Bacillus sphaericus Mosquito Pathogens in the Aquatic Environment

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The fate of Bacillus sphaericus *spores in the aquatic environment was investigated by suspending spores in dialysis bags in fresh and seawater. Spore viability was lost more rapidly in seawater. Neither* B. sphaericus *nor* B. thuringiensis *israelensis (B.t.i.) spores mixed with pond sediment appeared to attach to the sediment. However, rapid decrease in B.t.i. toxicity suggested attachment of parasporal bodies to sediment.* B. sphaericus *toxin settled more slowly and less completely.* B. sphaericus *spores fed to larvae of four aquatic invertebrates were mostly eliminated from the animal gut in less than one week. An exception was the cranefly* (Tipula abdominalis) *where spores persisted in the posterior gut for up to five weeks.*

Key words: *Bacillus sphaericus* - *Bacillus thuringiensis* israelensis - mosquito pathogenic bacteria - bacterial spores - *Chironomus riparius* - *Paragnetina media* - *Pteronarcys proteus* - *Tipula abdominalis* - biological control

Certain strains of *Bacillus sphaericus* are known to produce a binary protein toxin composed of 51 kDa and 42 kDa peptides. These proteins, synthesized at the time of sporulation, accumulate as a parasporal body in the sporangium. These same strains produce a 100 kDa toxin that is synthesized during vegetative growth. The location of the latter toxin in the cell is unclear (Baumann et al. 1991, Porter et al. 1993). Because of the high toxicity to mosquito larvae that consume these toxins, *B. sphaericus* may be used for control of susceptible mosquito species.

The dispersion of very large numbers of viable spores as well as toxins into the aquatic environment demands that the safety of these bacteria be assured. Strain 2362 has been the subject of several safety studies and it appears that the bacteria and the toxin do not pose any hazard (Lacey & Mulla 1990, Siegel & Shadduck 1990). Beyond the question of immediate infectivity and toxicity, there remains the longer range question of the impact on the aquatic animal and microbial communities of the introduction of large numbers of alien bacteria. To help answer this question, it is important to have at least a general knowledge of the fate of these bacteria after they have been delivered into the environment. To this end, we have examined the viability of *B. sphaericus* 2362 spores in water and also the fate of spores and toxin after they have been eaten by a variety of aquatic invertebrates.

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MATERIALS AND METHODS

Bacteria - Most studies were carried out with a rifampicin-resistant mutant of *B. sphaericus* (strain 2362-7). Spores were prepared by growth in NYSM broth (Lewis et al. 1987) with shaking at 30°C for 48 hr. Spores used in settling and attachment studies were commercial preparations from Abbott Laboratories, N. Chicago, IL, USA. The preparations were: *B. sphaericus* 2362 (ABG 6262) and *B. thuringiensis* serovar. israelensis (Vectobac 12AS, ABG 6193).

Spores were enumerated following heating of samples at 80°C for 12 min and plating on NYSM agar containing 50 µg/ml rifampicin and 0.002% cycloheximide. Commercial spore preparations were enumerated on NYSM agar.

Spore dormancy in dialysis bags - Twenty five ml of spore suspension prepared in filter sterilized pond or sea water was placed in each of three dialysis bags. For freshwater tests these bags were placed inside another nylon mesh bag for protection and submerged in a pond to a depth of 0.5 m. For seawater tests, bags were placed in a flow through aquarium that delivered filtered (30 µg) seawater from Santa Rosa sound (Gulf Breeze, Florida) at the rate of 750 ml/min. Bags were removed at intervals and samples taken to determine spore concentrations by heat resistant spore count and direct microscope count. Suspen-

sions were then placed into new bags and returned to their respective locations.

Spore settling and attachment - Two ml of Abbott preparations of either *B. sphaericus* 2362 or *B. thuringiensis* israelensis (B.t.i.) were mixed for 1 hr with 15 l of suspended organic pond sediment. Each suspension was poured rapidly into a column (178 cm height x 10 cm diameter) and samples withdrawn at intervals through a serum stopper port at the 83 cm depth.

Consumption of spores by aquatic insect larvae - Insects used in these experiments were obtained from wild populations. Larvae of the midge (*Chironomus riparius*), predatory stonefly (*Paragnetina media*), leaf shredding stonefly (*Pteronarcys proteus*), and cranefly (*Tipula abdominalis*) were reared as previously described (Yousten et al. 1992). Midge larvae were held in 200 ml sterile distilled water and fed Tetramin fish food and *B. sphaericus* spores (final conc. 9.6×10^6/ml) for 5 hr. Larvae were rinsed and placed in 200 ml water containing Tetramin but without spores. At intervals larvae were removed, homogenized and spore counts performed on the homogenate. Feces were collected by placing spore-fed larvae in a funnel blocked at the narrow end with a screen. The funnel outlet led into a rubber tube that dipped into an ice water bath. Larvae were fed fish food. Feces were recovered at intervals by centrifuging the cold water and resuspending the pellet for spore count.

