SUSTAINED RELEASE FORMULATIONS OF *BACILLUS SPHAERICUS* AND *BACILLUS THURINGIENSIS* (H-14) FOR CONTROL OF CONTAINER-BREEDING *CULEX QUINQUEFASCIATUS*[1]

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ABSTRACT. Aqueous suspensions of formulated *Bacillus thuringiensis* (H-14) wettable powder (Bactimos® WP) and *B. sphaericus* (1593) acetone precipitated spore powder were compared with slow release floating pellet formulations of each pathogen for the control of *Culex quinquefasciatus* breeding in urban settings. In 15 and 8 liter buckets both suspensions provided complete control at 1 mg of powder/liter but suppression was brief with the *B. thuringiensis* (H-14) WP. Bactimos® primary powder formulated into small, floating pellets (0.17 g primary powder/pellet), provided limited control for several weeks and was markedly inferior to the Bactimos briquette. One half briquette gave complete control for 3 weeks but its effectiveness declined as the briquette constituents sank. All *B. sphaericus* treatments provided continuous good to excellent control for the 8 weeks of observation. The best control in the 15 liter buckets was effected with 2 floating pellets each containing 0.21 g of *B. sphaericus* spore powder.

INTRODUCTION

The age of effective microbial control of mosquitoes literally began with the discovery of *Bacillus thuringiensis* Berliner var. *israelensis* (H-14) de Barjac and the highly larvicidal isolates of *Bacillus sphaericus* Neide. The literature is replete with references attesting to the efficacy of both bacteria. Despite the remarkable larvicidal activities of these agents, their full potential has not yet been realized partly because a broad diversification of formulations to meet specific needs is not currently available. Virtually no formulated material is available for *B. sphaericus*. Because there is little persistence under most field conditions and no recycling of *B. thuringiensis* (H-14), subsequent production of epizootics in target larvae is not observed when this agent is applied to mosquito habitats. Rapid settling of the *B. thuringiensis* (H-14) toxin prevents sustained contact with target populations (Vanková 1982, Ignoffo et al. 1981, Davidson et al. 1981), necessitating repeated treatments to effect continuous control. Residual activity of *B. thuringiensis* (H-14), however, has been observed at relatively high concentrations in clear shallow water (Larget 1981, Ramoska et al. 1981, Mulla et al. 1982). Even though *B. sphaericus* spores may persist for a considerable length of time (Hertlein et al. 1979), recycling in the environment and production of epizootics is seldom observed. A slow release formulation that provides a continuous high concentration of toxin within the feeding zone of mosquito larvae would enhance the control potential of both bacilli.

The controlled release of conventional larvicides and insect growth regulators has been successfully used against mosquito larvae in a number of experimental and practical situations (Raley and Davis 1949, Whitlaw and Evans 1968, Cardarelli 1976, Lewis 1981). Solid matrices with leaching or diffusion release mechanisms are the most commonly employed and can provide effective sustained release of larvicide for several months to years. However, because *B. sphaericus* spores and the parasporal crystals of *B. thuringiensis* (H-14) are active *per os*, such release mechanisms would be ineffective. Solubilization of the σ-endotoxin of *B. thuringiensis* (H-14) and spore toxin of *B. sphaericus* with retention of larvicidal properties is highly unlikely in most mosquito habitats. Continuous release of the toxins of both bacilli therefore must be accomplished by gradual dissolution of the carrier matrix. Excessively rapid dissolution will provide a high but evanescent level of toxin. A formulation that provides an adequate level of toxin for initial control of older mosquito larvae and continuous subsequent release of toxin for the control of younger larvae would be optimal.

In this paper we present information on the activity of sustained release formulations of *B. thuringiensis* (H-14) and *B. sphaericus* against peridomestic container-breeding *Culex quinquefasciatus* Say.

METHODS AND MATERIALS

The *B. thuringiensis* (H-14) formulations utilized were an aqueous suspension of Bac-

[1] This paper reports the results of research only. Mention of a pesticide, commercial or proprietary product does not constitute a recommendation or an endorsement of this product by the U.S. Department of Agriculture.

