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Toxin Accumulation and Biochemical Alterations in the Estuarine Crab *Neohelice granulata* (Decapoda, Brachyura) After Exposure to Microcystins

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ABSTRACT

Neohelice granulata were collected during a bloom dominated by *Microcystis* sp. in Patos Lagoon (RS, Brazil) and then sacrificed at different times of depuration in laboratory in order to verify microcystin (MC) content and toxic effects in hepatopancreas of the estuarine crab. Biochemical measurements were: lipid peroxidation (LPO), activity of glutathione-*S*-transferase (GST), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). No variation of crab biochemical parameters and MC content (mean value = 32 μg kg^{-1}) was verified during the depuration period. MC content of the lyophilized bloom sample was determined by HPLC (0.129 μg mg^{-1}). MC concentration in water at the sampling site was 1.92 μg L^{-1}. Experimental assays were also performed *via* oral exposure (by gavage), in doses and time exposure varying between 0 and 55 μg kg^{-1} and 48 and 96 h. Analyzed variables were: GST activity (remained unaltered in all experimental conditions), LPO (augmented after 48 h in doses higher than 5.5 μg kg^{-1} but the opposite was observed after 96 h at the same doses) and oxygen consumption (increased in all doses and times of exposure). We conclude that (1) there is an absent or low depuration rate of MC; (2) oxidative damage should be attenuated by antioxidant defenses other than GST; (3) higher oxygen consumption should favor reactive oxygen species generation.

Key words: *Neohelice granulata*, cyanobacteria bloom, depuration, microcystins, hepatopancreas, oxidative damage.

RESUMO

Acúmulo de toxina e alterações bioquímicas no caranguejo estuarino *Neohelice granulata* (Decapoda, Bracyura) após exposição às microcistinas

Neohelice granulata foram coletados durante uma floração dominada por *Microcystis* sp. na Lagoa dos Patos (RS, Brasil) e sacrificados em diferentes tempos de depuração em laboratório objetivando verificar o teor de microcistinas (MC) e efeitos tóxicos no hepatopâncreas do caranguejo estuarino. As análises bioquímicas foram: peroxidação lipídica (LPO), atividade da glutationa-*S*-transferase (GST), alanina aminotransferase (ALT) e aspartato aminotransferase (AST). Não houve variação nos parâmetros bioquímicos e no teor de microcistina (valor médio = 32 μg.kg^{-1}) durante o período de depuração. O teor de microcistinas na amostra liofilizada da floração foi determinado por HPLC (0,129 μg.mg^{-1}). A concentração de microcistinas no local de coleta foi de 1.92 μg.L^{-1}. Foram também realizados ensaios experimentais por ingestão forçada em doses e tempos de exposição variando entre 0 e 55 μg.kg^{-1} e 48 e 96 h. As variáveis analisadas foram: atividade da GST (permaneceu inalterada em todas as condições experimentais), LPO (aumentou após 48 h em doses maiores que 5.5 μg.kg^{-1}, porém o oposto ocorreu após 96 h nas mesmas doses de exposição) e consumo de oxigênio (aumentou em todas as doses e tempos de exposição). Concluímos que (1) a taxa de depuração de microcistina é baixa ou nula; (2) o dano oxidativo pode ser atenuado por outras defesas antioxiodantes que não a GST; (3) o aumento no consumo de oxigênio deve favorecer a geração de espécies reativas de oxigênio.

Palavras-chave: *Neohelice granulata*, floração de cianobactérias, depuração, microcistinas, hepatopâncreas, dano oxidativo.

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INTRODUCTION

The eutrophication of water bodies favor the generation of massive cyanobacterial blooms and the associated cyanotoxins, and it has been documented in many inlands waters worldwide (Carmichael, 1992). Among cyanotoxins, the hepatotoxic microcystins (MC) inhibits eukariotic protein phosphatases type 1 and 2A resulting in excessive phosphorylation of several cellular components (Carmichael, 1992). These toxins are cyclic heptapeptides being its generic structure cyclo-(D-Ala^{1}-X^{2}-D-MeAsp^{3}-Y^{4}-Adda^{5}-D-Glu^{6}-Mdha^{7}), where X and Y are variable L-aminoacids (Sivonen & Jones, 1999). Toxic blooms of cyanobacteria, usually dominated by *Microcystis aeruginosa*, have been registered in the Patos Lagoon (RS, Brazil) in the last two decades, sometimes reaching total microcystins concentrations as high as 289 mg.L^{-1} (Minillo *et al.*, 2000). In mammals, the hepatic damage caused by microcystins can be measured by some clinical enzyme markers, like alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). ALT and AST are members of the transaminase group of enzymes, which catalyzes the transfer of amino groups from amino acids to α-oxo-acids. Particularly ALT activity has been considered an important clinical indicator of hepatocellular injury (Solter *et al.*, 2000).

