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Environmental Contamination and Toxicology

Effects of Commercial Microbial Products on the Immune System of *Elliptio complanata* Mussels

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Commercial biotechnology products are gaining interest in our society for applications as diverse as pest control (biopesticides or transgenic crops), septic tank cleanup, organic stain removal and organic matter waste recycling. These products are often promoted as being safe for the environment because of their biological make-up and their supposed absence of persistent toxic products; hence they are not recognized as contaminants. For example, pests such as lepidopteran and dipteran insects are readily controlled with suspensions of *Bacillus thuringiensis* (Bt) containing spores and crystalline δ-endotoxins. The insecticidal properties of δ-endotoxin (Cry1Ab) have been available commercially for biological pest control for at least 40 years (Porcar, 2003). The continuous application of this biopesticide or other commercial microbial products could have unsuspected contaminating effects on freshwater environments (Ichimatsu, et al., 2000). Indeed, Bt was found in nearly half of the 107 water samples examined in Japan, with a relatively low environmental mean density of 45 counts of Bt per 100 mL. However, not all these Bt counts were necessarily toxic to dipterans (Iriarte et al., 2000). Bt could be toxic to non-target aquatic species, such as chironomids, as well. For example, Bt *israelensis*- (Bt*i*) inoculated pools along the Tai Tan River had significantly fewer chironomids and mosquito larvae than control ponds, with the possibility of selection of Bt*i*-resistant chironomid species (Dickman, 2000).

Applications of bacterial suspensions or mixtures could find their way into aquatic environments, thereby exposing aquatic species to higher loads of bacterium. Freshwater mussels are sessile endobenthic organisms that feed on phytoplankton and bacteria suspended in the water column. The immune system of bivalves is comprised of an open-circulating system called a hemolymph and it is in close contact with suspended material in surface water through the gills and in ingested material. The immune system in these bivalves largely depends on cellular immunity because they lack the immunoglobulins normally found in vertebrates (Glinski and Jarosz, 1997). The immune system has cytokine-like and opiate signalling pathways and it is subject to inflammatory conditions normally associated with disease (Hughes et al., 1992; Magazine et al., 1996; Gagné et al., 2005).

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The purpose of this study was to examine the *in vitro* effects of three commercial bacteria-based products on the immune function of the freshwater *Elliptio complanata* mussel. Three commercial products (Table 1) were evaluated for their capacity to disrupt hemocytic cellular integrity and their capacity to ingest fluorescent bacteria after an exposure period of 24 h to various dilutions of commercial microbial products in the laboratory.

MATERIALS AND METHODS

Mussels were collected by hand in a lake known to abound in *Elliptio complanata* mussels. They were transported at 4°C and placed in 300-L tanks filled with a continuous flow of charcoal-filtered and UV-treated tap water at 15°C. Mussels were allowed to acclimatize for one month and were fed twice a week with a commercial coral reef feed solution enriched with *Selenastrum capricornutum* algae (1–5 x 10^6 algae).

Table 1. Commercial microbial-based products examined.

Product	Bacterial composition	Use	Example
Product I	*Bacillus thuringiensis* subspecies *kurstaki*	Pest control of lepidopteran insects	Crop protection from infesting larvae
Product II	Bacterial consortia Nitrifying bacteria ?	Ammonia removal	Ammonia removal in ponds
Product III	Bacterial consortia Unknown composition	Sanitation product for eliminating odour and organic waste in aquatic environments	Cleans aquarium tanks

Hemolymph was collected from 10 mussels, pooled and mixed together at 4°C. These tissues were taken from the posterior adductor muscle with a syringe and aliquots of 200 µL were seeded in black polystyrene microplates. Bacterial suspensions, obtained commercially in Canada, were added (100 µL) to obtain a logarithmic dilutions of 0.04, 0.2 and 1% volume/volume (v/v) dilution in triplicates. The commercial bacterial suspensions were diluted in 25 mM NaCl containing 10 mM Hepes-NaOH, pH 7.4. The microplate was then left to stand in the dark for 24 h at 15°C under conditions of saturated humidity. At the end of the exposure period, the supernatant was removed by aspiration from cells adhered to the bottom of the wells and rinsed with 25 mM NaCl containing 10 mM Hepes-NaOH, pH 7.4 (200 µL). Immunocompetence was assessed on adhered cells using a miniaturized microplate assay developed by Blaise et al. (2002). Phagocytosis was determined by the amount of ingested *Escherichia coli* bacteria prestained with fluorescein isothiocyanate (Molecular Probes, USA) after incubating for 2 hours at room temperature. In separate wells, cell viability

and total proteins were also determined by the carboxy-fluorescein diacetate assay (fluorescein retention) and the fluorescamine assay, respectively. Standard solutions of fluorescein and serum bovine albumin were used for calibration. Lipid peroxidation in hemocytes was also determined by the thiobarbituric acid method (Wills, 1987). The data are expressed as μg thiobarbituric acid reactants (TBARS)/mg proteins using tetramethoxypropane for calibration.

