EVIDENCE FOR PERSISTENCE AND RECYCLING OF *BACILLUS SPHAERICUS*[1]

B. DES ROCHERS[2] AND R. GARCIA[2]

ABSTRACT. The relationship between the quality of water and the presence of mosquito larvae in the growth and maintenance of *Bacillus sphaericus* was examined. Persistence and growth of the bacteria was monitored in three water qualities: primary and secondary sewer and spring water. Results indicate that *B. sphaericus* not only persists and remains toxic in all water types for up to 30 days but that it is present in higher concentrations for significantly longer periods of time where larvae are present prior to inoculation. Tests indicate *B. sphaericus* grows in the larval cadaver and is released into the surrounding water as the cadaver disintegrates. Outdoor experiments support the above findings and indicate the cadaver provides protection for the bacteria from the deleterious effects of sunlight.

INTRODUCTION

In recent years investigators have reported the ability of *Bacillus sphaericus* Neide to recycle and persist in aquatic systems, particularly those containing moderate to high levels of organic matter. In 1979 Hertlein et al. successfully retrieved viable spores of *B. sphaericus* from a roadside ditch treated 9 months earlier and demonstrated larvicidal activity by infecting *Culex quinquesfasciatus* Say larvae in the laboratory. Singer (1980) was also able to retrieve *B. sphaericus* from dead *Aedes triseriatus* (Say) larvae collected from tree holes treated 9 months earlier.

Hornby et al. (1981) conducted tests in small sewer and fresh water ponds in Florida and found that spores of *B. sphaericus* strain 1593 persisted for periods ranging from 30 to 90 days depending on the quality of water. These investigators reported 100% control of *Cx. quinquefasciatus* and *Cx. nigripalpus* Theobald for 80–90 days in the sewer water with only one treatment of 2.5–7.5 $\times$ 10^4 spores/ml of *B. sphaericus*. Similar results were obtained in experiments conducted in fresh water but control lasted only 30–50 days. These workers speculated that the sewage habitat with its high organic debris maintained toxic levels of *B. sphaericus* for a longer period of time by protecting the bacteria from UV light. However, Mulligan et al. (1980) conducted studies with *B. sphaericus* 1593–4 and found that its activity was reduced in raw sewage effluent. Further, upon centrifuging the sewer water they found that the larvicidal activity of the bacillus was greatly reduced by the suspended solids component of the sewage as opposed to the supernatant. These researchers also examined the persistence capabilities of *B. sphaericus* in catch basins. Bioassays incorporating the bottom residues of the basins 28 days postinoculation produced 80–100% mortality in test larvae. However they found that although these spores persisted in the organic debris, only the initial larval populations were controlled with a single treatment. Subsequent populations were not controlled unless the basins were mechanically stirred to resuspend the spores. In more recent investigations it has been shown that *B. sphaericus* grows in the cadavers of *Culex* species (Davidson et al. 1984, Silapanuntakul et al. 1983, Des Rochers and Garcia 1983) in both clear and polluted waters.

The present work was conducted to further delineate the role of mosquito larvae in the maintenance and growth of *B. sphaericus* and to determine whether the organic content of the water influences this relationship.

MATERIALS AND METHODS

Laboratory experiments were conducted at a room temperature of 22±2°C and a 14 hr photoperiod. Second instar *Culex pipiens* Linn. (Berkeley strain) were used in all tests and were from colonies maintained in the laboratory under the above conditions. Three water qualities were used: primary and secondary sewer and bottled spring water. The sewer water was collected from a local sewer facility which handles mainly domestic sewage. Water analysis conducted at the facility on the collection day showed the primary sewer had a pH of 8.4 and a suspended matter content of 80 mgm/liter. The results for the secondary sewer were 7.2 and 12 mgm/liter, respectively. The bottled spring water (non-distilled) was purchased locally and used in the initial tests. Tap water, allowed to stand overnight, was used in the dilutions for the bioassays. *Bacillus sphaericus* strain 2362 was supplied in a power formulation containing approximately 1 $\times$ 10^7 spores/

[1] This research was funded in part by California Water Funds for Mosquito Research.

