FATE AND PERSISTENCE OF *BACILLUS SPHAERICUS* USED AS A MOSQUITO LARVICIDE IN DAIRY WASTEWATER LAGOONS[1]

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ABSTRACT. The fate and persistence of the mosquitocidal bacterium, *Bacillus sphaericus*, in dairy wastewater lagoons was evaluated in conjunction with trials of its larvicidal efficacy against *Culex stigmatosoma*. Two commercial formulations, BSP-2 (at 4.48 kg/ha) and ABG-6184 (at 2.24 kg/ha) gave about 90% reduction for up to 4 weeks, although surface water lost its insecticidal activity by 3 days posttreatment. Spores settled to the bottom within 3 days of treatment, but could be recovered in surface water after reflooding. Spore concentrations in bottom water varied widely, yet insecticidal activity remained high for from 3 days (BSP-2 at 4.48 kg/ha) to 2 weeks (ABG-6184 at 2.24 kg/ha). Spores persisted in the mud throughout the study period. These results indicate the extended control obtained was due primarily to the ingestion of spores from bottom water and mud by larvae, which routinely inhabit the shallow areas toward the edge of the pond and browse at the pond bottom.

INTRODUCTION

The microbial agent, *Bacillus sphaericus* Neide, contains numerous strains found in a variety of soil and water habitats. Strains of this species were not known as pathogens until Kellen et al. (1965) isolated a slightly larvicidal strain from moribund *Culiseta incidens* (Thomson) larvae in California. Since then, more than 30 strains have been isolated from a variety of mosquito breeding habitats and other sources (Weiser 1984, Davidson 1985).

Strains of *B. sphaericus* with various potencies have been evaluated in several studies against mosquito larvae (Mulligan et al. 1978, 1980; Mulla et al. 1984a, 1984b; Mulla 1986, Lacey et al. 1987). In general, species of *Culex* have been shown to be the most sensitive to *B. sphaericus*, with *Anopheles* spp. being susceptible to varying degrees, and activity against *Aedes* spp. being generally low (Singer 1985, Mulla 1986, Nicolas et al. 1987a, Wraight et al. 1987). An apparent advantage that *B. sphaericus* has over *B. thuringiensis* subsp. *israelensis* is that the former provides greater residual larvicidal activity, due apparently to its persistence or recycling. However, published reports regarding the extent of the residual activity provided by *B. sphaericus* present conflicting results and offer little insight into the mechanism underlying extended control (Hertlein et al. 1979, Mulligan et al. 1980, Davidson et al. 1984, Mulla et al. 1984b, Nicolas et al. 1987b).

Several years ago, Weiser (1984) isolated a strain of *B. sphaericus*, designated 2362, from an adult blackfly in Nigeria. This strain is now recognized as the most promising isolate of *B. sphaericus* for controlling mosquito larvae in clear as well as polluted waters (Lacey et al. 1986, Mulla 1986, Nicolas et al. 1987a). In the present study, in conjunction with larvicidal trials in dairy wastewater lagoons, we investigated the fate and persistence of the spores and insecticidal activity of *B. sphaericus* in surface water, bottom water and mud using 2 new formulations of 2362, ABG-6184 (Abbott Laboratories, N. Chicago, IL), a primary powder, and BSP-2 (Solvay Co., Brussels, Belgium), a flowable concentrate.

MATERIALS AND METHODS

The field trials were conducted in dairy wastewater lagoons at Kasbergen Dairy (Mira Loma, CA). The ponds averaged 0.12 ha (0.3 acres), and all contained some natural vegetation and variable populations of mosquito larvae as well as nontarget biota including aquatic insects, crustaceans and algae. The ponds were flooded 7 days prior to treatments. During the course of the field studies, normal water management practices were not altered.

