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Microcystin accumulation and antioxidant responses in the freshwater clam *Diplodon chilensis patagonicus* upon subchronic exposure to toxic *Microcystis aeruginosa*

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ABSTRACT

We investigated the accumulation and toxicity of microcystin-LR (MCLR) in the digestive gland of the freshwater clam *Diplodon chilensis patagonicus*. Treated clams were fed with a toxic strain of *Microcystis aeruginosa* (NPJB1) during 6 weeks and control clams received the non-toxic strain NPDC1. Filtration rate was estimated for both groups. Toxic effects were evaluated through the hepatosomatic index (HSI) and different oxidative stress biomarkers, lipid peroxidation (content of thiobarbituric reactive substances–TBARS), protein oxidation (carbonyl groups) and reduced glutathione (GSH) levels, and enzymatic activities of superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST). The extractable MCLR measured by ELISA in digestive gland extracts showed little or no change during the first 3 weeks and increased significantly at weeks 5 and 6. HSI was reduced by 30% in treated clams at weeks 5 and 6. No significant oxidative damage to lipids or proteins was. All the antioxidant defense parameters analyzed were significantly increased at week 5 or 6. GSH increased in treated clams at week 5, reaching 62% increase at week 6. SOD, CAT and GST activities were significantly increased in treated clams by 50%, 66% and 60%, respectively, at the end of the experiment. *D. chilensis patagonicus* can be exposed to prolonged cyanobacterial blooms accumulating significant quantities of MCLR, which could be a risk for mammals and birds, which feed on this species and, in a lesser extent, to humans.

1. Introduction

Microcystins (MCs) are a family of cyclic peptide toxins produced by brackish and freshwater cyanobacteria of the genera *Anabaena, Anabaenopsis, Chroococcus, Microcystis*, *Nostoc* and *Planktothrix* (Chorus, 2001; Pearson et al., 2010). These toxins, as well as other hepatotoxins and neurotoxins, are secondary metabolites that accumulate in the cytoplasm under certain situations (Paerl and Millie, 1996). The most widely studied MC variant is microcystin-LR (MCLR), which is characterized by presenting the amino acids leucine and arginine in positions 2 and 4. Cyanobacteria can form massive blooms in freshwater bodies causing serious water pollution and public health hazard to humans and livestock (Carmichael, 1994; Park et al., 1998; Falconer, 1999; Oudra et al., 2001).

Most research related to MCs toxicity has been focused on acute effects on mammals (Harada et al., 1996; Ito et al., 2002; Botha et al., 2004; Moreno et al., 2005) and fish (Kotak et al., 1996; Freitas de Magalhães et al., 2001; Malbrouck et al., 2003; Cazenave et al., 2005). Due to their filter-feeding habits and their use as a food resource for humans, the capacity of marine and freshwater bivalve mollusks to accumulate and depurate cyanotoxins has been extensively studied (Eriksson et al., 1989; Amorim and Vasconcelos, 1999; Dionisio Pires et al., 2004; Ibelings and Chorus, 2007). In contrast, there are few studies that describe the effects of cyanobacterial toxins on bivalve mollusks, especially with respect to freshwater species. However, it is important to notice that these animals are important links between primary producers and

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higher consumers, and that they often play key roles in structuring aquatic communities (Eriksson et al., 1989; Falconer et al., 1992).

In Argentina, toxic blooms of cyanobacteria, especially of the genus *Microcystis* have often been detected in temperate rivers, lakes and reservoirs (Amé et al., 2003; Andrinolo et al., 2007). In North-West Patagonia, potentially toxic blooms of *Anabaena* have often been reported (Puig, 1992).

Bivalves are good bioindicators due to their close association with water quality (Doherty et al., 1988; Warren et al., 1995), and they are efficient filter feeders being able to take up phytoplankton, organic matter and suspended particles from the water column (Turick et al., 1988; Hebert et al., 1991). *Diplodon chilensis* (Gray, 1828) is a common clam in lakes and rivers of central-southern Chile and Argentina, which is considered, due to their size and filtering habit, as a key influence on the phytoplankton communities in these environments (Lara and Parada, 1991; Soto and Mena, 1999).