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timos® WP [Biochem Products; factory determined toxicity of 3500 International Toxicity Units (ITU)/mg against *Aedes aegypti* (L.)], Bactimos briquettes (5% Bactimos primary powder, 95% inert ingredients) and Bactimos primary powder (7000 ITU/mg) incorporated into floating pellets. Pellets were made by mixing primary powder, sifted powdered sugar and polypropylene powder (Accurel® powder, Armak Co., McCook, IL; 75% void, 106–400μ diameter particles, 0.21 g/cm density) in a ratio of 1:1:1.33, respectively. The mixture was pelleted with a Parr® manually-operated pellet press. The resulting pellets were 1.25 cm in diameter by 0.7 cm and weighed an average of 0.57 ± 0.008 g/pellet (0.17 g primary powder/pellet).

The *B. sphaericus* 1593 isolate (serotype 5a5b; lot no. 109AP) was produced by Dr. H. Dulmage, USDA, Brownsville, TX, on peptonized milk with 0.2% yeast extract and harvested by the acetone precipitate-air drying procedure. The viable spore count was 5.77×10^7/mg. Formulations used for treatments consisted of an aqueous suspension of the spore powder and pellets produced in a manner identical to those made with *B. thuringiensis* (H-14). The average weight of the pellets was 0.7 ± 0.052 g (0.21 g spore powder/pellet).

Tests of the bacterial suspensions, pellets and briquettes were conducted against *Cx. quinquefasciatus* in 18.9 and 8 liter buckets from July 16 through September 10, 1982. Eight large and 3 small buckets were set in full or partial shade in each of 3 sites near the Insects Affecting Man and Animals Research Laboratory, Gainesville, FL, and provided with a handful of oak leaves and 15 and 8 liters of well water, respectively. Two of the sites were adjacent to laboratories and in close proximity of livestock. The third was in a mesic hammock near several occupied poultry barns. Within 1 wk natural populations of *Cx. quinquefasciatus* began ovipositing in the containers. When second and third instar larvae were observed, the buckets were treated with the various formulations. Each large bucket at each site received one of the following treatments: 1 ppm Bactimos WP or 1 ppm *B. sphaericus* powder; 1 or 2 pellets made with either *B. thuringiensis* (H-14) or *B. sphaericus* powder, or ½ Bactimos briquette for a total of 7 large treated buckets and 1 untreated control at each site. Each small bucket at each site received 1 pellet of either *B. thuringiensis* (H-14) or *B. sphaericus* or was left as an untreated control. After 2 wk the *B. thuringiensis* (H-14) pellets were cut in half and returned to the buckets to facilitate increased release of toxin.

Prior to treatment the density of larvae in each of the 24 large and 9 small buckets was sampled. Three aliquots of larvae were taken from each bucket in rotation at each site with 100 ml cups and subsequently returned after counting. In posttreatment samples, pupae were counted as surviving larvae and first instars were omitted from counts. After treatment, census counts were taken at 3 days, 1 wk and every week thereafter for 8 wk or until populations rebounded. Three wk after the test started, 5 egg rafts from the laboratory colony of *Cx. quinquefasciatus* were placed in each bucket each week to supplement natural oviposition.

In order to determine settling rates of *B. sphaericus* spores, an aqueous suspension of the powder was applied to 1.5 × 1.2 m, sod-lined, cement potholes at the rate of 0.5 kg/ha with a CO_2 powered sprayer through a flat fan nozzle. Water and mud samples (5 ml) were taken from the top, bottom and within the mud substratum of each pothole prior to treatment and at 2, 24, 48 and 96 hr after treatment. A 2 ml aliquot of each sample was pasteurized for 12 min at 80°C, diluted with sterile deionized water and plated on nutrient agar plates with 0.05% yeast extract and 0.01% streptomycin sulfate; a selective medium designed by A. A. Yousten (personal communication). The addition of antibiotic suppressed the growth of *B. thuringiensis* (H-14) and most other spore-forming bacteria while permitting germination and growth of *B. sphaericus*. The agar plates were incubated at 35°C for 48 hr and counted. Identification of *B. sphaericus* colonies was based on colony morphology and confirmed with precipitin tests using rabbit-generated *B. sphaericus* 1593 H-antiserum. Additionally, the sinking rate of *B. sphaericus* 1593 suspension applied at 0.1 mg spore powder/liter was compared with the spore count maintained by 2 floating pellets in 15 liter buckets at the 3 sites. The tests were conducted and samples were processed in the same manner as the above-mentioned pothole test except that samples were taken 2–3 cm below the surface at 2, 48 and 96 hr and at 1 and 2 weeks posttreatment.