[2] Present Address: Division of Biological Control, University of California, Berkeley, 1050 San Pablo Ave., Albany, CA 94706.

mgm by H. T. Dulmage.[3] An aqueous suspension of the powder was prepared to the desired dosage level using distilled water.

The experiment was conducted in 180 ml glass jars in which 100 ml of either primary or secondary sewer or bottled water was added. There were 100 jars set up for each type of water. The jars in each set were then divided into 3 groups: I, II and controls (see table 1). Twenty larvae were added to Group I only. With the exception of the controls, all jars were inoculated with *B. sphaericus* to a final concentration of 1×10^{-5} sp/ml on day 0. The larvae in Group I died and the cadavers were left in the containers. On days 2,6,10,14,22 and 30, 5 jars from each group (3 jars from the controls) were selected and bioassayed for the presence and level of *B. sphaericus* using the following procedure (see Table 2).

I. Group I: Jars with cadavers
   a. Cadavers were removed, ground-up with a mortar and pestle and diluted with 100 ml of tap water. This was called the stock solution. Ten-fold serial dilutions were prepared from this solution to final dilutions of 1×10^{-5}.
   b. After removal of the cadavers, the water remaining in the jars was diluted in 10-fold serial dilutions to a final concentration of 1×10^{-3}.

II. Group II: Jars without cadavers
   a. The water in the jars was treated in the same manner as "b" above.

III. Ten additional control jars containing the tap water used in the bioassay were set up along with the 3 jars from each water type.

IV. Twenty larvae were added to each of the test and control jars and the survivors remaining after 72 hrs were recorded.[4] One ml of a 5% mixture of liver powder and ground rabbit pellets was added to all containers after 24 hrs as a food source.

V. Statistical Analysis: All proportions were transformed using the arcsine-square root transformation ($2 \times$ arcsin [sqrt (proportion)]. Analysis of variance was conducted using Duncan's multiple range test to distinguish between the sample means ($P = 0.05$).

VI. Bottled spring water was added periodically to all of the stock solutions to maintain water volume.

Bacillus sphaericus Recovery: Samples of stock solutions, old cadavers, and recently killed larvae were plated on nutrient agar containing 0.05% yeast extract and a 0.01% solution of streptomycin. (Yousten et al. 1982).

Table 1. Experimental design for laboratory studies with *B. sphaericus*.

	Primary			Secondary			Water		
Group	I	II	C	I	II	C	I	II	C
Treatment:									
1. Larvae added pre-inoculation +	+	−	−	+	−	−	+	−	−
2. *B. spaericus* inoculated +	+	+	−	+	+	−	+	+	−

C = control.

A second series of experiments were conducted in a screenhouse where natural light was reduced by approximately 50%. These experiments used plastic dish tubs, 27 × 34 × 13 cm, containing No. 2 Monterey white sand and 3–4 cm of water. The water had been allowed to stand for several days prior to the experiments. The tubs were divided into 7 groups: I, II, III, IV, V, VI and controls. Group I contained 5 tubs and had 100 larvae added before the addition of the pathogen. Groups II–VI contained 3 tubs each and were inoculated with the pathogen without larvae. All tubs were inoculated with *B. sphaericus* strain 2362 to a final concentration of 1×10^{-5} sp/ml, with the exception of 4 tubs which were used for controls. Larvae which died in Group I were left in the tubs. After pathogen inoculation, 100 larvae were added 4 times to the various tubs over a 20-day period in a sequence described in Table 3.

The number of survivors remaining after 48 hrs was recorded and the mean percent mortality was determined for each of the groups. This experiment was conducted concurrently under conditions of shade and no shade where light intensity averaged < 2 and 2800 ft candles, respectively, at 1300 hr from July 9 to 16[5].

RESULTS

LABORATORY EXPERIMENTS. In Group I stock solutions (jars with cadavers) mortality in the bioassay remained high (100%) for all water types through day 30. Similar results were seen in Group II (jars without cadavers), however on day 30, mortality started to decline in the primary and secondary sewer water (Fig. 1a). In the 10^{-1} dilutions of spring and secondary sewer waters, a significant difference existed between Group I and Group II (Fig. 1b, c). However in

[3] H. T. Dulmage, U. S. Department of Agriculture, Brownsville, TX.

