

## FATE OF *BACILLUS* *SPHAERICUS* AND *BACILLUS* *THURINGIENSIS* SEROVAR *ISRAELENSIS* IN THE AQUATIC ENVIRONMENT<sup>1</sup>

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**ABSTRACT.** *Bacillus sphaericus* spores were suspended in bottles of filtered (0.45  $\mu\text{m}$ ) freshwater and seawater under various conditions of temperature, pH and salinity. Heat resistant culturable counts (spores) slowly decreased with time. Spores suspended in dialysis bags submerged in a freshwater pond or in flowing seawater underwent a more rapid drop in heat resistant spore counts than did spores held in bottles. Thus, laboratory studies may overestimate spore longevity in the environment. Spore settling rate was related to the nature of particulate material in the water column. Paraspores (or perhaps spores and toxin) of *B. thuringiensis* serovar *israelensis* (*B.t.i.*) had a greater tendency to adhere to and settle with suspended sediment and fine particulates than did paraspores of *B. sphaericus*. These observations may at least partially explain the greater persistence of *B. sphaericus* larvicidal activity in field tests than that of *B.t.i.*.

### INTRODUCTION

Some strains of *Bacillus sphaericus* produce a protein toxin that is lethal to several species of mosquito larvae upon ingestion. The toxin is produced as bacteria sporulate and aggregate as a parasporal inclusion or "crystal" inside the cells. The toxic inclusion body of *B. sphaericus* is held within the exosporium and tends to remain attached to the spore following lysis of the mother cell. This arrangement differs from that of *B. thuringiensis* serovar *israelensis* (*B.t.i.*) where the paraspore is independent of the spore upon sporangial lysis. When the *B. sphaericus* toxin is delivered into the aquatic environment for the control of mosquito larvae, large numbers of viable spores are also delivered (Yousten 1984).

The fate of the spores in aquatic environments is unclear. Hertlein et al. (1979) recovered viable spores from mud up to 9 months after application. Applying *B. sphaericus* 2362 to an experimental pond, Davidson et al. (1984) found about a 2-log decrease in spore numbers at 2 wk in the water column and recovered many viable spores from bottom mud after 3 weeks. Similar observations of spore settling and persistence in mud were reported by Nicolas et al. (1987). Karch and Charles (1987) found an approximately constant number of *B. sphaericus* 2362 spores present on the bottom of a treated cesspool from days 3 to 28 following their application. Counts of heat stable cells indicated that spore germination did not occur in the nutrient rich condi-

tions. However, bioassay indicated the toxic inclusion was apparently dissolved during this period. In none of these studies was it determined what fraction of the applied spores remained dormant in the mud or for how long they would persist.

Conditions that affect spore viability over a 308 day period were investigated by Lacey (1985). Spores held at 4° or 21°C lost heat resistance most rapidly (about 84–90 days) under acidic conditions. At 21°C they also lost heat resistance slowly at pH 9.2–10.8.

We report on the tendencies of *B. sphaericus* 2362 spores and toxin to adhere to certain defined and undefined particulate materials. These results are compared with those obtained with spores and toxin of *B. thuringiensis* serovar *israelensis* (*B.t.i.*). We also report the dormancy of *B. sphaericus* spores over time under various conditions of temperature, pH and salinity.

### MATERIALS AND METHODS

**Bacterial strains and spore preparations:** Spore dormancy studies used a spontaneous rifampicin-resistant mutant (2362-7) of *B. sphaericus* 2362 obtained from S. Singer (Western Illinois University, Macomb). Spores were prepared by growth of bacteria in NYSM broth (Lewis et al. 1987) for 48 h with shaking (175 rpm) at 30°C. Spores were recovered by centrifugation, washed twice with sterile distilled water, suspended in water and stored at 4°C. Spores used in settling and attachment studies were obtained from Abbott Laboratories, N. Chicago, IL. The preparations were: *B. sphaericus* 2362 (ABG 6262) and *B. thuringiensis* serovar *israelensis* (Vectobac 12AS, ABG 6193).