Predatory stoneflies were each fed three midge larvae that had been fed *B. sphaericus* spores overnight. Only stoneflies that consumed three midge larvae within 4 hr were used in the experiments. Immediately after consuming midge larvae, stoneflies were rinsed in sterile distilled water, the appendages removed, and the remaining bodies homogenized. The remaining stoneflies were held individually in chambers and the water was changed prior to the 24-hr sample and on alternate days thereafter. Each stonefly was fed one uncontaminated midge larva on days alternating with the water changes.

Leaf-shredding stoneflies held in individual chambers were each fed a 2-cm leaf disc that had been soaked overnight in 1×10^7 2362-7 spores and rinsed three times in sterile water. Animals were allowed to feed on discs overnight and were then placed in clean water and fed bits of uncontaminated leaves at 24, 48, and 120 hr. At intervals, guts were removed from three or four animals and homogenized for spore counts.

Cranefly larvae held in individual 400ml chambers were each fed one spore-soaked leaf disc (see above). Twenty-four hours after feeding on the leaf discs, chamber water was changed and an uncontaminated leaf disc added. Water changes and leaf disc additions were repeated on alternate days. At intervals, guts were dissected from three animals and either the three guts homogenized individually or, in a separate experiment, divided into anterior and posterior segments and each segment homogenized separately. The pH of isolated gut segments was determined using a model MI-413 needle electrode (Microelectrodes Inc., Londonderry, N.H.). Feces were collected from animal chambers during the first 24-hr period following feeding of leaf discs.

Bioassay of fecal material - Fecal material collected from cranefly chambers was homogenized and bioassayed for toxicity to second instar larvae of *Culex quinquefasciatus* as previously described (Yousten & Wallis 1987).

RESULTS

Maintenance of spore viability in freshwater and seawater - Viability of spores was judged by ability to survive heating and to give rise to colonies when plated on NYSM agar supplemented with rifampicin and cycloheximide. During the four weeks of the experiment, the dissolved organic carbon in the pond (that able to move into and out of the bags) averaged 5.3 ± 0.3 mg/l, the water temperature ranged from a low of 12.5°C to a high of 26°C, and the pH was approximately 8.3. Petroff microscope counts showed that about one-half of the spores placed into the bags at the beginning of the experiment were still present at the end, the remainder presumably having been lost during bag to bag transfer. Therefore, any decrease in number of spores beyond this represented loss of viability. Fig. 1 shows that during 28 days the number of viable spores decreased by about 20%.

A similar experiment was conducted in seawater in which the salinity ranged from 18 to 32 parts per thousand, the temperature ranged from 11°C to 16°C, and the dissolved organic carbon

