SUSTAINED RELEASE PELLETS FOR CONTROL OF *CULEX* LARVAE WITH *BACILLUS SPHAERICUS*[1]

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ABSTRACT. *Bacillus sphaericus* was formulated in pellets with partially hydrogenated vegetable oil, talc and a starch-based superabsorbent polymer. This formulation increased the residual activity against *Culex* spp. larvae in large and small plots, including polluted water. When the pellets were applied to dry artificial larval habitats 5 days prior to flooding, the *Psorophora columbiae* that hatched at the time of flooding were eliminated. After the pools were dried and reflooded, 611 *Bacillus* colony-forming units/ml were present in the surface water. An equivalent amount of primary powder suspension was ineffective as a preflood treatment, apparently due to solar inactivation of the toxin.

INTRODUCTION

Bacillus sphaericus Neide has proven to be an efficacious microbial control agent for larval mosquitoes, especially those of the genera *Psorophora* and *Culex*. In the case of *Psorophora*, preflood treatments of breeding sites would be advantageous because of their very rapid development in flood water. The fourth instar of *Ps. columbiae* (Dyar and Knab) can be reached in as little as 47 h after flooding with warm water (McHugh and Olson 1982). In the case of *Culex* spp., residual activity is desirable because of their continuous oviposition on organically rich water. The residual activity of both formulated and unformulated *B. sphaericus* has exceeded 3 wk in containers and habitats with solid substrates (Hornby et al. 1984, Lacey et al. 1984, Vankova 1984, Kuppusamy et al. 1987, Nicolas et al. (1987). In habitats with substrates of vegetation or soil or with high levels of pollution, reports of persistence of activity range from negligible (Davidson et al. 1981, Mulligan et al. 1978) to 2–4 wk (Mulla et al. 1984a, 1984b, 1987, 1988; Karch et al. 1988, Lacey et al. 1988, Ali et al. 1989, Matanmi et al. 1990). Clearly, a formulation that could improve its residual activity and be used for preflood treatments would make *B. sphaericus* more attractive as an operational control agent. This study evaluated such a formulation that was developed in our laboratory.

MATERIALS AND METHODS

A primary powder of *B. sphaericus* isolate 2362 produced by H. Dulmage, USDA-ARS, having a potency of 0.99 relative to the international standard RB80 (Bourgouin et al. 1984) was used throughout the study. Pellets were made from a mixture of 48.7% partially hydrogenated cottonseed and soybean oils (KLX®, Durkee International Foods, Louisville, KY), 24.4% talc, 24.4% bacterial powder and 2.4% Supersorb® (Superabsorbent Co., Lumberton, NC). The materials were mixed at 50°C to liquify the KLX. The cooled mixture was pelletized with a Parr® manually operated pellet press. Pellets of 2 sizes were prepared. The smaller weighed 1 g and measured 13 mm diam × 5 mm thick. The larger weighed 3 g and was 3 times as thick. Fresh pellets were prepared for each experiment.

A floating slab formulation was made with 100 ml of 3% type I carrageenan (Sigma, St. Louis, MO), 12 g corn oil, 3 g bacteria and 2 g cork particles. All components except the bacteria were mixed at 85°C to liquify the carrageenan. The bacteria were stirred in rapidly, and the mixture was poured into a cold petri dish. This produced slabs about 4 mm thick with 0.035 g of bacteria powder/g of slab.

Two small plot tests were carried out in 1.8 m^2 sod-lined, concrete artificial pools (Focks and Bailey 1983) in which the water level was maintained by adding well water. In the initial tests, a single 1-g pellet (0.24 g *B. sphaericus* 2362), 7.1 g of carrageenan slab (0.25 g *B. sphaericus* 2362), 0.25 g of primary powder, or no treatment was applied to 7 replicate pools each after flooding and invasion by *Culex* larvae. Pellets and slabs were placed in the plots by hand; powder was suspended in deionized water and applied with a hand sprayer. Immature mosquitoes were sampled with a 460-ml dipper on the day of treatment, 2, 4 and 7 days posttreatment and at weekly intervals for the next 6 wk, then at biweekly intervals. Neonate larvae were not

[1] Mention of a commercial or proprietary product in this paper does not constitute an endorsement of this product by the United States Department of Agriculture.

counted in any of the experiments. Surface water was sampled for determination of *Bacillus* colony-forming units (CFUs) on the day after treatment and at all mosquito sampling dates beginning on day 4 posttreatment.

In the second test, preflood treatments of 3-g pellets (0.73 g *B. sphaericus* 2362), 0.75 g of primary powder, or no treatment was applied to dry sod in 7 replicates per treatment. These plots were partially flooded by rain 5 days after treatment (September 11, 1990) and filled completely with well water one day later. Eight weeks after the initial flooding, the pools were dried for 4 days and reflooded. Larval and bacteriological samples were taken 2 and 7 days after flooding and at weekly or biweekly intervals thereafter.