During the pothole studies there were only trace amounts of rain. During the course of the bucket study natural rainfall kept the level of water high in the buckets but no overflow was produced.

Release rate of *B. sphaericus* spores from the floating pellets was studied in the laboratory (27°C) in the small buckets in 3 liters of distilled water. Samples (5 ml) of the water were taken prior to treatment for determination of bacterial background. After the addition of 1 pellet to each of 4 buckets, 5 ml samples were taken from the top 2–3 cm of the water and from the middle of the water column at 15, 30 and 45

min and 1, 1.5, 3, 4, 5 and 6 hr and every 6 hr thereafter until 48 hr posttreatment, after which sampling was conducted daily until the end of the first week. During the second week, samples were taken every other day and during the third and fourth weeks, samples were taken weekly. The samples were processed and plated in the manner described above for the spore settling study. Water was slowly added to the buckets daily to compensate for evaporation and that removed in sampling.

RESULTS

The effect of various formulations on the activity of *B. thuringiensis* (H-14) in large buckets is presented in Fig. 1. The two most effective treatments in terms of initial suppression of *Cx. quinquefasciatus* larvae, the 1 ppm aqueous suspension and the ½ Bactimos briquette treatments, differed sharply at 7 days posttreatment and throughout the remainder of the observation period with the briquette providing complete control for 3 weeks, The floating pellets gave fair (1 pellet) to good (2 pellets) control at 3 days posttreatment and although sustained suppression of *Cx. quinquefasciatus* larvae was greater than that afforded by the suspension it was markedly inferior to that of the briquette. Splitting the pellets in half noticeably improved efficacy in the 1 pellet treatments in both the large and small buckets (Figs. 1 and 2) but not in the 2 pellet treatments.

The *B. thuringiensis* (H-14) pellets performed more effectively in the small buckets (Fig. 2) than in the large, until after the census of the third week when numbers of *Cx. quinquefasciatus* returned to levels observed in the untreated checks.

All but a few of the *B. thuringiensis* (H-14) pellets remained floating throughout the test despite algal and fungal colonization of the pellet surface. The Bactimos briquette began to break up within the first week after treatment and by the third week most of the floating constituents had sunk, at which time numbers of larvae increased steadily until the termination of the test.

All of the *B. sphaericus* treatments gave nearly complete initial reduction of *Cx. quinquefasciatus* larvae and the suppression lasted considerably longer than with their *B. thuringiensis* (H-14) counterparts (Fig. 3). Similar control was obtained with 1 pellet in the small buckets (Fig. 4). Although the pellets provided longer lasting and greater control, the differences between the pellets and suspension were minimal.

Figure 5 depicts the rapid rate of settling of *B. sphaericus* spores when applied as an aqueous suspension to cement potholes. By 48 hr most of the spores had settled to the substratum.

The decline in spore count from the surface of the bucket on the other hand was not as sharp (Fig. 6). After 2 weeks sufficiently high numbers of *B. sphaericus* spores were detected to produce mortality in young *Cx. quinquefasciatus* larvae; however, a gradual decline in spore count was evident. Higher spore counts were initially apparent and maintained at an elevated level throughout the 2-wk observation period in the buckets treated with 2 pellets containing *B. sphaericus*. Data on the sustained maintenance of high spore counts by the *B. sphaericus* floating pellets in indoor buckets is presented in Fig. 7.

