Journal of Vector Ecology 22(1): 43-51

Efficacy of a Granular Formulation of *Bacillus sphaericus* against *Culex quinquefasciatus* and *Anopheles gambiae* in West African Countries

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Received 1 August 1996; Accepted 26 November 1996

ABSTRACT: The efficacy of a sustained released granular formulation of *Bacillus sphaericus* strain 2362 was compared to a flowable concentrate in containers, cesspools, and water ponds. Duration of control was dependent on formulation, dosage, exposure to sun, site, recycling, and target mosquito larvae. In a series of container tests with repeated additions of fourth-instar *Culex quinquefasciatus* larvae exposed to 0.3 or 3.0 g/m^2 when cadavers were not removed, more than 95 percent control was obtained for two and four days in containers that were exposed to the sun with sewage water treated with the flowable concentrate compared to four and seven days for those treated with the granule. In sun-exposed containers with sewage water, control persisted for two days for the flowable concentrate at both dosages and one and six days for the granule at 0.3 g/m^2 and 3.0 g/m^2, respectively. Compared to the above tests, more than seven weeks control was obtained with 0.3 g/m^2 of the flowable concentrate in closed containers where larvae were added weekly. In closed containers without weekly addition of larvae, the control was 15 percent when larvae were added five weeks after the treatment. Spore counts at the surface and bottom of the containers with lids showed an increase in number of spores at the surface where larvae were added weekly and a rapid decline where they were not. Spore counts at the bottom showed settling in both cases, but to a much higher level where larvae were added weekly. Nearly 100 percent control of *Cx. quinquefasciatus* larvae was obtained for at least 16 days in cesspools in Yaoundé, Cameroon, treated with the granule at 3.0 g/m^2 compared to approximately nine days for the flowable concentrate. At 0.3 g/m^2, the duration of this reduction was five days for both products. Nearly 100 percent control of *Anopheles gambiae* was obtained in sun-exposed water ponds near the village Kotiokh, Senegal, for at least 15 days with the granule at 3.0 g/m^2 compared to just five days for the flowable concentrate at the same dosage.

Keyword index: Bacillus sphaericus, Culex quinquefasciatus, microbial control, granules.

INTRODUCTION

Bacillus sphaericus (Neide) has been known for 20 years as a bacterium that forms a toxin during its sporulation that is active against certain species of mosquito larvae (Singer 1973). *Bacillus sphaericus* Strain 2362, isolated by Weiser in 1981 (Weiser 1984), is the strain commonly used for industrial production. It is very active against *Culex quinquefasciatus* Say, slightly less to *Culex pipiens* L., and more or less active against many other *Culex*, *Anopheles*, *Psorophora*, *Mansonia*, and *Aedes* species, but not against *Aedes aegypti* (reviewed by Yap 1990). *Bacillus sphaericus* has been used mostly in small scale programs for mosquito control, except in southern France where it has been used on an operational scale for nine years (Sinegre et al. 1993). In these programs and experimental tests, *B. sphaericus* has shown a residual effect unlike another mosquitocidal bacterium, *Bacillus thuringiensis var. israelensis* (Lacey et al. 1984). The amplification of the pathogen in cadavers of mosquito larvae may explain its residual effect (Lacey 1988a) which has been demonstrated both in the laboratory and the field (Davidson et al. 1984, Correa and Yousten 1995, Becker et al. 1995). This residual effect has not been proven to be important in the field except in tires that contain clean water (Kramer 1990) for *Culiseta incidens* Thomson. Nicholas et al. (1987b) reported that mosquito cadavers in cesspools settled at the bottom and prevented recycling of spores and toxins from the cadavers into the feeding zone close

to the surface. Settling of spores and toxins are among the factors that limited the effectiveness of the application (Davidson et al. 1984). Nicolas et al. (1987b) and Pailey et al. (1987) demonstrated that the number of spores and vegetative cells declined in the upper water layer in cesspools and increased at the bottom. The relatively slow decrease at the upper water layer combined with a high application rate (10 g/m^2) would then explain the residual effect.

