ROLE OF LARVAL CADAVERS IN RECYCLING PROCESSES OF *BACILLUS SPHAERICUS*

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ABSTRACT. The influence of larval cadavers of *Culex pipiens* on recycling processes of *Bacillus sphaericus* was investigated by bioassays and spore counts in the laboratory. Studies conducted with 3 different *B. sphaericus* concentrations (0.005, 0.01, 0.05 mg *B. sphaericus*/liter) indicated that the presence of cadavers in the water contributed to the maintenance of toxic levels of *B. sphaericus*. Larval cadavers seem to contain all the nutrients necessary both for vegetative multiplication and for toxin synthesis associated with the sporulation process. Bioassays of *B. sphaericus* revealed that the mortality of *Culex pipiens* remained on a high level over a period of 26 days when larval cadavers were added every second day to the test vessels. This result was supported by a sharp increase in spore density when cadavers were added at the same interval. The test series showed *B. sphaericus* recycles in intact cadavers of *Culex pipiens*, whereas this phenomenon could not be observed when crushed cadavers were used in the trials. Therefore, our results demonstrated that for successful recycling processes it seems of crucial importance that infected cadavers remain intact at least for a certain time and also that the dosage of the applied *B. sphaericus* plays a major role in recycling processes whereas larval density is only of minor importance to these processes.

INTRODUCTION

Bacillus sphaericus has been shown to be highly insecticidal, especially to larval *Culex* spp. mosquitoes, in numerous laboratory and field trials (Davidson 1984, Davidson and Yousten 1990, de Barjac 1990, Lacey 1990, Ragoonanansingh et al. 1992, Xu et al. 1992, Ludwig et al. 1994). Due to its high efficacy against *Culex* mosquitoes, *B. sphaericus* is a promising biocidal candidate for controlling larvae of *Culex quinquefasciatus* Say, the main vector of lymphatic filariasis in many areas of the world in which it is endemic.

A particularly attractive feature of *B. sphaericus* is its potential to persist and recycle under certain field conditions. Appropriate formulations have shown significant residual activity against *Cx. quinquefasciatus* and *Culex pipiens* Linn. in highly polluted breeding habitats (World Health Organization 1994).

Hertlein et al. (1979) found viable and infective spores of *B. sphaericus* 9 months after application in a roadside ditch. Tests of Des Rochers and Garcia (1984) indicated that *B. sphaericus* grows in larval cadavers and is released into the surrounding water as the cadavers disintegrate. According to Davidson et al. (1984), recycling in dead larvae leads to an increase of 100–1,000-fold in spore numbers in cadavers both in the laboratory and the field. These results were supported by Charles and Nicolas (1986), who found a similar increase in spore numbers in the laboratory. Field trials of Nicolas et al. (1987) in West Africa demonstrated that recycling took place in dead larvae but not in the mud.

The conditions for recycling of *B. sphaericus* are still not well understood. The present work was conducted to further elucidate the role of larval cadavers and the impact of different larval densities in recycling processes under defined conditions. The recycling effects were studied by means of bioassays and spore counts using crushed and intact cadavers.

MATERIALS AND METHODS

The experiments were conducted at a room temperature of 20 ± 0.5°C and a 16-h photoperiod. Third-instar *Cx. pipiens* from colonies maintained in the laboratory under conditions similar to the above mentioned were used in all tests. All tests were conducted in glass jars that were filled with 1 liter of distilled water.

In the first series of trials jars were inoculated with *B. sphaericus* (BSB 0004 primary powder, NOVO-Nordisk, Denmark) to final concentrations of 0.05, 0.01, and 0.005 mg/liter in 9 replicates each on day 0. Three untreated jars served as the control. Immediately after treatment (day 0), 50 3rd-instar larvae of *Cx. pipiens* were added to each jar. The jars of each concentration set were then divided into 3 groups and tests were run in 3 replicates each.