Previous studies suggest that oxidative stress may play a significant role in the mechanism of MCLR toxicity (Pinho et al., 2003; Li et al., 2003; Botha et al., 2004; Jos et al., 2005). Oxidative stress may be caused by either the overproduction of reactive oxygen species (ROS) or to the depletion of cellular antioxidant levels, particularly reduced glutathione GSH (Pflugmacher et al., 1998). Oxidative stress can lead to severe adverse effects on cells and tissues by causing lipid peroxidation and protein oxidation, possibly leading to enzyme inactivation and DNA damage (Winston and Di Giulio, 1991). Both ROS and lipid peroxides are known to be scavenged by the activity of antioxidant enzymes, such as gluthation peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) and non enzymatic defenses like GSH. GSH acts directly as a free radical scavenger (Halliwell and Gutteridge, 1999) and/or by conjugation to xenobiotics, playing an important role in the metabolic pathway leading to detoxification (Ding and Ong, 2003). Glutathione-S-transferase (GST) is an enzyme involved with in vivo MCs detoxification pathways, which catalyzes the conjugation of these toxins with GSH (Pflugmacher et al., 1998; Takenaka, 2001; Ito et al., 2002).

This study aims to investigate the accumulation of MCLR in the digestive gland of the freshwater clam *Diplodon chilensis patagonicus* (d'Orbigny, 1835) fed with a toxic strain of *Microcystis aeruginosa* during six weeks. The toxic effects of this treatment are evaluated through the hepatosomatic index (HSI), which has been reported as an indirect index of energy status in fishes and aquatic invertebrates (Chellappaf et al., 1995; Saborowski and Buchholz, 1996; Ahn et al., 2003) and as an indicator of contaminant stress (Pinkney et al., 2001; Mouneyrac et al., 2008).

Biochemical effects are evaluated through different oxidative stress biomarkers, lipid peroxidation, protein oxidation, GSH levels and activities of the enzymes SOD, CAT and GST. Since a pure cyanobacteria diet could affect the clams at an energetic level (e.g. providing more or less energy than the natural diet of *D. chilensis patagonicus*) and also the lipopolysaccharides present in the cell wall could produce biochemical effects, we used a non-toxic strain of *M. aeruginosa* as control diet.

2. Materials and methods

2.1. Organisms

The two *M. aeruginosa* strains used in these experiments were kindly provided by Dr. Raquel Soares from the Federal University of Río de Janeiro, Brazil. NPDC1 is a non-toxic strain and NPJB1 is a toxic strain that produces mostly microcystin-LR (Azevedo et al., 1994). The total (intracellular and extracellular) microcystin content of the culture used in this study, determined by ELISA assay (Brena et al., 2006), resulted in a concentration of 230 ± 27 ng MCLR equivalents/10^6 cells. The cells were lysed by three cycles of freezing and thawing prior to analysis. The determination was done in serial dilutions and each dilution was measured in triplicate. Other analytical details of the ELISA are described in Section 2.

M. aeruginosa was cultured in HSM-1 medium (Gorham et al., 1964) maintained at 24 ± 1 °C, with continuous cool-white fluorescent light illumination (15 μM photons m^{-2} s^{-1}). Cyanobacterial cultures were carried out in 50 mL flasks containing 30 mL of medium with an initial cell density of 30,000 cells/mL.

Individuals of *D. chilensis patagonicus* were collected by a diver at Yuco, an apparently pristine area of the Lacar Lake (40°10′S, 71°31′30″W) Neuquén province, Patagonia, Argentina, from the benthos at a depth of 6–8 m. The clams were transported alive at low temperature to the University of Buenos Aires, where they were used for laboratory studies. Clams identification was performed according to Vega Aguayo (1982).