However, in many cases, and especially in areas exposed to the sun, the residual effect is very short (Lacey et al. 1988b, Mulla 1986) and the product has to be reapplied at least weekly to control larvae (Aranda and Eritja 1992, Sinegre et al. 1993). Sustained release formulations may prolong the residual effect, but are limited under field conditions, especially in sun-exposed sites typical for anopheline larvae (Lacey 1990) and *Mansonia* larvae (Yap et al. 1991). An additional consideration for the field is that a product should be optimized for storage and handling. Fluid formulations of *B. sphaericus* are not stable in heat and cannot be stored for months under tropical conditions. This may be responsible for the failure of a mosquito campaign in Maroua, Cameron (Baldet 1995). Accordingly, there is a need for a product based on a dry formulation with a residual effect no less than the fluid formulation in covered sites and better in sun-exposed sites.

A granule based on this concept was developed and tested first in containers and then in the field in Cameroon and Senegal against *Cx. quinquefasciatus* and *Anopheles gambiae* Giles. The effect of the product in various test regimes was related to dosage, recycling and sustained release.

MATERIALS AND METHODS

As a reference product, the flowable concentrate Spherimos®, Novo-Nordisk (now Abbott Laboratories, Chicago) was used. The potency and spores/g were determined for this product and of a primary powder used for formulations. The granule products were formulated with 22 percent of a primary powder from the same company to make products that initially floated and spread on the water surface and then sank slowly while disintegrating into still smaller particles. After several days, 10 to 20 percent of the small particles were still floating in laboratory experiments.

Culex quinquefasciatus, strain S-lab was obtained from G. Georghiou, University of California, was used for bioassays and for the tests in containers. Bioassays were carried out in accordance with the protocol of de Barjac and Thierry (1984) with slight modifications: powder and granule samples were allowed to stand 30 min. in water with a droplet of Tween 80 after shaking. During this period, granule samples were homogenized several times with a Kinematic homogenizer with a Polytron PTA 20S rod for 30 sec. to break up the granules. All samples were homogenized with a Waring blender after dilution to 400 ml. No detergent was added to the fluid samples. After further dilution with tap water, 10 ml was transferred to three beakers with 90 ml dechlorinated tap water (in two tests: sewage water) with 20 L_4 larvae. The stock suspension was diluted to 50 percent, 10 ml was transferred to the next three beakers, etc. to make six to seven dilutions. An LC_{50} was calculated using a log-probit program with working probits as described by Finney (1971). Potencies of samples were calculated relative to the LC_{50} of a standard SPH88 from Institut Pasteur that was tested the same day. Each product was tested at least five times on different days.

The counting of spores included heating samples to 79°C, 12 min., application of ca. 20 µl of 1 percent Tween 80 to each 5 ml sample, sonication of the sample (Vibracell rodsonicator, 10 sec. at setting 80) and dilution in sterilized water containing 0.1 percent peptone and plating on MATS agar with 100 mg streptomycin/l (Yousten et al. 1985). Counting of spores in larval cadavers was carried out on two day old cadavers that were removed from the cups just after their death, transferred to distilled water, and sonicated. The mixture was then further diluted and plated as above.

The Spherimos flowable concentrate was diluted 1:10 and applied with a modified calibrated syringe in the container tests and in the cesspools in Cameroon. The flowable concentrate was diluted 1:10 and applied with a back sprayer in the field tests in water ponds in Senegal. The amount applied was determined by calibrating the outlet rate at a specified pressure and applying 1 g a.i./sec. Granules were weighed and applied by hand at 0.3 and 3.0 g/m^2.