[4] 72 hrs used to allow larvae in primary sewer water to consume a lethal does of pathogen (see Des Rochers and Garcia 1983).

[5] Light intensity measured with a Photovolt, model 200. 1115 Broadway, New York, NY 10010.

Table 2. Bioassay design for laboratory studies with *B. sphaericus*.

Group	Primary			Secondary			Water		
	I	II	C	I	II	C	I	II	C
Stock solution									
No. jars	5	5	3	5	5	3	5	5	3
Final dilution	10^{-3}	10^{-3}	–	10^{-3}	10^{-3}	–	10^{-3}	10^{-3}	–
Cadaver solution									
No. jars	5	–	–	5	–	–	5	–	–
Final dilution	10^{-5}	–	–	10^{-5}	–	–	10^{-5}	–	–

C = control.

the primary sewer water, with the exception of day 6, no significant difference existed between these two groups (Fig. 1d). Further, in Group I no significant difference existed between jars containing spring water and secondary sewer water; however, a significant difference did exist between these latter 2 water types and the primary sewer water (Fig. 1e). In Group II, there was no difference between the water types through day 14 at which time there was a significant difference between the spring water and sewer waters. This difference was also noted on days 22 and 30 (Fig. 1f).

Similar results were seen at 10^{-2}. However, at this level a significant difference was seen between all 3 water types in Group I on days 10 and 14 (Fig. 1g). This was not noted in Group II (Fig. 1h).

There was 0–0.05% mortality in the bioassays at 10^{-3} and in the controls for all water types.

In the stock solutions of the cadavers, mortality in the bioassays remained high (100%) for all water types for 30 days. At 10^{-1} and 10^{-2} dilutions, mortality in the bioassays increased between 2 and 6 days and remained high (85–100%) for all water types through day 14. Thereafter a decline in bioassay mortalities was noted for the sewer waters but not in the spring water (Fig. 2a). At the higher dilution levels, mortality in the bioassay was considerably less; but similar patterns of mortality, increasing between day 2 and 6 and decreasing after day 14 were noted for all water types (e.g., Fig. 2b). There was no mortality in the 10^{-5} dilutions. *Bacillus sphaericus* was identified by colony morphology and retrieved from all test situations.

Table 3. Experimental design for screenhouse studies with *B. sphaericus*.

Group	I	II	III	IV	V	VI	C
Larvae added pre-inoculation +	+	–	–	–	–	–	+
B. sphaericus added day 0	+	+	+	+	+	+	–
Larvae added:							
day 2	+	+	–	–	–	–	+
day 5	+	–	+	–	–	–	+
day 11	+	–	–	+	–	–	+
day 17	+	–	–	–	+	–	+
day 20	+	–	–	–	–	+	+

C = control.

SCREENHOUSE EXPERIMENTS. In the unshaded experiment in Group I (tubs with cadavers), high levels of mortality were seen in subsequent additions of larvae for 13 days. In Groups II–VI (tubs without cadavers) no mortality occurred in the added larval populations (Fig. 3a). Water temperatures ranged from 11.7 to 27.2° C. There was no mortality in control tubs.

In the shaded experiment in Group I, high levels of mortality were seen in subsequent additions of larvae for 20 days. In Groups II–VI a decline in mortality was noted after 13 days (Fig. 3b). Water temperatures ranged from 12.8 to 17.8° C. There was no mortality in control tubs.

DISCUSSION

The higher levels of mortality seen in the bioassays in Group I would indicate that the bacteria are using the cadavers as a medium for growth and are released into the surrounding water as the cadavers disintegrate. Decomposition of cadavers occurred more rapidly in the sewer waters and was particularly evident by day 30. This probably explains the sharp decrease in bioassay mortality seen at days 10 and 14 for these water types in the cadaver dilutions (Figs. 2a, b). It may also explain the increase in mortality noted on day 30 in the 10^{-2} dilution of secondary sewer water (Fig. 1g).