Recent evidences have shown that MC induce other toxicological effects, including oxidative stress and disruption of osmotic and ion-regulation in aquatic organisms (Pinho *et al.*, 2003; Vinagre *et al.*, 2003). In vertebrates, MC is taken up into the hepatocyte by multi-specific bile acid transporters (Eriksson *et al.*, 1990; Runnegar *et al.*, 1995) where it induces the production of reactive oxygen species (ROS) (Ding *et al.*, 2001), leading to augmented levels of lipid peroxides (LPO), DNA damage and induction of antioxidant enzymes activity (Ding *et al.*, 1998; Malbrouck *et al.*, 2003; Zegura *et al.*, 2003; Gehringer *et al.*, 2004). The detoxification of MC is known to occur via conjugation with glutathione (GSH), a reaction catalyzed by the enzyme glutathione-*S*-transferase (GST) (Pflugmacher *et al.*, 1998). Considering that MC seem to favor oxidative stress generation, it is important to note that GSH is considered one of the main non-enzymatic antioxidant and constitutes the first line of protection against ROS (Griffith, 1999; Sies, 1999). In this way, the conjugation of microcystin with GSH should represent a leak of this tripeptide from the intracellular pool, which could favor generation of oxidative stress through reduction of antioxidant (GSH levels) defenses.

In general, information available on MC content in crustaceans is scarce. In laboratory experiments that aimed to analyze MC accumulation and depuration kinetics, it was observed that the prawn *Penaeus monodon* depurated MC from hepatopancreas and muscle within a few hours (Kankanpää *et al.*, 2004). The crayfish *Procambarus clarkii* fed with toxic *Microcystis aeruginosa* strain containing 2.3 mg MC g^{-1}, showed a body accumulation up to 2.9 μg MC g^{-1} at the end of the exposure period (2 weeks) (Vasconcelos *et al.*, 2001).

The aim of the present study was to investigate MC retention and their possible toxic effects (in terms of LPO, enzyme activity of GST, ALT and AST) on the estuarine crab *Neohelice granulata* collected in an environment impacted by a *Microcystis sp.* bloom and then depurated up to 168 h under laboratory conditions. Experiments were also performed to evaluate oxygen consumption, oxidative damage (in terms of LPO) and GST activity in *N. granulata* orally exposed to MC.

MATERIAL AND METHODS

Adult male crabs were collected in salt marshes of Patos Lagoon round the Rio Grande city (RS, Southern Brazil, 30°20'S to 32°10'S) during a summer bloom dominated by the cyanobacteria *Microcystis sp.* They were immediately transferred to the Department of Physiological Sciences (Fundação Universidade Federal do Rio Grande, RS, Brazil). Fifteen crabs were immediately sacrificed and their hepatopancreas were dissected and stored at –80ºC for MC and biochemical parameters analysis (time 0 of depuration). The remaining crabs were maintained in tanks with clean water at controlled temperature (20°C) and salinity (2‰, the same that the registered at the sampling site, prepared by dilution of marine water in distillated water), under constant aeration. Photoperiod was fixed at 12L:12D. Crabs were sacrificed after 24, 96 and 168 h of the beginning of the depuration (n = 15 for each depuration time). Hepatopancreas were dissected and stored at –80ºC in order to perform the same measurements. The biochemical parameters analyzed were: lipid peroxidation (LPO), activity of the enzymes glutathione-*S*-transferase (GST) and alanine and aspartate aminotransferases (ALT and AST, respectively). The MC content retained in hepatopancreas and the MC concentration in water at the sampling area were also analyzed.