Container tests: 75 l plastic containers (42 cm and 47 cm diameter at bottom and top, respectively, 49 cm high) were filled with tap water plus 10 percent deionized water or sewage water similarly diluted. Deionized water was added to reduce the precipitation of calcium carbonate. The containers were wrapped in glasswool to reduce temperature fluctuations to 18° to 22°C and filled with water nearly to the edge in outdoor experiments. Containers in the greenhouse were put in boxes of polystyrene (60 x 60 x 60 cm) to reduce heating of the containers by the sun (temperature fluctuation reduced to 26°C ± 1°C in test before June and to 28±1°C in tests in July until September).

Five tests were performed in containers with or without lids. For the latter type of tests, deionized water

was cautiously added to compensate for evaporation. The tests were carried out in the French Organisation for Cooperation in Development and Research (ORSTOM) facilities in Montpellier, southern France. It did not rain during the period of the outdoor experiments. In the container studies, 50 *Cx. quinquefasciatus* L_4 were added with fixed daily intervals and live larvae were removed after two days exposure. Larvae were removed from the surface when they appeared to breath with sterile pipettes not to resuspend sediment product. Cadavers were not removed. Larvae not found were counted as dead. In some tests, surviving larvae were brought to the laboratory and their mortality was followed until adult emergence. Water samples for bacterial counting were taken after removal of live larvae. Samples from the water surface were taken with a 5 ml sterile plastic pipette. In the tests where samples were taken from the bottom also, water was filled to 25 cm height only and samples were taken with a 30 cm sterile glass pipette. Bottom samples of mud were taken along a median line, and a few samples were also taken at the bottom along the sides.

Field Tests

These were carried out in sewage systems in two military camps in the capital Yaoundé, Cameroon. The city and its climate are described in Hougard et al. (1993). The tests were conducted in the beginning of the rainy season where the temperature was between 22°C (night) and 30°C (day) with daily heavy showers. The sewage systems consisted of one to three tanks connected to the outlet tubes and delivering to a cesspool open to the soil. All tanks were closed to the air except for holes made by rats, mechanical damage, and eventual air tubes. The highest concentration of larvae were found in the cesspool, or when the admittance to this was clogged, in the sewage tank before the clog. Tanks with more than five larvae per dip (250 ml) were used. Not more than one tank was treated and used in the experiment per sewage system. Forty-five sewage systems were placed into five groups according to the tank where most larvae were found, and these five groups were distributed between six treatment regimes: the flowable concentrate or the big granules: 3.0 g/m^2 (10 tanks in each group), the flowable concentrate or the big granules: 0.3 g/m^2 (5 and 10 tanks, respectively), and the small granules: 3.0 g/m^2 (5 tanks), control (5 tanks, no treatment). Big granules were 1-2 mm, whereas small granules ranged from 0.5-1 mm. Products were applied as in the container tests.

Control effect was measured as the number of *Cx. quinquefasciatus* L_2 to L_4 recovered in three dips that did not survive the next two days in the laboratory in untreated water compared to the initial number of larvae per site. This method was preferred to one comparing the numbers of larvae in the sites before and after treatment, because of some cases of migrating larvae from untreated neighboring chambers. First instars were not included since their life span is shorter than the killing rate of the products. Coexisting *Culex decens* (Theobald) larvae were not included.

In the Senegal study, the test sites consisted of 18 unconnected holes (5-25 m^2) dug into a dry river basin, thus excluding immigration of larvae between basins. Effect was measured as mean number of *Anopheles gambiae* larvae caught with a 200 ml tray sampling along the edge of the ponds (3 samples in small ponds, 5 to 10 in larger, ponds) compared to numbers before treatment. First-instar larvae were ignored. *Anopheles* larvae were not evenly distributed along the edges and care was taken to sample at the highest densities. There were no other mosquito species in these sites. Five ponds were treated with the big granules at 0.3 g/m^2, four with the big granules at 3.0 g/m^2, initially three, but reduced to one site with the flowable concentrate at 0.3 g/m^2 and five with the flowable concentrate at 3.0 g/m^2 (of these two with former 0.3 g/m^2) and three were kept untreated as controls. One of these sites dried out during the test period and data from this pond were omitted. The experiments were stopped before the effect of the high dosage of the granule product started to decline.