In general, the results of the primary sewer water bioassays suggest that, compared to other water types, this particular medium was detrimental to the pathogen. The fluctuations seen, not only in the primary sewer water (Fig. 1d) but also in the other water types, particularly notable on day 10, are difficult to explain. Unknown factors associated with either the larval populations used or the deteriorating sewer waters may have influenced the results. There is also the possibility that the fluctuations are

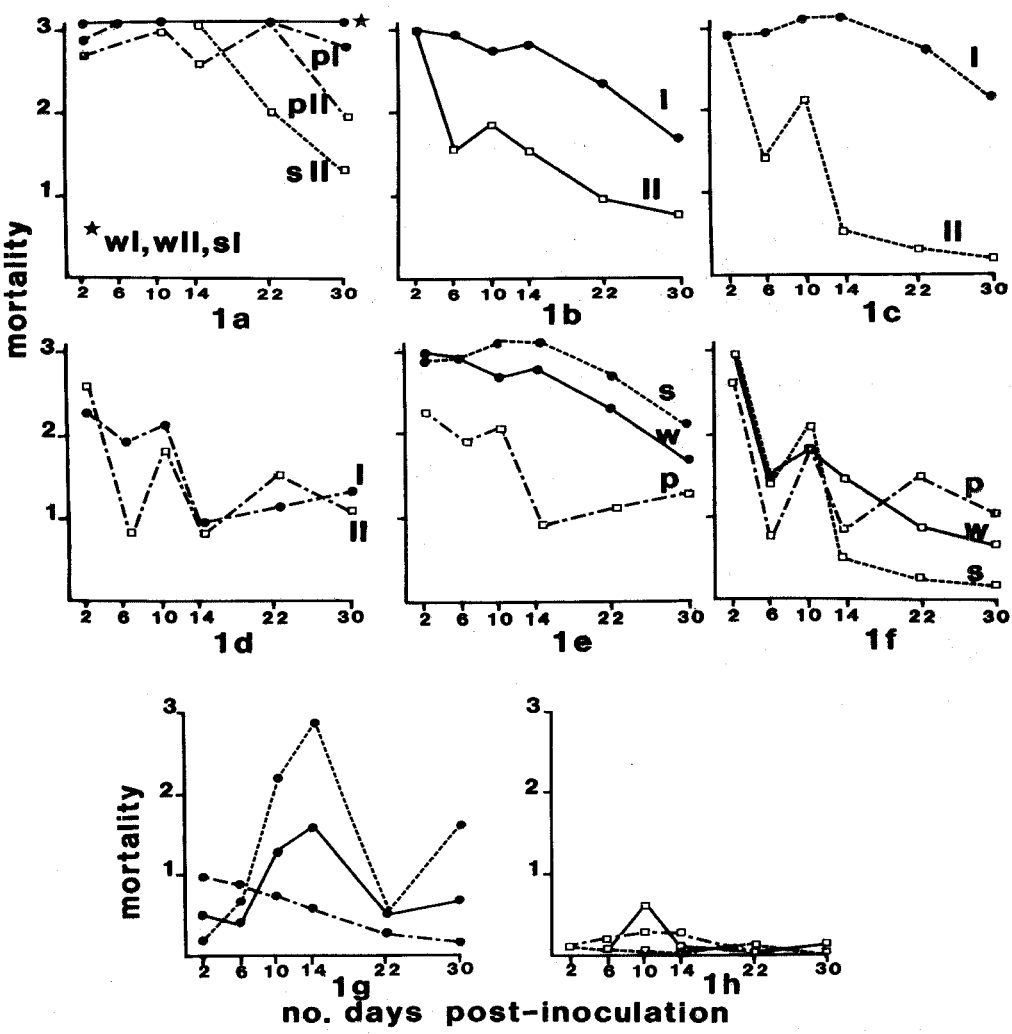

Fig. 1. Results of stock solution dilutions: bottled water (——), secondary sewer (– – –), primary sewer (—·—); • = Group I, □ = Group II. (a) result of stock solutions for Groups I and II; (b–f): 10^{-1} dilution (b) water (c) secondary sewer (d) primary sewer (e) Group I combined (f) Group II combined; (g–h): 10^{-2} dilution (g) Group I combined (h) Group II combined. Mortality is expressed in radians.