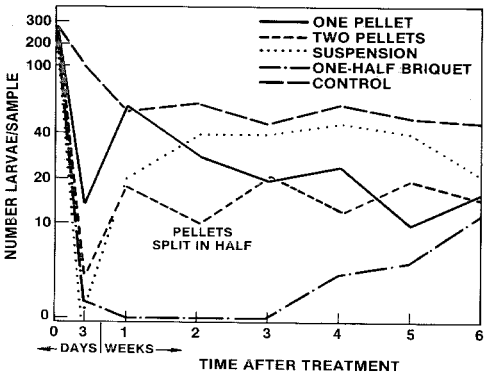

Fig. 1. Efficacy of Bactimos® WP aqueous suspension and slow release formulations of *Bacillus thuringiensis* (H-14) against *Culex quinquefasciatus* larvae breeding in 15 liter buckets.

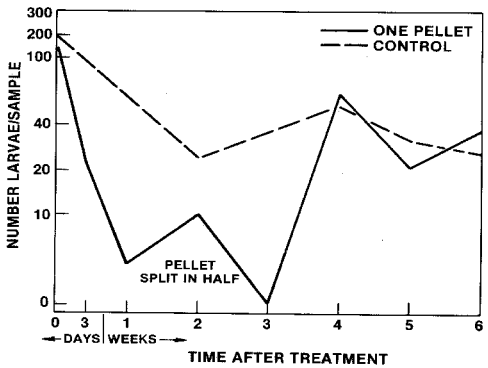

Fig. 2. Efficacy of a slow release pellet formulation of *Bacillus thuringiensis* (H-14) against *Culex quinquefasciatus* larvae breeding in 8 liter buckets.

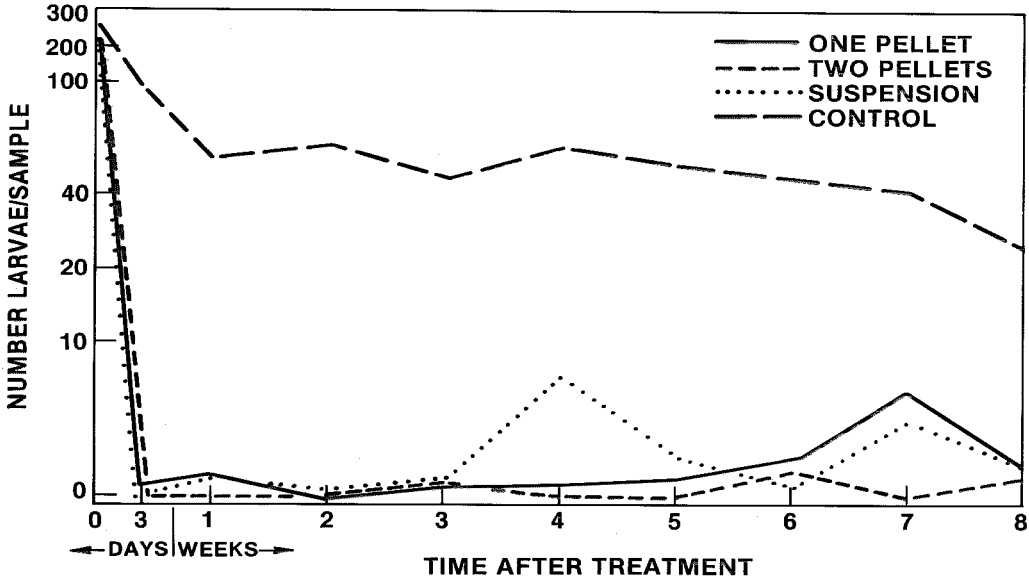

Fig. 3. Efficacy of an aqueous suspension and slow release formulation of *Bacillus sphaericus* (1593) against *Culex quinquefasciatus* larvae breeding in 15 liter buckets.

