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Evaluating the Elimination of Brazilian Entomopathogenic *Bacillus* by Non-target Aquatic Species: An Experimental Study

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Abstract Ecotoxicity tests are key to predict environmental hazards resulting from chemical and biological pesticides in non-target species. In order to assess the effects of microbial pesticides it is important to determine if they cause infection in test organisms. At present the microbial elimination rate or clearance is not included in ecotoxicological regulatory protocols. This study evaluated the elimination of *Bacillus thuringiensis* and *Bacillus sphaericus* from fish and snails, after 30 days' exposure to commercial formulations of such entomopathogens. Data obtained showed that in clean water the tendency to eliminate microbial agents from the body of the exposed organisms is gradual over time but after 7 days the fish and snails were free of the two tested *Bacillus* spp.

Keywords *Bacillus thuringiensis* · *Bacillus sphaericus* · Aquatic persistence · *Danio rerio* · *Biomphalaria glabrata* · Biopesticides

Ecotoxicity tests have been increasingly used for the determination of harmful effects on aquatic organisms, due mainly to the potential risk of transfer of pollutants to these species, as well as to assess water quality to maintain aquatic life.

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Microbial pesticides have been presented as an alternative to chemical pesticides. Several studies have shown significantly higher efficiency and selectivity of Brazilian entomopathogens, which generate a lower environmental impact (Oliveira-Filho 2005; Grisolia et al. 2009; Oliveira-Filho et al. 2011). The use of microbial products for mosquito control has also been of great importance to developing countries, where the return of diseases transmitted by mosquitoes has become a public health problem in recent years (Sabbatini et al. 2010). In this context, the United States Environmental Protection Agency (USEPA) has developed several guidelines for evaluating adverse effects from microbial pesticides on aquatic species (USEPA 1996a, b). The protocols do not differ much from conventional toxicological tests, since the main objective is to determine the presence of any hazardous toxin in the strains of microorganisms and estimate the effects of these strains and their toxins on non-target species (Oliveira-Filho 2005). In several mammalian test guidelines, the persistence of entomopathogens is an important point to investigate the potential infectivity of microbial pesticide control agents. However, this is one of the points neglected by guidelines for evaluating adverse effects on aquatic species.

Thus, the aim of this study was to evaluate the presence and the time for eliminating microbial agents from non-target aquatic species, after exposure to biopesticides composed of two entomopathogenic strains of *Bacillus* sp.

Materials and Methods

The non-target species chosen were the native benthic snail *Biomphalaria glabrata*; Gastropoda (Say, 1818); the exotic zebrafish *Danio rerio*; Cyprinidae (Hamilton, 1822); and

the native mato-grosso fish *Hyphessobrycon eques*; Characidae (Steindachner, 1882). Adult snails with shell diameter of 12–15 mm were obtained from the colony maintained at the Laboratory of Ecotoxicology of Embrapa Cerrados. *D. rerio*, a wild type, weighed 0.2–0.4 g with an average length of 2.0–3.0 cm, and *H. eques* weighed 0.3–0.5 g and had an average length of 2.5–3.5 cm; both were purchased from a commercial supplier in Brasília, Brazil.