Validation of effects was carried out by analysis of variance of the number of larvae per treatment and the effect of each product. Probit analysis of mortality as a function of time was carried out in the test groups where the mortality declined significantly. Because the number of mosquito larvae in all treated sites initially was reduced to ~0, controls were not considered in the variation analysis so as not to obtain high total variances that were not related to the effects. It was first determined whether pretreatment numbers of larvae were the same in all groups, including controls and if the control group changed with time.

RESULTS

Bioassays of the flowable concentrate, the granule, and the primary powder showed them to contain 235±64, 150±50, and 1450±275 ITU /mg, respectively (mean values ± S.D.). The slope of the dose mortality curve was 2.01±0.26 for the standard SPH88 and 2.20±0.62, 1.79±0.59, and 2.30±0.39 for the flowable concentrate, primary powder, and the granule, respectively. Analysis of variance indicated that these slopes were different ($P<0.01$, $F=122$, $df = 34,3$) and that bioassays with the granule gave a lower slope than for the other products. The flowable concentrate contained $5x10^9$ spores/g.

SPH88 is reported to contain $1.3x10^{11}$ spores/g. The granule and the flowable concentrate were applied at 0.3 and 3.0 g/m^2 in the field test and in the container test.

Bioassays of flowable concentrate of the granule yielded LC_{50} values depending on water type: 1.2 µg Spherimos/g sewage water and 1.3 µg granulate/g sewage water compared to 0.028 µg/g clean water and 0.035 µg/g clean water, respectively.

The container tests indicated that residual efficacy was positively influenced by dosage (TABLE 1) and negatively by sunlight (TABLE 1, lower part, compared to TABLE 2). In sewage water with no sunlight, the effect of one low dosage treatment with the flowable concentrate increased to 100 percent in two weeks when larvae were added weekly (TABLE 2) and just 15 percent control after five weeks where larvae were not added weekly. Bacterial counts from the containers in samples taken at the surface showed a steady increase in spore counts where larvae were added weekly and a decrease to zero where larvae were not added (Fig. 1).

Bacteria were also counted in mud from the bottom of the containers. Within the first five weeks in treatments with lids on, spore counts increased from near zero to $1.8x10^5$ spores/g mud where larvae were added weekly ($1x10^6$ spores/g along the edge where most cadavers were found) and to $1.8x10^3$ spores/g where larvae were not added weekly.

The mean number of larvae in pretreatment counts in the cesspool in Cameroon was 85 larvae/3 dips (range 9-900). Analysis of variance of the control sites showed that initially they had the same numbers as the other sites ($P>0.05$, $F=2.36$, $df=4,15$) and that the number of larvae in the control sites did not change significantly during the test period ($P>0.05$, $F= 1.89$, $df= 5,33$). Analysis of variance of the treated sites showed that after two days the same level of control was obtained at all sites, but after that the level of control declined as a function of dosage and product type (TABLE 3). There was no significant decline in control of the big granules at the high dosage ($P>0.05$). Analysis of the decline of activity per treatment group (slope of mortality-time functions) showed a significant and logarithmic decrease in activity of the flowable concentrate at both levels ($P<0.01$, $F=45$ and 35, $r^2 = 0.96$ and 0.95, respectively).

The tests in Senegal showed a more pronounced dosage and product dependent effect. There was no effect of the flowable concentrate at 0.3 g/m^2 in three sites after two days, and two of these were then treated

TABLE 1. Percent mortality of mosquito larvae after treatment with a flowable concentrate and a granular formulation in 75 l outdoor, sun exposed containers with clean water and sewage water. Numbers without brackets are mortality of larvae transferred from the containers to the laboratory after two days and kept in nontreated water with food, whereas numbers in brackets are the mortality at sampling. First series (0-18 days) represents a trial in clean water, second series (0-11 days) a trial in sewage water. Each column in the table represents one container. Control mortality may be lower than indicated in the table since all larvae not found were counted as dead and it was not easy to find larvae alive in a container with sewage water.

