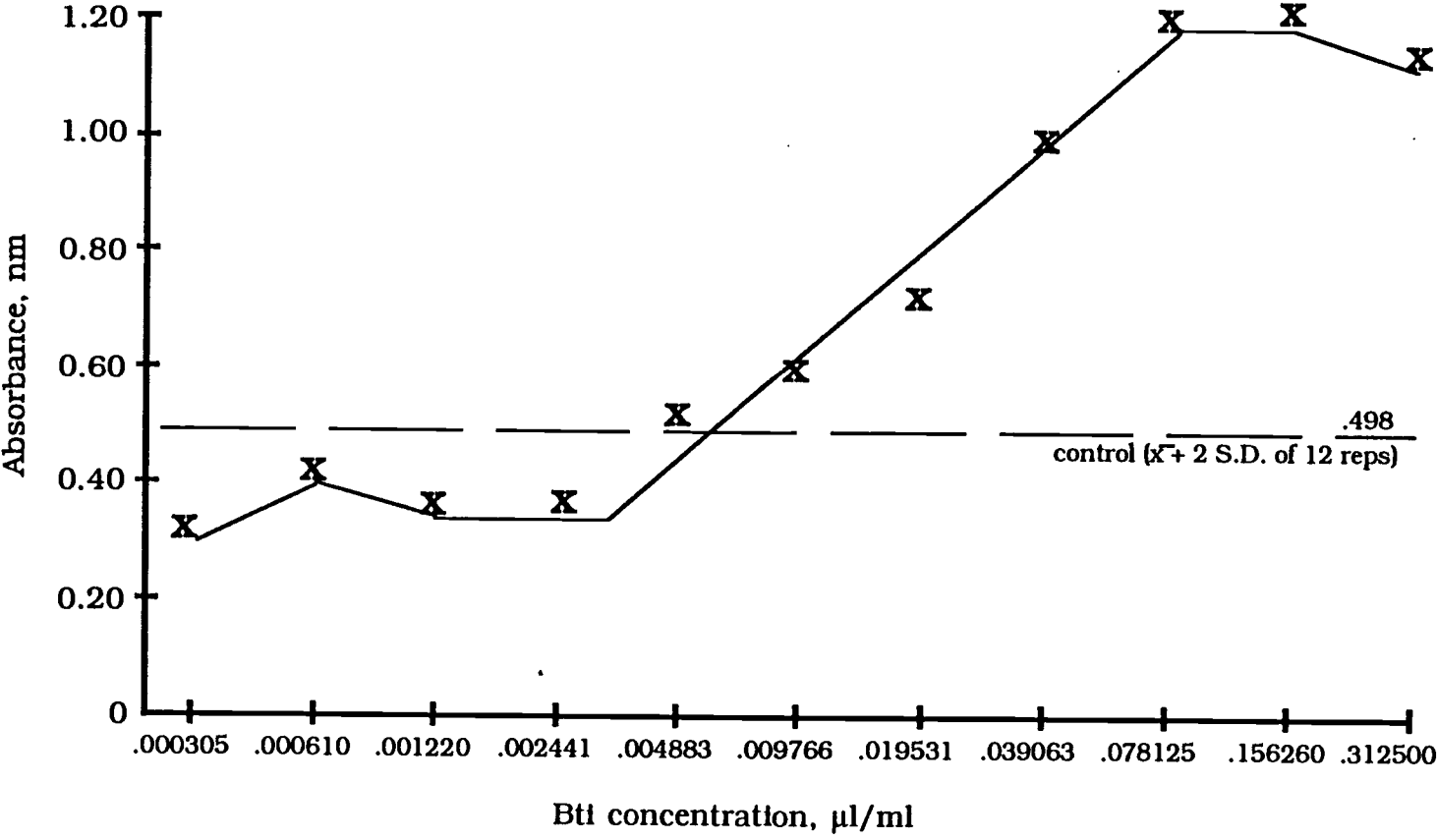Standard curve for Bti endotoxin

Figure 10