For experimental exposure to MC, organisms of *N. granulata* were collected in fall and summer and acclimated during one month to the same conditions described above. To obtain aqueous MC extracts, cells of *M. aeruginosa* (toxic strain RST 9501, which produces [d-Leu^{1}]microcystin-LR; Matthiensen *et al.*, 2000) were cultured in BG11 medium (plus 8.82 mM of $NaNO_3$) at 25 ± 1°C (Rippka *et al.*, 1979). Cells were sonicated for three min in 100 Hz and centrifuged (10,000 × g, 10 min.) at room temperature. The supernatant was collected and stored at –80°C until use. MC content of the extracts was determined using a commercial enzyme-linked immunoassay (ELISA) with polyclonal antibodies (EnviroLogix Inc., Portland, ME). After acclimation, crabs were employed in two different bioassays. In bioassay 1 (crabs collected in fall, mean weight = 8.66 ± 2.19 g; n = 43), crabs were exposed during 48 h to MC doses of 0; 0.55; 5.5 and 55 μg kg^{-1} body weight. In bioassay 2 (crabs collected in summer, mean weight = 9.25 ± 2.43g; n = 72), organisms were exposed during 96 h to MC doses of 0; 0.05; 0.55; 5.5 and 55 μg kg^{-1} body weight. The different doses were obtained by diluting the stock solution of MC (obtained as described above) in a crustacean

physiological solution (Vinagre *et al.*, 2003). The MC exposed groups and control group received, respectively, 100 µL of the cyanobacterial extract (resulting in equal doses per group, since there was not difference between the mean weight of groups) and 100 µL crustacean physiological solution by gavage. At the end of the bioassays, crabs were sacrificed and their hepatopancreas dissected immediately. Oxygen consumption was measured immediately in samples of about 50 mg of tissue. Other pieces of hepatopancreas were stored at –80ºC for biochemical analysis. Crabs were not fed during the exposure period.

Oxygen consumption was determined according to Nithart *et al.* (1999) through the determination of oxygen concentration in time 0 and 30 min post incubation of tissue at 20ºC in physiological solution plus 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Sigma).

Lipid peroxide levels were determined according to Hermes-Lima *et al.* (1995) and modified for microplate reader (Biotek ELx 800, Winooski, VT). Hepatopancreas samples were homogenized (9% W/V) in methanol 100% and centrifuged at 1000 g (4ºC) for 10 min and the supernatants kept for bioassay. LPO were determined using 90 µL of $FeSO_4$ (1 mM), 35 µL of H_2SO_4 (0,25 mM), 35 µL of xylenol orange (1 mM; Sigma), 170 µL of MilliQ water, and 20 µL of the methanolic extract. All reagents were added following a sequential order mentioned above. Samples were incubated at room temperature until the reaction was completed (75 min), and then the absorbance (550 nm) was registered. Cumene hidroperoxide (CHP; 0,1 mM; Sigma) was employed as standard. Lipid peroxides were quantified in terms of CHP equivalents g^{-1} of wet weight (ww) of tissue.

For analysis of enzyme activity (GST, ALT and AST), microcystin and protein content the hepatopancreas was homogenized (20% w/v) in a buffer containing Tris base (20 mM), EDTA (1 mM), dithiothreitol (DTT 1 mM, Sigma), sucrose (0.5 mM) and KCl (150 mM), and the pH adjusted to 7.60. Samples were centrifuged at 9,000 × g (4ºC) for 30 min and the supernatants employed as enzyme source. Enzyme activities were measured by spectrophotometric techniques, as described in Dewes *et al.* (2006), employing commercial kits (Doles Reagents, Ltda; Goiania, GO, Brazil).

For quantification of MC in hepatopancreas, after homogenization samples were sonicated (3 min, 100 Hz), centrifuged (10 min, 10,000 g) and diluted with an equal volume of chloroform. The supernatants (aqueous phase) were collected and the microcystin content determined by immunoassay (EnviroLogix Inc., Portland, ME). MC content in hepatopancreas was expressed in terms of µg of microcystin.kg^{-1} of wet weight (ww) tissue. Water collected at the sampling area was sonicated (3 min, 100 Hz), centrifuged (10 min, 10,000 g) and MC concentration determined by immunoassay, being the result expressed in terms of µg of microcystin L^{-1} of the water sample.

Total protein content in the homogenate was determined using a commercial kit (Doles Reagents Ltda., Goiânia, GO, Brazil), employing the Biuret reagent, which reacts with sample proteins, resulting in a violet colored complex proportional to protein sample concentration. Determinations were done at 550 nm at least in duplicate.

Microcystin quantification in lyophilized bloom sample was performed by high performance liquid chromatography (HPLC), in a HPLC-UV equipment (Shimadzu, Japan) following the technique described by Harada *et al*. (1999). The whole system comprises a reversed-phase C18 silica column pressured by a water (0.05% TFA) – acetonitrile (0.05% TFA) gradient from 30 to 70%. Ultraviolet detection (UV) was set at 238 nm. A 1-D-Leu Microcystin-LR standard was used for a quantititative reference as this is the main MC produced by the strain RST9501. Before analysis by HPLC, samples were sonicated (3', 50 Hz) and filtered (acetate cellulose filter of 0.45 µm pore).