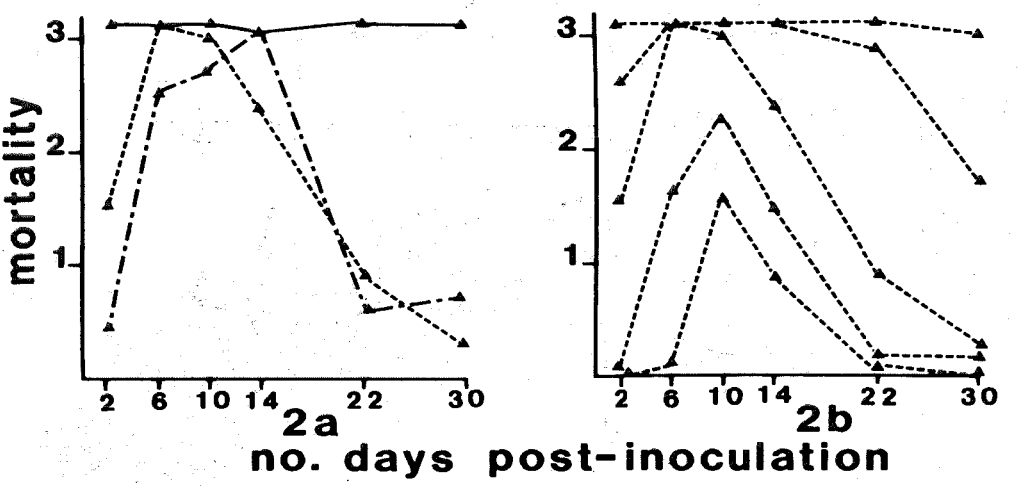

Figure. 2. Examples of cadaver solution dilutions (a) 10^{-2} dilution for bottled, secondary and primary sewer water (b) Dilutions of secondary sewer water. Mortality is expressed in radians.

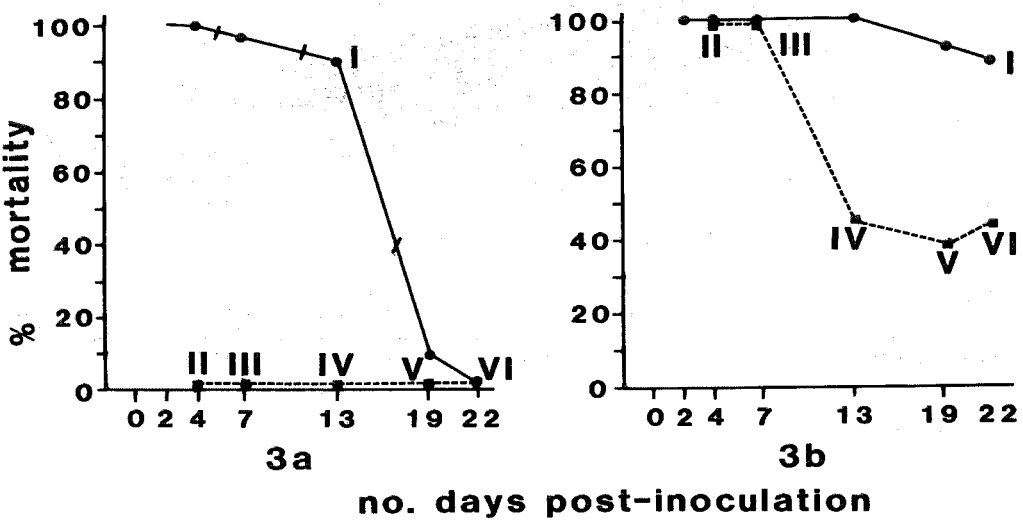

Fig. 3. Results of screenhouse experiments (a) no shade, light intensity 2800 ft candles (b) shade, light intensity < 2 ft candles.

triggered by some factor, possibly cyclic in nature, associated with the pathogen. This is difficult to assess in these experiments due to the 4 to 8 day interval between bioassays.

There is no firm evidence from these data that would indicate secondary sewer water is a more or less favorable medium for growth for *B. sphaericus* than spring water. In Group I at the 10^{-1} dilution, a slightly greater bioassay mortality was seen in secondary sewer water as opposed to spring water but the differences are not significant (Fig. 1e). The differences, though, are significant in the 10^{-2} dilution but only on days 10 and 14 (Fig. 1g); however in Group II the results are essentially the same for all three water types (Fig. 1h).

