Ecotoxicology and Environmental Safety 120 (2015) 136–141

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Oxidative effects and toxin bioaccumulation after dietary microcystin intoxication in the hepatopancreas of the crab Neohelice (Chasmagnathus) granulata

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ARTICLE INFO

Article history:
Received 23 February 2015
Received in revised form
22 May 2015
Accepted 26 May 2015

Keywords:
Estuarine crab
Toxin bioaccumulation
ELISA
Microcystin depuration
Hepatopancreas

ABSTRACT

We studied the accumulation and depuration of microcystin-LR (MCLR) in the hepatopancreas of the crab *Neohelice granulata* fed twice weekly with either non toxic or MCLR-producing *Microcystis aeruginosa* (strain NPDC1 or NPJB, respectively) during seven weeks. We also analyzed MCLR effects on the oxidative stress- and detoxification-related variables, superoxide dismutase and glutathione-S-transferase activities, and the levels of reduced glutathione and lipid peroxidation (as thiobarbituric acid reactive substances, TBARS).

Hepatopancreas MCLR content slightly increased during the first three weeks, up to 8.81 ± 1.84 ng g^{-1} wet tissue mass (WTM) and then started to decrease to a minimum of 1.57 ± 0.74 ng g^{-1} WTM at the seventh week ($p < 0.05$ with respect to that in the first week). TBARS levels were about 55% higher in treated than in control *N. granulata* ($p < 0.001$ and $p < 0.05$) during the first three weeks of the experimental period. GSH content became 50% lower than in control individuals ($p < 0.01$) during weeks 6 and 7. SOD activity was increased by about 2-fold ($p < 0.05$ or $p < 0.001$) from week 3 to 7 in treated crabs with respect to control ones, while GST activity was about 70% higher in treated than in control crabs from week 4 to week 7 ($p < 0.05$).

Our data suggest that in the hepatopancreas of *N. granulata* MCLR accumulation and oxidative damage are limited and reversed by detoxification-excretion and antioxidant mechanisms. The activation of these defensive mechanisms becomes evident at 3-4 weeks after the start of the intoxication.

1. Introduction

Cyanobacteria blooms can affect water quality and cause intoxication on a great variety of aquatic and terrestrial species (Wiegand and Pflugmacher, 2005), including humans (Azevedo et al., 2002). Among the great variety of cyanotoxins known, microcystins are the most frequently reported as causing intoxication events (Sivonen and Jones, 1999; Dietrich and Hoeger, 2005; Dörr et al., 2010). In aquatic animals, although there are reports on the uptake of microcystin through other organs, e.g. fish gills; the main rout of microcystin uptake is the digestive system (Bury et al., 1998; Cazenave et al., 2005; Malbrouck and Kestmont, 2006). Despite the general trend towards biodilution along the trophic chain reported in several works, high toxin levels have been also detected in carnivorous fish (Negri and Jones, 1995; Li et al., 2003; Xie et al., 2005; Ferrão Filho and Koslowsky-Suzuki, 2011, for a review). This means that microcystin accumulation in invertebrates and planktivorous fish can be associated to a risk of human intoxication through direct consumption or through the consumption of predator fish.

Microcystin can be accumulated in different methanol-extractable forms (native microcystin, or bound to glutathione (GSH)

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http://dx.doi.org/10.1016/j.ecoenv.2015.05.041
0147-6513/

or cysteine), as well as bound to proteins (Carmichel and An, 1999; Kaya and Sano, 1999). Since most of the literature is focused on effects and accumulation of extractable microcystin and there is some consensus that trophic transference would be limited to that form (Ibelings and Chorus, 2007; Ferrão Filho and Koslowsky-Suzuki, 2011), only extractable microcystin will be considered in this work. Aquatic species accumulate a low proportion of the ingested microcystin, e.g *Daphnia* accumulates only 2.7% of the applied dose (Rohrlack et al., 2005) while in fish like rainbow trout 1.5% of the dose has been detected in liver after forced feeding with cyanobacteria cells (Tencalla and Dietrich, 1997). Once absorbed, microcystin is transported in the blood and accumulated in tissues, where it is subject to detoxification (Pflugmacher et al., 1998; Davies et al., 2005) and excretion, leading to the rapid reduction of the toxin burden (Williams et al., 1997a, 1997b; Pflugmacher et al., 1998; Chen and Xie, 2005; Contardo-Jara et al., 2008; Campos and Vasconcelos, 2010; Bieczynski et al., 2014).