2.2. Filtration rate

A filtration rate experiment was performed with the objective of determining the maximum concentration at which *D. chilensis patagonicus* could filter non-toxic and toxic cells of *M. aeruginosa* at similar rates. Twelve clams (6.07 ± 1.56 cm shell length) were put into individual beakers with 500 mL of dechlorinated tap water, with constant aeration and a temperature of 20 ± 1 °C. After 48 h of acclimation with no food, when each individual had the valves opened, *M. eruginosa* strains were added at a final concentration of 4×10^5 cells/mL. This concentration was chosen because in preliminary experiments it was the highest cell concentration of the NPJB1 strain at which all the clams kept their valves open. Samples were taken after 3 h and cell number was estimated by direct counting, using an Olympus light microscope at 1000×, with 0.1 mm deep counting hemocytomer (improved Neubauer chamber). Filtration rate for each individual, expressed as L/h per dry soft tissue mass (g), was calculated according to Jorgensen (1990). Dry soft tissue mass was measured after removal, dissection and drying in an oven for 48 h at 60 °C until constant mass.

2.3. Feeding experiments

Prior to the experiments, adult *D. chilensis patagonicus* (6.13 ± 1.49 cm shell length) were acclimated at constant temperature (20 ± 1 °C), photoperiod 12-h light: 12-h dark cycle and fed with the green alga *Chlorella kessleri* for three weeks. Following the acclimatization period, two groups of 36 animals were weighed and placed in 5 L containers with aerated dechlorinated tap water. The control group was fed with *M. aeruginosa* strain NPDC1 (non-toxic). The treated group received the toxic *M. aeruginosa* strain NPJB1. Both groups were allowed to filter cyanobacterial cells for 24 h, twice a week. The treated group received an estimated mean weekly dose of 0.625 μg MCLR/g clam. MCLR content of the cyanobacteria supplied to the clams at each feeding time was measured as described above. This procedure was repeated for six weeks. Water was changed before adding the cyanobacteria. Six individuals were sacrificed at the end of each week, and their body mass and shell length were recorded. Then, the digestive glands were dissected, weighed, thoroughly washed in ice-cold saline and homogenized individually in 0.134 M KCl, pH 7.4 (1:5 w/v) containing freshly prepared protease inhibitors (0.05 mM PMSF and 0.05 mM benzamidine). The same homogenate was used for all the biochemical determinations. The hepatosomatic index (HSI) was calculated as digestive gland mass/fresh body mass.

2.4. Quantitative analysis of microcystin in exposed clams

Digestive glands were dissected, weighed, washed in ice-cold saline, homogenized individually in 0.134 M KCl and treated with equal volume of chlorophorm prior to the determination of microcystins content by ELISA. The ELISA assay was performed essentially as described by Brena et al. (2006) using a polyclonal antiserum of broad specificity developed at the Faculty of Chemistry, Republic's University, Montevideo, Uruguay. We defined the MC detected by ELISA as "extractable MC". In animal tissues extractable MC is mostly comprised by free MC and conjugation products such as MC–glutathione, MC–cysteine–glycine and MC–cysteine (Metcalf et al., 2000). Protein-bound MC is not detected by our technique while detection of most degradation products is uncertain.

To evaluate the matrix effect of the analytical method we performed blank determinations in non-exposed *D. chilensis patagonicus* and studied the recovery of spiked samples. Background microcystin values determined in non-exposed clams were very low and the detection limit, calculated as the average result of the blanks plus three standard deviations ($n=6$) was 0.0040 μg MC/g of tissue. Spiked recoveries were very satisfactory, in the range 76–123%.

The coating antigen was prepared by coupling MCLR to thiol groups introduced in cationized and thiolated bovine serum albumin (BSA). ELISA plates were coated with the MCLR–microcystin–BSA conjugate (100 μL per well containing 70 ng/mL of conjugate in phosphate buffered saline, (PBS)); then they were blocked with 200 μL 1% BSA (m/v) in PBS, for 45 min and thoroughly washed with PBS containing 0.05% Tween.

The mixture containing microcystin standards (0.2; 0.6; 1.0; 1.5 and 2.5 μg/L) or adequately diluted samples in PBS containing 1% BSA and 0.5 g/L sodium azide