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FIELD EVALUATION OF NEW WATER-DISPERSIBLE GRANULAR FORMULATIONS OF *BACILLUS THURINGIENSIS* SSP. *ISRAELENSIS* AND *BACILLUS SPHAERICUS* AGAINST *CULEX* MOSQUITOES IN MICROCOSMS

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ABSTRACT. A variety of formulations of *Bacillus thuringiensis* var. *israelensis* de Barjac (*B.t.i.*) and *Bacillus sphaericus* Neide (*B.s.*) have been studied for mosquito control under laboratory and field conditions. High efficacy, specificity, low risk of development of resistance, long shelf-life, and transportability, as well as the safety to nontarget organisms of these 2 microbial agents have been well documented. Some of the currently available formulations of *B.t.i.* and *B.s.* have low potency per unit mass. Research and development efforts are focusing on commercializing formulations with high potency and low minimum effective dosage that are suitable for long-distance shipment. To achieve this goal, new water-dispersible granule (WDG) formulations of both microbial agents were prepared and made available by Abbott Laboratories for evaluation. The newly developed WDGs of *B.t.i.* and *B.s.* with high potency dispersed readily in water with gentle agitation. These WDGs were evaluated and the minimum effective dosages were determined in microcosms against natural populations of *Culex* mosquitoes. The minimum effective dosage for *B.t.i.* WDGs with 4,000 International Toxic Units (ITU)/mg was 0.27–0.53 lb/acre which yielded significant control for up to 7–12 days. The minimum effective dosage for *B.s.* WDGs with 350–630 ITU/mg was 0.05–0.10 lb/acre, which yielded significant control of immature mosquitoes for up to 14–20 days.

KEY WORDS *Bacillus thuringiensis* var. *israelensis*, *Bacillus sphaericus*, water-dispersible granules, *Culex* mosquitoes, microcosms

INTRODUCTION

Since the discovery of the mosquitocidal property of *Bacillus thuringiensis* var. *israelensis* de Barjac (*B.t.i.*) and *Bacillus sphaericus* Neide (*B.s.*), various commercial formulations of these agents have been developed and evaluated. The attributes of *B.t.i.* and *B.s.* preparations such as high efficacy, specificity, relatively low risk of development of resistance, large-scale production, long shelf-life, and transportability, as well as their safety to nontarget organisms (Mulla 1990, Siegel and Shadduck 1990) ensure a promising future for these agents in practical application for mosquito control.

Persistence, recycling, and suspension of the particulate toxins and spores in the feeding zone of mosquito larvae are some of the major considerations for efficacy of *B.t.i.* or *B.s.* formulations. Recycling of *B.s.* preparations has been indicated in laboratory or field tests where cadavers of mosquito larvae served as growth medium for the bacteria (Aly et al. 1985, Nicolas et al. 1987, Kramer 1990, Becker et al. 1995, Correa and Yousten 1995, Skovmand and Bauduin 1997). Regarding the distribution of toxins and spores in aquatic habitats after application of these microbial agents, the toxin particles and spores of most current formulations settle to the bottom, rather than remain in the feeding zone of the larvae for extended periods (Mulla et al. 1988, Matanmi et al. 1990). To overcome some of these problems, and to develop more potent formulations with low minimum effective dosage that are suitable for long-distance shipment, new water-dispersible granule (WDG) formulations of *B.t.i.* and *B.s.* with high potency were made available for testing by Abbott Laboratories, North Chicago, IL. The current studies were carried out to assess the efficacy and longevity of these formulations against *Culex* mosquitoes, as well as to determine the minimum effective dosages of these new WDG formulations in microcosms.

MATERIALS AND METHODS

Test materials

The test materials were one WDG formulation of *B.t.i.* (designated as ABG-6490, lot 30-067-BR) and 2 WDG formulations of *B.s.* (designated as ABG-6491, lot 30-073-BR and lot 32-094-BR), provided by Abbott Laboratories. The *B.t.i.* WDG formulation had a potency of 4,000 International Toxic Units (ITU)/mg. The potencies of *B.s.* WDG formulations were 350 ITU/mg for lot 30-073-BR and 630 ITU/mg for lot 32-094-BR. The WDG formulations were brownish fine-sized granules with loose appearance that dispersed readily when mixed with water.

Bioassay

In order to compare the activity of newly developed WDG formulations with technical powders (TPs) of *B.t.i.* and *B.s.*, bioassays were set up for all test WDG formulations against early 4th instars of colonized *Culex quinquefasciatus* Say. VectoBac TP (lot 81-634-W5) had a potency of 7,000 ITU/mg. VectoLex TP (strain 2362, ABG-6184, lot

13-194-W5) had a potency of 2,000 ITU/mg. To prepare suspensions, 200 mg of the test material was suspended in 20 ml of distilled water by vigorous shaking, yielding a 1% (w/v) suspension, which was then diluted serially 10-fold in a total volume of 20 ml, giving suspensions of 0.1, 0.01, and 0.001% (w/v). The needed aliquots of appropriate diluted suspensions were added to 120-ml waxed paper cups containing 100 ml of tap water and the test larvae (see below).