Days after application	Control	Granule 3.0 g/m^2	Fluid Conc. 3.0 g m^2	Granule 0.3 g/m^2	Fluid Conc. 0.3 g/m^2
0	-	100 (100)	100 (96)	100 (100)	100 (96)
2	-	100 (87)	100 (57)	100 (72)	100 (57)
4	-	100 (72)	100 (39)	95 (45)	80 (18)
7	-	100 (82)	66 (19)	90 (40)	74 (26)
18	-	66 (48)	8 (0)	-	-
0	12 (8)	100 (98)	100 (96)	100 (24)	100 (38)
2	6 (0)	100 (100)	100 (100)	66 (34)	100 (98)
6	10 (0)	100 (90)	78 (32)	56 (24)	56 (20)
11	4 (0)	86 (66)	12 (6)	-	16 (2)

TABLE 2. Mean number of dead L_4 with a flowable concentrate and a granular formulation in 75 l indoor containers with sewage water and lid on. Mean number of dead larvae are given for three containers per group (two in control). Fifty L_2 added to all containers weekly except for one treatment group, where larvae were added day 34.

Days After Application	Control	Fluid Conc. 0.3 g/m² Larvae Applied Weekly	Fluid Conc. 0.3 g/m² Larvae Applied Day 34
0	5[1]	44.3 ± 7.4	No larvae added
5	5[1]	47.7 ± 3.2	No larvae added
11	1.5	47.3 ± 3.8	No larvae added
16	0	50	No larvae added
26	0.5	50	No larvae added
34	1	50	6.3 ± 2.5

[1]*Notonecta* sp was found in one control container with initial high control mortality and was removed on day 7.