The organisms were exposed for 30 days to solutions of two commercial biopesticides, products registered and marketed for use in Brazil. These were, a *Bacillus thuringiensis* product (BT1) serotype *israelensis*, encoding Cry4A, Cry4B, Cry11 and cyt1 proteins, toxic to dipteran larvae (Monnerat et al. 2005), and a *Bacillus sphaericus* product (BS1) serotype H5 encoding 51 and 42 kDa proteins, toxic to dipteran larvae (Monnerat et al. 2004). The first step was counting the number of spores present in the tested commercial products. To obtain these data 100 µL of each solution was submitted to heat shock (12 min—80°C and 5 min—0°C) and spread with a glass hockey stick spreader on Petri dishes with Embrapa medium (Monnerat et al. 2007) and penicillin (100 µg/mL) for BT1 and streptomycin (25 µg/mL) for BS1. Plates were incubated for 48 h at 22°C for determination of colony forming units (CFU), which is an estimate of the number of viable bacteria or spores. To perform the present study the formulations containing around 10^8–10^{10} CFU/mL were submitted to serial dilutions to achieve a maximum hazard concentration of 10^6 CFU/mL as proposed by USEPA (USEPA 1996a) for toxicity tests with non-target aquatic species. The dilution water used was a non-sterilized synthetic soft water with hardness between 40 and 48 mg/L as $CaCO_3$ and pH in the range of 7.2–7.6, as standardized by the Brazilian Association for Technical Standardization (ABNT 2004). The static-renewal exposures were performed with the formulation solutions renewed twice a week. In the renewal of each solution, an aliquot was collected for the initial quantification of CFU/mL. In all cases the quantification of the initial concentration in each renewal procedure was between 10^6 and 10^7 CFU/mL. Dissolved oxygen was maintained in the range of 6.5 ± 1.0 mg/L. Fish were fed with commercial fish chow and snails with pieces of lettuce leaf daily. Ten fish of each species and ten snails were exposed individually in glasses of 300 mL. A control group of each species was maintained in the same conditions, exposed only to the synthetic softwater. After 30 days of exposure to the biopesticide, organisms were transferred to clean water, renewed each day, where they remained for 18 h until the first isolation, for 68 h until the second isolation and 168 h until the third and last bacterial isolation. At each time of CFU counting three organisms were removed from the solution. For counting at 168 h the last four organisms were removed. Each counting period was performed with a pool of all organisms taken from each species, and together macerated in a porcelain recipient and homogenized for subsequent addition of 2 mL of sterile distilled water. The macerated suspensions were divided into three replicates, pipetted in eppendorf microcentrifuge tubes and labeled. The isolation procedure was the same as that described for the formulations, but in this case three dishes for each pool of organisms were placed in an incubator for approximately 22 h for further observation and to count the CFU number. Final results of each dish were divided by the number of organisms used in the pool for the estimation of CFU/individual. For all dilution procedures CFU counting was performed manually with reverse lighting.

Results and Discussion

The results obtained show that the decrease in CFUs in non-target species for BT1 and BS1 was significant at 7 days (168 h) after the end of exposure. After 18 h of exposure *D. rerio* did not present any CFU of BT1 or BS1 in its body. On the other hand, at 18 h *B. glabrata* had 120 CFUs of BT1 (Fig. 1) and 200 CFUs of BS1 (Fig. 2) in its body, falling to 0 CFU of both microrganisms after 168 h. *H. eques* did not present CFUs of BS1 in its body after the end of exposure. After 18 h exposure 609 CFUs of BT1 were detected per individual. This number was reduced to 225 in 68 h, and finally the species was totally clear after 168 h (Fig. 1). The organisms maintained in the control group did not present any CFUs of tested *Bacillus* in their body.

For BS1 only *B. glabrata* presented CFUs in its body after 18 h of exposure. At this time 200 CFUs/individual were found, and at 68 h the number increased to 280, decreasing to 0 in 168 h (Fig. 2).

The elimination rate is an important parameter and needs to be included in microbial toxicity guidelines because this evaluation with entomopathogens is similar to the bioaccumulation factor for chemicals. As shown in other publications, *Bacillus thuringiensis* and *B. sphaericus* in the concentration of 10^6 spores/mL were not lethal to *Biomphalaria glabrata* (Oliveira-Filho et al. 2011) or to the fish *Danio rerio* (Grisolia et al. 2009). In the present study it was observed that the tested entomopathogens were not acutely toxic to *Hyphessobrycon eques* either. It should be noted that to evaluate the environmental hazard of biopesticides, acute tests and clearance evaluation are not enough. Snarski (1990) and Oliveira-Filho (2008) warn of the need to consider the potential effects of the formulation components and not only the quantification of microbial spores or CFUs for environmental risk assessments of

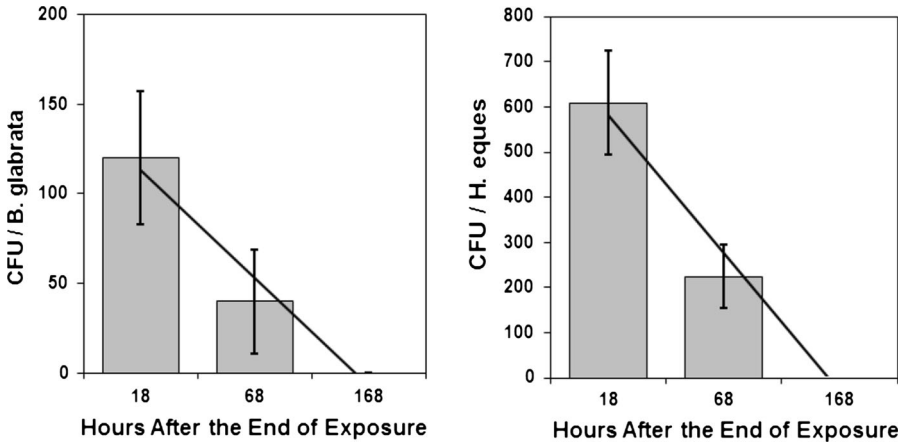

Fig. 1 Seven days' quantification of CFU from *Bacillus thuringiensis* in the snail *B. glabrata* and in the fish *H. eques* after the end of 96 h' exposure. Data are presented as means (±SD) of CFU/individual. The initial inoculum for exposure of both species was 1.6×10^6 CFU/mL