Microcystis aeruginosa is one of the most commonly cited toxin-producing cyanobacteria (Chorus and Bartram, 1999; Ferrão Filho and Koslowsky-Suzuki, 2011) and the most frequently reported as toxic bloom forming in Argentina. Blooms of this species were detected in the Rio de la Plata since 1944 (Guarrera, 1950; Gómez and Bauer, 1997). However, a *M. aeruginosa bloom* with toxin production was first reported in 2001 (De León and Yunes, 2001).

Microcystin LR (MCLR) is a potent inhibitor of serine-threonine phosphatases, such as PP1 and PP2A, with the consequent effects on various cell functions (Eriksson et al., 1990; Honkanen et al., 1994; MacKintosh et al., 1990, 1995; Runnegar et al., 1993). In addition, as many other toxic compounds, microcystins can induce ROS formation (Wiegand et al., 1999; Li et al., 2003) and/or reduce the capacity of the organism's antioxidant system to respond against either endogenously or exogenously generated ROS (Amado and Monserrat, 2010). Several works show that, besides protein phosphatase inhibition, oxidative stress could play an important role in the toxicity of microcystins (Ding et al., 1998; Towner et al., 2002; Ding and Ong, 2003; Zegura et al., 2003; Amado and Monserrat 2010; Sabatini et al., 2011). It has been demonstrated that microcystin can be conjugated with the tripeptide glutathione (GSH), in a reaction catalyzed by the enzyme glutathione-S-transferase (GST) (Kondo et al., 1992; Pflugmacher et al., 1998; Takenaka, 2001; Ito et al., 2002). This reaction could reduce the intracellular pool of GSH, lowering the protection against oxidative stress (Storey, 1996). Evidences on the role of oxidative stress in the pathogenic effect of microcystins include increased ROS production and lipid peroxidation in rat hepatocytes exposed to lyophilized toxic cyanobacteria (Ding et al., 1998) and increased lipid peroxidation in rat liver exposed to microcystin (Guzmán and Solter, 1999).

The grapsoid crab, *Neohelice granulata* is a dominant species in estuarine intertidal environments of Brazil, Uruguay and Argentina, e.g. in the extensive salt marshes located in both margins of the Río de la Plata estuary and tributary rivers (Boschi, 1964; Botto and Irigoyen, 1979), where adults and larvae of this species constitute an important alimentary item for several commercially exploited fish. According to its deposit feeding habits (Iribarne et al. 1997), *N. granulata* could be exposed to ingest toxic cyanobacteria during cyanobacterial blooms. Previous works have demonstrated that microcystin LR can be absorbed in the digestive tract of this species (Vinagre et al., 2003; Pinho et al., 2003, 2005; Dewes et al., 2006). Thus, the possible accumulation of this toxin in *N. granulata* could imply a serious risk for fisheries and also for human health.

The aim of this work was to evaluate the accumulation and depuration of MCLR in the hepatopancreas of *N. granulata* fed twice weekly with cells of MCLR-producing *M. aeruginosa* during seven weeks. We also evaluated the possible oxidative stress produced by MCLR through the measurement of superoxide dismutase and glutathione-S-transferase activities, and the levels of reduced glutathione and lipid peroxidation.

2. Materials and methods

2.1. Organisms and exposure conditions

Two strains of *M. aeruginosa* were cultured in ASM-1 medium (Gorham et al., 1964), with continuous cool-white fluorescent light illumination (15 μM photons m^{-2} s^{-1}) and maintained at 24 ± 1 °C. Both, NPDC1, a non-toxic strain and NPJB, which produces mostly microcystin-LR (Azevedo et al., 1994) were kindly provided by Dr. Raquel Soares from the Federal University of Río de Janeiro, Brazil. Cultures were carried out for 15 days in flasks with an initial cell density of 30,000 cells/mL and then used for the experiment. Microcystin content in NPJB1 strain cultures, measured by ELISA, was 230 ± 27 μg MC 10^6 $cells^{-1}$ (Sabatini et al., 2011). The number of cells per mL was determined by cell counting in a Neubauer's chamber. Before being used cyanobacteria cells were centrifuged and resuspended three times in culture medium, in order to remove dissolved MCLR.