Data were subjected to a variance analysis (ANOVA) followed by the Newman-Keuls test. Normality and variance homogeneity were previously checked and logarithmic transformation applied when needed. In all statistical tests, a significance level of 5% was adopted ($\alpha = 0.05$). 95% confidence limits (CI) were estimated for LD_{50} values.

Table 1 – Lipid peroxides (LPO, nmol CHP.g $tissue^{-1}$), glutathione-S-transferase activity (GST, units), alanine aminotransferase activity (ALT, units), aspartate aminotransferase activity (AST, units) and microcystin content (µg MC.kg $tissue^{-1}$) in hepatopancreas of *Neohelice granulata* submitted to depuration time of 0, 24, 96 and 168 hours after exposure to a *Microcystis* bloom in their natural environment. Data are expressed as mean value ± 1 standard deviation (sd). n = 5 for LPO, GST, ALT and AST analysis and n = 2 for microcystin analysis. CHP: cumene hydroperoxide, employed as standard. $\alpha = 0.05$.

	0 H	24 H	96 H	168 H
LPO	44.3 ± 13.8	49.9 ± 14	48.3 ± 13.6	39.6 ± 9.2
GST	0.33 ± 0.02	0.26 ± 0.07	0.24 ± 0.04	0.33 ± 0.03
ALT	1.9 ± 0.1	2.1 ± 0.6	1.0 ± 0.8	1.7 ± 0.9
AST	3.2 ± 1.2	3.1 ± 0.4	2.5 ± 1.2	2.4 ± 0.7
MC	32.2 ± 0.8	29.6 ± 6.3	33.3 ± 0.9	34 ± 0.9

RESULTS

As showed in Table 1, no differences ($p > 0.05$) in LPO levels and GST, ALT and AST activities were observed after all the depuration times tested. Hepatopancreatic MC remained unaltered ($p > 0.05$) up to 168 h of depuration, being the mean value of 32.3 ± 3.02 μg kg^{-1} (mean of all depuration times). MC concentration in water at the sampling site was 1.92 μg L^{-1}.

Crabs exposed by gavage to MC showed higher oxygen consumption ($p < 0.05$) in both bioassays (48 and 96 h) even at the lowest dose of MC (0.05 μg kg^{-1}) (Figures 2a and 2b). LPO levels increased ($p < 0.05$) in 48 h exposure bioassay in doses higher than 5.5 μg kg^{-1} (Figure 3a). In 96 h exposure bioassay, an opposite result was observed, since significantly ($p < 0.05$) lower LPO levels were observed in the same doses (Figure 3b). GST activity remained unaltered ($p > 0.05$) in all experimental conditions (Figures 4a and 4b).

DISCUSSION

The absence of biochemical responses in the depuration experiment, despite MC retention in hepatopancreas of *N. granulata*, could be related to a quick response, since Dewes *et al.* (2006) observed a rapid and transitory induction of GST activity only after 12 h of MC exposure with the same species (the induction of GST activity was not observed in exposure times longer than 12 h). These previous results suggest that crabs were exposed to toxins released during the bloom for more than 12 h, leading the biochemical parameters close to those observed for control organisms. The MC accumulated in hepatopancreas of *N. granulata* showed a mean value of 32.3 ± 3.02 μg kg^{-1}, a toxin concentration lower than those found for other crustacean species impacted by MC-producing cyanobacteria blooms. For example, Kankaanpää *et al.* (2004) detected 130 μg kg^{-1} in hepatopancreas of the shrimp *Penaeus monodon* and Magalhães *et al.* (2003) determined 103 μg kg^{-1} in muscle tissue of a crab (the species is not cited by the authors of the referred article).

Considering that TDI (tolerable daily intake) for humans is 0.04 μg of MC.kg^{-1}.day^{-1} (value calculated by WHO – World Health Organization, according to Chorus & Bartram, 1999), MC accumulation detected in the hepatopancreas of *N. granulata* correspond to a value of 0.008 μg of MC kg^{-1} day^{-1}, leading to a TDI values < 0.04, which is acceptable. In the calculation it was considered an adult of 60 kg, who ingests, on the average, 300 g of crab a day, which approximately corresponds to 15 g of hepatopancreas. Although usually crab hepatopancreas is not ingested by humans, two possibilities must be considered: (1) accumulated MC in the hepatopancreas could be transferred along the trophic web, since it is known that *N. granulata* is predated by the blue crab *Callinectes sapidus* and by the band-tailed gull *Larus belcheri* (Palomo *et al.*, 2003); (2) MC can be retained in others tissues ingested by humans, like muscle. Also is important to note that MC concentration in the water where the bloom occurred was 1.92 μg L^{-1}, a relatively low concentration, if we consider that WHO recommend 1.0 μg L^{-1} as maximum concentration of MC in drinking water. The fact of *Microcystis aeruginosa* blooms have been registered in Patos Lagoon with MC concentrations of 289 μg L^{-1} (Yunes *et al.*, 1998) indicate that exist the possibility of *N. granulata* retaining values of MC higher than those found in the present study.