**Spore counts:** Spore numbers were determined by plate and direct microscope counts. For plate count determinations, 2-ml samples were heated at 80°C for 12 minutes. In experiments using spores of 2362-7, plating was carried out in

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NYSMRC agar (NYSM broth supplemented with 50  $\mu\text{g}/\text{ml}$  rifampicin, 0.002% cycloheximide and 2.0% agar). Plating of the Abbott *B. sphaericus* and *B.t.i.* spores was on NYSM agar. Pond water samples (1 ml) which were at pH 8.4 or which had been adjusted to pH 6.5 were mixed with 1 ml of 0.5 M potassium phosphate buffer (pH 7.0) prior to heating for spore counts. Seawater samples (2 ml at pH 8.0) were heated without dilution in buffer. Plates were incubated at 30°C for 48 hours. Direct microscope counts were performed in freshwater samples using a Petroff Hauser chamber and in seawater samples using the acridine orange microscope count (AODC) (Francisco et al. 1973).

*Spore dormancy in bottles:* Pond water from Shadow Lake (Blacksburg, VA) was centrifuged (9800  $\times g$ ) for 15 min and the supernatant passed through an 0.45  $\mu\text{m}$  membrane filter. The dissolved organic carbon in this water was 1.5 mg/liter. A second sample obtained from the bottom of the same pond contained finely divided sediment of rotting leaves and other decomposing organic matter. Total carbon in the sediment-containing water at the beginning of the experiment was 158 mg/liter. Part of each water sample was left at the initial pH of 8.4 and part of each used to prepare 0.05 M pH 6.5 MOPS buffer. One ml of 2362-7 spore stock was added to each of the water samples and 100 ml aliquots of each was placed in bottles. Two bottles containing water at pH 8.4 or 6.5, filtered or with sediment, were incubated at 15° and 30°C. At intervals the bottles were well shaken and samples taken for spore counts.

Synthetic seawater was used to examine the effects of salinity (10, 20 and 30%) and temperature (4, 15 and 30°C) on spore persistence. The effect of salinity was determined at 30°C; the effect of temperature was determined at a salinity of 20%. Synthetic seawater (Instant Ocean, Aquarium Systems, Mentor, OH) was made to the desired salinity and placed into bottles. Duplicate bottles for each variable were filled with 100 ml of seawater and autoclaved. Bottles were inoculated with 0.35 ml of *B. sphaericus* spore stock. The bottles were shaken prior to sampling.

*Spore dormancy in dialysis bags:* Spores from the stock suspension were added to water taken from Hoge Pond (Blacksburg, VA) or Santa Rosa Sound (Gulf Breeze, FL), which had been previously centrifuged and filtered (0.45  $\mu\text{m}$ ). For both fresh and seawater experiments, three dialysis bags were filled with 25 ml of spore suspension. Freshwater bags were placed in a nylon mesh bag for protection and submerged in Hoge Pond to a depth of approximately 0.5 m. The saltwater bags were placed in a flow

through aquarium that delivered filtered (30  $\mu\text{m}$ ) seawater from Santa Rosa Sound at a rate of 750 ml/min. The bags were removed at intervals and samples taken to determine spore concentration by heat resistant plate count and direct microscope count. The suspensions were then placed into new bags and returned to their respective locations.

*Spore settling and adherence to substrates:* Two ml of either *Bacillus sphaericus* 2362 or *B.t.i.* (Abbott Laboratories) spores were mixed for 1 h with 15 liters of suspended organic sediment (Shadow Lake). The suspension was poured rapidly into a column (178 cm height  $\times$  10 cm diam) and samples were withdrawn at intervals through serum stopper ports at the 8, 83 and 158 cm depths.

Settling studies were also performed using defined substrates of sand (Mystic White®, No. 45, New England Silica, Inc. South Windsor, CT), clay-silt (ASP® 400, Engelhard Corp., Edison, NJ) and activated charcoal N.F. powder (Merck and Co., Inc. Rahway, NJ). According to the manufacturers, the sand, clay-silt and activated charcoal had particle size ranges of 150–840, 4–62 and 30–40  $\mu\text{m}$ , respectively. The settling experiments were carried out as described for pond sediment except that spores were mixed with 6 mg/ml of each substrate and then poured into 1 liter graduated cylinders. Samples were removed by pipet at a depth of 8 cm and spore counts were performed as described above. Weights were determined on triplicate samples after drying at 110°C. Bioassays were performed as previously described against 2nd instar *Culex quinquefasciatus* Say larvae (Yousten and Wallis 1987).