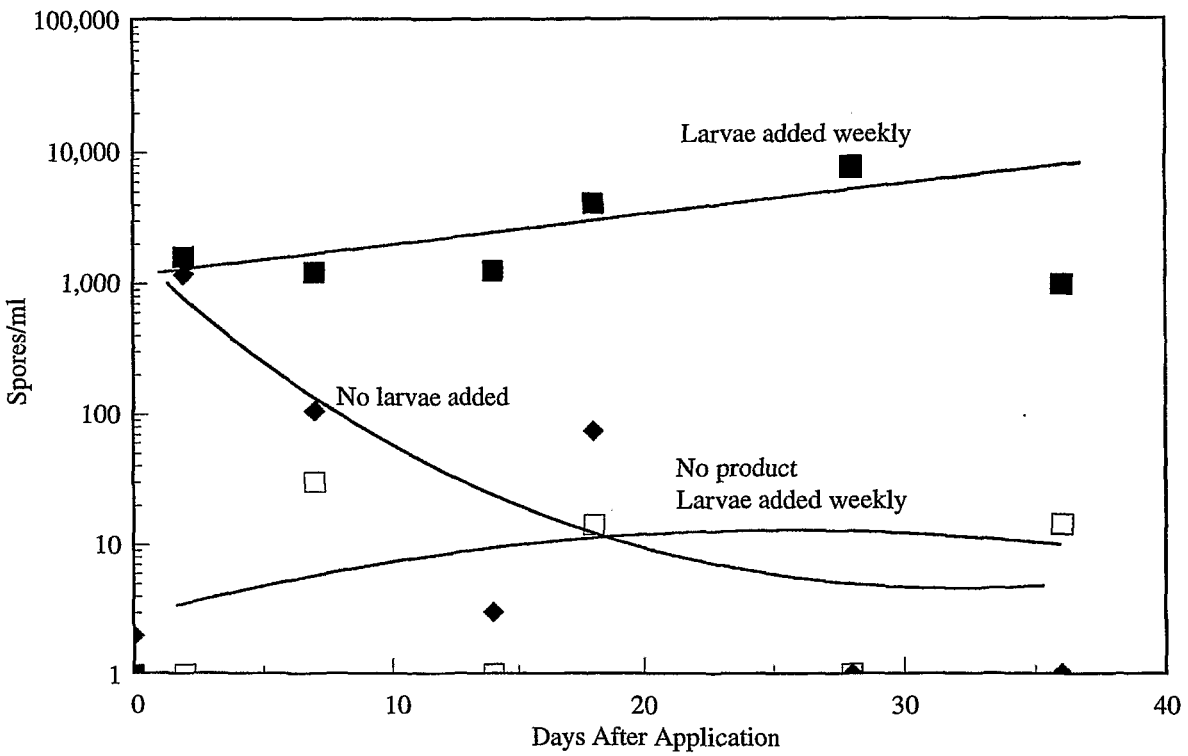

Figure 1. Mean number of *B. sphaericus* spores per ml at the surface of containers treated with the flowable concentrate and with or without weekly addition of larvae.

TABLE 3. Mean number of larvae per treatment in cesspools treated at two dosages. Numbers with the same indexes are not significanty different (P<0.05; a,b: analysis of variance per day across products, x,y,z: variance analysis per treatment group across days).

Treatment	Number of sites	Pre-treatment	Day 2	Day 5	Day 8	Day 16
Fluid Conc. 0.3 g/m^2	5	110^{ax}	0.8^{ay}	4.8^{az}	21.2^{ax}	67.6^{ax}
Fluid conc 3.0 g/m^2	10	127^{ax}	1.0^{ay}	1.3^{aby}	4.3^{by}	14.4^{bz}
Gran, Big 0.3 g/m^2	10	34^{ax}	1.9^{ay}	5.4^{az}	9.8^{by}	6.9^{byz}
Gran, Big 3.0 g/m^2	10	108^{ax}	0.1^{ay}	0.4^{by}	0.3^{bx}	5.1^{by}
Gran, Small 3.0 g/m^2	5	52^{ax}	0.2^{ay}	0.2^{ay}	3.4^{bz}	5^{by}
Control	5	65^{ax}	37^{x}	21^{x}	22^{x}	45^{x}

with Spherimos at the high dosage. In the third, the number of larvae increased above pretreatment levels; 3.0 g/m^2 of the flowable concentrate gave control above 90 percent for six days only and no control at all after 15 days (Fig. 2). At this time, there was still 50 percent control by the granule at the low dosage and 100 percent control at the high dosage.

DISCUSSION

A granular formulation of *B. sphaericus* was compared to a flowable concentrate in trials in southern France and in West Africa. The container trials revealed a major influence of sun exposure on the control effect on both formulations (TABLE 1), at least in southern France in spring and summer where the UV-irradiation is high and much higher than in Africa south of Sahara (Monteny, 1990). Without sun exposure, a single application of 0.3 g/m^2 Spherimos initially resulted in 90 percent control increasing to 100 percent for five weeks when larvae were added weekly (TABLE 2). Without the weekly addition of larvae, control declined to 15 percent after the five weeks. Spore counts at the bottom and surface of containers confirmed the amplification of *B. sphaericus* at the water surface where larvae were added compared to the decline where they were not (Fig. 1). This demonstrates the recycling of *B. sphaericus* through host larvae in polluted water as demonstrated previously for mosquito larvae in clean or distilled water (Kramer 1990, Becker et al. 1995, Correa and Yousten 1995). Contrary to that found by Nicolas et al. (1987b), we found black mosquito cadavers floating at the surface of the sewage water for several days, which may explain why we obtained effect of recycling. Bacterial counts at the bottom confirmed sedimentation as also reported in other studies (Davidson et al. 1984, Nicolas et al. 1987b).