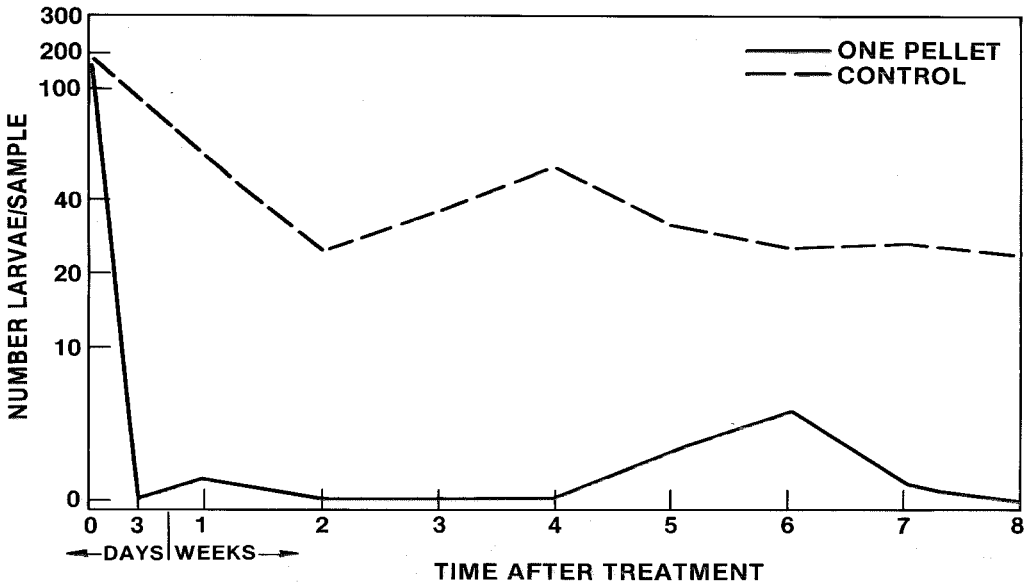

Fig. 4. Efficacy of a slow release pellet formulation of *Bacillus sphaericus* (1593) against *Culex quinquefasciatus* larvae breeding in 8 liter buckets.

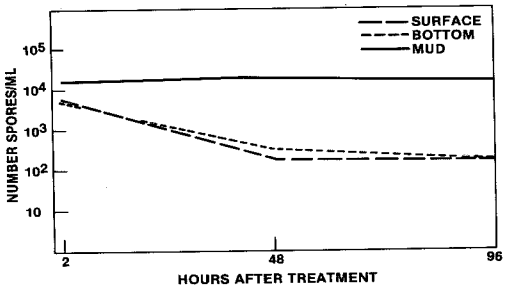

Fig. 5. Settling rate of *Bacillus sphaericus* (1593) spores applied at the rate of 0.5 kg/ha in cement potholes.

At 30 days, spore counts were continuing to rise but the water had become rather cloudy, ostensibly due to the release of sugar into the medium and growth of background microbes.

DISCUSSION

Both *B. thuringiensis* (H-14) and several isolates of *B. sphaericus* are highly larvicidal to *Cx. quinquefasciatus* (Tyrell et al. 1979, Mulligan et al. 1980, Davidson et al. 1981, Lacey and Singer 1982). Toxicity of these bacilli to *Cx. quinquefasciatus* and other target species may vary depending on a variety of extrinsic biotic and abiotic factors (Davidson 1982). The negative correlation of density of suspended particles and level of larvicidal activity is one of the most striking (Ramoska and Pacey 1979, Mulligan et al. 1980, Ignoffo et al. 1981, Mulla et al. 1982, Ramoska et al. 1982, Van Essen and Hembree 1982). Ostensibly the binding of suspended materials to the toxin containing spores of *B. sphaericus* and the parasporal crystalline inclusions of *B. thuringiensis* (H-14) expedites settling. Other mechanisms of particulate interference with the toxins of *B. thuringiensis* (H-14) and *B. sphaericus* are postulated by Ramoska et al. (1982) and Ramoska and Pacey (1979). Rapid settling in clear water is also observed with *B. thuringiensis* (H-14) (Vanková 1982, Ignoffo et al. 1981). Once settled from the feeding zone of the larvae, the parasporal crystalline inclusions of *B. thuringiensis* (H-14) are apparently rapidly denatured by microorganisms in the substratum. *Bacillus sphaericus*, however, may persist as viable spores for several months (Hertlein et al. 1979) and larvicidal activity may again be observed if the spores are resuspended in the feeding zone of the larvae (Mulligan et al. 1980). Unlike the σ-endotoxin of *B. thuringiensis*, the *B. sphaericus* toxin is contained within the protective spore wall and is seemingly more resistant to degradation than the parasporal crystalline inclusions of *B. thuringiensis* (H-14).

