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PERSISTENCE OF *BACILLUS SPHAERICUS* IN CADAVERS OF MOSQUITO LARVAE

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ABSTRACT

Factors influencing the long-term efficacy of *Bacillus sphaericus* larvicide were studied in *Culex pipiens* larvae from a laboratory colony. The toxicity of *B. sphaericus-poisoned* larval cadavers significantly increased within a week elapsed since larval death and strongly depended on the number of cadavers per test. The toxicity did not depend on the concentration of the larvicide used to produce these cadavers. Dried larval cadavers harboring *B. sphaericus* spores remained toxic to the fresh larvae for at least a year, probably even more. Disintegration of the cadavers, that gradually occurs during this period, did not influence their toxicity. The correlation between the spore number and the larval mortality indicates that the propagation of *B. sphaericus* and a prolonged preservation of its spores in the dead larvae are the main sources of larvicide persistence. Dry 3-month-old *B. thuringiensis* subsp. z.vrae/e».vi.s-poisoncd cadavers were not toxic for *C. pipiens* larvae. Larvae that had survived at least a week-long contact with *B. sphaericus-poisoned* cadavers were able to pupate but produced only half as much adults as the control or larvae that were in short-term contact with cadavers.

KEY WORDS: *Bacillus sphaericus, Bacillus thuringiensis* subsp. *israelensis, Culex pipiens,* mosquito larvicide, larvicidal effect, spore recycling, larvicide persistence, sublethal effects, mosquito control.

INTRODUCTION

Some strains of *Bacillus sphaericus* are highly toxic to mosquito larvae of many species, especially of the genera *Culex* and *Anopheles.* This fact, as well as the safety of *B. sphaericus* to mammals and birds, and its minimal impact on natural enemies of mosquitoes render this bacterium a promising agent for long-term mosquito control (Hougard and Back, 1992; Lacey and Orr, 1994; Charles et al., 1996; Uspensky, 1996). Recently, *B. sphaericus* larvicide has been successfully applied in various mosquito control programs (Mulla et al., 1988; Karch et al., 1992; Hougard et al., 1993; Kumar et al., 1994). At the same time, the first commercial formulations appeared on the market (Bauer and Sinegre, 1995). However, a number of questions concerning *B. sphaericus* efficacy has not been answered yet. One of them is related to the persistence of this larvicide.

It is believed that *B. sphaericus* larvicide is able to persist in the environment for much longer time intervals than *Bacillus thuringiensis* subsp. *israelensis (Bti),* especially in waters polluted with organic material (Davidson, 1989; Lacey and Orr, 1994). However, by direct measurements of water samples' toxicity following *B. sphaericus* application, low persistence has been demonstrated (Davidson et al., 1984; Becker et al., 1995), unless the larvicide was appropriately formulated. At the same time, *B. sphaer icus* spores were recovered several months after treatment (Hertlein et al., 1979). A prolonged larval mortality after a single larvicide application was observed under a variety of conditions (Nicolas

polluted with organic material (Davidson, 1989; Lacey and Orr, 1994). However, by direct measurements of water samples' toxicity following *B. sphaericus* application, low persistence has been demonstrated (Davidson et al., 1984; Becker et al., 1995), unless the larvicide was appropriately formulated. At the same time, *B. sphaericus* spores were recovered several months after treatment (Hertlein et al., 1979). A prolonged larval mortality after a single larvicide application was observed under a variety of conditions (Nicolas et al., 1987; Karch et al., 1988). In this regard, spore recycling in larval cadavers has been considered as one of the possible factors responsible for larvicide persistence (Hertlein et al., 1979; Davidson et al., 1984; Karch and Coz, 1984, 1986; Charles and Nicolas, 1986; Becker et al., 1995; Correa and Yousten, 1995). This phenomenon has attracted the attention of researchers for the last 10–15 years, and the number of papers concerning this subject is constantly increasing.

The recycling phenomenon has also been observed in *Bti* (Aly, 1985; Aly et al., 1985; Z. Barak, personal communication), although *Bti* recycling has never become a subject of similar interest.

Bacterial recycling is the germination of ingested spores with subsequent multiplication of vegetative cells, and their sporulation in dead mosquito larvae (Charles and Nicolas, 1986). The following facts may be accepted as well established: (1) Mosquito larvae killed by *B. sphaericus* maintain the toxic level of the larvicide in the water reservoir. (2) *B. sphaericus* recycles in larval cadavers producing a 100–1,000-fold increase in spore count. (3) The recycling is not a species-specific phenomenon; it has been demonstrated in larvae of several *Culex, Anopheles* and *Culiseta* species. (4) The most effective recycling takes place in *B. sphaericus* strains producing the spore-associated binary toxin.