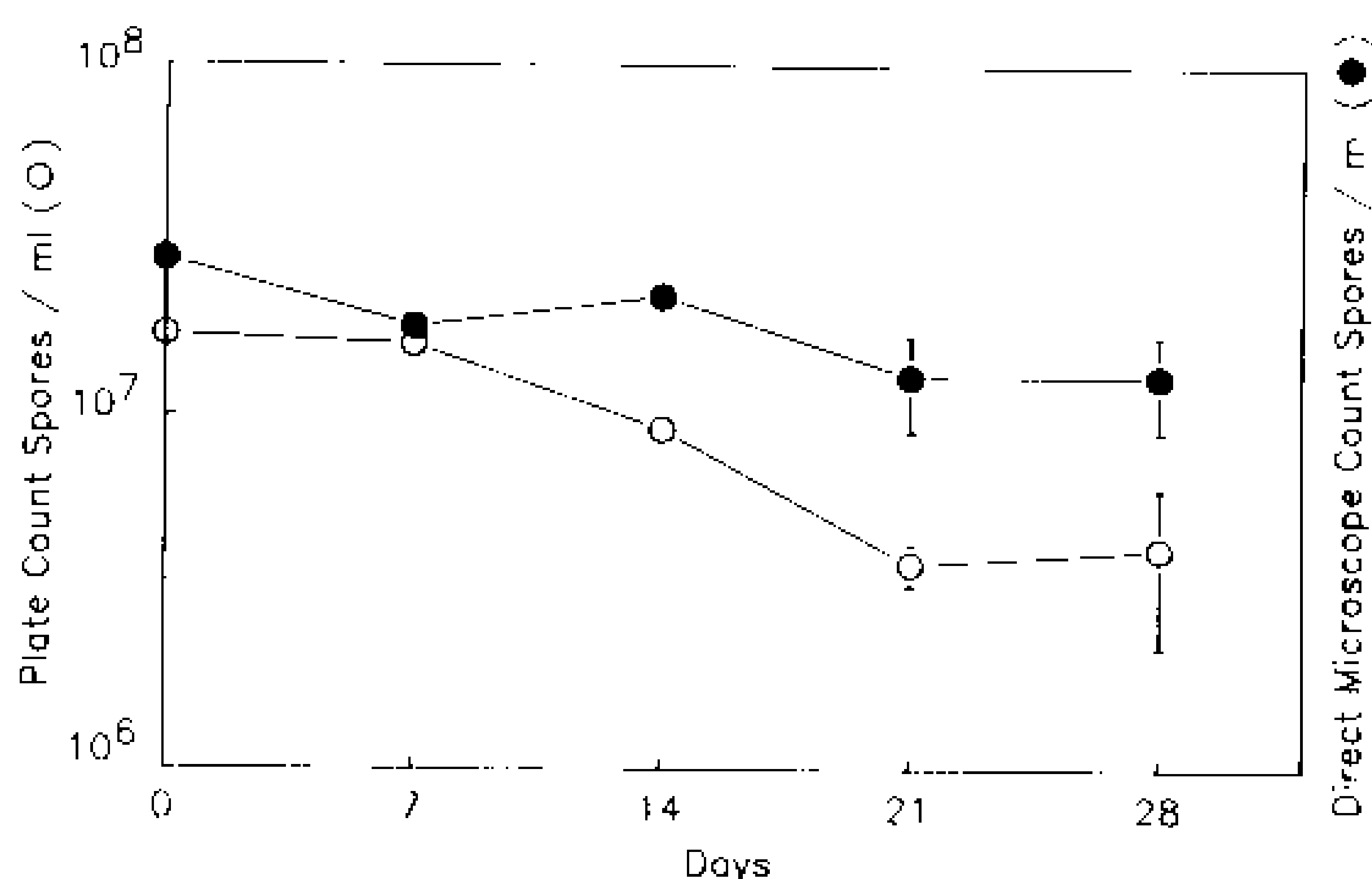

Fig. 1: *Bacillus sphaericus* spores in dialysis bags submerged in a freshwater pond. Suspensions were plated for heat resistant spores, counted microscopically for total spores and transferred to new bags at weekly intervals. (O) heat resistant spores; (●) total spores. Each point represents the mean of three bags and error bars denote standard deviation from the mean.

ranged form 3 to 10 mg/l. In this experiment the spore viability decreased by about 80% in 28 days and by about 97% by 50 days.

Settling of spores and toxin mixed with pond sediment - Spore counts of *B. sphaericus* 2362 declined only slightly ($1.37x10^6$/ml to $1.20x10^6$/ml) during 24 hr in the column. Similarly, there was only a small decline in B.t.i. spore count ($4.45x10^6$/ml to $3.8x10^6$/ml). In both cases the pond sediment had settled in 30 min. The *B. sphaericus* toxicity decreased slightly during the first 30 min of settling but then remained about constant during the following 24 hr. *B. thuringiensis* toxicity decreased sharply during the first 30 min and continued to decline during the next 24 hr (Fig. 2).