Group 1: Every 2nd day dead and/or surviving larvae were removed, the mortality was determined, and 50 new larvae were added.

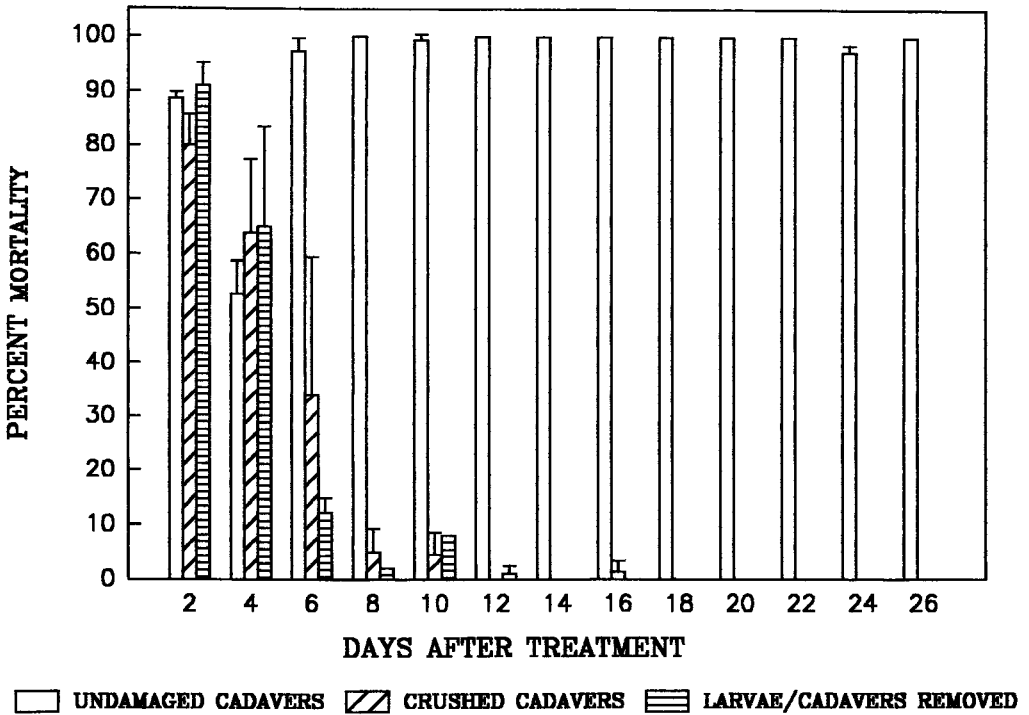Group 2: Dead larvae were not removed. Surviving larvae were killed by drying them on

Fig. 1   Efficacy of *Bacillus sphaericus* (0.05 mg/liter) against 3rd instars of *Culex pipiens* (mean ± SD).

filter paper for 3 h. Then the cadavers were returned to the jars. Afterwards 50 fresh larvae were added. This procedure was repeated every 2nd day when the mortality rate was assessed.

Group 3: Dead and/or living larvae were removed from the jars each 2nd day when the mortality was recorded. Then larvae and cadavers were ground up with a mortar. The crushed larvae were then returned to the jars and 50 fresh larvae were added.

The trials lasted 26 days.

In a 2nd test series the influence of larval density on recycling processes was investigated. Therefore, 5, 20, 60, and 100 3rd-instar larvae of *Culex pipiens* were placed at day 0 in 1-liter jars treated with 0.01 mg *B. sphaericus*/liter. Mortality was evaluated after 48 h. Tests were run with 3 replicates per concentration. Dead and surviving larvae were treated like those of Group 2. This procedure was repeated every 2nd day. Jars containing distilled water only were used as controls.