*Organic carbon analysis:* Organic carbon in seawater was determined before inoculation and during the tests using the Carlo Erba Instruments NA1500 nitrogen/carbon analyzer. Organic carbon in freshwater was determined with a Dohrman model DC80 carbon analyzer.

## RESULTS

*Spore persistence under laboratory conditions:* *Bacillus sphaericus* 2362-7 spores suspended in bottles of filtered (0.45  $\mu\text{m}$ ) pond water at pH 6.5 or 8.4 and at 15° or 30°C showed about a 30% decrease in heat resistant spore counts after 238 days. Rates of decrease and total decrease were about the same at both pHs and at both temperatures. Spores suspended in unfiltered, sediment-containing water showed a greater decrease in count than in filtered water (Figs. 1 and 2). The loss was most rapid at 30°C, pH 8.4 and represented about 90% of the initial population after 63 days. However, the loss only

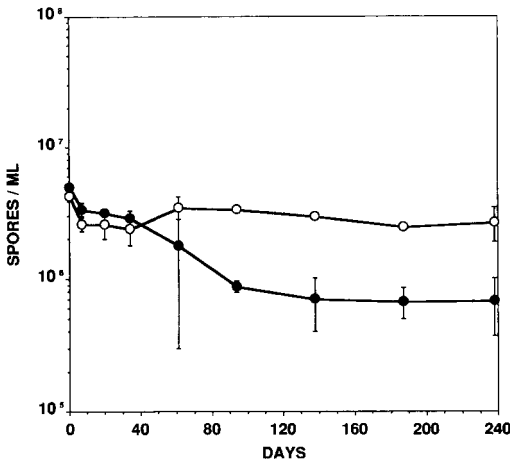


Fig. 1. Dormancy of *Bacillus sphaericus* spores suspended in unfiltered pond water (pH 6.5) in bottles held at 15°C (○) or at 30°C (●). Each point represents the mean of 2 bottles, and error bars denote standard deviation from the mean.

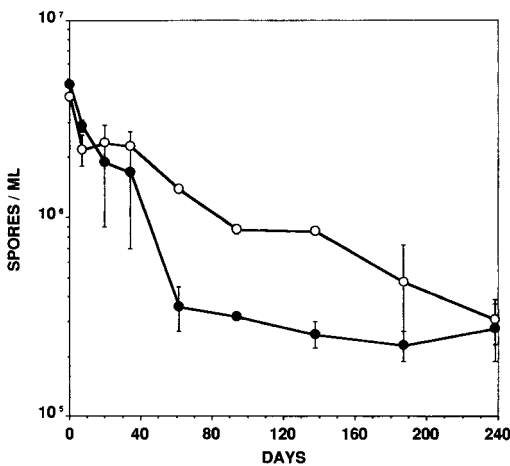


Fig. 2. Dormancy of *Bacillus sphaericus* spores suspended in unfiltered pond water (pH 8.4) in bottles held at 15°C (○) or at 30°C (●). Each point represents the mean of 2 bottles, and error bars denote standard deviation from the mean.

increased to 92% after 238 days. The spores appeared to retain heat resistance (dormancy) only at pH 6.5 and 15°C.

*Spore persistence in dialysis bags in freshwater:* The number of heat resistant spores suspended in dialysis bags submerged in a freshwater pond declined somewhat more rapidly than in the laboratory experiment where spores were suspended in bottles of unfiltered water held at pH 8.4, 30°C (the condition promoting the highest rate of loss of heat resistance) (Fig. 3). A drop of 74% in 4 wk was observed in the

dialysis bags versus 64% in 5 wk in the laboratory. The dissolved organic carbon in the pond averaged  $5.3 \pm 0.3$  mg/liter during the 4 wk of the experiment (samples taken at the initiation of the experiment and at weekly intervals). The water temperature ranged from a low of 12.5°C to a high of 26°C during that period. Petroff microscope counts that enumerated both heat resistant (dormant) and heat sensitive spores (germinated) verified that most of the spores placed in the bags at the initiation of the experiment were still present at the end. Therefore, the decline in the number of heat resistant spores detected by plating was not due to leakage from the bags or to loss during transfer to new bags.