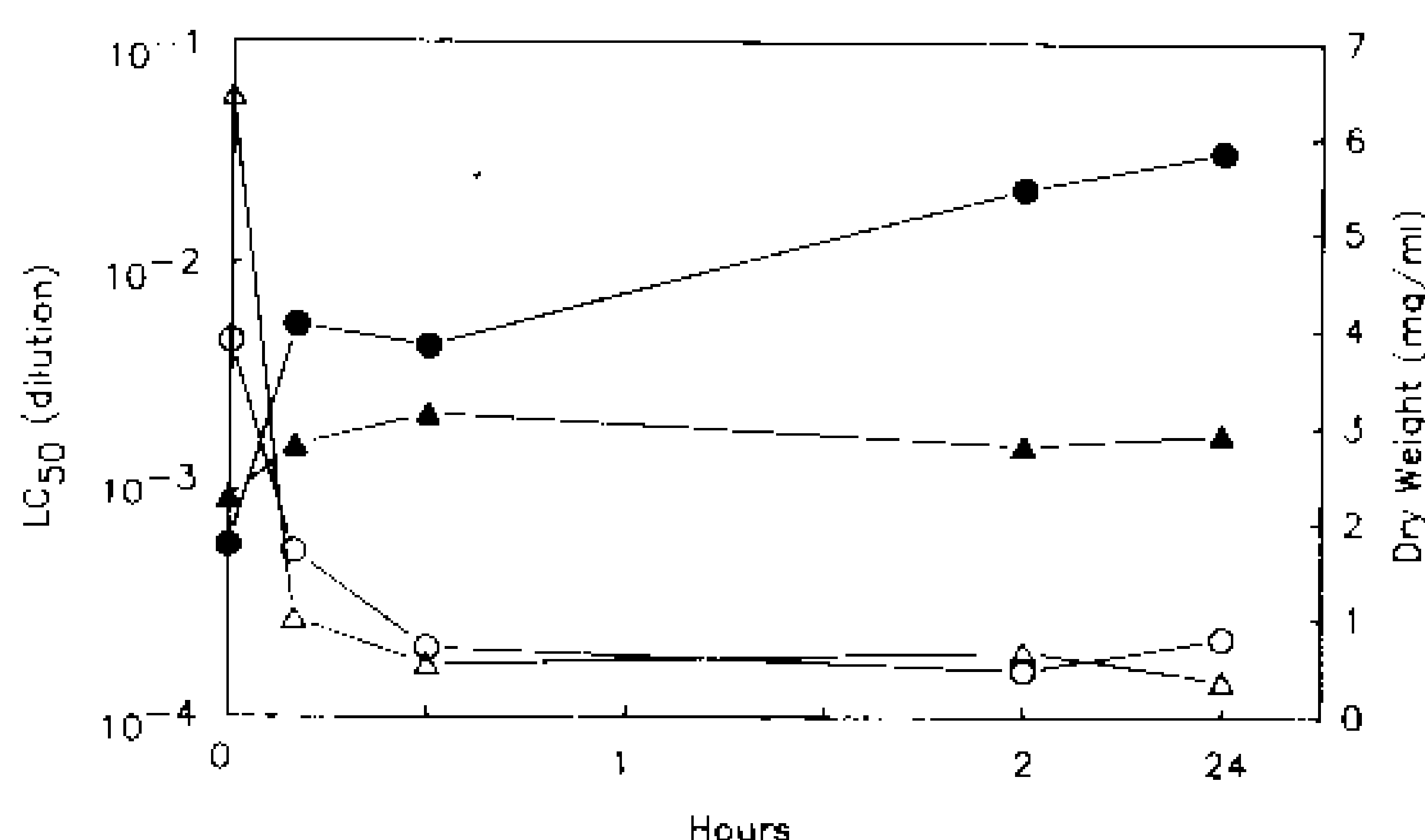

Fig. 2: *Bacillus sphaericus* and *B. thuringiensis* israelensis spores mixed with pond sediment and allowed to settle in a column. Symbols: (○) dry weight of B.t.i.-sediment mixture; (Δ) dry weight of *B. sphaericus*-sediment mixture; (●) LC_{50} of B.t.i. suspension; (▲) LC_{50} of *B. sphaericus* suspension.

Elimination of spores by aquatic invertebrates - Midges acquired spores directly from water and rapidly (96 hr) eliminated the spores following transfer to clean water (data not shown). Large numbers of spores were present in midge feces during the 24 hr immediately after feeding on spores, and this number declined in parallel to the decline in spores in the whole animal. Leaf shredding and predatory stonefly larvae acquired spores from spore-bearing leaf discs or from spore-laden midge larvae respectively and eliminated the spores from their guts within one week (Figs 3, 4). However, cranefly larvae that ingested spores from contaminated leaf discs retained a high number of spores in the gut for up to four weeks (Fig. 5). Feces collected from these larvae contained spores during the first week after feeding but the fecal spore content then declined. When the alkaline anterior and neutral posterior gut segments were examined separately, it was found that most of the spores were retained in the posterior segment (Fig. 6). Spores were removed from the anterior (high pH) section of the gut gradually over four weeks.