In both tests in the experimental pools, 4 dips were taken from the corners of the pools, except for the initial sampling after preflood treatment when 8 dips were taken. At the outset of the first test (November 22, 1988), the composition of mosquito larvae was 51% *Culex nigripalpus* Theobald, 44% *Cx. quinquefasciatus* Say and 5% *Cx. salinarius* Coquillett. Later in the course of the experiment, *Cx. nigripalpus* became rare, and *Cx. restuans* Theobald was present in varying numbers. A small number of anopheline larvae were found during the sampling but not in sufficient numbers for statistical evaluation. In the test of preflood treatment, only *Psorophora columbiae* larvae were present in dip samples taken 2 days after flooding. One week after flooding, the larvae were 58% *Cx. quinquefasciatus* and 42% *Cx. nigripalpus*; 2 wk after flooding the larvae were 96% *Cx. nigripalpus* and 4% *Cx. territans* Walker.

Three-gram pellets were tested in residential sewage clarifiers of approximately 10 m^2 surface area and at water replacement rate of 8 turnovers per day. Two clarifiers each were treated with 3-g pellets suspended in mesh bags or 0.75 g of primary powder, and 2 clarifiers served as controls. Ten dips were taken from each clarifier before treatment and 2 days after treatment, when it became clear that the bacteria were washing away.

Larger scale trials were carried out at the University of Florida Swine Research Unit. The plots were set in 2 concrete-walled ponds 4.6 × 25 m and 1 m deep with mud and leaf litter substrata. The ponds had been dug for treatment of animal waste effluent. Pond 2 received effluent overflow and was highly polluted and nearly clear of floating vegetation throughout the course of the experiments. The other pond (1) did not receive effluent directly, but only ground and rain water. Pond 1 was partially covered with duckweed (*Lemna* sp.). At the initiation of the experiments, the biological oxygen demands (determined with a Hach model 2173B BOD apparatus, Hach Co., Loveland, CO) were 210 and 410 mg/liter in ponds 1 and 2, respectively. Turbidities (measured with a HF model DRT 15C NTU turbidimeter, HF Scientific, Ft. Myers, FL) were 26 and 92 NTU for ponds 1 and 2, respectively. Both ponds periodically produced large numbers of *Cx. nigripalpus*.

The ponds each were divided in half with plastic sheeting. Treatments were applied to the side of each in which an overflow pipe was located, with the opposite ends serving as controls. First, pond 1 was treated with 5.5 g of primary powder and pond 2 was treated with 22 1-g pellets (ca. 0.1 g primary powder/m^2 for both). After the initial treatments ceased to reduce larval populations and the number of bacterial CFUs were the same in treated and control areas, the treatments were reversed; i.e., pond 1 was treated with pellets, and pond 2 was treated with powder.

Twenty-four dips and 8 surface water samples were taken from each plot at 2 and 7 days after posttreatment and at weekly intervals thereafter. Nearly all larvae were *Cx. nigripalpus*. *Uranotaenia* and *Anopheles* larvae were present in small numbers in pond 1 and were not affected by the treatments.

Water samples for determination of *Bacillus* CFUs in all tests were taken at the water surface with autoclaved test tubes. Water samples were diluted as necessary and heat shocked at 75°C for 25 min to select for spore-forming bacteria. One-tenth ml of each sample was spread on nutrient agar containing 0.01% streptomycin. Colonies with the morphology of *B. sphaericus* were counted at 24–48 h.

Duncan's multiple range test was used for comparisons of means from small plot tests; Student's *t*-test was used to compare means from the pond tests. For all comparisons $\alpha = 0.05$.

RESULTS AND DISCUSSION

When the artificial pools were treated after flooding and colonization by *Culex*, all 3 treatments maintained the *Culex* populations significantly below ($P < 0.05$) control levels from 2 days posttreatment through 84 days (Fig. 1). Over all of the sample dates taken together, all of the treatments differed significantly ($P < 0.05$) from all others. The lowest *Culex* populations were present in pools treated with pellets, followed in order by those treated with powder, carrageenan slabs and the control pools. The number of *Culex* larvae and pupae in pools