*Effects of salinity on spore persistence under laboratory conditions:* Varied levels of salinity had no effect on spore dormancy (data not shown). Heat resistant counts of spores suspended in sterile seawater, adjusted to salinities of 10, 20 and 30‰ and incubated in bottles at 30°C, declined at statistically similar rates. The half life of spores was 10 days and the double half-life (75% loss) was 41 days. Temperature had no effect on spore dormancy in saline suspensions (data not shown). Heat resistant counts of spores contained in bottles of sterile seawater (20‰ salinity) incubated at 4, 15 and 30°C declined at significantly similar rates. A 98% reduction in heat resistant spore counts was observed after 131 days. The effect of pH

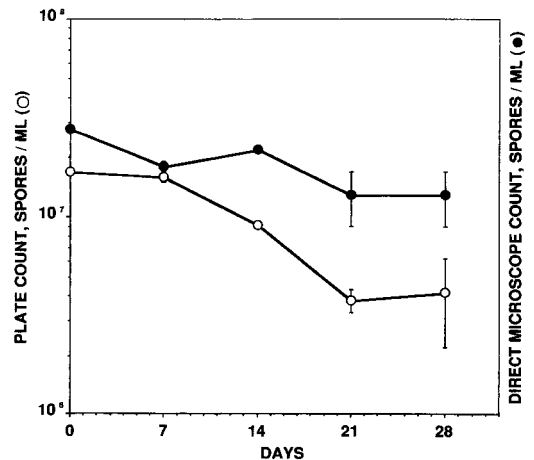


Fig. 3. Dormancy of *Bacillus sphaericus* spore suspensions placed in dialysis bags and submerged in a freshwater pond. At weekly intervals the suspensions were plated for heat resistant spores, counted microscopically for total spores, and transferred to new bags. Symbols: (○) heat resistant spores; (●) total spores by microscopic count. Each point represents the mean of 3 bags, and error bars denote standard deviation from the mean.

was not determined in the estuarine environment because seawater is buffered within a narrow range remaining close to pH 8.0 (Nybakken 1983).

*Spore persistence in dialysis bags in seawater:* Spore counts in seawater resembled those in freshwater. In seawater the heat resistant spore counts in dialysis bags declined more rapidly than the spore counts in bottles (Fig. 4). After 7 wk, heat resistant spore counts in dialysis bags declined 97.5% versus 84% in bottles. Total microscopic counts remained constant throughout the experiment, indicating that spores were not lost from the dialysis bags. During this experiment the salinity ranged from 18 to 32‰, the temperature ranged from 11° to 16°C and the dissolved organic carbon in the seawater ranged from 3 to 10 mg/liter.

*Spore and toxin adherence to defined particulates:* Settling rates of 3 defined particulates mixed with commercial preparations of spores and toxin are presented in Table 1. Sand settled most rapidly (less than 1 min), followed by charcoal and clay-silt. All had settled below the 8 cm sampling depth by 24 hours. Sand had no effect on settling of bacteria or toxin. Clay-silt produced detectable settling of spores and toxin of 2362 and a very large loss of spores and toxin of *B.t.i.* Charcoal produced a marked drop in both

spores and toxin of 2362 and an even larger drop in spores and toxin of *B.t.i.*

*Spore and toxin adherence to undefined substances in pond sediment:* Spore counts of *B. sphaericus* 2362 declined only slightly in 24 h at the 8, 83 or 158 cm sampling depths of the column ( $1.37 \times 10^6$  to  $1.20 \times 10^6$ /ml at 83 cm). However, the sediment had settled past all the ports by 30 min, indicating a lack of attachment of spores to sediment (Fig. 5). The toxicity (expressed as the dilution of the water achieving an  $LC_{50}$ ) declined slightly (data from the 83 cm port in Fig. 5). The *B.t.i.* spores also settled only slightly in 24 h ( $4.45 \times 10^6$  to  $3.8 \times 10^6$  at 83 cm) although the sediment had settled by 30 minutes. However, the toxicity of the *B.t.i.* preparation decreased by 1 log within 10 min and decreased by almost another log by 120 minutes. There was only a slight additional decrease between 120 min and 24 hours.