Fourth-instar *Cx. quinquefasciatus* larvae given a lethal dosage of *B. sphaericus* produced very variable number of cells (10^3-10^5) per cadaver. Davidson et al (1984) reported 10^5 to 10^6 spores/cadaver for *Cx. tarsalis* and Paily et al (1987) found 2.5 x 10^3 spores/cadaver for *Cx. tritaeniorhynchus*. Provided all larvae died and the bacterium proliferated in the cadavers, 50 larvae per week for five weeks would add 2.5 x 10^7 to 10^8 spores compared to the 2.5 x 10^7 spores applied with the product. In comparison, 1.3 x 10^9 of spores/g was estimated in one mm mud at the bottom or more than 10 times the bacteria added or reproduced in mosquito larvae. That may indicate that *B. sphaericus* not only recycled in the cadavers, but was multiplying in the sewage water or in the mud also.

Sustained effect due to recycling in cadavers is counteracted by settling and other inactivations of spores and crystals and requires a continuous presence of larvae as provided in this study. On account of this, Arrendondo-Jimenez et al. (1990) found longer residual activity in field tests where the control was below 100 percent.

When sun exposed containers were treated, the

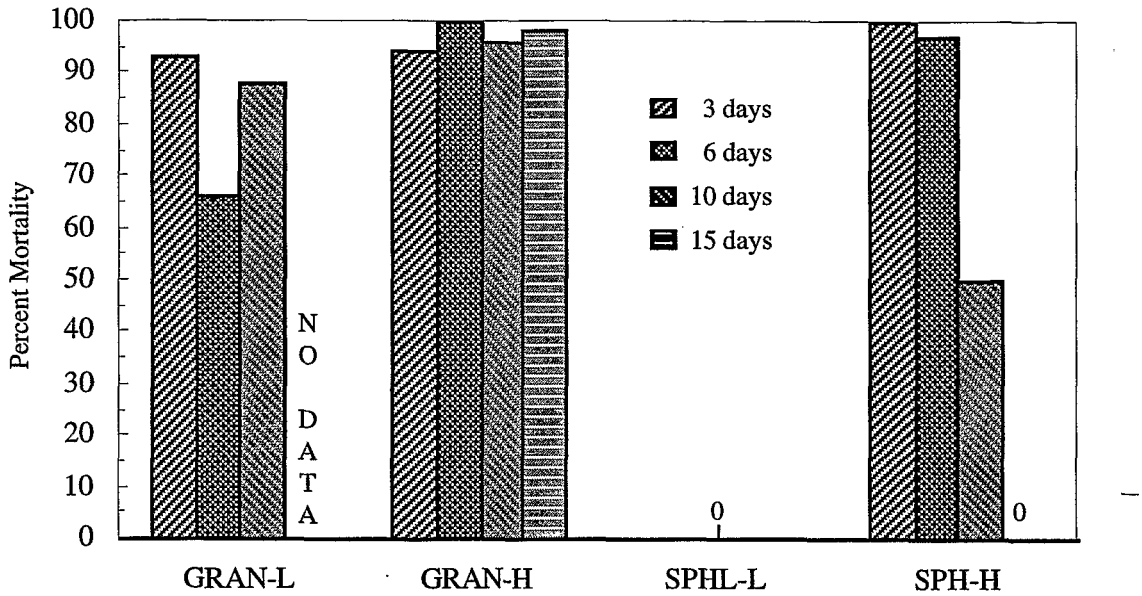

Figure 2. Control of *Anopheles gambiae* larvae in water ponds in a dry river bed in Senegal. Gran-L are results of treatment with the granular formulation , 0.3 g/m2, Gran-H is the same product, 3.0 g/m2, SPH L and H are results obtained with a flowable concentrate, dosage 0.3 and 3.0 g/m2, respectively. Tests were stopped after 18 days.

residual effect of 0.3 g/m was just a few days (TABLE 1), showing that the UV-inactivation of *B. sphaericus*, at least in the feeding zone close to the water surface, was more important than amplification through recycling. Only the granule applied at 3 g/m^2 resulted in control for a week or longer. Shorter larvicidal efficacy in the sewage water than in the tap water may be related to a higher dosage needed to kill larvae in polluted water where many other food particles are present. A bioassay with sewage water instead of dechlorinated tap water gave ~40 times higher LC_{50} values. In field tests, 600 to 800 spores/ml was found to be necessary to prevent the survival of mosquito larvae in polluted water compared to 100 spores/ml in clean water (Nicholas et al. 1987b, Pailey et al. 1987).