Prior to and at intervals after application, mosquito larval populations present in the ponds were determined by sampling using dippers. The extent of population reduction was calculated on the basis of posttreatment versus pretreatment comparisons of the 3rd and 4th instar larvae recorded from these samples (20 dips per pond, 5 from each side). Control ponds, which were not treated with any larvicides, were sampled in the same manner. Water temperature, pH and dissolved oxygen were also monitored with a minimum/maximum thermometer, an oxygen/temperature meter (YSI Model 51B) and a pH meter.

Bacillus sphaericus 2362 (ABG-6184 primary

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powder, 7.6×10^{10} spores/g, lot no. 86-95-BD) was applied at the rate of 1.12 and 2.24 kg/ha (1 and 2 lb/acre), respectively, while BSP-2 (flowable concentrate) was applied at 2.24, 4.48 and 5.6 kg/ha of the formulations. For each treatment and the check, one randomly selected pond was used. The formulations were applied by suspending them in 7.56 liters (2 gal) of water and applied to each pond from the edge towards the center by 3.78-liter (1-gal) hand-pumped sprayer fitted with no. 0006 Tee Jet nozzle.

Fate and persistence of B. sphaericus in water as determined by bioassay: To determine presence and persistence of *B. sphaericus* 2362 in treated water, duplicate composite water samples (1 gal each) were obtained from the check and treated ponds prior to treatment, and 1 h, 1 day, 3 days, 1, 2, 3 and 4 weeks after treatment. One of the samples was obtained from the surface, while the other was siphoned from the bottom of the pond via a hand operated pump. To avoid contamination, samples were first taken from the control pond, then from the treated ponds. Each sample was obtained from 8 different locations, covering 4 sides of each pond. Collected water was transported to the laboratory in 7.8-liter buckets (2-gal), and each bucket was lined with double plastic bags. Bioassays of water samples in the laboratory were conducted as follows:

One hundred ml of water from control plots, and 25, 50 and 100 ml of water from treated plots, on each sampling interval was transferred into 120-ml disposable cups, in triplicate. Then 75 or 50 ml of distilled water were added to cups containing 25 or 50 ml, respectively, of treated field water, to yield 100 ml of water per cup. Twenty early 4th instars of *Culex quinquefasciatus* Say from a laboratory colony were added to each cup. Test cups were placed in a holding room where temperature was maintained at 25.5 ± 1°C, and mortality was determined after 48 h.

Microbiological assessment: Water and mud samples were taken from treated and control ponds pretreatment (0 day), 1 h, 1, 3, 7, 14, 21 and 48 days posttreatment. Undisturbed surface water was sampled using a clean mosquito dipper, while bottom water, 2.5 cm (1 inch) above the bottom hydrosoil, was sampled with a plastic hand-cranked siphon pump fitted with 1 cm diam. Tygon tubing (Dynamic Classics Ltd., New York). The water was kept in sterile 120-ml Nasco Whirl-Pak sampling bags. Water and mud samples were taken from the sides of the ponds beginning with the control, and in increasing order of the treatment rates. The surface mud was sampled by means of a scoop, with a 2-m aluminum handle, which removed a 15- × 15- × 5-cm (6- × 6- × 2-inch) block of undisturbed mud. The scoop was pushed into surface mud and gradually dragged and lifted out of the water. A subsample of 150 g of the surface mud was scraped with a clean spatula and placed in unused Sweetheart® cups with tight-fitting plastic lids (Maryland Cup Corp., Baltimore, MD). This constituted an individual random sample; 3 such samples were taken in each pond while subsamples thereof were taken during the microbiological analysis in the laboratory. Following each sampling exercise, all equipment was washed in hot soapy water to remove any adhering particles.

The samples, kept cool at 5°C until processed, were analyzed in the laboratory a few hours after collection. Prior to plating the samples on BATS microbiological medium (Yousten et al. 1985), 10-ml aliquots of water, or 1 g of the mud samples (wet weight basis) in 10 ml of sterile water, were placed in sterile 30-ml screw-cap vials. The samples were pasteurized at 80°C for 12 min (Davidson et al. 1984) in a precalibrated waterbath (Matheson Scientific, Los Angeles, CA).