For each material, a preliminary test was conducted in 3 replicates by using 3 or 4 concentrations from 0.005 to 0.010 ppm and a control to determine the activity range. Tests used 25 larvae in 100 ml of distilled water in each 120-ml test cup, replicated 3 times, totaling 75 larvae in each concentration. One drop of 10% larval food was added to each test cup after treatment. The bioassay was carried out in a holding room maintained at 27–29°C. Mortality was recorded at 24 and 48 h after treatment for *B.t.i.* and *B.s.* preparations, respectively. Moribund larvae were considered dead. If mortality in the control was more than 5%, the test was discarded. Using the concentration range found in the preliminary test as a reference, the final test was set up by using 3 or 4 concentrations yielding 10–95% mortality and 9 replicates for each concentration and control. A total of 225 larvae in each concentration and 675–900 larvae for each material was bioassayed.

The dose–response data were subjected to probit regression analysis using POLO-PC (LeOra Software 1987). The quality of the data was controlled by the values of heterogeneity, which was calculated by χ^2/df. The slope, χ^2, median lethat concentration (LC_{50}), 90% lethatl concentration (LC_{90}), and their 95% confidential intervals were calculated using this software.

Field tests

Test facilities and treatments: To test the materials, 25 fiberglass tubs were placed on a concrete slab in an open sunlit area at the Aquatic and Vector Control Research Facility, University of California, Riverside, CA. The tubs measured 1.0 × 1.0 × 0.4 m deep. Before flooding, the tubs were enriched with rabbit pellets (crude protein ≥ 17%, Brookhurst®, Brookhurst Mill, Riverside CA) at the rate of 100 g per tub to provide continuous and sustained oviposition by the mosquitoes *Culex stigmatosoma* Dyar, *Cx. quinquefasciatus,* and *Culex tarsalis* Coquillett (Rodcharoen et al. 1997). The tubs were filled to a depth of 30 cm with 236 liters of water from an irrigation reservoir. The water level was kept constant by float valves. Each of the treatments and the control were assigned at random using 5 replicates. The 1st test was carried out using WDGs of *B.t.i.* and *B.s.* at 1.1–2.7 lb/acre from August 14 to September 15, 1997, followed by the 2nd test using the same materials at 0.27–0.53 lb/acre from September 19 to October 13, 1997. The 3rd test was conducted using *B.s.* WDGs at 0.05–0.10 lb/acre from May 4 to June 4, 1998. The tubs were treated 7 days after flooding, when 3rd and 4th instars and a few pupae were present. The WDG formulations were suspended in distilled water at 1% (w/v) just before application. The required aliquots based on the treatment rates were applied to the water surface using a 1- or 5-ml pipette.

Efficacy evaluation: The sampling for density determination of mosquito larvae and pupae was conducted on day 0 (pretreatment, 7 days after flooding), and at different intervals after treatment by taking 5 dip samples per tub, one from each corner, and an additional one from an area of aggregated mosquito larvae. In each test, 25 dips were taken for all the replicates in the control or each treatment on every sampling day. Immature mosquitoes were counted in 3 categories: early (1st and 2nd) instars, late (3rd and 4th) instars, and pupae. The average numbers of total immatures (larvae and pupae), late instars, and pupae were compared among the control and treatments on each sampling day by one-factor analysis of variance (ANOVA) (Scheffé F test). At the same time, the species composition of the mosquitoes from the control tubs was also determined by identifying about 150 4th instars on each sampling day, and compared by a chi-square test among species.

Water quality determinations: During the test period, water quality parameters were determined for all replicates of the control and one selected treatment at high rate in each test. Water temperature was determined using a submerged minimum–maximum thermometer (Taylor Instruments, Arden, NC). Dissolved oxygen (O_2), electrical conductivity (EC), and salinity were measured by a YSI Model 85 Water Quality Meter (YSI Incorporated, Yellow Spring, Ohio). The pH values of the water were estimated by pH test strips (Gallard-Schlesinger Industries, Inc., Carle Place, NY).

RESULTS AND DISCUSSION

Bioassays

Bioassays against early 4th instars of colonized *Cx. quinquefasciatus* showed that the LC_{50} values were 0.014 and 0.024 ppm and the LC_{90} values were 0.030 and 0.059 ppm for VectoBac TP and *B.t.i.* WDGs, respectively, indicating that the TP of *B.t.i.* was more active than the WDG preparation (Table 1). For the *B.s.* preparations, VectoLex TP and 2 formulations of WDG (lot 30-073-BR and lot 32-094-BR), the LC_{50} values were 0.024, 0.049, and 0.025 ppm and the LC_{90} values were 0.085, 0.088, and 0.065 ppm, respectively. The *B.s.* WDG formulation with the potency of 350 ITU/mg was less active than the TP and the high-potency WDGs with 630 ITU/mg. The activity of *B.s.* WDGs with high potency was equal to or better than the TP, as

Table 1. Bioassay of test materials against early 4th instars of a laboratory colony of *Culex quinquefasciatus*, along with the comparisons with VectoBac and VectoLex technical powders.[1]

Materials	LC_{50} (ppm) (95% CL)	LC_{90} (ppm) (95% CL)	Slope ± SE	χ^2/df
VectoBac TP (7,000 ITU/mg)	0.014 (0.013–0.016)	0.030 (0.025–0.037)	3.9 ± 0.24	6.02
B.t.i. WDGs (4,000 ITU/mg)	0.024 (0.022–0.026)	0.059 (0.053–0.066)	3.3 ± 0.18	0.06
VectoLex TP (2,000 ITU/mg)	0.024 (0.016–0.032)	0.085 (0.060–0.153)	2.3 ± 0.14	1.89
B.s. WDGs (350 ITU/mg)	0.049 (0.044–0.055)	0.088 (0.075–0.111)	5.1 ± 0.34	11.34
B.s. WDGs (630 ITU/mg)	0.025 (0.022–0.030)	0.065 (0.051–0.094)	3.1 ± 0.21	9.67