It is of obvious interest and importance to establish for how long larval cadavers may maintain the toxic level of the larvicide under different conditions. The duration of laboratory observations in the papers published on this subject was limited to a maximum of 3 months. We are reporting some data that extend the observation period to 18 months.

MATERIALS AND METHODS

Various aspects of the *B. sphaericus* recycling process were studied by bioassays with second- and third-instar *Cx. pipiens* from our laboratory colony. The colony was maintained and laboratory tests were conducted at a temperature of 28 ± 1°C with a natural photoperiod. All tests were carried out with *B. sphaericus* 2362 received from the Pasteur Institute, Paris.

The influence of the following factors on the toxicity of larval cadavers was tested: the concentration of larvicide used to produce these cadavers; the age of the cadavers; and the number of cadavers per test. To obtain a sufficient number of larval cadavers, larvae were placed in plastic jars filled with 500 ml of de-ionized water containing *B. sphaericus* larvicide at toxic concentrations. Larval cadavers were removed from these jars, washed twice with de-ionized water, and placed into new jars filled with fresh water and containing 50 intact larvae. Every second day, larvae were fed powdered dog food (supplemented with amino acids) dispersed over the water. Larval mortality was recorded after 48 and 96 hours. The fate of pupae emerging from surviving larvae was followed. Pupae were transferred into net-covered plastic glasses with fresh water and observed until death or emergence of adults. All tests were made in 3–4 replicates. The mean mortality values were compared using Student's *t*-test.

The preservation of *B. sphaericus* toxicity in larval cadavers was tested in the following manner. About 15–20 larvae per jar were killed with a toxic concentration of *B. sphaericus* larvicide. Further observations were made under two different conditions. Some cadavers were kept in a plastic jar from which the toxin-containing water was allowed to evaporate (complete drying took ca. 10 days). The jar was covered with a net to prevent cadavers from being damaged mechanically. Other cadavers were kept in a jar under water; more water was occasionally added to compensate for evaporation. Bioassays were made 1, 3, 6, 12 and 18 months after larval death. Jars with dry cadavers were filled with fresh water and 50 intact larvae were added. The same number of larvae was introduced into the jars where the cadavers were kept under water. The larvae were observed until death or pupation of the last individual, and the fate of pupae was followed as above. Food was given every second day. These tests were made in 2 replicates at each time-point. Spore counts that supplemented the results of bioassays were conducted according to Yousten et al. (1985). A limited number of tests with *Bti*-poisoned larval cadavers (3 and 6 months after larval death) were made using the same methodology.

A number of dry larval cadavers was placed in a closed glass jar and kept in the refrigerator. After one year of storage, the remains were milled into a fine powder; the powder (0.25 mg/L) was added to a container (10 L) with de-ionized water into which 200 larvae were introduced. The larvae were observed for 1 week; food was given every second day. The test was made in 2 replicates. The test was repeated with a standard *B. sphaericus* larvicide powder that was also kept in the refrigerator. The dose of the larvicide was 0.1 mg/L. The doses were chosen according to the preliminary data that showed that the standard larvicide is 2 to 3 times as toxic as the experimental powder.

RESULTS

B. sphaericus-poisoned cadavers placed in fresh water caused death of mosquito larvae. The mean mortality did not depend on the concentration of the larvicide used to produce these cadavers (Table 1). It depended on the age of cadavers (1–2 days [young] or 6–8 days [old] after death) and of their number in the jar (2, 10 or 25 cadavers). Higher mortality was observed with older cadavers; it increased with the number of cadavers in the jar (Table 1). There was a highly significant difference (t = 5.7–24.0; $P < 0.001$) between mortality elicited by cadavers of different age, as well as by different number of cadavers in the jar after 96 h of contact. The differences between the larval mortality after 48 and 96 h were highly significant (t = 4.6–9.35; $P < 0.01$ or even ≤ 0.001).

Some larvae that were in a prolonged contact (at least a week) with the *B. sphaericus*-poisoned cadavers survived and successfully pupated. Only about 40% of these pupae produced viable adult mosquitoes which was significantly different from the control ($t = 13.2$; $P < 0.001$) (Table 2). When the contact took place for less than 5 days, the emergence of adults from pupae was only slightly, insignificantly lower than in the control ($t = 1.06$; $P > 0.3$).