The BATS selective medium was prepared after Yousten et al. (1985). Petri dishes were inoculated with 0.1 ml of the water or diluted mud samples collected from the respective treated and control ponds. The plates were wrapped in clear plastic bags and incubated at 30°C for 48 h. Colony-forming units (CFU) of *B. sphaericus* were counted under a stereomicroscope. Spore counts were expressed as CFUs per ml of water or grams of mud on wet weight basis.

The same process was repeated during the second test, which involved the application of *B. sphaericus* 2362 flowable concentrate (BSP-2 containing 2×10^7 spores/ml) at 2.24 and 4.48 kg/ha. In the third test, BSP-2 was applied at a rate of 5.6 kg/ha (5 lb/acre).

The microbiological data were transformed into logarithmic values and the curves plotted using the Cricket Graph Program (Cricket Software, Inc., Philadelphia, PA).

RESULTS

Detailed data on reduction of mosquito larval populations treated with the ABG-6184 and BSP-2 formulations of *B. sphaericus* in the field were presented by Mulla et al. (1988), and information relevant to the present study is summarized in Fig. 1, A and B. Basically, a single application of ABG-6184 at 1.12 or 2.24 kg/ha (1 and 2 lb/acre, respectively) reduced populations of *Cx. stigmatosoma* by an average of greater than 90% for at least 4 weeks (Fig. 1A). A similar level of control was obtained with BSP-2 at the rate of 4.48 kg/ha (4 lb/acre), whereas at the lower rate of 2.24 kg/ha (2 lb/acre), effective control lasted for only 7 days (Fig. 1B).

Analysis of treated water in the laboratory using mosquito larval bioassays demonstrated that surface water lost insecticidal activity within 3–7 days with both formulations at all rates tested, but that bottom water in lagoons treated with the ABG-6184 formulation remained moderately to highly toxic for at least 2 weeks (Tables 1 and 2). More specifically, bioassays of surface water obtained 1 day after treatment from ponds treated with the primary powder ABG-6184 at the rates 1 and 2 lb/acre (1.12 and 2.24 kg/ha), displayed high insecticidal activity against 4th instar larvae of *Cx. quinquefasciatus* in the laboratory. However, no activity was detected in treated surface water samples obtained 3, 7, 14, 21 and 28 days after treatment. Water samples obtained from the bottom, however, exhibited a high level of activity for 14 days, but activity at both rates began to decline 21 days after treatment (Table 1).

The persistence pattern of the flowable concentrate formulation (BSP-2) differed some-

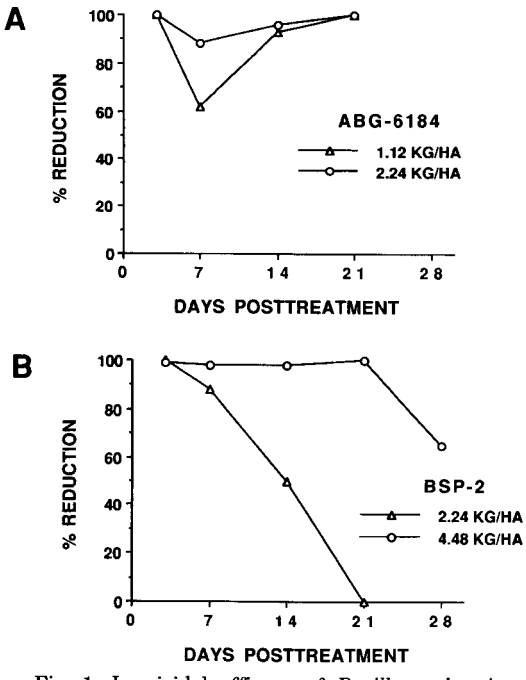


Fig. 1. Larvicidal efficacy of *Bacillus sphaericus* formulations against *Culex stigmatosoma* in dairy wastewater lagoons. (A) ABG-6184, a wettable powder; (B) BSP-2, a flowable concentrate.