## DISCUSSION

The longevity of spores delivered into the field is important because of the role of viable spores in the recycling potential of *B. sphaericus*. Spore longevity will also be of importance when predicting the fate of genetically engineered strains of this bacterium (Trisrisook et al. 1990). We found spores to be stable for up to 238 days when incubated in the laboratory in filtered freshwater having a low dissolved organic carbon content. However, in water with high total organic carbon, the number of spores declined as the water temperature and pH increased. The very small decrease in spore count after 63 days in the unfiltered water at 30°C suggests a sub-population of spores that is viable but dormant under the conditions in the unfiltered water. When considering plate counts for spores, it is not possible to distinguish between loss of heat resistance (spore germination) and loss of the ability of spores to germinate since both would result in decreased plate counts after heating of the spores.

Spores incubated in seawater having a low dissolved organic carbon content were stable; heat resistant, culturable counts decreased slowly with time. Temperature and salinity had no effect on spore dormancy. Perhaps, as was observed in the dialysis bags, fluctuations in these variables may affect spore longevity. The decrease in heat resistant plate counts in the filtered seawater was, however, greater than that observed in freshwater. The reason for this difference is not clear. Perhaps the ionic components in seawater, even at a low salinity of 10‰ caused the more rapid decrease in heat resistant spore counts.

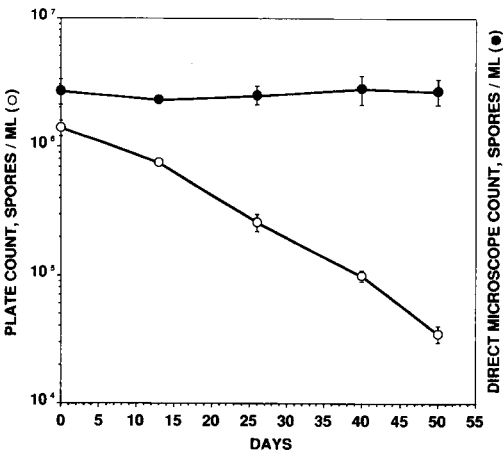


Fig. 4. Dormancy of *Bacillus sphaericus* spores in the estuarine environment. Spore suspensions in filter-sterilized (0.45  $\mu$ m) seawater were placed into dialysis bags. The bags were placed in an aquarium which received and discharged water (750 ml/min) from Santa Rosa Sound, Gulf Breeze, FL. Numbers of spores per ml were determined twice monthly. Symbols: (○) numbers of spores per ml as determined by heat resistant plate count; (●) numbers of spores per ml as determined by AODC direct count. Each point represents the mean of 2 bags, and error bars denote standard deviation from the mean.

Table 1. Settling of *Bacillus sphaericus* and *B. thuringiensis* spores and toxin mixed with particulate material.

Particulate	Time	Dry weight (mg/ml)	Spores/ml	LC <sub>50</sub> <sup>a</sup>
<i>B. sphaericus</i> 2362				
Sand	0	6.0	$2.0 \times 10^6$	$4.1 \times 10^{-4}$
	24 h	0.01	$1.9 \times 10^6$	$5.7 \times 10^{-4}$
Clay-silt	0	6.0	$1.4 \times 10^6$	$5.8 \times 10^{-4}$
	24 h	0.04	$8.2 \times 10^5$	$1.6 \times 10^{-3}$
Charcoal	0	6.0	$8.3 \times 10^5$	$2.4 \times 10^{-4}$
	24 h	0.02	$7.0 \times 10^4$	$3.7 \times 10^{-2}$
<i>B. thuringiensis</i>				
Sand	0	6.0	$7.8 \times 10^5$	$4.9 \times 10^{-4}$
	24 h	0.03	$9.6 \times 10^5$	$1.2 \times 10^{-4}$
Clay-silt	0	6.0	$4.8 \times 10^5$	$5.4 \times 10^{-4}$
	24 h	0.04	$1.8 \times 10^3$	$2.0 \times 10^{-1}$
Charcoal	0	6.0	$6.2 \times 10^5$	$3.7 \times 10^{-4}$
	24 h	0.01	$9.5 \times 10^2$	$2.0 \times 10^{-1}$

<sup>a</sup>LC<sub>50</sub> is expressed as the dilution of the sample which killed 50% of *Culex quinquefasciatus* second instar larvae.