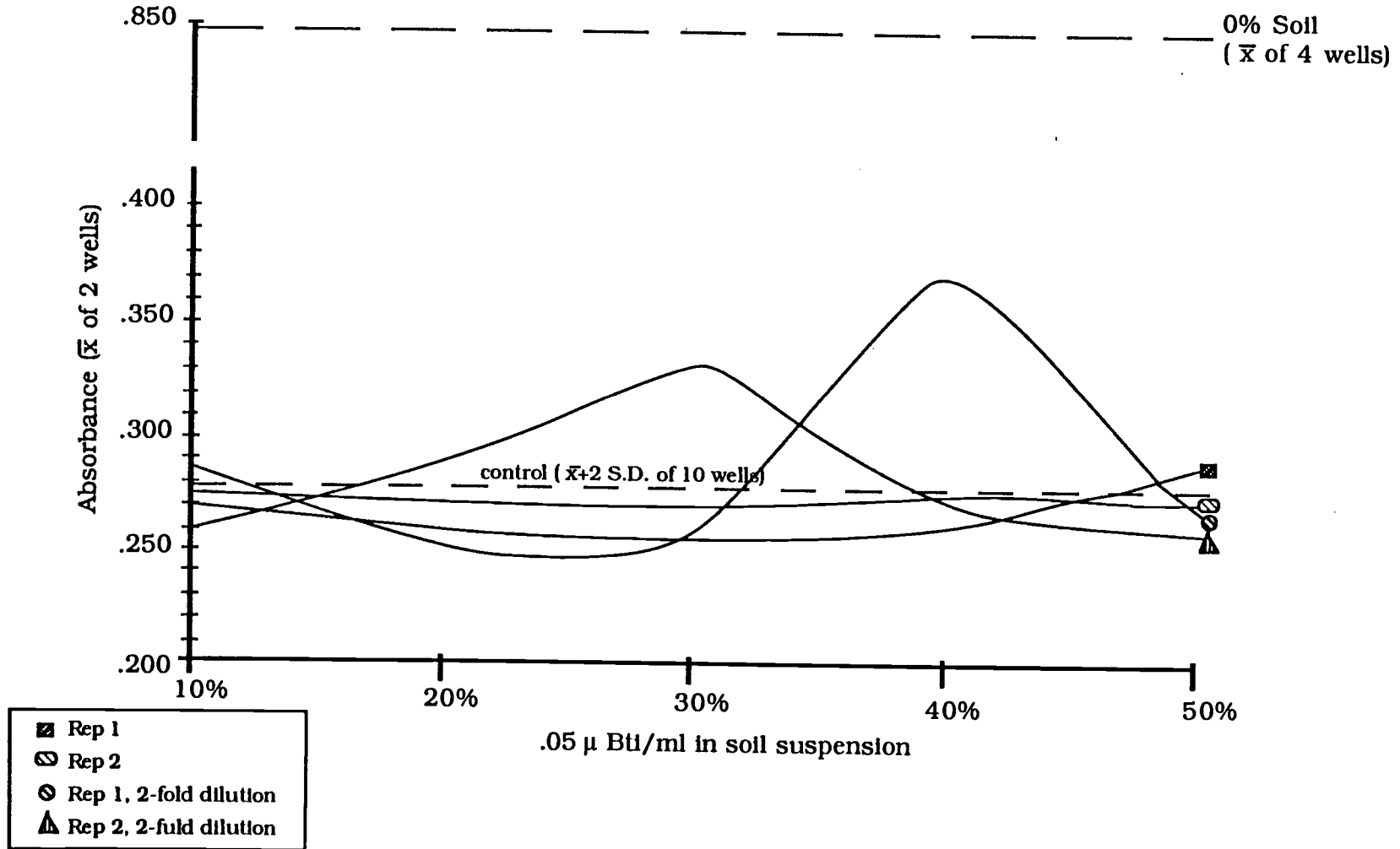Effect of soil on Bti endotoxin after 1 week