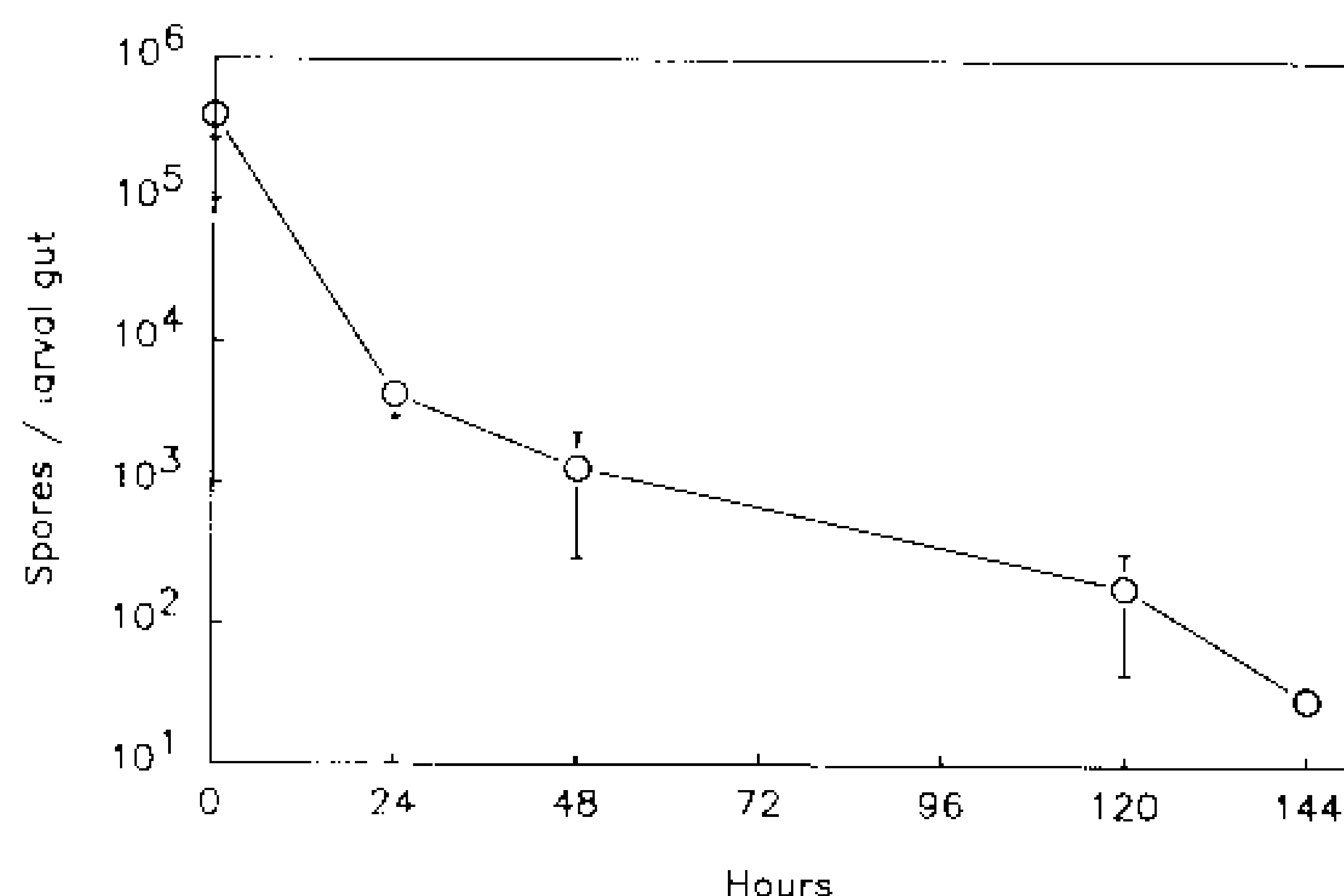

Fig. 3: elimination of *Bacillus sphaericus* spores from *Pteronarcys proteus* larvae. Error bars represent standard deviation from the mean.