Table 2. Bioassay of treated dairy wastewater against 4th instar larvae of *Culex quinquefasciatus* in the laboratory to determine activity of the BSP-2 formulation of *Bacillus sphaericus* 2362.

Rate kg/ha	(%) Field water	Pretreatment	Mean (%) mortality pre- and posttreatment (days) 1	3	7	14	21	28
			Surface water					
2.24	50	—	67	63	2	10	2	0
	100	5	77	78	5	10	3	2
4.48	50	—	80	100	0	3	6	0
	100	—	82	100	2	2	0	0
Check	100	7	3	5	2	0	0	0
			Bottom water					
2.24	50	—	65	85	15	5	0	0
	100	8	63	82	57	5	0	0
4.48	50	—	83	65	0	7	2	0
	100	7	82	70	0	18	2	0
Check	100	5	3	3	7	0	3	0

Table 1. Bioassay of treated dairy wastewater against 4th instar larvae of *Culex quinquefasciatus* in the laboratory to determine activity of *Bacillus sphaericus* 2362 (ABG-6184 formulation).

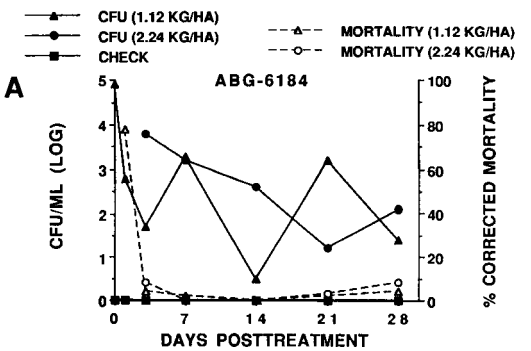
Rate kg/ha	(%) Field water	Mean (%) mortality after treatment (days)[a] 1	3	7	14	21	28[b]
		Surface water					
1.12	50	82	2	5	8	8	0
	100	90	2	2	2	3	2
2.4	25	48	0	2	—	—	—
	50	78	3	5	5	2	2
	100	—	3	3	0	3	7
Check	100	10	0	7	8	2	0
		Bottom water					
1.12	50	62	92	100	90	62	Drying
	100	65	95	100	90	63	Drying
2.4	25	92	98[c]	50	—	—	Drying
	50	97	100	62	83	0	Drying
	100	—	100	70	97	13	Drying
Check	100	10	2	2	8	0	Drying

[a] Mortality readings were taken after 48 h of exposure.
[b] Water in the lagoons was spotty and ranged in depth from 6–12 cm.
[c] Water from barn wash was running into treated lagoon at time of sampling.

what from that of the primary powder preparation (ABG-6184). In the surface water from BSP 2-treated pond, mortality was high at the rates of 4 and 5 lb/acre (4.48 kg/ha), until 3 days after treatment, but had declined significantly by 7 days after treatment (Table 2). This is in contrast to ABG-6184, where little activity was detected in surface water 3 days posttreatment. Similarly, little activity was detected in the bottom water after 3 days posttreatment. This again is in contrast to ABG-6184, where persistent activity in bottom water was detected for 14 days posttreatment.

In general, the data obtained in regard to the presence of spores in surface water and bottom water (Figs. 2 and 3) correlated with the data on the presence of insecticidal activity in these waters (Tables 1 and 2). With both ABG-6184 and BSP-2, spore counts in surface water decreased rapidly within 2–3 days of treatment, and concomitantly rose in the bottom water samples. Spore counts in the mud samples increased quickly after treatment and then remained relatively stable in lagoons treated with both formulations (Fig. 4).

During the trials with the primary powder formulation, the pH recorded in the treated as well as check ponds ranged from 7.5 to 8.7, while those recorded in the trials with the flowable concentrate ranged from 7.3 to 8.5. During the same period, dissolved oxygen in the ponds ranged from nearly zero to 8.4 ppm and 7.3 to 8.5 ppm, respectively. There was no observed correlation between the pH, dissolved oxygen and spore counts or their activity.