In addition to the evaluation of mortality, spore counts were conducted according to Yousten et al. (1985) once a week. To eliminate the vegetative cells of *B. sphaericus* a sample of 1 ml of each vessel was taken and heated for 12

min at 80°C in a water bath. Next the samples were diluted with sterile distilled water and plated on BATS medium according to Yousten et al. (1985). To assess the total spore number of BSB 0004, 50 mg of the powder were suspended in 10 ml sterile distilled water and homogenized with 15 glass beads (6 mm) on a Vortex® shaker for 10 min at maximum speed. Dilutions of this stock solution were also heated for 12 min at 80°C and plated on an unselected complex medium (CASO-Agar, Merck, Darmstadt) and on BATS medium to determine the percentage of retrieval. For statistical purposes data were subjected to Duncan's multiple range test and/or Student's *t*-test.
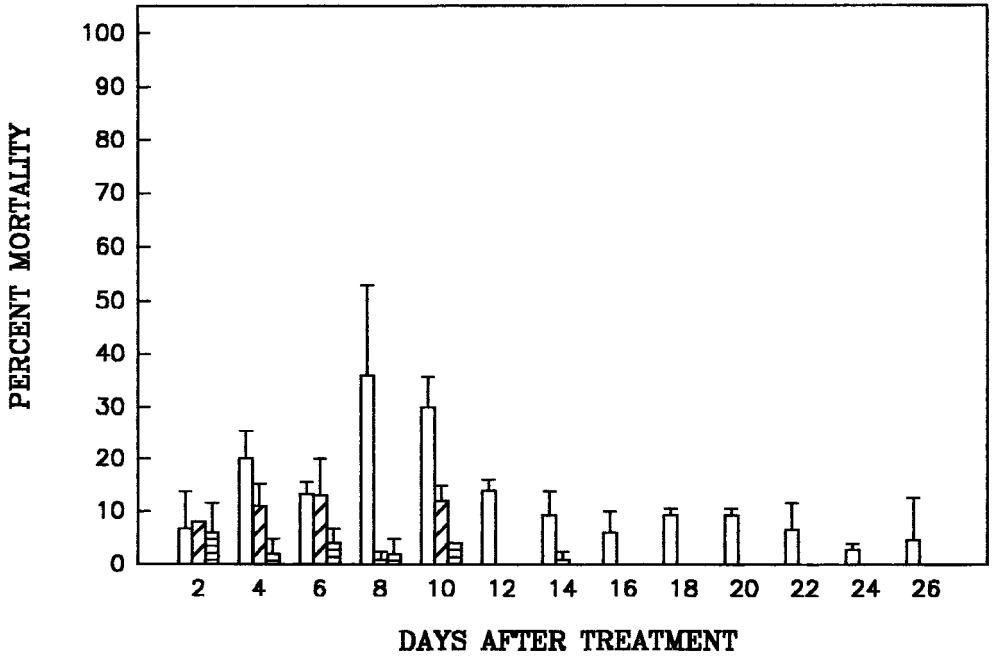
RESULTS