## Figure 11

### Effect of Bentonite on Bti endotoxin after 1 week
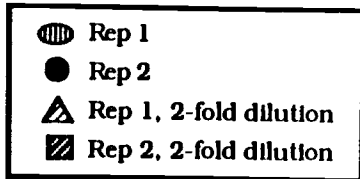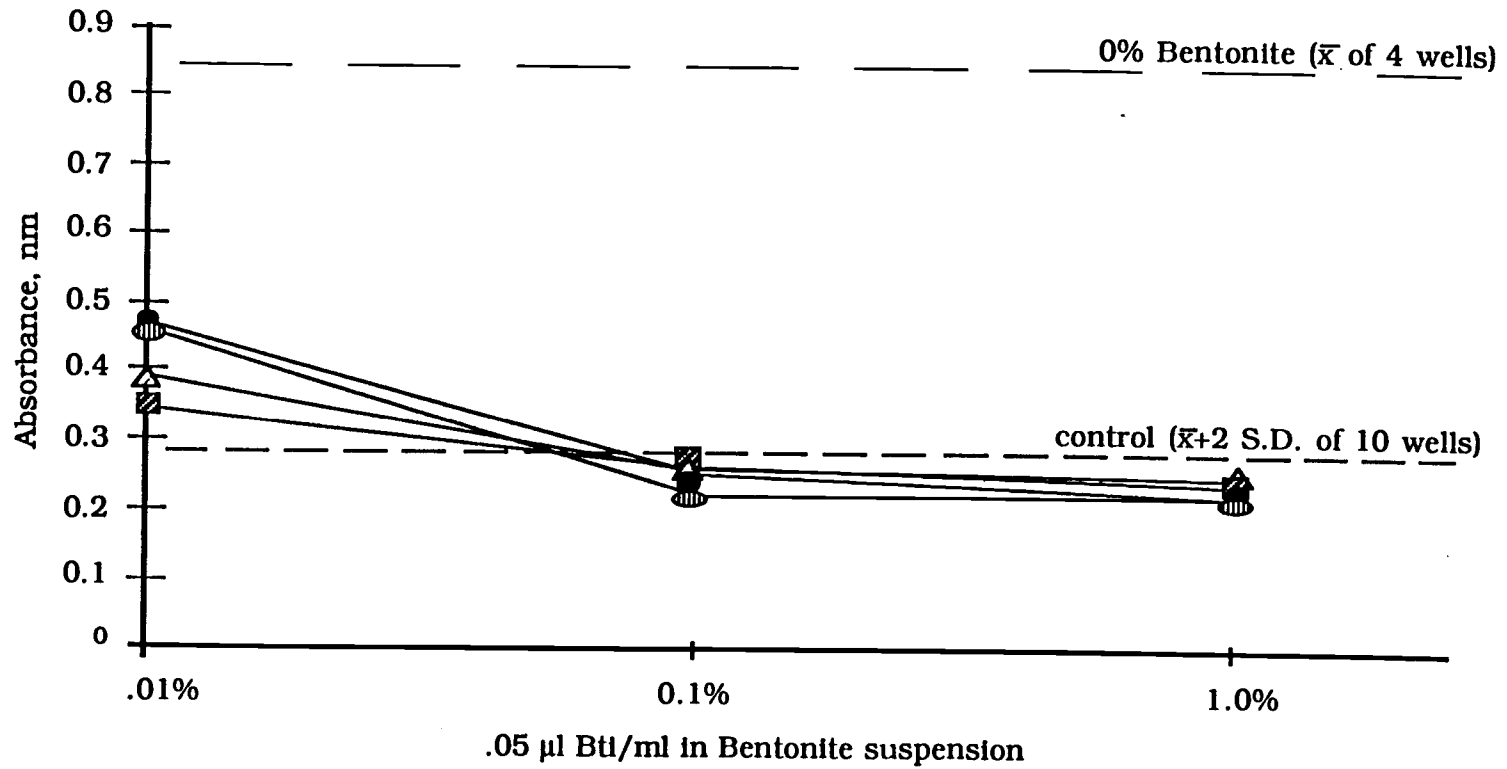
# Figure 12