Exposure experiments were replicated n = 3 times and critical differences between control and treated cells were determined by an analysis of variance followed by Dunnett's *t* test. A correlation analysis was performed with the Pearson-moment method. The threshold concentrations were obtained by the geometric mean of the lowest effect concentration and the no effect concentration in % v/v: (lowest effect concentration x no effect concentration)$^{1/2}$. Significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

A general description of products I, II, and III is shown in Table 1. The safety and efficacy of bacteria-based products, especially those containing complex bacterial consortia, are difficult to assess (Dubois et al., 2004). Moreover, there is considerable uncertainty about the relative proportion of bacteria added during the manufacturing process, which makes accurate risk assessment for aquatic biota difficult. Product I consists of a *Bacillus thuringiensis-kurstaki* (Btk) suspension for the control of lepidopteran (biting) insects (i.e. mosquitoes), while products II and III are commercial bacterial consortia products whose microbial composition is unknown.

Exposure of hemocytes to increasing concentrations of Btk suspension (Product I) produced significant changes on some of the immune parameters (Figure 1). Phagocytic activity was significantly induced at 0.2 % v/v with a threshold concentration of 0.07%, while intracellular esterase activity (cell viability) was significantly reduced at 1 % with a threshold concentration of 0.4%. Phagocytic activity was positively correlated with intracellular esterase activity ($R = 0.49$, $p = 0.05$), but phagocytic efficiency (phagocytosis/cell viability ratio) declined significantly at a threshold concentration < 0.04% (results not shown), indicating the potential of Bt suspensions to decrease phagocytic efficiency in immunocytes. No change in oxidative stress of hemocytes was observed, as determined by the content of TBARS in hemocytes. This suggests that Btk suspensions have opsonizing properties in hemocytes, but decrease the efficiency of phagocytosis. The toxic potential of bacterial consortia was also examined (Table 2). For product II, phagocytic activity was significantly reduced at a threshold concentration of 0.04%, while intracellular esterase activity was readily induced at a threshold concentration of 0.08%. No significant change was obtained in lipid peroxidation (LPO) or cell adherence. Intracellular esterase activity was not significantly correlated with phagocytic activity, but phagocytic efficiency was significantly reduced at a threshold concentration < 0.04%.

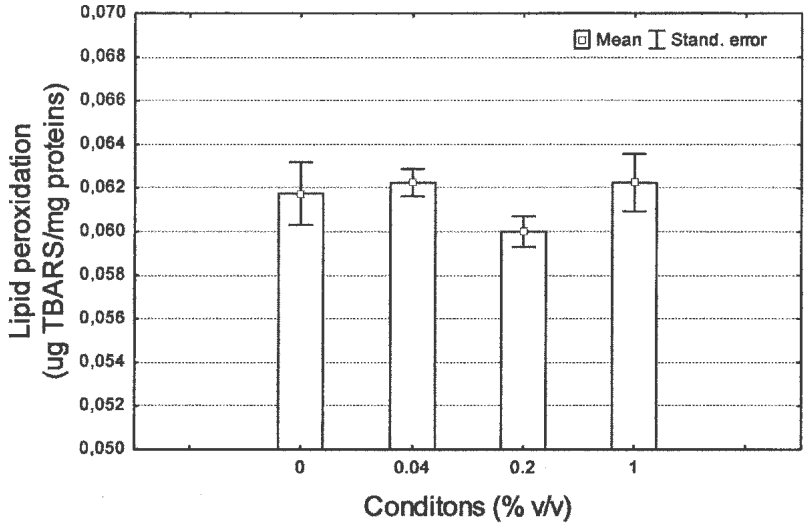
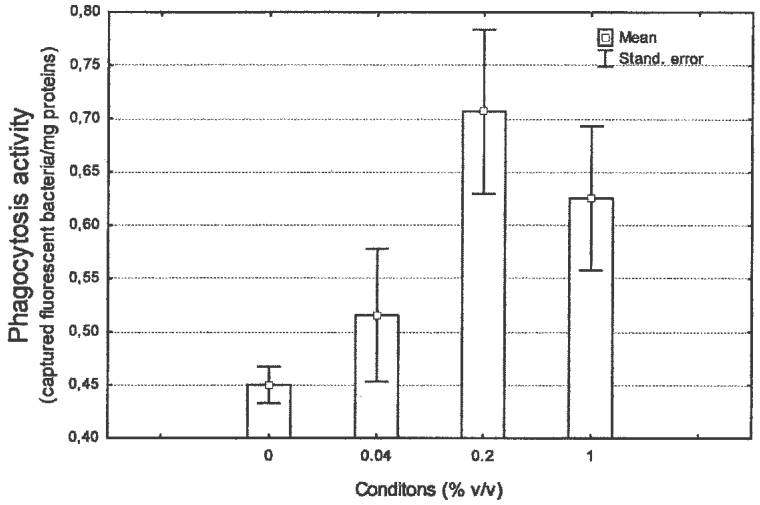
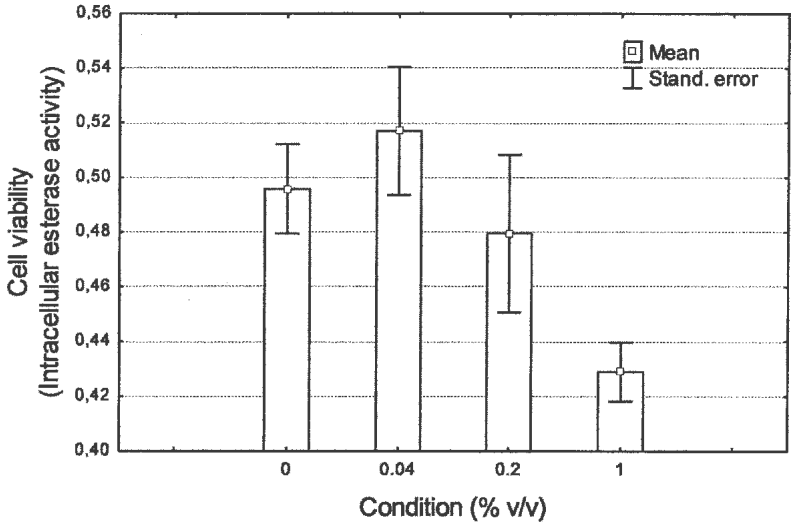