In Group 1 (jars without cadavers) mortality within the jars with 0.05 and 0.01 mg *B. sphaericus*/liter decreased significantly ($P \leq 0.1$) after 4 days (Figs. 1 and 2). After 8 and 12 days, respectively, through to day 26, no significant activity of *B. sphaericus* could be observed. Within the lowest *B. sphaericus* concentration of Group 1 (0.005 mg/liter) none to only very low levels of mortality (0–6%) occurred. In Group 2 (jars with undamaged cadavers) mortality within
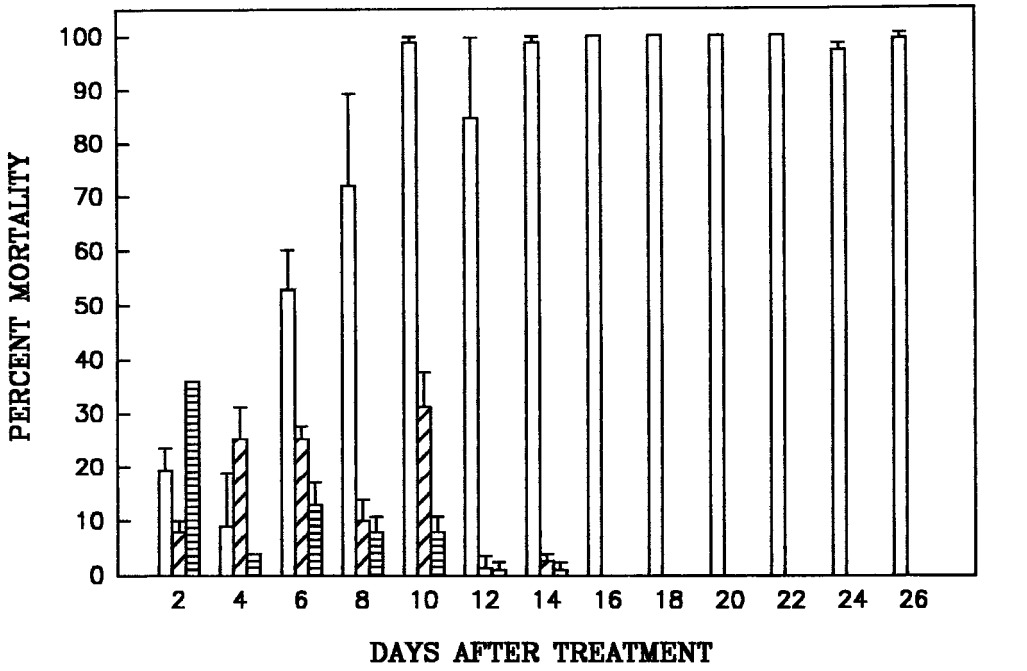
Fig. 2.   Efficacy of *Bacillus sphaericus* (0.01 mg/liter) against 3rd instars of *Culex pipiens* (mean ± SD).

Fig. 3.   Efficacy of *Bacillus sphaericus* (0.005 mg/liter) against 3rd instars of *Culex pipiens* (mean ± SD).
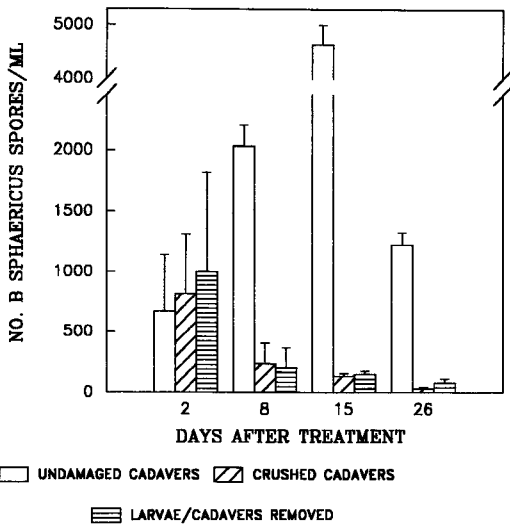
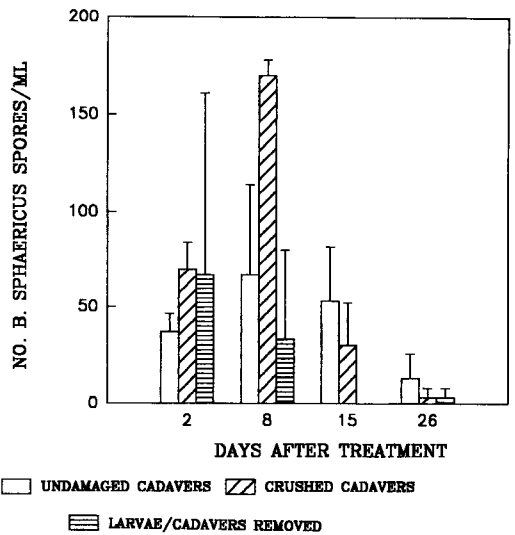Fig. 4. Spore density of *Bacillus sphaericus* in jars treated with 0.05 mg. *B. sphaericus*/liter (mean ± SD).