[1] LC_{50}, median lethal concentration; CL, confidence limits; LC_{90}, 90% lethal concentration; TP, technical powder; ITU, International Toxic Units; *B.t.i., Bacillus thuringiensis* var. *israelensis*; WDG, water-dispersible granule; *B.s., Bacillus sphaericus*. The bioassay was carried out in a holding room at 27–29°C. The LC_{50s} and LC_{90s} were generated from the mortality of 675–900 larvae (25 larvae/cup) exposed to the materials for 24 h in *B.t.i.* and for 48 h in *B.s.* within the range of 0.005–0.100 ppm.

indicated by the LC_{50} and LC_{90}, respectively (Table 1). These results suggest that formulation technology can enhance the activity of low potency products, such as the case of *B.s.* WDGs with 630 ITU/mg compared with Vectolex TP with 2,000 ITU/mg.

Field mosquito control

Immature mosquitoes were sampled and categorized into early instars, late instars, and pupae. For assessment of effectiveness of the test materials, counts of total immatures, late instars, and pupae were made in control and treatments in each test. Late instars and pupae provided a more reliable indication of efficacy than the total immatures because newly hatched larvae will have had little time to ingest lethal amounts of the toxins. The number of total immatures in all controls showed a natural decline posttreatment (Figs. 1–3). This was probably the result of a progressive reduction in ovipositional attractancy of the artificially enriched breeding sites (Rodcharoen et al. 1997).

WDGs of B.t.i. *and* B.s. *(lot 30-073-BR) at the high rates of 1.1–2.7 lb/acre:* For efficacy evaluation on newly developed WDG formulations of *B.t.i.* and *B.s.* with high potency and determination of their minimum effective dosages, the 1st test was initiated using the dosages of 1.1–2.7 lb/ac. In this test, control and treatment plots had statistically the same population densities before treatment. From the data of total immatures (Fig. 1), it is evident that the 2 WDG formulations at 1.1 lb/acre and 2.7 lb/acre were equally effective on day 2 posttreatment, yielding almost 100% control. Statistically, all the treatments showed significant reduction on day 7 posttreatment, but formulation-based differences in level of efficacy occurred, indicating that the *B.t.i.* WDG formulation was much less effective than *B.s.* WDGs at both rates. The *B.t.i.* WDG formulation lost its efficacy further at both rates on day 12 posttreatment, whereas the *B.s.* WDGs still had a high level of control at this time. On day 19 posttreatment, no significant control was indicated in the *B.t.i.* WDG formulation at the low rate. The *B.t.i.* WDG formulation at the high rate and *B.s.* WDGs at both rates were equally effective, yielding significant control. On day 25 posttreatment, all treatments gave significant control, but the *B.t.i.* WDG formulation at the low rate was less effective than other treatments, which exhibited equal level of control. All treatments yielded almost 100% control of late instars on day 2 posttreatment (Fig. 1). On days 7 and 12 posttreatment, significant reduction was achieved in all treatments, but the *B.t.i.* WDG formulation was less effective than *B.s.* WDGs at both rates. On days 19 and 25 posttreatment, the *B.t.i.* WDG formulation was no longer effective at the low rate, whereas all other treatments were equally effective, showing significant control.

As a result of larval control, the pupation level was very low in the treated regimens (Fig. 1). On day 2 posttreatment, all treatments showed almost 100% reduction in presence of pupae. On day 7 posttreatment, all treatments still exhibited significantly lower pupal numbers than in the control. The *B.t.i.* WDG formulation at the low rate was less effective than other treatments, which showed equal level of control. No efficacy was indicated on day 12 posttreatment, and significantly higher numbers of pupae prevailed in *B.t.i.* WDG treatments at both rates than in the control. On days 19 and 25 posttreatment, the *B.t.i.* WDG formulation at the low rate was no longer effective; all other treatments showed equal efficacy (Fig. 1). Mosquito oviposition decreased in the control and treatments at this time, when the test was terminated.

Proportions of *Cx. stigmatosoma* and *Cx. quinquefasciatus* in controls decreased from 53.3 to 6.5%, and 34.8 to 6.9%, respectively, whereas *Cx. tarsalis* increased from 11.9 to 86.5% (Fig. 4). These changes in species composition might be attributed to oviposition attractancy of enriched habitats and oviposition behavior of the prevailing species. Newly polluted water attracted gravid *Cx. stigmatosoma* and *Cx. quinquefasciatus,* whereas gravid *Cx. tarsalis* preferred to oviposit 2–3 weeks after enrichment, when the organic materials were degraded and the water became clearer.