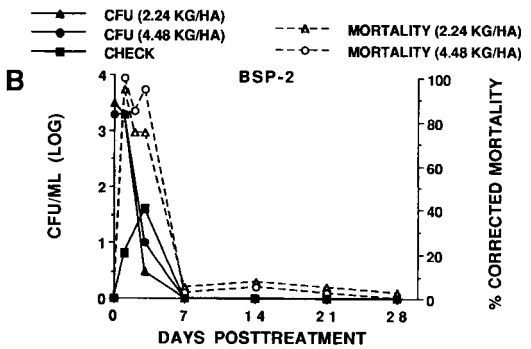


Fig. 2. Spore counts (CFU/ml) recorded in surface water samples versus larvicidal activity in these samples as determined by laboratory assays. (A), ABG-6184; (B), BSP-2.

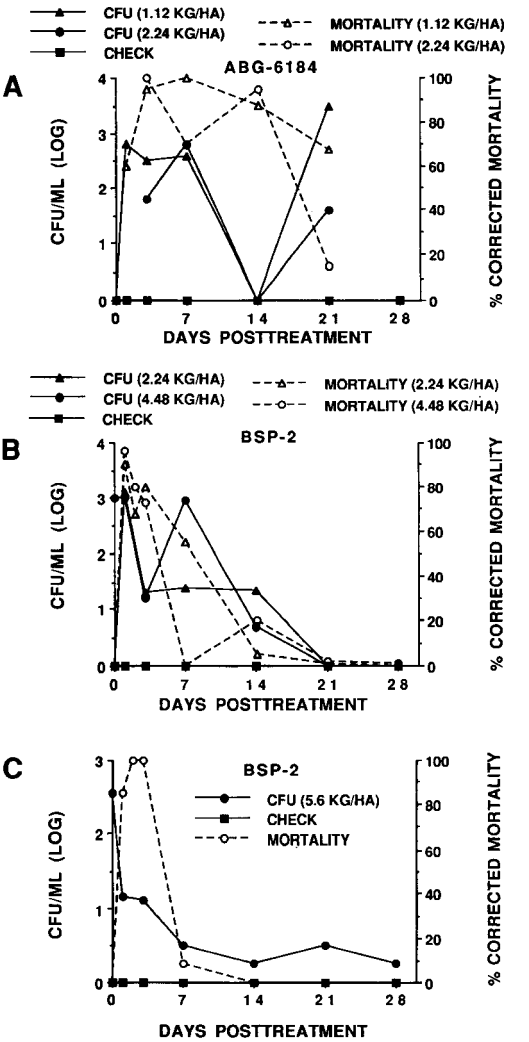


Fig. 3. Spore counts (CFU/ml) recorded in bottom water samples vs. larvicidal activity in these samples as determined by laboratory bioassays. (A), ABG-6184; (B and C), BSP-2.

DISCUSSION

Analysis of the results obtained from spore count data (Fig. 4) and laboratory bioassays of treated lagoon water (Tables 1 and 2), in conjunction with larval reductions obtained in the field and observations of larval feeding behavior