Fig. 6. Spore density of *Bacillus sphaericus* in jars treated with 0.005 mg *B. sphaericus*/liter (mean ± SD).

the jars containing 0.05 mg/liter *B. sphaericus* decreased after 4 days of exposure. Six days after exposure mortality increased significantly ($P \leq 0.01$) compared with days 2 and 4 and remained at *ca.* 100% through day 26. At the lesser concentration level of 0.01 mg *B. sphaericus*/liter, similar patterns of mortality, decreasing between days 2 and 4 and increasing after day 6, were noted. Also at this dosage the mortality remained high (97.3–100.0%) from day 8 through day 26. In the jars with the lowest *B.*
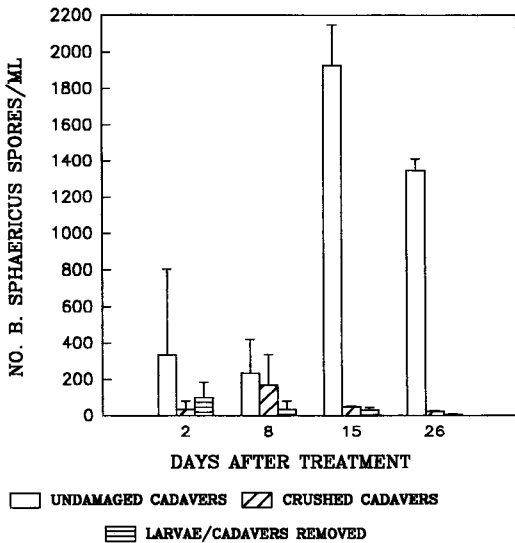

Fig. 5. Spore density of *Bacillus sphaericus* in jars treated with 0.01 mg *B. sphaericus*/liter (mean ± SD).

sphaericus concentration (0.005 mg/liter), mortality increased significantly after 4 days of exposure up to 36% on day 8 (Fig. 3). Thereafter a decline in mortality was noted. From day 14 to day 26 mortality was recorded between 3 and 9%. Results in Group 3 (jars with crushed cadavers) were similar to those observed in Group 1: 12 days after exposure no (or only very weak) mortality occurred in all 3 *B. sphaericus* concentrations. The results of the spore counts (Figs. 4–6) support the findings of the bioassays. Up to 7 days in all groups similar numbers of *B. sphaericus* spores were recorded. Although the number of spores decreased in Groups 1 and 3, in Group 2 a sharp increase in spore density occurred after 14 days in the jars treated at concentrations of 0.01 and 0.05 mg/liter. The mentioned process started earlier at a dosage of 0.05 mg/liter. In the lowest concentration (0.005 mg/liter) this phenomenon was not observed.

Larval density had a significant impact on the efficacy of *B. sphaericus* only up to day 4. Mortality after 2 days with 5 larvae/liter was 100%; with 20 larvae/liter, 96.7%; with 60 larvae/liter, 83%, and with 100 larvae/liter, only 69%. Six days after application mortality in all test series was 100%. Mortality stayed at this level until the end of the investigation (26 days). Spore counts support these results. In all test jars the number of spores increased steadily from the 2nd day until day 17 (Fig. 7).