## Effect of temperature
## on Bti endotoxin after 1 week



1 week temperature treatment, .05 µl Bti/ml
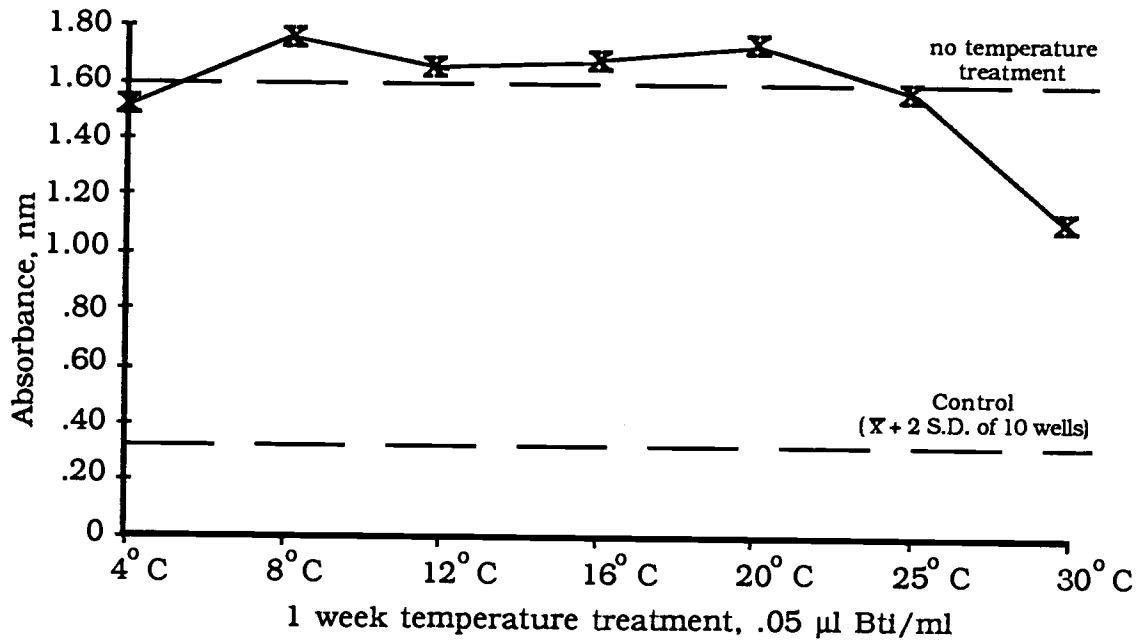
# Figure 13