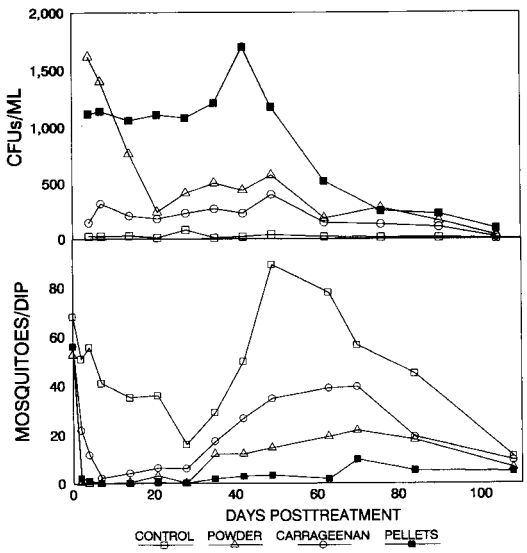

Fig. 1. *Culex* larvae and pupae and *Bacillus* colony-forming units in 1.8 m^2 artificial pools after postflooding treatment with *Bacillus sphaericus* in lipid-based pellets, carrageenan slabs and primary powder.

treated with pellets was significantly lower ($P < 0.05$) than the numbers in the controls and in the pools treated with carrageenan slabs on all posttreatment sample dates except the last. There were fewer *Culex* in pools treated with pellets than in those treated with powder on all posttreatment sample dates, but the differences were significant ($P < 0.05$) only at 35, 42 and 84 days.

As expected during the first week after treatment, the number of *Bacillus* CFUs/ml at the surface was highest in pools treated with powder. One day posttreatment, there were more than 6 times as many CFUs than in those that received the other treatments (not shown on Fig. 1), but most of this material was rapidly lost, probably due mainly to settling (Davidson et al. 1984, Matanmi et al. 1990). From 2 wk through 104 days posttreatment, significantly more ($P < 0.05$) CFUs were obtained from pellet-treated pools than those with the other treatments. There were significantly fewer ($P < 0.05$) CFUs/ml in the pools treated with carrageenan slabs than in those treated with powder on all but 3 sample dates.

Preflood treatment was first attempted in the 1.8 m^2 pools using 1-g pellets or the equivalent amount of powder (0.25 g), but these were ineffective. Accordingly, the pools were drained and left dry for 1 wk, and a second preflood treatment was made with 3-g pellets or 0.75 g of primary powder. In plots treated with these larger pellets, no *Ps. columbiae* were found after flooding, while small numbers were found in control plots and those treated with 0.75 g of primary powder (Table 1). The mean CFUs/ml counts in powder-treated plots did not differ significantly ($P > 0.05$) from the controls at any point during the experiment. Evidently, the spores and toxin in the powder were destroyed by sunlight, and most of the toxin in the pellets remained active and was released into the water.

A large increase in the number of CFUs/ml and a reduction in the number of *Culex*/dip in the pellet-treated pools occurred between 28 and 42 days posttreatment. This was probably due to concentration of the bacteria as the water evaporated.

After the experimental pools had been dried, exposed to sunlight for 4 days and refilled, those treated with the pellets contained an average of 611 CFUs/ml. Whether that amount of bacteria would be larvicidal could not be evaluated because of low mosquito populations. However, 611 CFUs/ml was more than the number that gave 100% control of *Culex* spp. in the pellet-treated plots 7 days after the initial flooding. This indicates that *B. sphaericus* formulated in these pellets could be used for residual control even in ephemeral *Culex* habitats.

The 3-g pellets were also applied to residential sewage clarifiers. Both pellets and powder provided initial control of *Culex* larvae with no residual effect. There was, however, better initial control with pellets than powder. Two days after treatment, *Culex* larvae were reduced by 98% in clarifiers treated with pellets and 82% in those treated with powder, while the control populations increased by 82%. Replacement of the water 8 times per day prevented any residual effect.

The results of treatment of the swine farm ponds are presented in Fig. 2. Although the pellets and powder were applied to the ponds at different times and consequently cannot be compared directly, it is clear that the pellets provided residual mosquito control that was lacking with the unformulated powder. Both materials persisted longer in pond 1 than pond 2. Pond 2 was the more polluted and lacked the duckweed that may have limited solar degradation of the toxin and spores.

The pretreatment mosquito counts were higher in the control half than the treatment half of pond 1 when the pellets were used, but 1 wk later, all *Culex* were eliminated from the treatment plot. The population remained significantly lower ($P < 0.05$) than the control population through 6 wk posttreatment.

Table 1. Mosquito larvae and pupae and *Bacillus* colony-forming units (CFUs) in 1.8 m^2 artificial pools treated with *Bacillus sphaericus* pellets and powder prior to flooding.