WDGs of B.t.i. *and* B.s. *(lot 30-073-BR) at the medium rates of 0.27–0.53 lb/acre:* In order to determine the minimum effective dosages of these

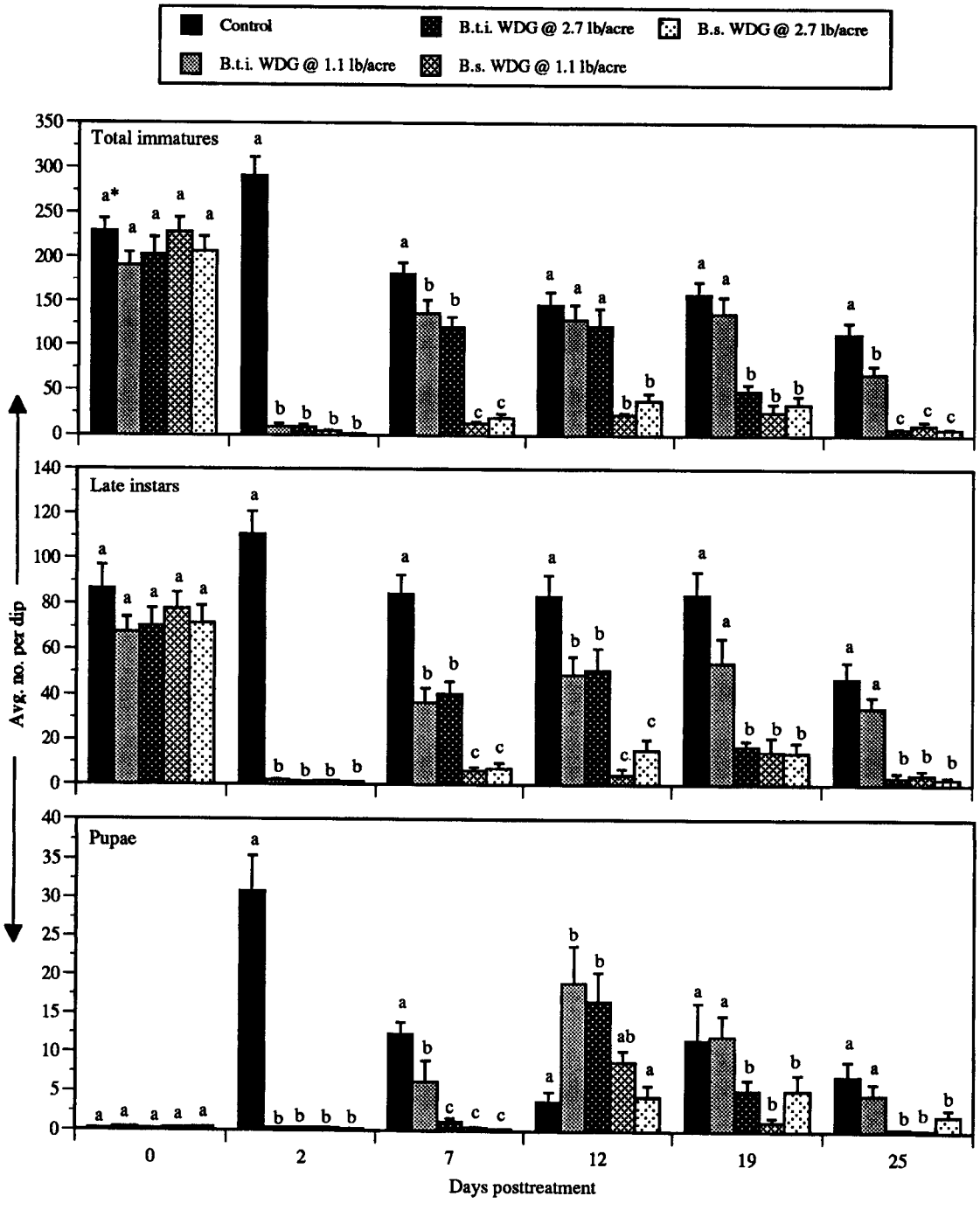

Fig. 1. Population trends of total immatures, late instars, and pupae of mosquitoes in tubs treated with water-dispersible granule (WDG) formulations of *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) (4,000 International Toxic Units[ITU]/mg) and *Bacillus sphaericus* (*B.s.*) (350 ITU/mg) at 1.1–2.7 lb/acre (Aquatic and Vector Control Research Facility, University of California, Riverside, CA, August 14–September 15, 1997). *Different letters on a given sampling day indicate significant differences among control and treatments by one-factor analysis of variance (Scheffé *F* test) at the 0.05 level.