Adult male crabs (*N. granulata*) of similar body size (27–33 mm carapace width; 14.05–16.52 g body mass) were collected in a mud–sand flat at Faro San Antonio beach, near the Southern edge of the Río de la Plata estuary (Argentina). Animals were acclimated to the laboratory conditions for three weeks. Laboratory conditions were set at 20 ± 2 °C, 2‰ salinity and 12:12 light / dark photoperiod. Water of 2‰ salinity was prepared by adding HW Marine mix salts to dechlorinated tap water. Water was changed twice a week after animals were fed. Dissolved oxygen and pH varied from 5.1 ± 0.17 to 3.98 ± 0.64 and from 7.12 ± 0.26 to 6.86 ± 0.07, respectively, between water changes (YSI Professional Plus multiparameter instrument). Ammonia nitrogen varied from 0.77 ± 0.44 to 6.52 ± 0.56 mg/L (Hach DR 2800, method 343, modified from Reardon et al., 1966) between water changes.

Seventy crabs were randomly sorted into two groups, control and treated, placed individually in 2 L glass containers with 500 mL of aerated 2‰ water. The containers were tilted in order to allow the crabs to breath air.

Both experimental groups were fed with commercial rabbit feed (16% protein, 3% fat, 17% fiber, 42.5% nitrogen free extract, 10% ash, 1% calcium, 0.55% Phosphorous, 12% humidity) supplemented with *M. aeruginosa* cells (4×10^5 $cells\ mL^{-1}$ final cell concentration) of either NPDC1 or NPJB1 strain. In a previous study, we tested the lack of effects of NPDC1 cells effects on the oxidative balance of *N. granulata* (Sabatini, unpublished results). The rabbit feed pellets were ground, mixed with the corresponding cyanobacteria suspension and re-pelletized with a commercial pasta maker. Crabs were fed twice a week at a total ration of 1% body mass, and an approximate mean dose of 28 ng MC g^{-1} crab per week (0.15 ng MC g^{-1} feed) in the treated group. No crabs died during the 7 weeks of exposure to microcystin. Groups of 5 control and 5 treated crabs were sacrificed at 1, 2, 3, 4, 5, 6 and 7 weeks of treatment, each crab was analyzed individually.

2.2. Tissue dissection and sample preparation

Before dissection, crabs were weighed and rapidly killed by destroying the nervous system with a spike. The hepatopancreas was carefully removed, weighed and homogenized in 0.134 mol L^{-1} KCl (1:5 m/v) containing 0.5 mmol L^{-1} PMSF and 0.2 mmol L^{-1} benzamidine (protease inhibitors). The homogenates were centrifuged (microcentrifuge Hermle, Model Z216 MK) at 11,000g for 20 min at 4 °C, and the resulting supernatants were used for the biochemical determinations. Total soluble protein content was determined

according to Bradford (1976), using bovine serum albumin as standard. Results were expressed as mg total protein per mL. Hepatosomatic index (HSI) was calculated as hepatopancreas fresh mass/fresh body mass.

2.3. Microcystin quantification

Crabs' hepatopancreas were dissected out, weighed, washed in ice-cold saline, homogenized individually in 0.134 mol L^{-1} KCl and treated with equal volume of chloroform prior to the determination of microcystin content by ELISA, as described by Pirez et al. (2013) and Sabatini et al. (2011), using a broad specificity polyclonal antiserum developed at the Faculty of Chemistry, University of the Republic, Uruguay. The coating antigen was prepared by coupling MCLR to thiol groups and introduced in cationized and thiolated bovine serum albumin (BSA). The MCLR detected by ELISA was defined as "extractable MCLR". Data were expressed as nanograms of MCLR per gram of hepatopancreas wet tissue mass (ng g^{-1} WTM).

2.4. Oxidative damage

Lipid peroxidation was determined according to Beuge and Aust (1978) by the thiobarbituric acid reactive substances (TBARS) method. TBARS concentration was estimated using an extinction coefficient of 156 mM^{-1} cm^{-1}. Results were expressed as nmol TBARS per mg of protein.

2.5. Reduced glutathione (GSH) content

GSH levels were measured following the procedure of Anderson (1985). Absorbance (Shimadtzu UV160A UV–visible spectrophotometer) at 412 nm was read after 30 min incubation at room temperature and data were referred to a standard GSH curve. Results were expressed as nmol thiol (GSH equivalents) per mg of protein.

2.6. Enzyme assays

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured using the procedure of Beauchamp and Fridovich (1971). Samples were exposed for 15 min to intense cool-white light, and then kept in the dark until absorbance was read at 560 nm. Results were expressed as enzymatic units per mg of protein. One SOD unit was defined as the enzyme amount necessary to inhibit 50% the reaction rate.