## Effect of temperature
## on Bti endotoxin after 3 weeks



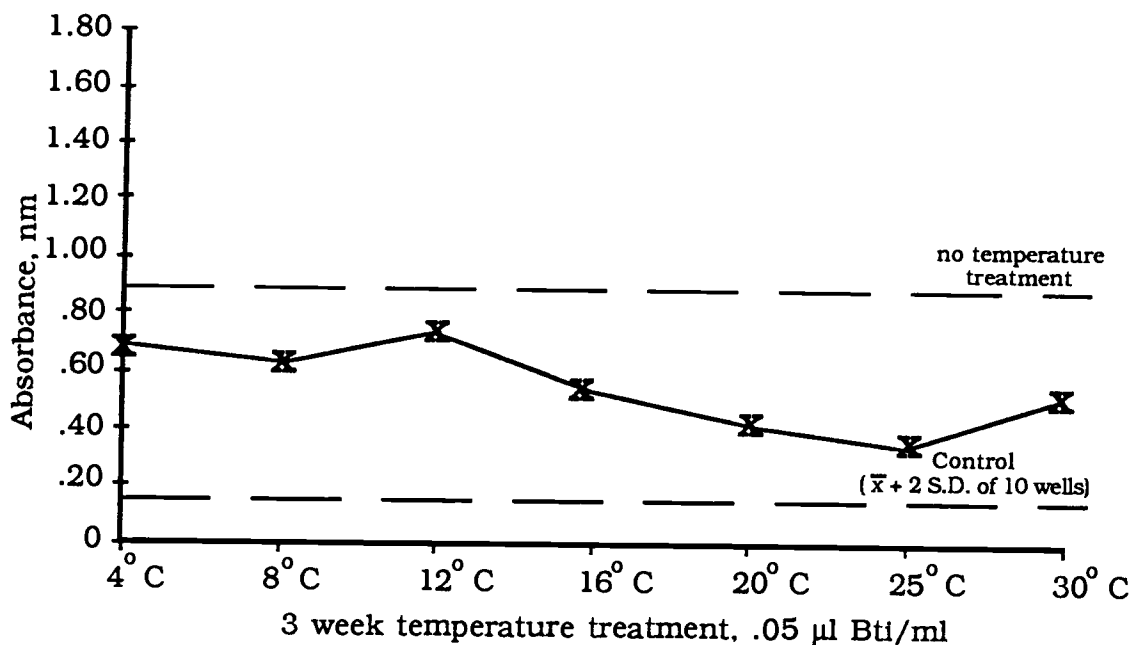3 week temperature treatment, .05 µl Bti/ml

TABLE 1

Effect of Bti (ITU/ml) on B. sphaericus (micrograms/ml) lethal dose values

| PERCENT MORTALITY | B. sphaericus .001 Bti | B. sphaericus .001 Bti | B. sphaericus .01 Bti | B. sphaericus 0.1 Bti | B. sphaericus 1.0 Bti |
|---|---|---|---|---|---|
| 30 | .0015 (.0013-.0017) | | | | |
| 40 | .0145 (.0143-.0147) | .0128 (.0126-.0130) | | | |
| 50 | .0266 (.0265-.0268) | .0262 (.0260-.0264) | .0129 (.0127-.0131) | | |
| 60 | .0380 (.0386-.0389) | .0396 (.0394-.0398) | .0270 (.0268-.0272) | .0127 (.0125-.0129) | |
| 70 | .0517 (.0515-.0519) | .0540 * (.0537-.5420) | .0421 (.0419-.0423) | .0289 (.0287-.0290) | |
| 80 | .0669 (.0667-.0671) | .0708 * (.0705-.0710) | .0597 (.0590-.0599) | .0477 (.0476-.0477) | .0050 (.0047-.0052) |
| 90 | .0880 (.0877-.0883) | .0941 * (.0937-.0944) | .0842 (.0840-.0845) | .0739 (.0737-.0742) | .0436 (.0434-.0438) |
| 95 | .1054 (.1050-.1057) | .1133 * (.1129-.1137) | .1045 (.1041-.1048) | .0956 (.0952-.0959) | .0755 (.0753-.0758) |
| 99 | .1380 (.1376-.1385) | .1499 * (.1488-.1499) | .1424 (.1491-.1429) | .1361 (.1357-.1366) | .1354 (.1349-.1359) |

* .95 Confidence Interval.
+ LD value greater than LD value of single treatment.