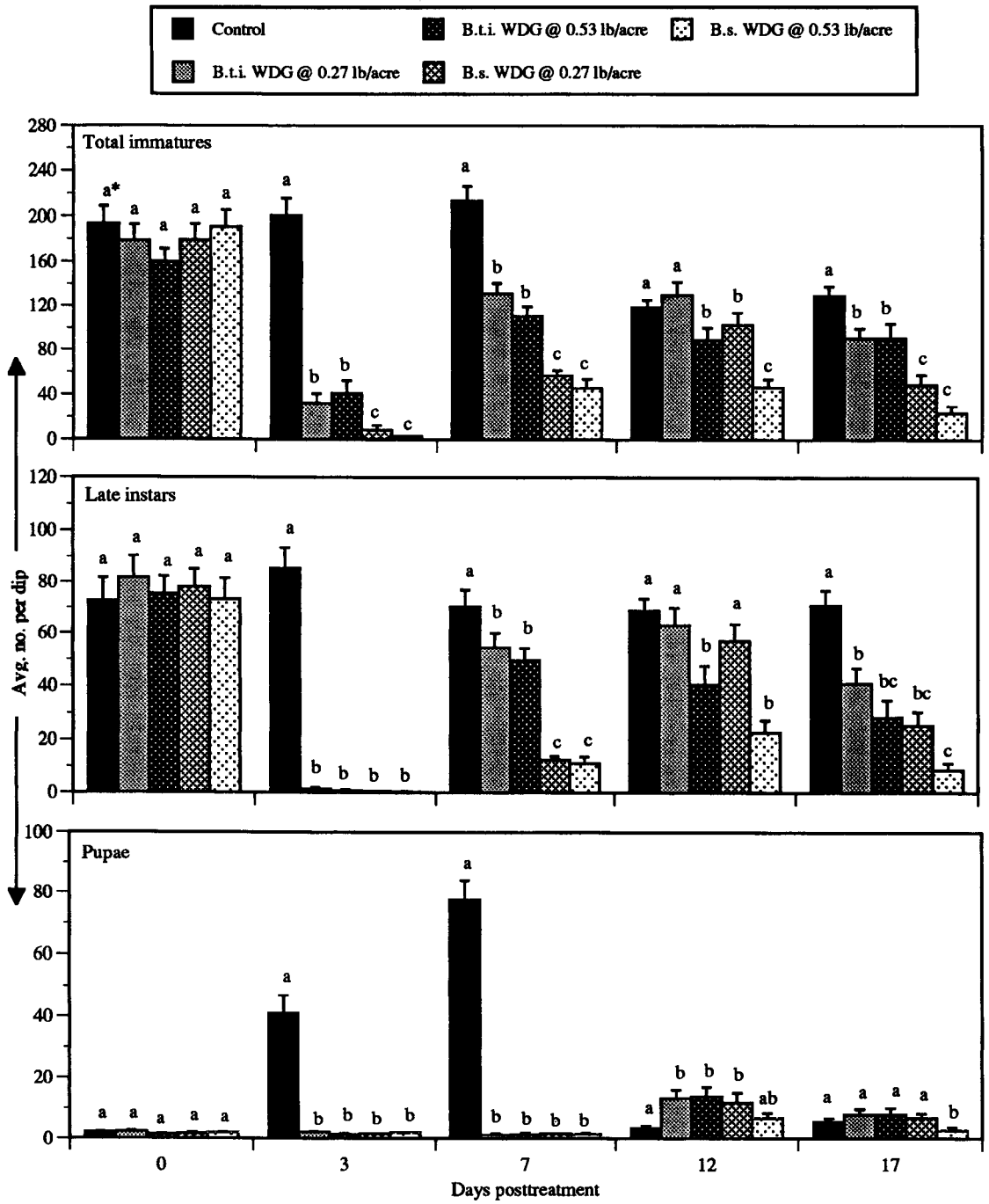

Fig. 2. Population trends of total immatures, late instars, and pupae of mosquitoes in tubs treated with water-dispersible granule (WDG) formulations of *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) (4,000 International Toxic Units[ITU]/mg) and *Bacillus sphaericus* (*B.s.*) (350 ITU/mg) at 0.27–0.53 lb/acre (Aquatic and Vector Control Research Facility, University of California, Riverside, CA, September 19–October 13, 1997). *Different letters on a given sampling day indicate significant differences among control and treatments by one-factor analysis of variance (Scheffé *F* test) at the 0.05 level.