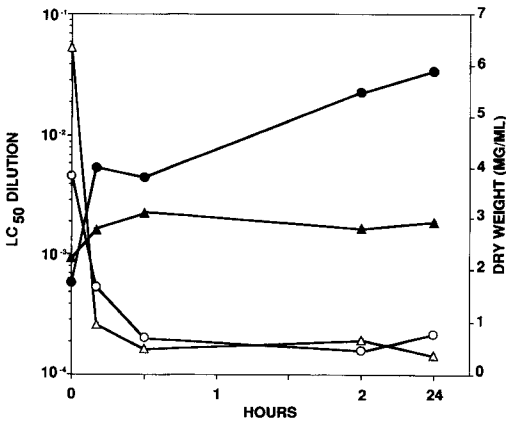


Fig. 5. Fate of *Bacillus sphaericus* and *B. thuringiensis* serovar *israelensis* spores mixed with pond sediment and allowed to settle in a column. Symbols: (○) dry weight of *B.t.i.*—sediment mixture; (△) dry weight *B. sphaericus*—sediment mixture; (●) LC<sub>50</sub> of *B.t.i.* suspension; (▲) LC<sub>50</sub> of *B. sphaericus* suspension.

Spores suspended in dialysis bags in a freshwater pond and in flowing seawater underwent a more rapid loss of heat resistance than spores held in bottles in the laboratory. In the bags the spores were exposed to varying combinations of temperature, salinity, pH, light and 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. This suggests that studies conducted in the laboratory may overestimate spore longevity in the environment where a combination of physical factors as well

as biological factors such as predation will remove spores.

Following delivery of *B. sphaericus* spores and toxic parasporal inclusions into water, these bodies 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. Our data indicate that the rate of sinking depends in part on the nature of the particulate materials which the spores encounter suspended in the water. In calm water most particulate material will be at the bottom when the spores are delivered; however, under more turbulent conditions a considerable amount may be in suspension. Neither *B. sphaericus* spore/parasporal (usually present as a single particle) nor the spore and independent *B.t.i.* parasporal showed any immediate tendency to attach to sand. Clay-silt removed some of the *B. sphaericus* spores and a disproportionate amount of toxicity. The *B.t.i.* spores and toxin were even more severely sedimented by clay-silt, resulting in the water being effectively detoxified. Charcoal also removed most of the *B.t.i.* spores and toxin and, like clay-silt, removed some *B. sphaericus* spores and a disproportionate amount of toxicity. Perhaps more of the *B. sphaericus* toxin occurs independent of the spore than previously suspected.

Neither *B. sphaericus* nor *B.t.i.* spores were removed from suspension by the settling of decaying pond sediment. This is in contrast to the report of Ohana et al. (1987), who found that *B.t.i.* spores were sedimented by 20 mg/ml of suspended mud. The difference may lie in the amount of sediment used (20 mg/ml by Ohana

et al. (1987) and 3.9 mg/ml in this study) or in the chemical nature of the sediment. However, both studies found the toxin to be removed by sedimentation with particulates. In contrast to *B.t.i.*, we observed that the toxin of *B. sphaericus* was only slightly diminished by settling sediment (even though almost twice as much sediment was mixed with *B. sphaericus*). There was a significant difference in the tendency of the *B.t.i.* paraspore and the *B. sphaericus* paraspore to adhere to and be carried to the bottom of the water column by suspended sediment under these test conditions. This undoubtedly depends upon the nature of the sediment, e.g., sand was ineffective with either bacterium. Perhaps of greater practical importance is that adherence of toxin to particulates 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 at least partially explain the greater persistence of *B. sphaericus* larvicidal activity compared with that of *B.t.i.*.

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