Glutathione-S-transferase (GST, 1.11.1.9) activity was measured by the technique of Habig et al. (1974), monitoring changes in absorbance at 340 nm during 90 s. One GST unit was defined as the amount of enzyme needed to catalyze the formation of 1 μmol of GS-DNB/min at 25 °C. All the reagents used were of analytical grade.

2.7. Statistical analyses

Microcystin accumulation in crab hepatopancreas was compared among weeks of treatment by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Results from the other variables were compared statistically by two-way analysis of variance (ANOVA) followed by a Dunnett's post hoc test. Differences were considered significant with $p < 0.05$. Normality and homogeneity of variance were tested with Lilliefors and Bartlett tests, respectively (Sokal and Rohlf, 1999). Graph Pad Prism 3 software was used for statistical analysis.

3. Results

3.1. Hepatopancreas index (HSI) and microcystin content

There was no significant effect of MCLR or time on HSI (data not shown). MCLR was accumulated in the hepatopancreas of *N. granulata* during the first three weeks, reaching a peak of 8.81 ± 1.84 ng/g WTM at the third week of treatment and then started to decrease (Fig. 1). The lowest hepatopancreas MCLR content was detected in the seventh week of treatment (1.57 ± 0.74 ng g^{-1} WTM, $p < 0.05$ with respect to that in the first week).

3.2. Lipid peroxidation

MCLR exposure induced an increase of ca. 55% with respect to control individuals in TBARS levels in the hepatopancreas of *N. granulata* during the first three weeks ($p < 0.001$ or $p < 0.05$). This effect was reversed to control values from the fourth week to the end of the experiment (Fig. 2).

3.3. Reduced glutathione (GSH) content

Animals exposed to microcystin LR showed a decreasing trend in hepatopancreas GSH content. This decrease reached minimum values during weeks 6 and 7, at which GSH content was 50% lower than in control individuals ($p < 0.01$) (Fig. 3).

3.4. Enzyme activities

SOD activity was increased by about 2-fold in MCLR treated crabs with respect to control ones. Significant effects ($p < 0.05$ or $p < 0.001$) were detected from the third week of treatment (Fig. 4). GST activity was about 70% higher in treated than in control crabs ($p < 0.05$) from the fourth week on (Fig. 5).

4. Discussion

The results obtained in our study reveal that orally administered MCLR accumulates in the hepatopancreas of *N. granulata* at a concentration enough to induce biochemical changes, without causing mortality. The MCLR accumulation pattern in *N. granulata* hepatopancreas is biphasic, with a maximum value at the third week of treatment and a minimum at the seventh week. In accordance, Vasconcelos et al. (2001) fed the crayfish *Procambarus*

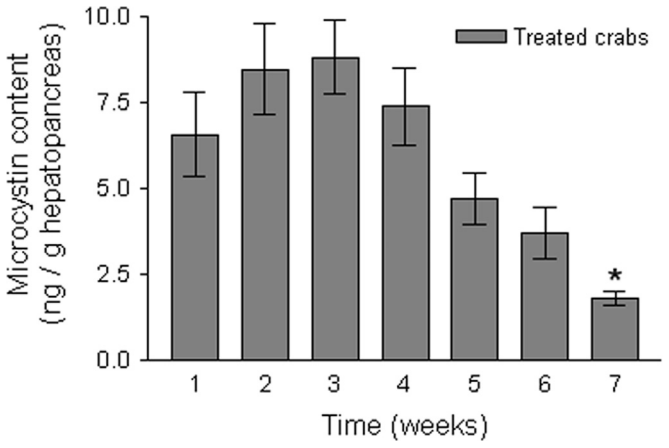

Fig. 1. Microcystin content (ng MCLR g^{-1} hepatopancreas wet tissue mass) in *Neohelice granulata* fed for seven weeks with toxic *Microcystis aeruginosa* cells. Data are expressed as means ± S.D. ($n = 5$). * indicates significant differences at $p < 0.05$ between weeks one and seven.