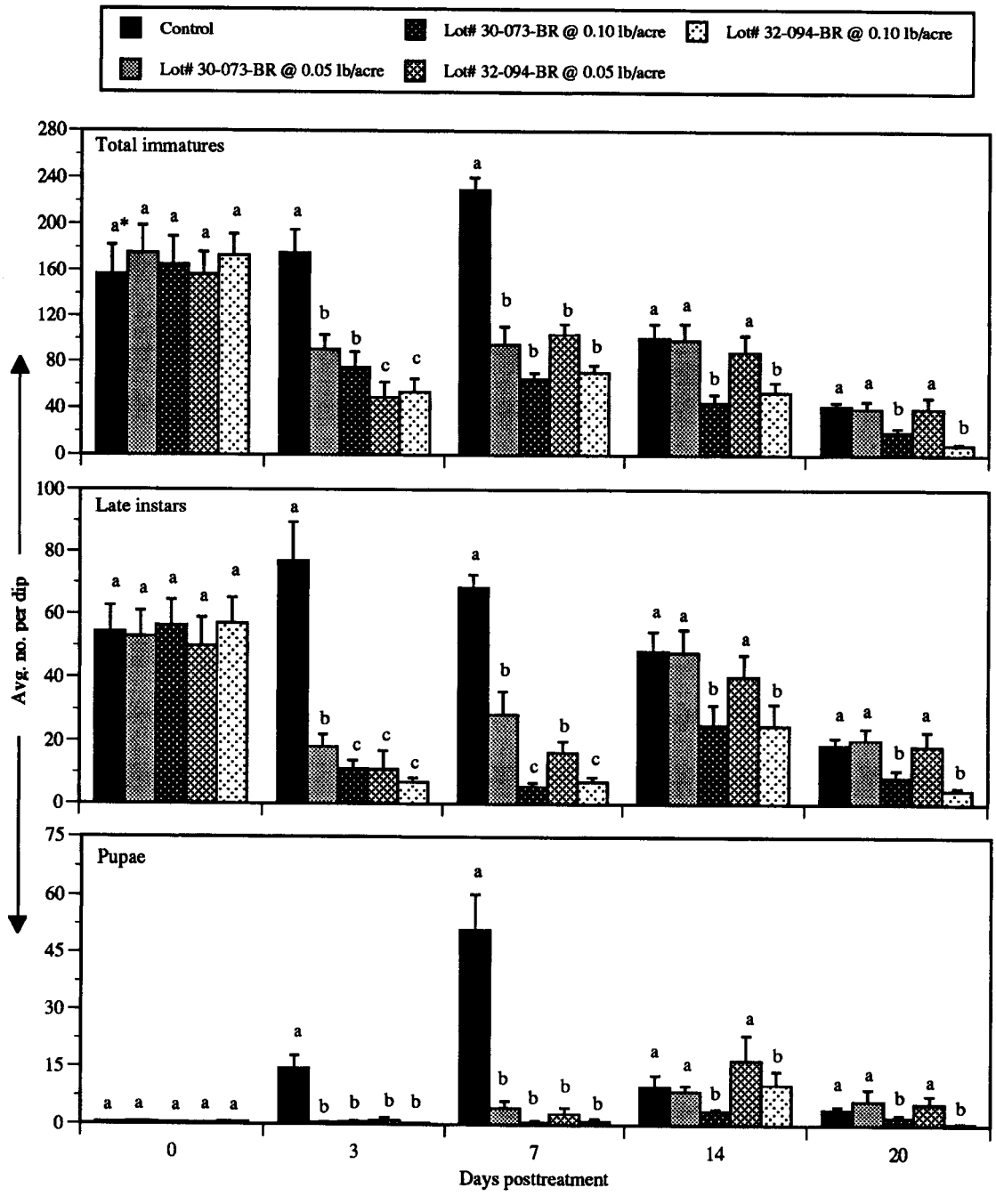

Fig. 3. Population trends of total immatures, late instars, and pupae of mosquitoes in tubs treated with 2 *Bacillus sphaericus* (*B.s.*) water-dispersible granule (WDG) formulations (lot 30-073-BR, 350 International Toxic Units [ITU]/mg and lot 32-094-BR, 630 ITU/mg)) at 0.05–0.10 lb/acre (Aquatic and Vector Control Research Facility, University of California, Riverside, CA, May 4–June 4, 1998). *Different letters on a given sampling day indicate significant differences among control and treatments by one-factor analysis of variance (Scheffé *F* test) at the 0.05 level.