Larval cadavers caused death of intact larvae long after drying. When fresh water was added into jars where cadavers were kept for 1, 3, 6 and 12 months, the mortality of intact larvae reached 90 to 100% in a week (Table 3). Only after 18 months, larval mortality seemed to decline. If, however, larval cadavers were kept under a layer of water, their toxicity decreased

TABLE 1
Mortality of *Cx. pipiens* larvae elicited by the contact with *B. sphaericus*-poisoned larval cadavers: dependence upon the age and number of cadavers

Larvicide concentration,* mg/L	Age of cadavers, days	No. of cadavers per jar	Larval mortality, % Mean ± SD	
			After 48 h	After 96 h
0.1	1–2	2	2.5 ± 2.2	12.0 ± 5.1
		10	7.5 ± 3.8	38.3 ± 9.8
		25	47.5 ± 6.7	86.5 ± 10.1
	6–8	2	5.0 ± 3.6	33.8 ± 8.3
		10	82.0 ± 7.9	96.5 ± 4.4
		25	100	
5.0	1–2	2	3.3 ± 4.2	12.0 ± 2.0
		10	8.0 ± 3.5	35.3 ± 12.1
		25	50.8 ± 11.0	85.3 ± 6.1
	6–8	2	4.0 ± 3.3	35.3 ± 5.0
		10	80.8 ± 7.1	97.5 ± 2.6
		25	100	

*The concentration of larvicide in the jars from which the cadavers were taken.

TABLE 2
Emergence of adult mosquitoes from *Cx. pipiens* larvae that survived the contact with *B. sphaericus*-poisoned larval cadavers

Duration of contact with cadavers, days	No. of pupae (No. of experiments)	Emergence of adults, % Mean ± SD
<5	44 (6)	81.7 ± 10.2
>7	53 (7)	42.2 ± 5.8
0 (control)	76 (7)	86.9 ± 6.8

already after 3 months of storage and even more after 6 months. No mortality was observed 12 months after the beginning of the test (Table 3). The larval mortality correlated with spore counts (Fig. 1).

No larval mortality was observed in similar tests with a prolonged (3 and 6 months) storage of *Bti*-poisoned larval cadavers of *Cx. pipiens* either dry or under water.

A powder prepared from the remains of cadavers that were kept in a refrigerator for 1 year, caused high mortality of intact mosquito larvae in the 10-L container. The mortality after 7 days was the same as obtained with the standard *B. sphaericus* larvicide that was also kept in the refrigerator for the same time (Table 4).

TABLE 3
Larval mortality elicited by *B. sphaericus*-poisoned cadavers after prolonged storage of these cadavers under different conditions

Conditions of storage	Time of storage, months	Larval mortality within a week, % Mean ± SD
Dried	1	100
	3	95.0 ± 5.0
	6	100
	12	92.0 ± 4.0
	18	75.0 ± 25.0
Under water	1	100
	3	78.0 ± 12.0
	6	43.0 ± 9.0
	12	5.0 ± 5.0

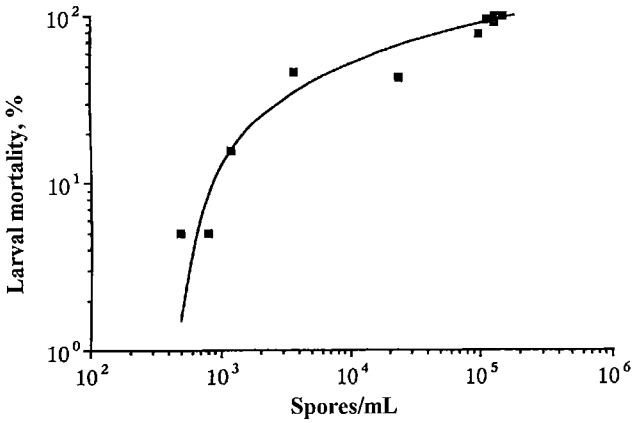

Fig. 1. Relationship between larval mortality elicited by *B. sphaericus*-poisoned cadavers and spore count in these cadavers.

TABLE 4
Efficacy of the powder prepared from *B. sphaericus*-poisoned larval cadavers and of *B. sphaericus* larvicide that were stored in the refrigerator for a year

Sample	Dose, mg/L	Replicate	Larval mortality, % After 3 days	Larval mortality, % After 7 days
Experimental powder	0.25	1	58	97
		2	63	99
Standard larvicide	0.10	1	85	98
		2	86	97

DISCUSSION

The toxicity of *B. sphaericus*-poisoned larval cadavers was found to depend only on the number of cadavers per test and the length of time passed since their death. It did not depend on the concentration of the larvicide that had caused the death leading to the cadavers. It was demonstrated in previous studies that the number of spores in a *B. sphaericus*-treated larva decreases during the first day after larvicide ingestion. Then it starts to increase rapidly reaching a plateau approximately one week after death of the larva (Davidson et al., 1984; Charles and Nicolas, 1986). The ability of cadavers to kill fresh larvae observed in our tests seems to follow the same pattern. The correlation between the spore number in the cadavers of different age and the larval mortality indicates that the propagation of *B. sphaericus* and preservation of its spores in the dead larvae is the main source of the larvicide persistence.