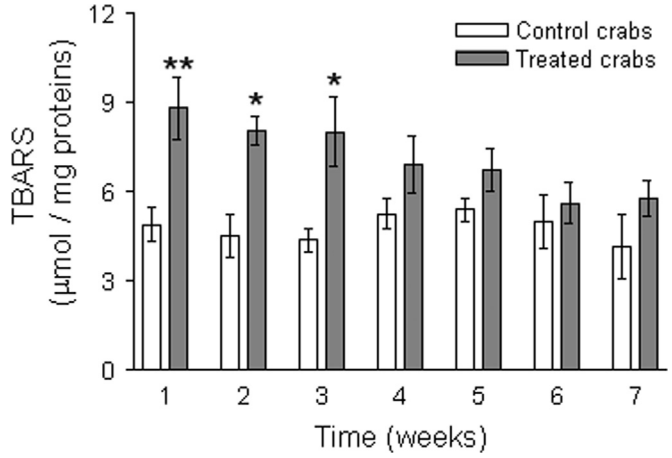

Fig. 2. TBARS content (µmol mg^{-1} proteins) in hepatopancreas of *Neohelice granulata* fed for seven weeks with either toxic or nontoxic cells of *Microcystis aeruginosa*. Data are expressed as means ± S.D. ($n=5$). Significant differences between control and MCLR treated crabs at each time of exposure are indicated by * ($p<0.05$) or ** ($p<0.001$).

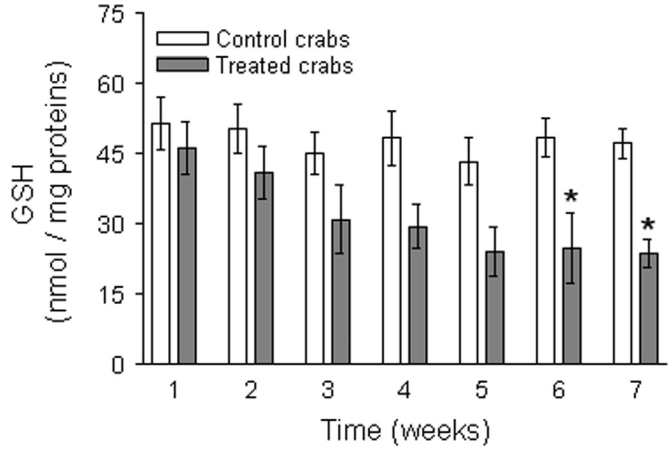

Fig. 3. Reduced glutathione (GSH) content (nmol mg^{-1} proteins) in hepatopancreas of *Neohelice granulata* fed for seven weeks with either toxic or nontoxic (control) cells of *Microcystis aeruginosa*. Data are expressed as means ± S.D. ($n=5$). * indicates significant differences between control and MCLR treated crabs at each time of exposure at $p<0.05$.

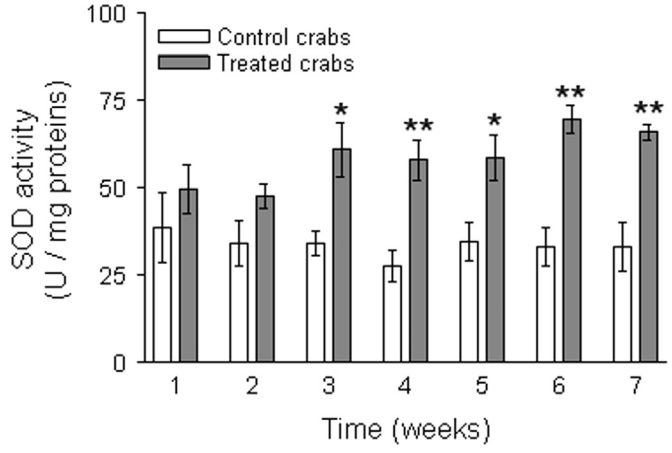

Fig. 4. Superoxide dismutase activity (U SOD mg^{-1} proteins) in hepatopancreas of *Neohelice granulata* fed for seven weeks with either toxic or nontoxic (control) cells of *Microcystis aeruginosa*. Data are expressed as means ± S.D. ($n=5$). Significant differences between control and MCLR treated crabs at each time of exposure are indicated by * ($p<0.05$) or ** ($p<0.001$).

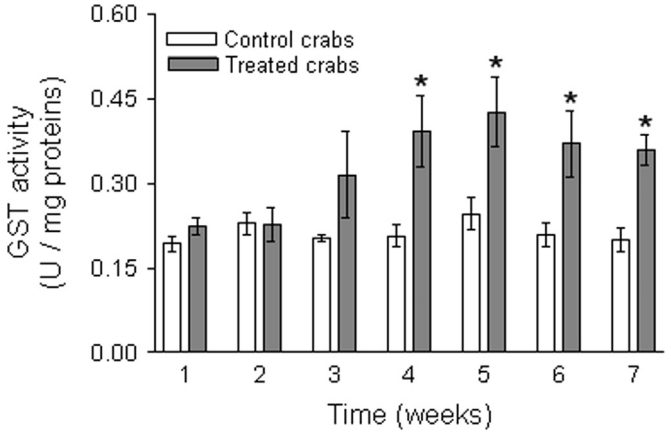

Fig. 5. Glutathione-S-transferase activity (U GST mg^{-1} proteins) in hepatopancreas of *Neohelice granulata*. Data are expressed as means ± S.D. ($n=5$). * indicates significant differences at $p<0.05$ between treated and control crabs at each time of exposure.