DISCUSSION

Our results demonstrate that under laboratory conditions the presence of cadavers in the water
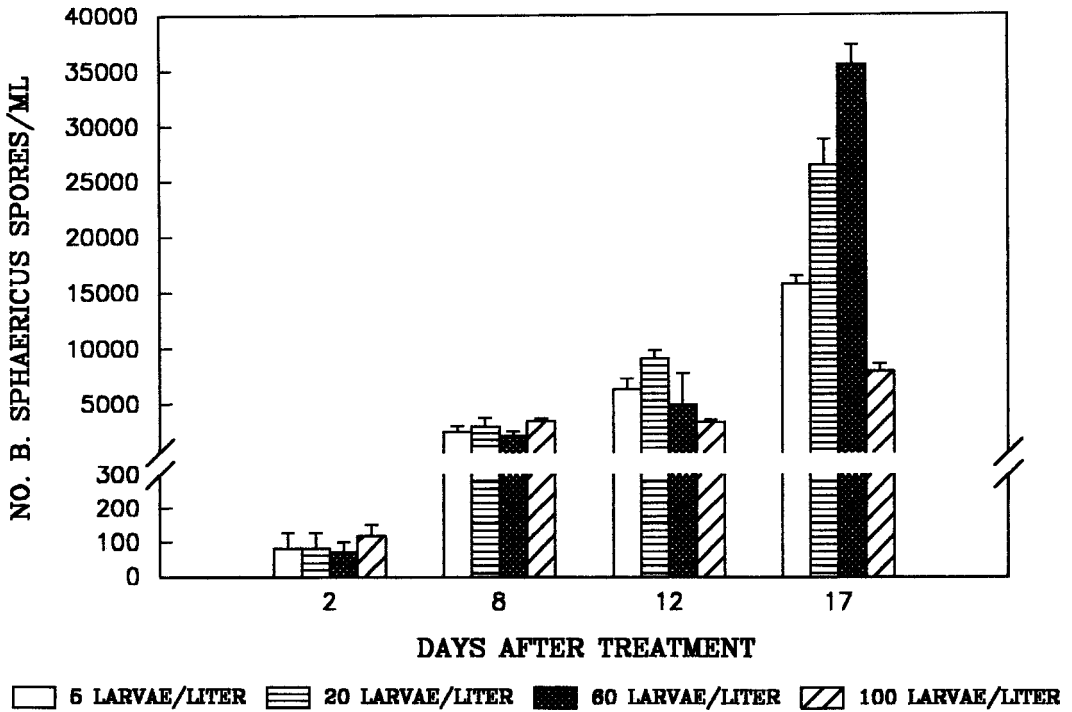
Fig. 7.   Spore density of *Bacillus sphaericus* in jars (treated with 0.01 mg *B. sphaericus*/liter) with different densities of *Culex pipiens* larvae (mean ± SD).

contributes to the maintenance of toxic levels of *B. sphaericus*. Larval cadavers seem to contain all the nutrients necessary both for vegetative multiplication and for toxin synthesis associated with the sporulation process, as suggested by Charles and Nicolas (1986). Similar results were found by Des Rochers and Garcia (1984). For successful recycling processes it seems important that cadavers remain intact for a certain time in order to provide sufficient concentrations of nutrients for recycling processes. Des Rochers and Garcia (1984) speculated that *B. sphaericus* utilizes the larval cadaver as a medium for growth and is released into the surrounding water as the cadavers disintegrate. In our test series with crushed larvae no recycling effect could be observed. This effect demonstrates that the productivity of each larval habitat is of crucial importance for recycling processes of *B. sphaericus*. The higher the number of larval cadavers the more effectively is *B. sphaericus* recycled. It seems to be important that the initial dosage of the treatment should be sufficiently high in order to provide a number of germinating spores in dying larvae.

An influence of larval density on recycling was not found, at least when a concentration of 0.01 mg *B. sphaericus*/liter was used. Obviously

a relatively low number of larval cadavers already leads to an effective recycling process. Thus on days 2 and 4 after exposure, significant differences in mortality between the different larval densities were found, but these differences are obviously due to the effect of larval density on the efficacy of microbial agents, as observed by Becker et al. (1992, 1993). From day 6 after exposure when the recycling processes showed their influence, mortality rates of 90–100% were observed at all different larval densities. According to Charles and Nicolas (1986) in the field, recycling might significantly enhance the efficacy of a treatment only if larval cadavers containing recycled spores remain in the larval feeding zone (e.g., the upper water layers) to kill additional hatching larvae. So in deep larval habitats, where cadavers disappear from the feeding zone, as for instance in rainwater barrels, the recycling effect will be significantly less important than in shallow larval habitats.

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