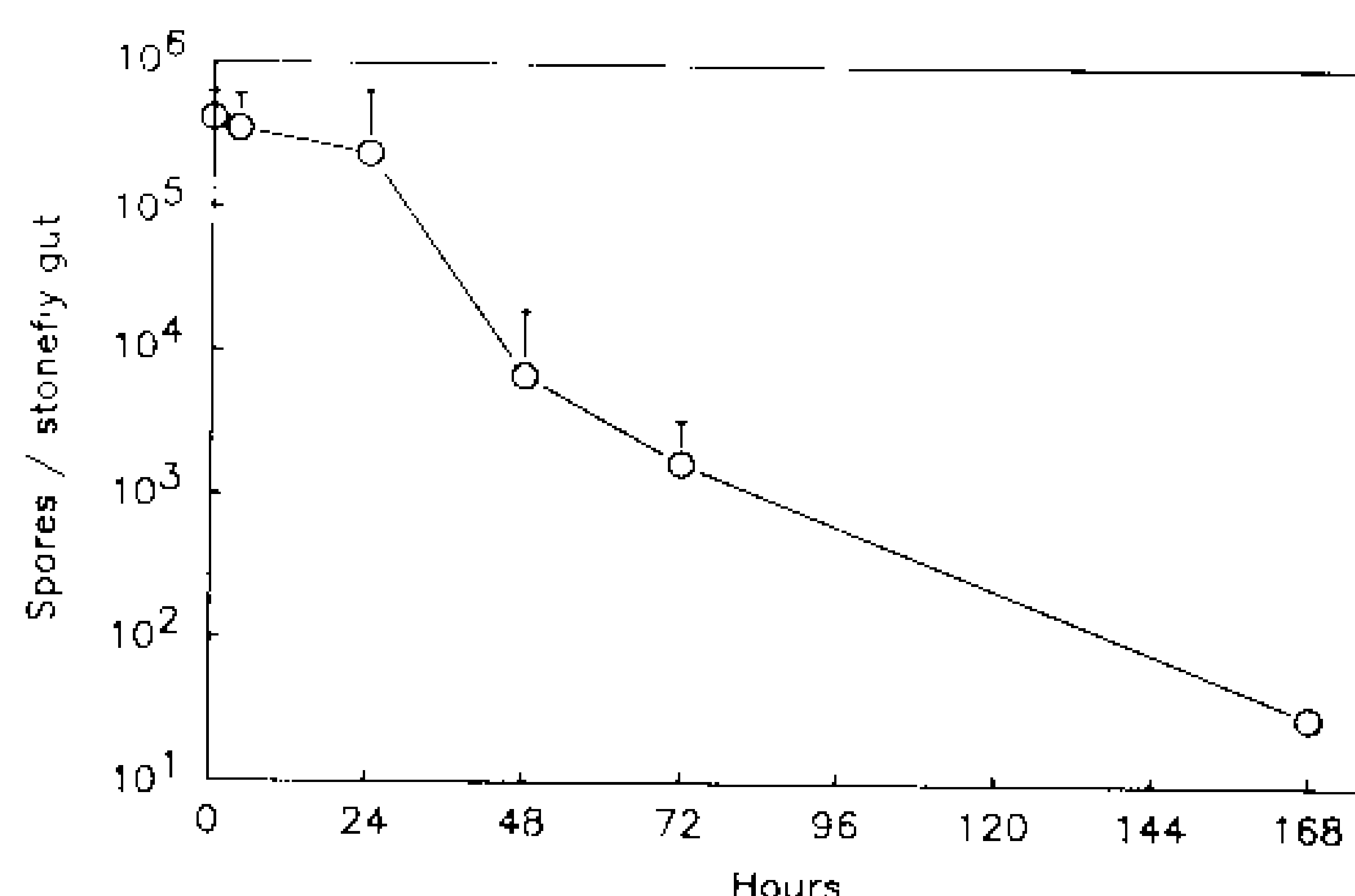

Fig. 4: elimination of *Bacillus sphaericus* spores from *Paragnetina media* larvae. Error bars represent standard deviation from the mean.