clarkii with toxic *M. aeruginosa* cells for two weeks and recorded increasing MCLR accumulation. In the following depuration period, the crayfish eliminated most of the toxin in one week. Although the design and the duration of the experiments is not completely comparable, both in *P. clarkii* and in *N. granulata* MCLR is increasingly accumulated during the first 2-3 weeks and both species show MCLR depuration capacity. Particularly, *N. granulata* reduces its hepatopancreas MC-LR content from the fourth to the seventh week of the feeding period. This suggests that after the accumulation peak at the third week, toxin depuration is faster than toxin accumulation. This capacity would be important to protect the individuals of this species and to limit the trophic transference of MCLR during persistent cyanobacteria blooms.

Several works on fish report an increase in lipid peroxidation levels in soft tissues after oral exposure to toxic cyanobacteria (Jos et al., 2005; Prieto et al., 2007; Bieczynski et al., 2013). Pinho et al. (2005) have reported no change in SOD activity and increased lipid peroxidation in *N. granulata* after one week of MCLR daily administration. Accordingly, in this work, TBARS level is significantly higher in the hepatopancreas of treated than in control crabs between one to three weeks of feeding with toxic *M. aeruginosa cells*, coinciding with the period of increasing MCLR accumulation. However, at the fourth week, whereas MCLR content is still high, the hepatopancreas TBARS level becomes not significantly different from that in the controls. This compensation seems to respond to the increase of SOD and GST activities at the third and fourth weeks, respectively. These results indicate that the antioxidant defense system of this species is activated after exposure to the toxin and is efficient to restore oxidative balance, although the GSH level decreases to the end of the experiment. Similar timing in the antioxidant response to MCLR has been reported for the tilapia, *Oreochromis niloticus* by Jos et al. (2005). These authors detected increased liver SOD, catalase and glutathione peroxidase activity at the third week of oral exposure to *M. aeruginosa* cells.

GST is involved in detoxification reactions, catalyzing the enzymatic conjugation of GSH to toxic compounds, including microcystins (Storey, 1996; Takenaka, 2001). This reaction would neutralize the electrophilic sites of MC, increasing its water solubility and favoring its excretion (Kondo et al., 1992; Wiegand et al., 1999; Ito et al., 2002). Pflugmacher et al. (1998) have demonstrated the existence of a microcystin-GSH complex formed through GST catalysis in several aquatic organisms, suggesting that GST plays an important role in microcystin detoxification. In our study, GST activity is significantly higher in MCLR treated crabs with respect to the control, from the fourth week to the end of the

experiment, coinciding with the start of a declining trend in MCLR and GSH content. Increased GST activity has also been reported for other aquatic organisms exposed to MCLR (Wiegand et al., 2002; Sotton et al., 2012; Bieczynski et al., 2013) and also for *N. granulata* (Pinho et al., 2005). Bieczynski et al. (2014) proposed that in rainbow trout intestine MCLR is exported from the enterocytes to the lumen through multixenobiotic resistance active transporters belonging to the ATP binding cassette family (ABC), such as ABCC/Mrp proteins, after conjugation of the toxin with GSH, catalyzed by GST. In the present work, the coincidence of increased GST activity with decreasing MCLR and GSH contents in *N. granulata* hepatopancreas suggests that MCLR detoxification in this species could involve conjugation with GSH catalyzed by GST and excretion of the MCLR-GSH conjugate through ABCC proteins.

5. Conclusions

N. granulata accumulates MCLR from ingested intact cells of *M. aeruginosa*. Toxin accumulation is limited and reversed by a depuration mechanism probably based on GST-mediated conjugation with GSH. Dietary exposure to MCLR in this species produces oxidative damage to lipids, which can be reversed by increased activity of antioxidant and detoxifying enzymes like SOD and GST.

Acknowledgments

This work was partly supported by grants PIP 6244 from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) and 20020100100985 from Universidad de Buenos Aires.

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