The prolonged activity of the pellet formulations in our studies may be related to overcoming some of the problems associated with set-

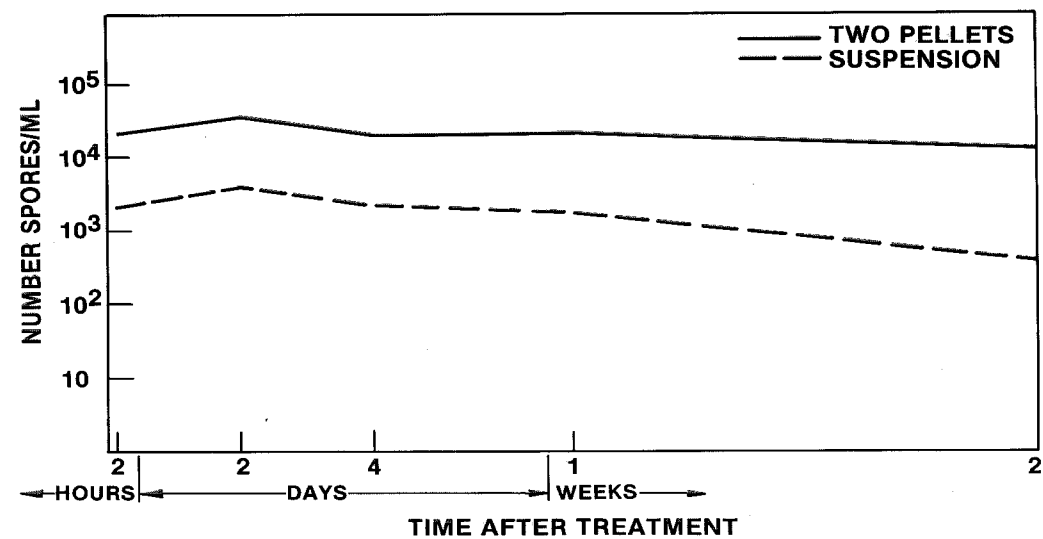

Fig. 6. Average spore counts in 15 liter buckets treated with 0.1 mg/liter of the *Bacillus sphaericus* (1593) spore powder and 2 floating pellets containing *B. sphaericus* spore powder.

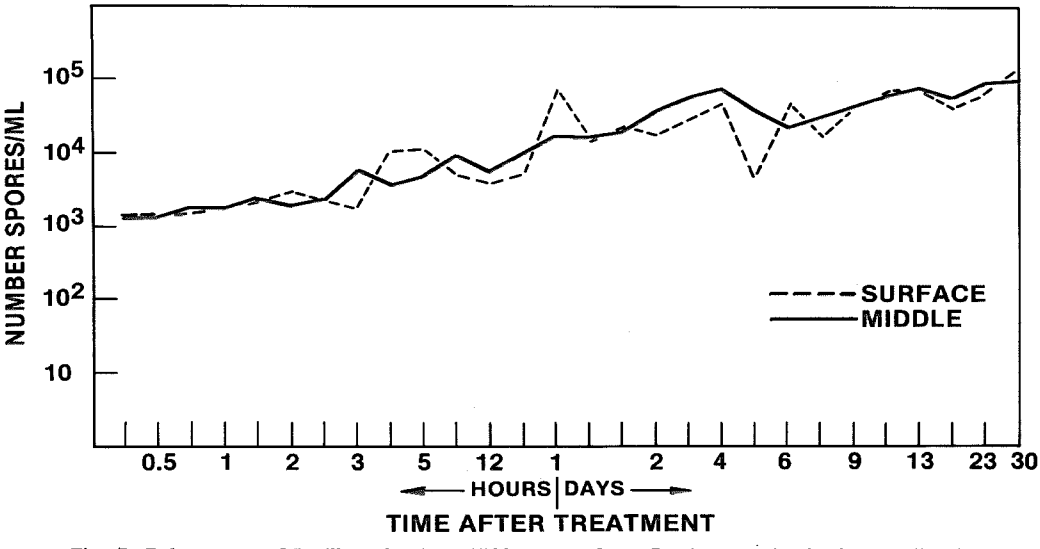

Fig. 7. Release rate of *Bacillus sphaericus* (1593) spores from floating sustained-release pellets in 3 liters of water.