Days postflood	CFUs/ml			Mosquitoes/dip[a]		
	Pellet	Powder	Control	Pellet	Powder	Control
2	1,439	50	52	0	1.3	3.4
7	510	8	3	0	23.4	27.8
14	1,104	89	77	0.1	4.9	7.3
21	1,200	21	5	0.2	0.3	2.2
28	918	29	11	5.0	21.4	21.8
42	2,566	85	42	0.4	11.9	8.0
56 (reflood)[b]	611	78	24			

[a] *Psorophora columbiae* on day 2, *Culex* spp. on subsequent dates.
[b] Plots were dry for 4 days; samples taken 4 days after reflooding.

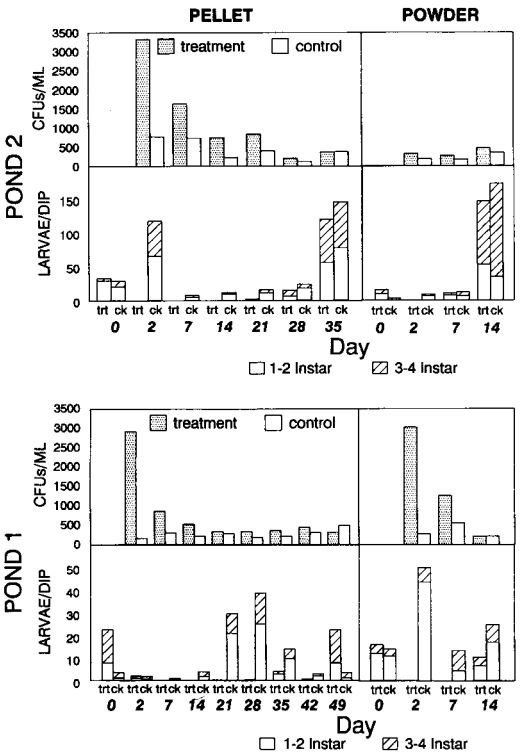

Fig. 2. *Culex* larvae and *Bacillus* colony-forming units in treated and control halves of 4.6 × 25 m ponds treated with *Bacillus sphaericus* in lipid-based pellets or primary powder. Pond 2 received swine sewage; pond 1 did not.

When primary powder was applied to this pond, the number of *Culex* remained significantly lower ($P < 0.05$) in the treatment plot than in the control plot for 2 weeks. However, the plots yielded nearly equal CFU counts from samples taken 14 days posttreatment, indicating that the low treatment plot population was probably not due to the *B. sphaericus* powder, but to a low level of oviposition.

In pond 2, the more polluted pond, the powder showed no effect by 1 wk posttreatment. In contrast, when the pellets were used, there were significantly fewer *Culex* ($P < 0.05$) for 4 wk and significantly more ($P < 0.05$) CFUs for 3 weeks.

Differences in sampling methods, in the aggregation of spores in various formulations and in the characteristics of larval habitats undermine comparisons among studies of CFU counts and their implications. In previous studies, Davidson et al. (1984) reported that at least 100 spores/ml in surface water were needed for control of third and fourth instar *Culex tarsalis* Coq. in small plot tests, and Nicolas et al. (1987) found that 100–500 CFUs/ml were needed to control *Cx. quinquefasciatus* in cesspools. Among the results presented here, a range of 100–500 CFUs/ml provided control of *Culex* spp. in most cases. A single exception occurred in one set of samples in the artificial pools where 918 CFUs/ml provided only 77% control. It appears that maintenance of CFU counts in the hundreds is an appropriate target for formulators.

In all of the experiments, *Bacillus* CFUs were obtained from control plots. In the artificial pools, the number of CFUs was low, and approximately half of these were insecticidal when tested against second instar *Cx. quinquefasciatus*. The noninsecticidal colonies were formed by other *Bacillus* spp., and the presence of insecticidal colonies may be due to the movement of animals among the pools and possibly indigenous *B. sphaericus*. In the larger ponds, there were substantially more control plot CFUs obtained. Apparently, small spaces at the edges of the plastic barriers were enough to allow some mixing of treatment and control water. This is a troublesome consequence of dividing aquatic plots, but it demonstrates that *B. sphaericus* has considerable dispersal capacity.

Bacillus sphaericus can provide considerable residual control in most larval habitats. Improvement of its residual activity and development of preflood treatment will make it more competitive with other control measures.

The *B. sphaericus* pellet described here offers a gradual release of its toxic moieties. The inclusion of a very small amount of the superabsorbent polymer allows the pellets to break up without the immediate dispersion of the bacteria that occurs with powder and liquid formulations. An important factor in the limitation of residual activity is the settling of the bacteria into the substrate (Davidson et al. 1984, Matanmi et al. 1990). The lipid component is intended to lend buoyancy to the released material. Although no control plots with bacteria-free lipid were used in these tests, it is unlikely that this material, which melts at 40°C, would affect larvae by interfering with respiration. The inclusion of similar materials in bacterial larvicide formulations will enhance their efficacy and utility for mosquito control.

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