TABLE 2

Effect of B. sphaericus (micrograms/ml) on Bti (microliters/ml) lethal dose values

| PERCENT MORTALITY | 0 B. sphaericus | 0.0001 B. sphaericus | 0.001 B. sphaericus | 0.01 B. sphaericus | 0.1 B. sphaericus |
|---|---|---|---|---|---|
| 30 | .0737 (.0686-.0788)* | | | | |
| 40 | .2945 (.2901-.2989) | .1402 (.1348-.1456) | | | |
| 50 | .5007 (.4961-.0268) | .4017 (.3967-.4067) | .1511 (.1469-.1552) | | |
| 60 | .7070 (.0386-.0389) | .6632 (.0394-.0398) | .3887 (.0268-.0272) | .2183 (.0125-.0129) | |
| 70 | .9278 (.9205-.9351) | .9431 + (.9347-.9515) | .6430 (.6383-6476) | .4525 (.4490-.4561) | |
| 80 | 1.186 (1.176-1.195) | 1.270 + (1.259-1.282) | .9405 (.9342-.9469) | .7266 (.7222-.7310) | |
| 90 | 1.544 (1.532-1.557) | 1.725 + (1.709-1.741) | 1.353 (1.344-1.363) | 1.107 (1.100-1.113) | |
| 95 | 1.840 (1.825-1.856) | 2.100 + (2.080-2.120) | 1.694 (1.682-1.706) | 1.421 (1.412-1.429) | .4377 (.4347-.4407) |
| 99 | 2.395 (2.374-2.416) | 2.8035 + (2.776-2.831) | 2.333 (2.316-2.350) | 2.009 (1.996-2.022) | 1.762 (1.752-1.771) |

* .95 Confidence Interval.
+ LD value greater than LD value of single treatment.

TABLE 3

Mantel-Haenszel analysis

<u>Effect of *B. sphaericus* (mg/ml) on Bti (ml/ml):</u>

| | | | |
|---|---|---|---|
| 0.10 *B. sphaericus* | +* | + | + | + |
| 0.01 *B. sphaericus* | + | + | + | + |
| 0.001 *B. sphaericus* | + | - | - | - |
| 0.0001 *B. sphaericus* | - | - | - | - |
| | 0.001 Bti | 0.01 Bti | 0.10 Bti | 1.0 Bti |

<u>Effect of Bti (ml/ml) on *B. sphaericus* (mg/ml):</u>

| | | | |
|---|---|---|---|
| 1.0 Bti | + | + | + | + |
| 0.1 Bti | + | + | + | + |
| 0.01 Bti | + | - | - | - |
| 0.001 Bti | - | - | - | - |
| | 0.0001 *B. sphaericus* | 0.001 *B. sphaericus* | 0.01 *B. sphaericus* | 0.1 *B. sphaericus* |

\* + = chi-square value significant at .01.

TABLE 4

Residual Activity

of Bti and *B. sphaericus* as determined by bioassay

One Week Post-Treatment:

| Null Hypothesis | P-Value | Significance Level |
|---|---|---|
| Ho: Trial 4 - Trial 1 = 0 | .003906 | .01 |
| Ho: Trial 4 - Trial 2 = 0 | .003906 | .01 |
| Ho: Trial 4 - Trial 3 = 0 | .054688 | accept Ho |
| Ho: Trial 3 - Trial 1 = 0 | .030000 | .05 |
| Ho: Trial 3 - Trial 2 = 0 | .003906 | .01 |
| Ho: Trial 2 - Trial 1 = 0 | .15625 | accept Ho |

48 Hours Post-Treatment:

| Null Hypothesis: | P-Value | Significance Level |
|---|---|---|
| Ho: Trial 4 - Trial 1 = 0 | .007813 | .01 |
| Ho: Trial 4 - Trial 2 = 0 | .003906 | .01 |
| Ho: Trial 4 - Trial 3 = 0 | .007816 | .01 |
| Ho: Trial 3 - Trial 1 = 0 | .030000 | .05 |
| Ho: Trial 2 - Trial 2 = 0 | .003906 | .01 |
| Ho: Trial 2 - Trial 1 = 0 | .218750 | accept Ho |