tling by providing a continuously available source of toxin. Rapid settling of the initially effective Bactimos WP suspension and/or degradation of the σ-endotoxin may have been responsible for the relatively brief period of control. The ½· Bactimos briquette probably provided more effective control than did the pellets because the primary powder of *B. thuringiensis* (H-14) was more available to the larvae. The same mechanism that was responsible for the greater observed level of control, rapid break-up of the briquette, was also responsible for its decline in activity after 3 weeks.

In programs where indoor water receptacles require control measures, a sustained release formulation would not only have to be effective but it would also have to remain somewhat intact and not produce a noticeable amount of particulate matter in the drinking water. The amount of particulates produced by the briquette, while of no consequence in peridomestic containers, would be undesirable in water for human consumption.

The greater activity of the *B. sphaericus* pellets over that of the *B. thuringiensis* (H-14) pellets may have been due to slightly more primary powder in the *B. sphaericus* formulation and/or greater larvicidal activity against *Cx. quinquefasciatus*. The residual activity of both *B. sphaericus* suspensions and pellets could also have been aided by the continuous addition of recently hatched larvae to the buckets. These may have promoted the type of cycling observed by M. Urbina and E. W. Davidson (unpublished data) when infected cadavers are accessible to subsequent cohorts. Persistent control might also have been facilitated by the high treatment rate utilized for both the *B. sphaericus* suspension and pellet treatments. Under laboratory conditions, 100% mortality in *Cx. quinquefasciatus* resulted from exposure to concentrations of lyophilized *B. sphaericus* in the vicinity of 0.009 ppm (Lacey and Singer 1982). Rapid degradation of the parasporal crystalline inclusion of *B. thuringiensis* (H-14) may be responsible for the absence of residual activity under identical conditions in the buckets.

Although our studies in the sod-lined potholes confirm other authors' observations that *B. sphaericus* spores rapidly sink from the feeding zone of mosquito larvae (Davidson et al. 1981, Mulligan et al. 1978, 1980), our results in the buckets indicate that larvicidal levels of spores ($\geqslant 10^2$ spores/ml) are evident after 2 weeks, even when treated with concentrations as low as 0.1 mg spore powder/liter. It is possible, in addition to other factors, that rainfall could be responsible for stirring up settled spores. The sod substratum in the potholes and paucity of rain during that study might account for lack of a similar phenomenon there. Additionally, full exposure to sunlight in the potholes and not in the buckets may have resulted in differential spore counts because of

lowered spore viability in the surface water of the potholes due to exposure to ultraviolet radiation. In studies conducted by Burke et al. (1983), rapid loss in *B. sphaericus* spore viability resulted from exposure to sunlight and UV radiation.

Modification of the formulation constituents and pellet structure could further improve performance for both bacteria. For example, the pellet might be constructed in the form of a ring thereby creating a larger surface area and eliminating the waste of material that makes up the inaccessible core of the pellet. A more effective carrier/dispersing agent than powdered sugar could enhance release of the active moieties and may reduce colonization of the pellet surface by fungi and microflora. Although extracted polypropylene powder eventually degrades, a more easily degraded flotation agent may be more desirable. Future research into the optimization of a floating sustained release formulation for the control of container breeding mosquitoes will have to compromise between sustained release of toxin and preservation of floating properties to maintain effective levels of toxin within the feeding zone of mosquito larvae.

The use of such pellets may be advantageous in labor intensive programs in developing countries for the long lasting control of container-breeding culicine vectors of elephantiasis, dengue and yellow fever without concomitant pollution of drinking water and the additional labor inherent in spray application.

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