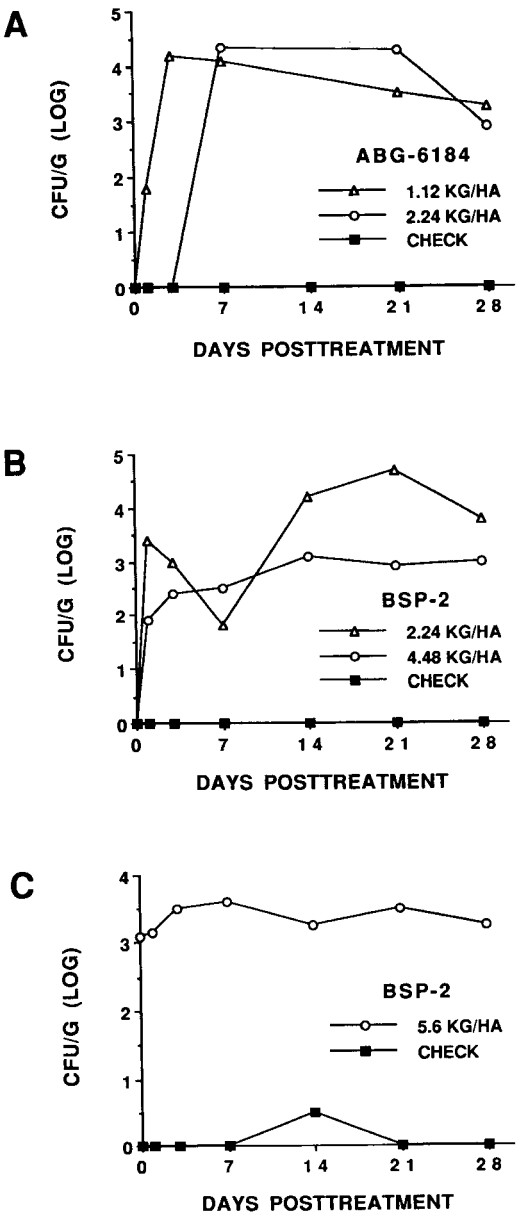


Fig. 4. Spore counts (CFU) in bottom mud from treated dairy wastewater lagoons. (A), ABG-6184; (B and C), BSP-2. Spore counts were determined per gram of mud on a wet weight basis.

(Mulla et al. 1988), provide considerable insight into the dynamics of mosquito control obtained with *B. sphaericus* 2362. Although differences in efficacy were found between ABG-6184 and BSP-2, the trends observed in the fate and persistence of the bacterial spores in these preparations were similar. The bioassays and spore counts indicate that spores settle out of the surface water rather quickly, typically within 24 h, with virtually no mosquitocidal activity being present in the surface water by 7 days posttreatment. Davidson et al. (1984), working with a powder preparation of *B. sphaericus*, have also shown that spores sink to the bottom. After settling to the bottom of the pond, the data from spore counts and bioassays of bottom water demonstrate the presence of significant spore levels and larvicidal activity for up to 14 days posttreatment (Tables 1 and 2; Figs. 2 and 3). However, even though spores could be detected until 28 days posttreatment in bottom water (Fig. 3), as well as in mud samples (Fig. 4) for up to 28 days posttreatment, the longest period tested, the bioassays of bottom water against 4th instars of *Cx. quinquefasciatus* in the laboratory indicated the presence of only moderate to low larvicidal activity after 14 days posttreatment.

Though the bioassays of treated bottom water showed only relatively short-lived mosquitocidal activity, these results are in contrast to data collected in the field on population reduction, which demonstrated good to excellent control for as long as 4 weeks after a single application of ABG-6184 or BSP-2 (Mulla et al., 1988). The longer period of control obtained in the field has several possible explanations. First, larvae in the field were exposed continuously to the spore from hatching onward, and therefore most probably consumed amounts sufficient to induce mortality prior to pupation. Moreover, younger instars are more susceptible than older instars. Second, and another aspect of continuous exposure, is that larvae in the field were observed to congregate near the edges of the lagoons where the water was shallow, and periodically dove to the bottom and grazed in the hydrosoil zone shown to have a relatively high spore load. In addition, the lagoons at Kasbergen Dairy were reflooded with wastewater at least once during the study period; agitation of the water during reflooding could have led to a resuspension of spores, at least temporarily, into the surface water.

The extended level of control obtained with *B. sphaericus* is thought to be due to persistence and/or recycling (reproduction) of this bacterium. In the present study, we have provided evidence for persistence, based primarily on the spore counts found in bottom water, and particularly in the mud. Though this study does not rule out the possibility of recycling as an important factor in extended mosquito control, the stability of the spore counts in mud, and the lack of any significant increase in spore counts in surface and bottom water or mud over time, indicates persistence of the spores is probably more important for mosquito control than recycling.

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