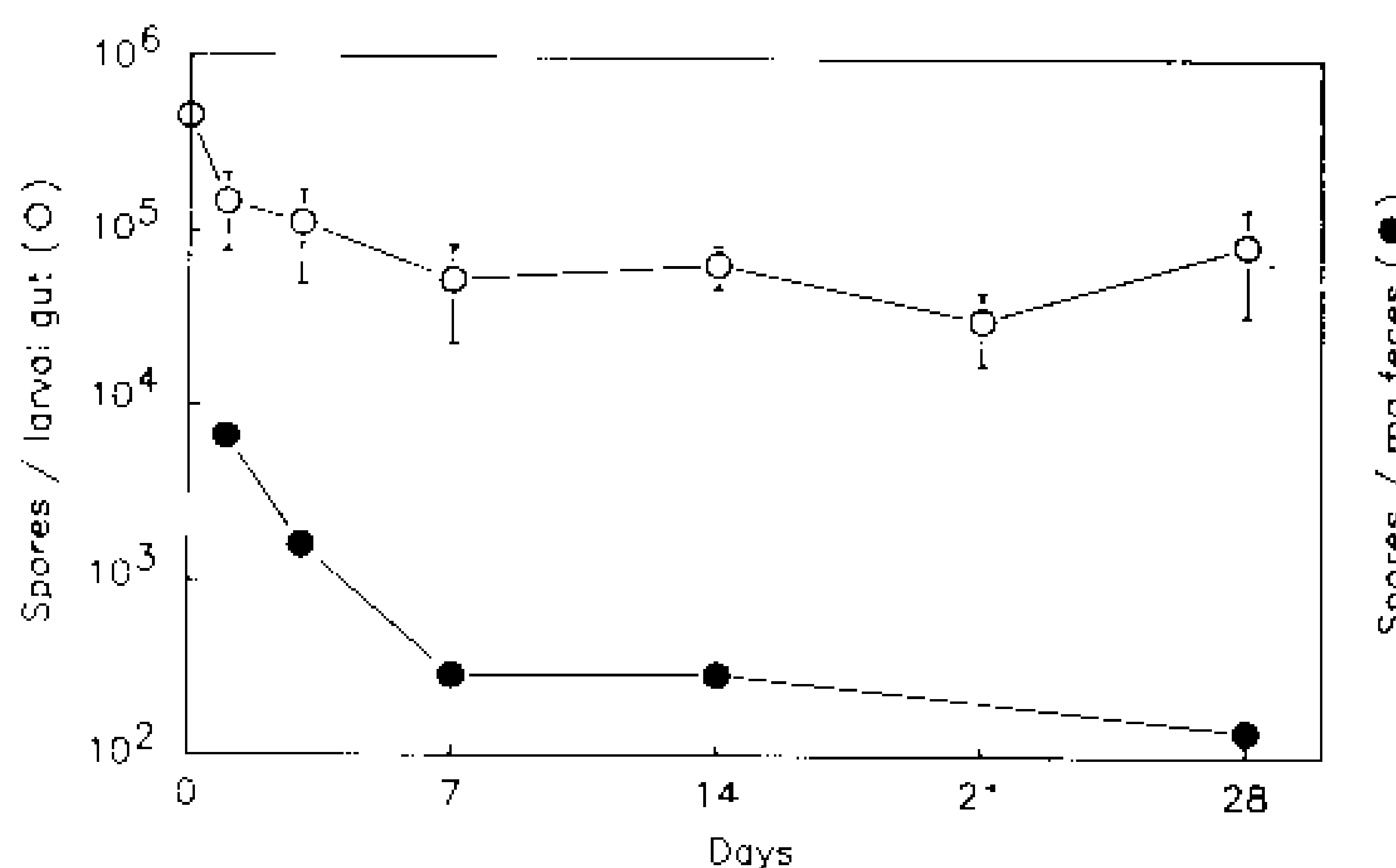

Fig. 5: elimination of *Bacillus sphaericus* spores from *Tipula abdominalis* larvae. (○) Spores/larval gut; (●) spores/mg feces. Error bars represent standard deviation from the mean.

Feces collected from cranefly larvae 24 hr after feeding on spore-laden leaf discs were bioassayed for toxicity to mosquito larvae. The LC_{50} of the spore stock suspension fed to larvae was $5.1x10^2$ spores/ml, but we were unable to obtain 50% kill of mosquito larvae with the highest number of spores available from feces ($3.0x10^4$ spores/ml).