In the bioassays of ground cadavers an increase in mortality was noted between day 2 and 6 (e.g., Fig. 2a, b) in all water types and suggests the concentration of *B. sphaericus* is increasing during this period. This supports observations by Davidson et al. (1984) which indicate that *Culex* larvae fatally infected by this bacterium produce, on the average, 10^5–10^6 *B. sphaericus* cells/larva 3 days after treatment.

The results in the screenhouse experiment suggest that mosquito larvae are not only taking up the bacteria at the time of inoculation and serving as a medium of growth, but that they are also protecting the pathogen from the deleterious effects associated with sunlight. In Group I exposed to sunlight, complete mortality was seen in the first addition of larvae 2 days after the pathogen was inoculated; whereas in Group II, no mortality was seen suggesting the pathogen had already been destroyed.

The results in the screenhouse and in the laboratory indicate that the presence of cadavers in the medium contribute to the maintenance of toxic levels of the pathogen. However, both experiments were conducted in shallow water with relatively large numbers of larvae. In field use, water depth along with larval population size at the time of inoculation may be important factors. Other considerations include the quality of water which would not only affect the pathogen directly but would also influence the rate of cadaver decomposition. Cadavers remaining relatively intact for longer periods would not only provide, for a while, a concentrated source of the bacteria for subsequent populations of mosquito larvae, but would also be more susceptible to complete removal by scavengers. The effects in general of other filter feeders, predators and scavengers on the bacteria in the medium has yet to be assessed.

CONCLUSIONS

These experimental results indicate that *B. sphaericus* utilizes the larval cadaver as a medium for growth and is released into the surrounding water as the cadavers disintegrate. Further, the presence of larvae at the time of inoculation outdoors (in clear water) appears to contribute to the maintenance of the bacteria by protecting it from the deleterious effects of sunlight. There was no significant difference seen between tests conducted in secondary sewer water and spring water, but the primary sewer water used in these experiments appeared detrimental to *B. sphaericus*.

ACKNOWLEDGMENTS

We wish to thank Mr. David Rowney for the statistical analysis conducted on the laboratory experiments and Dr. H. T. Dulmage and his colleagues for supplying the *Bacillus sphaericus*.

References Cited

Davidson, E. W., M. Urbina, J. Payne, M. S. Mulla, H. Darwaszeh, H. T. Dulmage and J. A. Correa. 1984. Fate of *Bacillus sphaericus* 1593 and 2362 spores used as larvicides in the aquatic environment. Appl. Environ. Micro. 47:125–129.

Des Rochers, B. and R. Garcia. 1983. The efficacy of *Bacillus sphaericus* in controlling mosquitoes breeding in sewer effluent. Proc. Calif. Mosq. Vector Control Assoc. 51:35–37.

Hertlein, B. C., R. Levy and T. W. Miller, Jr. 1979. Recycling potential and selective retrieval of *Bacillus sphaericus* from soil in a mosquito habitat. J. Invertebr. Pathol. 33:217–221.

Hornby, J. A., B. C. Hertlein, R. Levy and T. W. Miller Jr. 1981. Persistent activity of mosquito larvicidal *Bacillus sphaericus* 1593 in fresh water and sewage. WHO/VBC/81.830.

Mulligan F. S., III, C. H. Schaeffer and W. H. Wilder. 1980. Efficacy and persistence of *Bacillus sphaericus* and *B. thuringiensis* H-14 against mosquitoes under laboratory and field conditions. J. Econ. Entomol. 73:684–688.

Silapanuntakul, S., S. Pantuwatana, A. Bhumiratana and K. Charoensiri. 1983. The comparative persistence of toxicity of *Bacillus sphaericus* strain 1593 and *Bacillus thuringiensis* serotype H-14 against mosquito larvae in different kinds of environments. J. Invertebr. Pathol. 42:387–392.

Singer, S. 1980. Use of entomogenous bacteria against insects of public health importance. Dev. Ind. Microbiol. 20:117–392.

Yousten, A. A., Jones, M. E. and Benoit, R. E. 1982. Development of selective/differential bacteriological media for the enumeration of *Bacillus thuringiensis* serovar. *israelensis* (H-14) and *Bacillus sphaericus* 1593. WHO mimeograph sheet: WHO/VBC/82.844, WHO Geneva.