Figure 1. Effects of a *Bacillus thuringiensis kurstaki* suspension on the immune function of hemocytes. Hemocytes were exposed to increasing concentrations of a biopesticide suspension for 24 h at 15°C. After the exposure period, hemocytes were harvested for assessment of phagocytosis, cell viability (intracellular esterases) and lipid peroxidation.

For product III, all parameters were affected: phagocytic activity and intracellular esterase activity were induced at a threshold concentration of 0.45%, while LPO was induced at a threshold concentration of 0.09%. Cell adherence was significantly reduced at 0.45% v/v. Phagocytic activity was significantly correlated with intracellular esterase activity ($R = 0.72$, $p = 0.002$), but phagocytic efficiency, defined as the ratio of phagocytosis/esterase activity, was significantly reduced at < 0.04%. These results suggest that bacterial suspensions and mixtures could influence some of the immune functions of mussels. Phagocytic efficiency was always reduced when hemocytes were exposed to these bioproducts. On the one hand, the observed effects were found at concentrations around 0.04% — concentrations that are not supposed to be found in aquatic environments. However, mussels could accumulate bacteria during feeding and respiration and reached relatively high tissue concentrations. On the other hand, the behaviour of these bacterial mixtures in aquatic environments is not well understood at the present time. It is possible that these bacterial products could proliferate in surface water or sediment compartment, thus reaching levels that could harm the organisms. Their continuing use could contribute to exposing aquatic organisms like mussels to Bt products, but the extent of the exposure is difficult to characterize at present.





....Figure 1 continued

Table 2. Immune effects of selected bioproducts on *Elliptio complanata* hemocytes.

Products	Phagocytic activity (%)[1]	Cell viability	Adherence	Phagocytosis/ Esterase	LPO
Product I	0.07 (increase)	0.4 (decrease)	ND	< 0.04 (decrease)	ND[2]
Product II	0.04 (decrease)	0.08 (increase)	ND	0.25 (decrease)	ND
Product III	0.45 (increase)	0.45 (increase)	0.45 (decrease)	0.3 (decrease)	0.09 (increase)

1. Threshold concentration expressed in % v/v dilution: (lowest observable effect concentration x no-effect concentration)$^{1/2}$. 2. ND: not detected.

Mussels readily concentrate bacteria during feeding and perhaps even during respiration, which makes them species at risk to bacterial based products. Moreover, it is not known if the bacteria could proliferate in the water column at levels sufficient to elicit effects in hemocytes or provoke disease. This hypothesis should be further examined. Spores from Bt-*israelensis* adhere more strongly to suspended sediments and fine particles, on which mussels feed, than those of *Bacillus sphaericus* (Yousten et al., 1992). The occurrence of Bt is relatively rare in soils and sediments, as shown by Cr1Ab determinations (Douville et al., 20005). In another study, only 0.2% of Btk occurred in field isolates (Delucca et al., 1981). The first step in any risk assessment strategy, even before examining the biological effects, would be to characterize the environmental exposure of these bioproducts. However, the fate of these microbial products in the aquatic environment is difficult to predict at present; not only can they disappear by dilution or by degrading in the environment, but they may also proliferate in ecosystems and selectively accumulate in mussels. Bacteria have developed various strategies to evade recognition by hemocytes in mussels and our understanding about the factors eliciting phagocytosis if fairly limited in bivalves (Canesi et al., 2002). This would certainly help to understand the persistence of potentially pathogenic bacteria in mussel tissues. The development of a DNA-based (microarray-based) assay to characterize the bacterial make-up of these commercial microbial products (Dubois et al., 2004) would help determine if mussels are being exposed to these compounds in the environment including aquatic organisms.

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