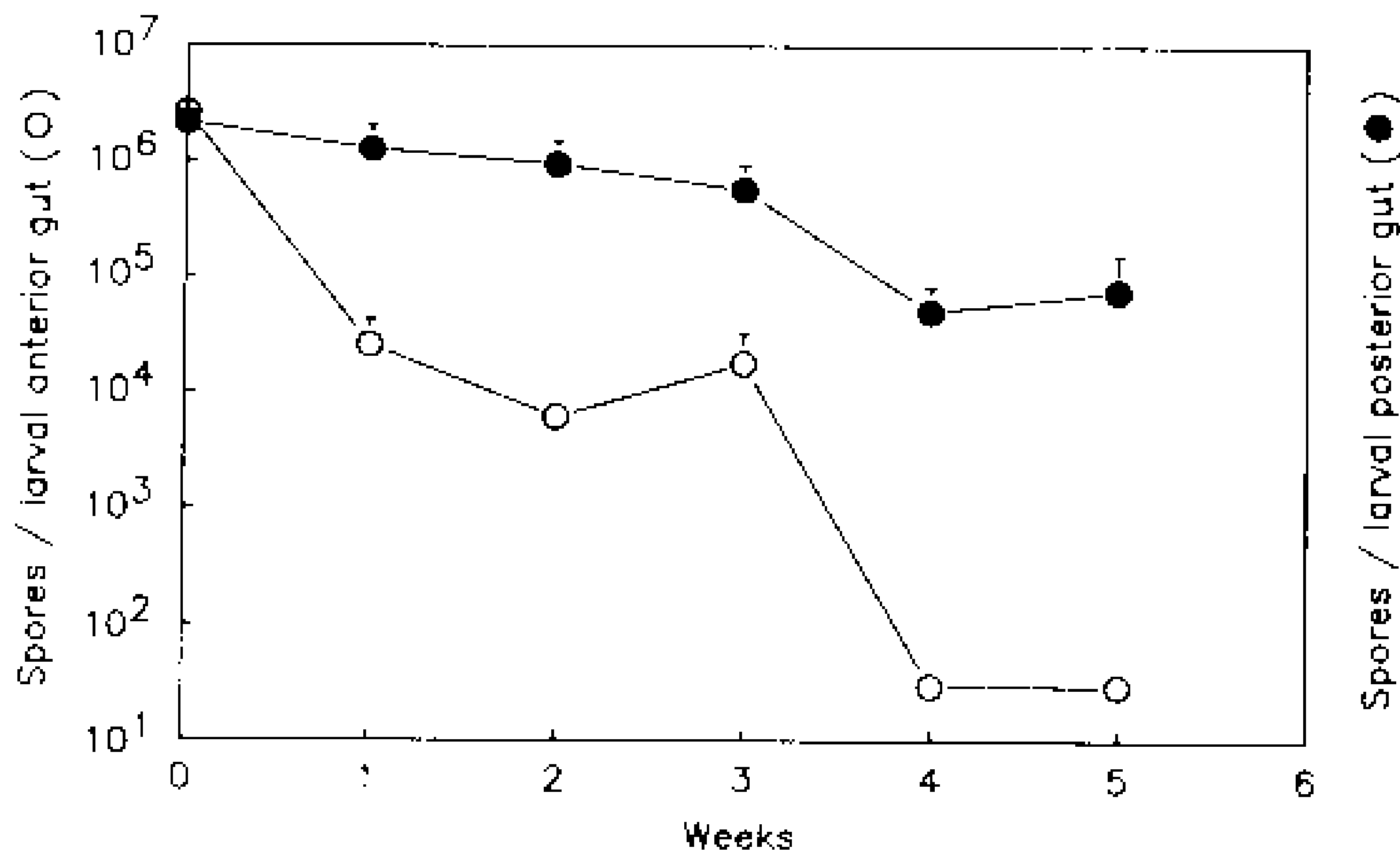

Fig. 6: elimination of *Bacillus sphaericus* spores from anterior and posterior gut segments of *Tipula abdominalis*. (O) spores/anterior gut segment; (●) spores/posterior gut segment. Error bars represent standard deviation from the mean.

DISCUSSION

B. sphaericus spores suspended in dialysis bags in fresh or seawater were exposed to varying combinations of temperature, salinity, pH, light, dissolved organic carbon, and other nutrient fluctuations that normally occur in the environment. Bags were permeable to substances of about molecular weight 10,000 to 12,000 or less. The decline in spore viability was more rapid in seawater than in freshwater. This decline could have resulted from loss of viability of the dormant spore, loss of ability of spores to germinate (indistinguishable from loss of viability), or death of spores that had germinated in the water. It is not known which factors in the environment are most important in influencing loss of spore viability. It has been reported (Hertline et al. 1979) that viable spores could be recovered up to nine months after introduction into soil. However, it is unknown what percentage of the spores originally introduced were still viable and what percentage resulted from bacterial growth in larval cadavers.

After spores and toxic paraspores are delivered into water, they sink to the bottom. The rate at which this occurs and the degree to which they remain on the bottom are factors in determining the persistence of the larvicidal effect. In calm water, most particulate matter will be at the bottom when spores are delivered. However, under more turbulent conditions a considerable amount of sediment may be in suspension. Neither *B. sphaericus* spores nor B.t.i. spores were carried to the bottom of the column by the settling of decaying pond sediment. The latter settled within 30 min, but most spores remained in suspension past this time indicating that spores had not attached to the sediment particles. During the 30 min of sediment settling, there was also a decline in toxicity of both B.t.i. and *B. sphaericus* preparations. The B.t.i. preparation lost more toxicity than *B. sphaericus* and the B.t.i. toxicity continued to decline over the following 24 hr. This indicates a difference in the tendency of the toxin of these bacteria to adhere to sediment and to be carried to the bottom with it. This undoubtedly depends on the nature of the sediment. Of practical importance is that adherence of toxin to sediment may limit the extent to which the toxin may be resuspended by water disturbances. The action of wind or animals is less likely to resuspend toxin that is adhering to particles than it is to resuspend toxin that is not attached. Also, once resuspended by turbulence in water, toxin adhering to particles will sink more rapidly. These observations may be a partial explanation for the greater persistence of *B. sphaericus* larvicide compared to B.t.i.

The four aquatic insects studied in these experiments were unharmed by eating *B. sphaericus* spores and toxin. Three of the four insects eliminated spores from the gut in one week or less. Viable spores were demonstrated in both midge and cranefly feces. This indicates that spore digestion was not the reason for removal of spores from these animals and that viable spores survive transit through the gut. Predation was shown to be a possible route for entry of spores into the predatory stonefly (*P. media*).

Cranefly larvae were found to be unusual in that viable spores were present in high numbers in the posterior larval gut for up to five weeks. One possible explanation for this apparent retention of spores is that some spores had germinated and given rise to an actively growing vegetative bacterial population in the gut. This population might produce the spores detected in the gut. However, total viable plate counts gave a number about equal to the spore counts. Thus, if vegetative *B. sphaericus* were present, their numbers were about equal to or less than the number of spores present. We cannot eliminate the possibility that a small *B. sphaericus* population became established in the cranefly posterior gut. However, it seems more likely that spores were lodged in some part of the posterior portion (perhaps the rectal lobe described by Sinsabaugh et al. 1985) and remained there in a dormant form. The anterior portion of the cranefly gut is sufficiently alkaline to solubilize the *B. sphaericus* parasporal toxin. The reason for cranefly immunity to the toxin is most likely due to the absence of suitable binding sites for toxin. This is apparently the reason for the resistance of many insects to *B. thuringiensis* toxin (Gill et al. 1992) and appears to be the reason for resistance of *Aedes aegypti* larvae to *B. sphaericus* toxin (Davidson 1988). Bioassay of cranefly feces revealed no residual toxicity for mosquito larvae. Thus, the solubilized toxin must either be destroyed by passage through the gut or have been lost from the fecal material. This suggests that consumption of

toxin by nontarget invertebrates that happen to have an alkaline gut may be one path by which *B. sphaericus* toxin is eliminated from the ecosystem.

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