A delayed and reduced pupation in mosquito larvae that survived after *B. sphaericus* treatment was observed by Lacey et al. (1987) and Mulla et al. (1991). These authors recorded even lower emergence of viable adults, 10 to 25%. Our results demonstrate that sublethal effects of *B. sphaericus* appear only after a prolonged contact between cadavers and live larvae. It follows, that if the larvicide is applied at a point of time when mainly fourth-instar mosquitoes are present in the water reservoir, the impact of sublethal effects would not be high.

Dry larval cadavers harboring *B. sphaericus* spores remain toxic for at least a year, probably even more. Disintegration of the cadavers, that gradually occurs during this period, did not influence their toxicity. It is difficult to say whether the toxicity of cadavers begins to decline 18 months after larval death, since our results were not clear-cut. In one replicate, complete larval mortality was observed, whereas in the other only 50% of the larvae died (average, 75%). In control jars that initially contained *B. sphaericus* larvicide-treated water but not cadavers, the mortality of fresh intact larvae was similar to that of the pure control (without larvicide), not more than 10% (not shown in Table 3). This and the increase of mortality with the time of contact between larvae and cadavers indicates that *B. sphaericus*-poisoned larval cadavers were the only cause of death among fresh mosquito larvae.

Dried *B. sphaericus*-poisoned larval cadavers, that were kept in the refrigerator, preserved their toxicity for at least a year. The slower onset of larval mortality observed with powdered cadavers and the higher dose of the powder necessary to kill all larvae, as compared with the standard larvicide, may be explained by the presence in the powdered larval cadavers of an inert material such as chitin.

The cadavers that were kept under a layer of water, lost their toxicity after about 3 months. This phenomenon may be attributed to the ability of *B. sphaericus* spores to germinate and vegetate again in the spent medium containing the autolysis products. *B. sphaericus* is able to cycle in spent media resulting from sporulation-associated lysis of other *Bacilli* such as *Bti* or *B. subtilis* (S. Braun, unpublished data). This so-called cycling results in gradual decrease of the spore count in the medium with each cycle. Not only spores but the larvicide crystals are produced as well. These repeated cycles of germination, vegetative growth and sporulation are interrupted by drying. Thus, the spore count in dry cadavers remains relatively constant. It is unlikely that the protein crystal or the products of its proteolysis may be as stable as the spore under the conditions of microbial degradation following the death. Thus, our results may support the idea of the major importance of spores as compared with crystals in *B. sphaericus* toxicity and persistence.

Culex larvae predominantly employ a collecting-filtering feeding mode (Clements, 1992; Merritt et al., 1992). They remove particles suspended in the water column or associated with the water surface. However, most mosquitoes are believed not to be restricted to a single feeding mode and can employ 2 or more strategies. Larvae of some *Culex* species supplement the collecting-filtering with shredding and, sometimes, feed off dead or live invertebrates, even of their own species (Clements, 1992; Merritt et al., 1992; I. Uspensky, unpublished data). Biting small particles of cadavers' residues carrying *B. sphaericus* spores seems to be the main mode of poisoning of fresh *Cx. pipiens* larvae.

Our results demonstrate that larval cadavers are an important factor in the persistence of *B. sphaericus* larvicide. The phenomenon of *B. sphaericus* recycling should be taken into account for the improvement of the practical mosquito control efficiency. Under the conditions of high larval density in a water reservoir, the larval population is continuously replenished by egg-batches laid by adult mosquitoes. Larval cadavers settle down for as long as about 48 h after death (Porter et al., 1993). Thus, there is always a sufficient number of cadavers, retained in the feeding zone of mosquito larvae, to ensure toxicity. When, however, larval cadavers settle to the bottom of the reservoir without rapid replacement by fresh cadavers in the upper layer of the water, one would not observe persistence. This may explain the conflicting reports on *B. sphaericus* persistence. One may observe that most of the reports on field persistence were made in tropical or subtropical areas where mosquito populations are dense during most of the year or even all year round.

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