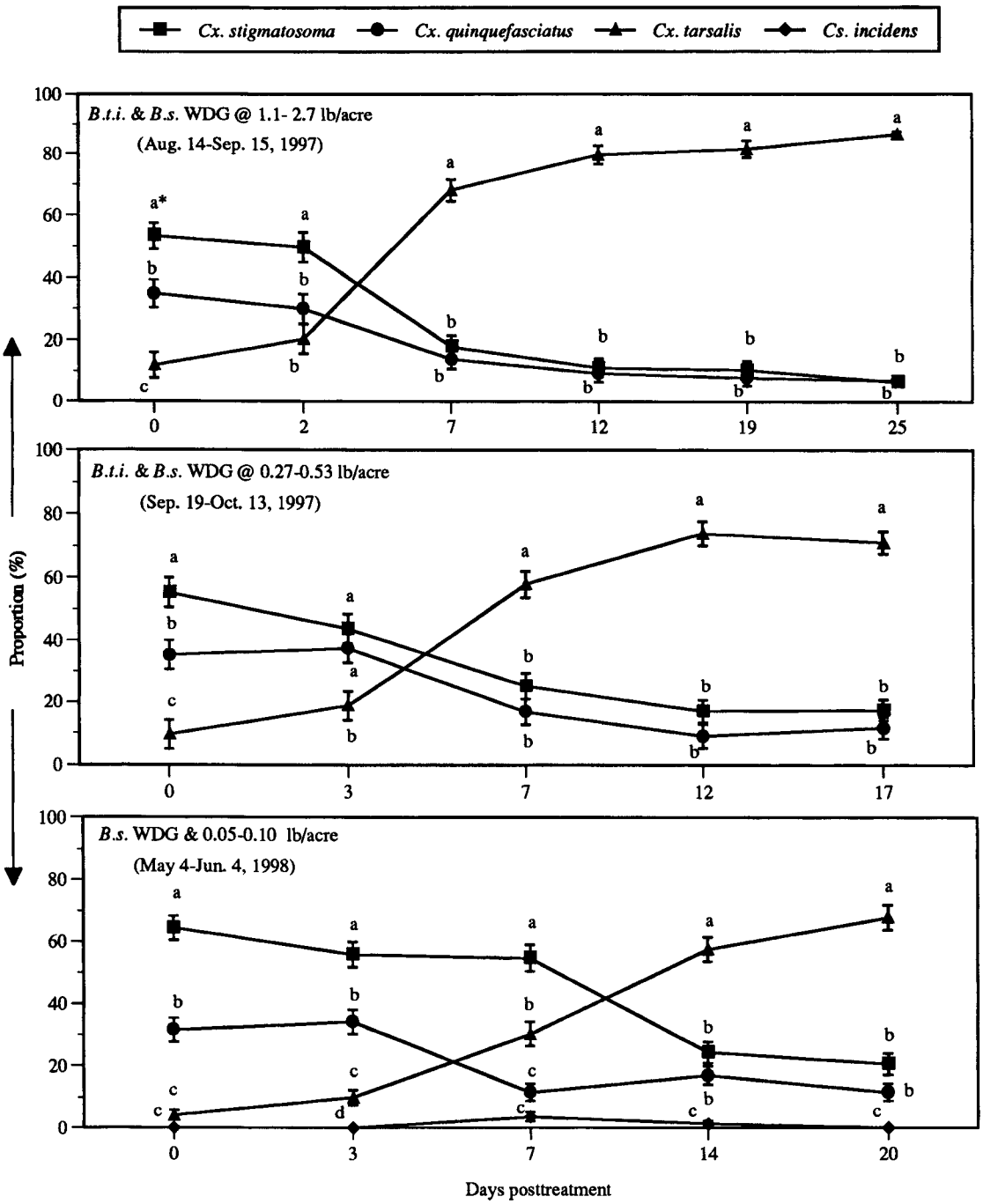

Fig. 4. Species composition of mosquitoes in check tubs as indicated by identification of about 150 4th instars (Aquatic and Vector Control Research Facility, University of California, Riverside, CA). *Different letters on a given sampling day in each test indicate significant differences among species by a chi-square test at the 0.05 level.

WDG formulations and the difference in efficacy between *B.t.i.* WDGs and *B.s.* WDGs, dosages approximately one fourth of those of the 1st test were applied. The pretreatment densities of total immatures, late instars, and pupae were essentially the same in assigned control and treatments. A significant reduction in the number of total immatures was achieved in all treatments on days 3 and 7 post-

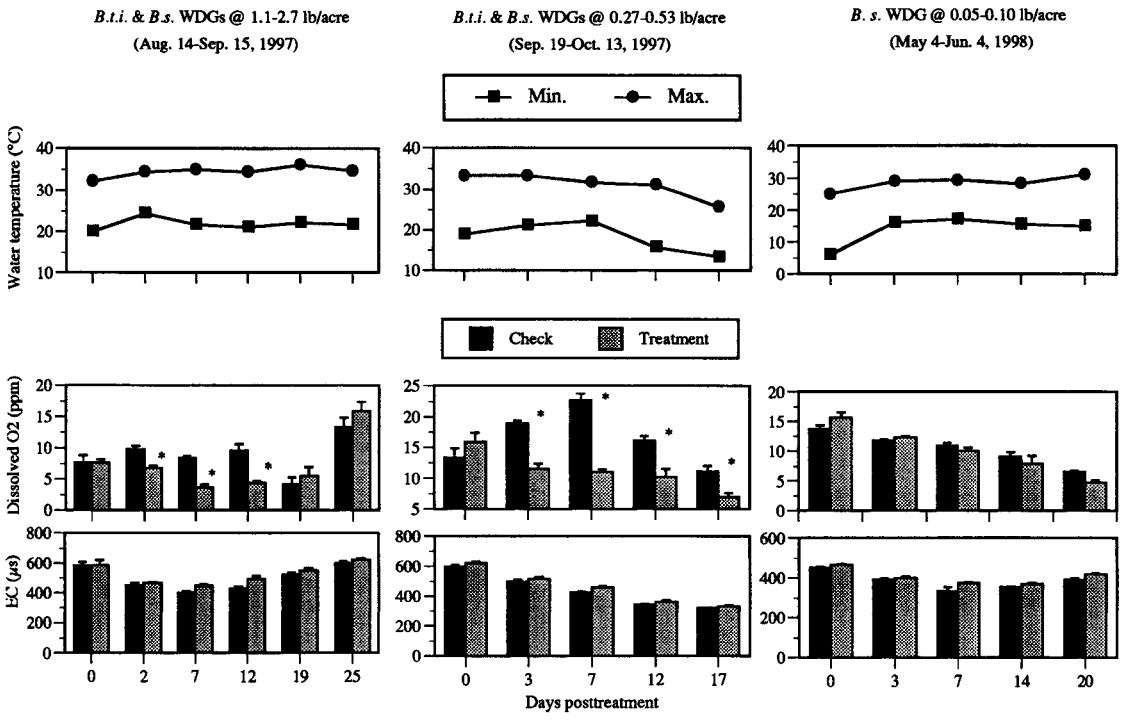

Fig. 5. Minimum and maximum water temperature (upper) in a tub located in the center of the tub arrangement, and dissolved oxygen and electrical conductivity (EC) (lower) in control and treated tubs during the test period (Aquatic and Vector Control Research Facility, University of California, Riverside, CA). An * indicates significant differences between control and treatment on each sampling day by *t*-test at the 0.05 level.

treatment (Fig. 2). Formulation-dependent differences were apparent as early as day 3, and became more obvious on day 7 posttreatment, indicating that the *B.t.i.* WDG formulation was less effective than *B.s.* WDGs at both rates. The 2 rates of both materials were equally effective on this sampling day. However, on day 12 posttreatment, the *B.t.i.* WDG formulation at the low rate lost its efficacy. The *B.t.i.* WDG formulation at the high rate and *B.s.* WDGs at the low rate were equally effective, but were less effective than *B.s.* WDGs at the high rate. The situation on day 17 posttreatment was similar to that on days 3 or 7 posttreatment.

With regard to the densities of late instars (Fig. 2), which provide a better indicator for evaluation of efficacy than numbers of total immatures, these 2 formulations were equally effective at both rates, yielding almost 100% control on day 3 posttreatment. On day 7 posttreatment, all treatments still yielded significant reduction, and formulation-related differences were noted. The *B.t.i.* WDG formulation was less effective than *B.s.* WDGs at both rates. Some differences were also revealed between the 2 rates of either *B.t.i.* WDGs or *B.s.* WDGs on day 12 posttreatment; the high rates showed partial control, but the low rates lost their efficacy at this time. On day 17 posttreatment, all treatments showed partial control; the *B.t.i.* WDG formulation at the low rate was less effective than *B.s.* WDGs at the high rate, whereas *B.t.i.* WDGs at the high rate and *B.s.* WDGs at the low rate were equally effective.