TABLE 5

ELISA absorbance (nm) values
for water samples of field bioassays

| POND | TREATMENT | PRE | TRIAL 1 | 2 | 3 | 4 |
|------|-----------|-----|---------|---|---|---|
| 1 | low Bti | .432 (.505)** | .869* (.433) | .329 (.338) | .309 (.322) | .712* (.459) |
| 3 | mid Bti | .254 (.294) | .387 (.399) | .349 (.365) | .320 (.288) | .389 (.303) |
| 4 | low *B.sphaericus* | .317 (.309) | .342 (.405) | .312 (.277) | .320 (.329) | .338 (.299) |
| 6 | mid Bti mid *B.sphaericus* | .419 (.404) | .352 (.318) | .347 (.362) | .288 (.323) | .366 (.317) |
| 7 | mid Bti low *B.sphaericus* | .358 (.348) | .381 (.321) | .322 (.333) | .365 (.382) | .365 (.325) |
| 8 | control | .391 (.314) | .450 (.356) | .333 (.302) | .376 (.363) | .347 (.324) |
| 10 | mid *B.sphaericus* | .308 (.297) | .350 (.354) | .320 (.313) | .389 (.374) | .306 (.297) |
| 11 | low Bti low *B.sphaericus* | .298 (.305) | .413 (.341) | .349 (.418) | .325 (.353) | .298 (.406) |
| 13 | low Bti mid *B.sphaericus* | .359 (.374) | .344 (.401) | .306 (.269) | .374 (.410) | .315 (.267) |
| 14 | control | .435 (.500) | .353 (.319) | .337 (.269) | .363 (.365) | .491 (.398) |

RECOVERY OF KNOWN Bti CONCENTRATIONS

| Bti microliters/ml | 0.5 | .05 | .005 | .0005 |
|--------------------|-----|-----|------|-------|
| Absorbance value | 1.40 | .358 | .424 | .336 |

* larger than control value of .498 and with reasonable
  value for two-fold dilution.

** value for absorbance of two-fold dilution.

TABLE 6

ELISA absorbance (nm) values
for sediment samples of field bioassays

| POND | TREATMENT | PRE | 1 | TRIAL 2 | 3 | 4 |
|------|-----------|-----|---|---------|---|---|
| 1 | low Bti | 1.30* (.606)**| .631 (.691) | .355 (.424) | .512* (.385) | .255 (.231) |
| 3 | mid Bti | .791* .360) | .877* (.545) | .588 (.642) | .833* (.500) | .241 (.355) |
| 4 | low *B.sphaericus* | .839* (.522) | .655 (1.03) | .573* (.535) | .472 (.586) | .349 (.250) |
| 6 | mid Bti mid *B.sphaericus* | .605* (.478) | .566* (.545) | .300 (.421) | .715* (.601) | .259 (.273) |
| 7 | mid Bti low *B.sphaericus* | .465 (.432) | .912* (.518) | .915 (1.10) | .253 (.276) | .249 (.457) |
| 8 | control | .366 (.368) | .664* (.537) | .589 (.721) | .466 (.326) | .339 (.270) |
| 10 | mid *B.sphaericus* | .340 (.394) | .837* (.551) | .505* (.438) | .408 (.298) | .278 (.248) |
| 11 | low Bti low *B.sphaericus* | .361 ('.369) | .530 (.562) | .471 (.437) | .292 (.272) | .283 (.250) |
| 13 | low Bti mid *B.sphaericus* | .324 (.302) | .438 (.493) | .352 (.354) | .382 (.377) | .442 (.308) |
| 14 | control | .269 (.322) | .569* (.368) | .495 (.497) | .368 (.331) | .257 (.309) |

RECOVERY OF KNOWN Bti CONCENTRATIONS

| Bti microliters/ml | 0.5 | .05 | .005 | .0005 |
|--------------------|-----|-----|------|-------|
| Absorbance value | .346 | .304 | .306 | .278 |

* larger than control value of .498 and with reasonable
  value for two-fold dilution.

** value for absorbance of two-fold dilution.

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