In the cesspool systems all products initially gave nearly 100 percent control (TABLE 3), and this activity declined in a log-linear manner for the fluid products indicating that a single process of inactivation took place, e.g., sedimentation or dilution. For the granules, there was no log-linear decline. For the bigger granules at the high dosage, there was no significant decline at all within the 16 days after application. This was probably due to the sustained release of spores and toxin. Sunlight could not enter the cesspools, but the water in these systems is probably not as undisturbed as in the containers, which may explain the limited control of 0.3 g/m^2 of the flowable concentrate in the field tests compared to the long residual control in the covered containers at the same dosage.

Against *An. gambiae*, 0.3 g/m^2 (50,000 ITU/m^2) of the flowable concentrate failed to give any control compared to 65 to 80 percent control obtained with the granule on day 10 at the same dosage (Fig. 2). At 3.0 g/m^2, there was still 97 percent control with the granule after 18 days compared to no control with the flowable concentrate. These differences might be due to the different behaviors of the two products. After application to the surface, the flowable concentrate starts to sediment slowly, whereas a substantial part of the granule floats and disintegrates on the surface into smaller floating particles. Since *Anopheles* larvae feed at the surface film (Dahl 1988) the flowable concentrate was probably inaccessible to the larvae within short time due to a combination of sedimentation and UV-inactivation.

No other studies reported residual effect for 15 days as obtained (at least) with the granules in this study. The relatively short durations of control obtained in this study with the flowable concentrate against *Anopheles* species are in the range of what has been reported elsewhere. To make a fair comparison, applied amounts are recalculated as applied dosage in ITU/m^2 in the

following. In our study on *An. gambiae*, the high dosage of the granule and of the fluid concentrate was 500,000 ITU/m^2 and 750,000 ITU/m^2, respectively, and the low dosage 10 times lower. Testing on *An. stephensi* Liston, Kumar et al. (1994) reported continuous control by weekly application of powder of 450.000 ITU/m^2 in covered water tanks, while tests with *An. gambiae* at dosages from 2,500 to 2,500,000 ITU/m^2 in sun exposed areas gave at the most two days control (Darriet et al. 1985, Nicholas 1987a, Karch et al. 1991, Karch et al. 1992). Very short effects or practically no reduction was observed for other anopheline species (Balaraman et al. 1987, Lacey et al. 1988b), which might be due to lower sensitivity. *Anopheles quadrimaculus* Say is about 20 times less susceptible than *An. albimanus* Wiedemann (Mulla 1986), and *An. gambiae* is three times more susceptible than *An. albimanus*. The long control obtained in this study is not due to a higher dosage than in other studies.

Acknowledgments

The authors wish to thank Philip Barbazan and Vincent Roger for identifying field sites in Cameron and Senegal, respectively, and providing laboratory facilities to support the field test; Patchoke Salomon for helping with the field tests in Cameron; and Lawrence Lacey for valuable comments on the manuscript. The study was financed by a Danida Research Grant 104.Dan8/659 and carried out in cooperation between the Danish Bilharzia Laboratory and ORSTOM, Montpellier, Laboratoire de Lutte contre les Insectes Nuisible.

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Efficacy of a Granular Formulation of *Bacillus sphaericus* against *Culex quinquefasciatus* and *Anopheles gambiae* in West African Countries

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Journal of Vector Ecology
Society for Vector Ecology
Vol. 22, No. 1: 43-51, June, 1997