As an indication of larval control, pupal density provides the most important parameter for efficacy assessment. These 2 formulations at the 2 rates were equally effective on days 3 and 7, producing almost 100% reduction of pupae. On day 12 posttreatment, no reduction was indicated in all treatments. Partial reduction occurred in *B.s.* WDGs at the high rate on day 17 posttreatment (Fig. 2). Mosquito oviposition decreased in the control and treatment tubs by day 17 posttreatment, when the test was terminated. From this test, the minimum effective dosage for the *B.t.i.* WDG formulation seemed to be 0.27–0.53 lb/acre, which yielded good control for up to 7–12 days. In this test, we were not able to determine the minimum effective dosage of *B.s.* WDGs and because of this an additional experiment (see below) was carried out. The same trend in species composition in control was indicated as in the previous test (Fig. 4).

Comparison of 2 B.s. *WDGs at the low rates of 0.05–0.10 lb/acre:* Because the less potent *B.s.* WDG formulation (lot 30-073-BR, 350 ITU/mg) at 0.27–0.53 lb/acre provided excellent control in the last test, the dosage was reduced here to 0.05–0.10

lb/acre to determine the minimum effective dosage and to compare it with the WDG formulation having high potency (lot 32-094-BR, 630 ITU/mg). The pretreatment populations of total immatures, late instars, or pupae were essentially the same among the control and treatment tubs. In terms of the overall control (Fig. 3), on day 3 posttreatment, all treatments gave good control. The less potent *B.s.* WDG formulation (350 ITU/mg) was significantly less effective on day 3 than the high potency (630 ITU/mg) product at the same rates. No dosage-related differences were indicated on this sampling day. On day 7 posttreatment, all treatments showed equally significant reduction. However, on days 14 and 20 posttreatment, only high rates (0.10 lb/acre) in both formulations yielded partial control. Both materials no longer were effective at the low dosage of 0.05 lb/acre.

In terms of densities of late instars (Fig. 3), on day 3 posttreatment, all treatments yielded excellent control. The low-potency formulation at the low rate was less effective than at the high rate and the high-potency formulation at both rates. Significant reduction was still noted on day 7 posttreatment in all treatments. The low rates were less effective than the high rates in both formulations. However, on days 14 and 20 posttreatment, the low rates of both formulations were no longer effective, and only the high rates gave partial control. On days 3 and 7 posttreatment, all treatments were equally effective in producing lowered pupal populations resulting from larval control. On days 14 and 20 posttreatment, pupal populations declined in the control as well. No additional reduction occurred in the low rates, whereas the high rates still gave significant reduction in pupae (Fig. 3). Thus, the rates of 0.05–0.10 lb/acre are the minimum effective dosages for both formulations, yielding good control for 14–20 days (Fig. 3). In terms of suppressing late instars and the resulting pupae, the high potency material was more effective. A similar relationship was noted for the efficacy of these 2 formulations evaluated in polluted habitats in Thailand (Mulla et al. 1999).

The changes in species composition in control tubs during this test was similar to those in previous tests. The species *Culiseta incidens* Thomson was present at low percentage during the test period (Fig. 4).

Water quality

Water quality parameters were measured in all control tubs and in tubs of one treatment at the high dosage in each test. In the 1st test for *B.t.i.* and *B.s.* WDGs at 1.1–2.7 lb/acre carried out from August 14 to September 15, 1997, minimum and maximum water temperatures ranged from 20.0 to 24.4°C and 32.2 to 36.1°C, respectively. In the 2nd test for the same materials at 0.27–0.53 lb/acre conducted from September 19 to October 13, 1997, minimum and maximum water temperatures were 13.3–22.2°C and 25.6–33.3°C, respectively. In the 3rd test for 2 *B.s.* WDGs at 0.05–0.10 lb/acre conducted from May 4 to June 4, 1998, minimum and maximum water temperatures were 6.1–15.0°C and 25.0–31.1°C, respectively (Fig. 5). On days 2, 7, and 12 posttreatment in the 1st test and days 3, 7, 12 and 17 posttreatment in the 2nd test, the O_2 levels in control tubs were significantly higher than in the treated tubs. No significant difference in O_2 levels was shown between the control and treatment in the 3rd test (Fig. 5). For all 3 tests, no significant difference in EC was detected between control and treatment (Fig. 5), and the pH values were 7.5–8.0 and the salinity readings were 0–0.3 ppt in both control and treated tubs during the test periods.

CONCLUSIONS

In the current studies, the test materials yielded excellent control of *Culex* mosquitoes in microcosms for up to 7–12 days at the dosage of 0.27–0.53 lb/acre for *B.t.i.* WDGs and 14–20 days at the dosage of 0.05–0.10 lb/acre for *B.s.* WDGs. These newly developed WDG formulations of *B.t.i.* and *B.s.* have high potency per unit mass and are highly effective against mosquito larvae. These materials disperse in water readily by gentle agitation and have an additional advantage of being easy to apply to mosquito breeding sources as the aqueous sprays that are the preferred methods in most vector control programs.

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