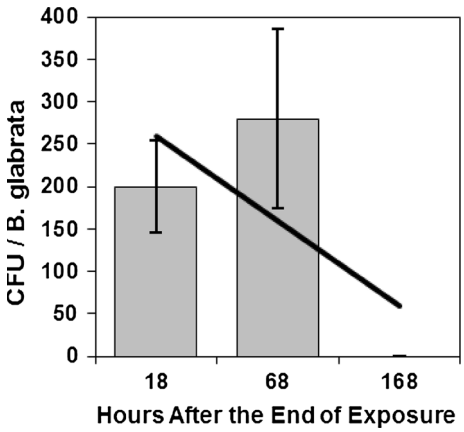

Fig. 2 Seven days' quantification of CFU from *Bacillus sphaericus* in the snail *B. glabrata* after the end of 96 h' exposure. Data are presented as means (±SD) of CFU/individual. The initial inoculum for exposure of this species was 5.0×10^6 CFU/mL

biological insecticides. Several studies reinforce the need to evaluate the toxicity of both the active ingredient and the formulation of chemical pesticides, because in many cases the formulation or some components may have greater toxicity than the active ingredient of the product (Cox and Surgan 2006; Pereira et al. 2009; Puglis and Boone 2011). However, there are no records of studies assessing this differential toxicity regarding biopesticides.

Snarski (1990), studying the persistence of *B. thuringiensis* subsp. *israelensis* in the fish *Pimephales promelas*, reported that the spore count was rapidly reduced after transferring fish to clean water, with a decrease of around 3 orders of magnitude in 1 day. This author shows that spores were rarely detected in fish after 8 days (192 h). As observed in Fig. 2, the initial count was lower than the second count. A similar effect was found with the same strain of *B. sphaericus* in mice (Oliveira-Filho et al. 2009). In the present study this increase can be related to a potential adsorption of spores in the shell of snails. In this case, a minimum difference in adsorption or in relation to shell size of the organisms can be responsible for an increase in the recovery of CFUs. It should be noted that in any case at the end of the observation time (168 h) was recorded absence of CFUs in the last group (Fig. 2). Yousten et al. (1995) reported that after feeding larvae of four aquatic non-target insects with *B. sphaericus* spores, three species eliminated all spores in less than 1 week. In research with the crustacean *Gammarus lacustris*, Brazner and Anderson (1986) observed that on the 7th day post-exposure to *B. thuringiensis* subsp. *israelensis* a larger number of spores were still found in the whole body of organisms. These authors suggested that the gut of this species seems to be rapidly free of spores, but the persistence can be related to the spores adsorbed in their exoskeleton. Although crustaceans were not tested in the present study, the results obtained from snails suggest that adsorption in the shell may be the best explanation for the persistence of CFUs in *B. glabrata*, considering that the experiments were conducted using the whole body of the organism. It should be pointed out that the USEPA (1996b) affirms that certain forms of microorganisms (e.g. spores) may be cleared from the host animal at a slower rate than vegetative forms (CFUs) of the same microorganism, and then the elimination of CFUs does not mean clearance, or a disappearance of the microorganism, but a step to reach it. For the Agency the data should be interpreted in function of the decline curves generated.

This study demonstrated that the fish and snails eliminated the tested *Bacillus* from their body after 7 days of recovery in clean water. In snails it was observed that CFUs of *B. sphaericus* were found in higher number after 68 h of recovery, but in any case, the tendency presented by the obtained data shows that total elimination is imminent and that the persistence of spores or the bacteria for a time does not seem to be deleterious to the organism. It can, however, be of importance in spreading the

entomopathogen throughout the aquatic ecosystem, favoring the control of target species. Overall, the presented methodological proposal was efficient for detection and quantification of entomopathogens persistence in non-target aquatic species, and the regulatory agencies should consider including this endpoint in the current assessment protocols.

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