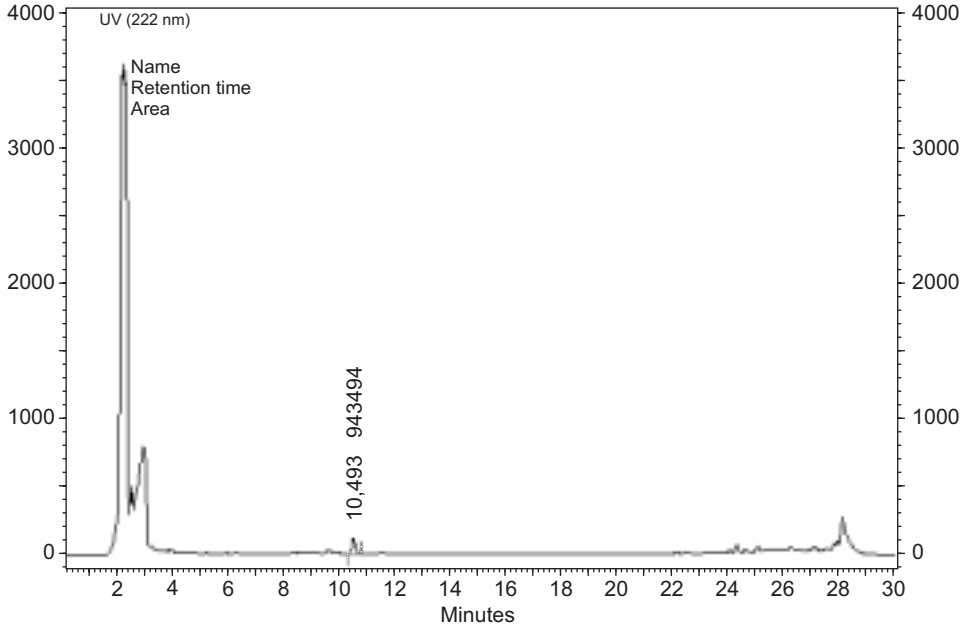

Figure 1 – Chromatogram of microcystin analysis through HPLC. Values of 10.493 and 943494 corresponds to the retention time and the peak area (equivalent to a microcystin concentration of 0.129 μg mg^{-1}), respectively.

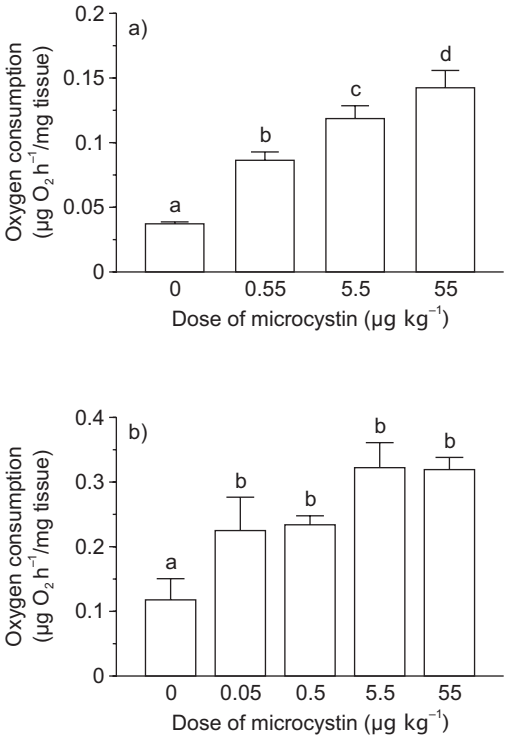

Figure 2 – Oxygen consumption (µg O_2 h^{-1}.mg tissue^{-1}) in hepatopancreas of the estuarine crab *Neohelice granulata* exposed to 0; 0.55; 5.5; and 55 µg of microcystins.kg^{-1} during 48 h (a) or after exposure to 0; 0.05; 0.55; 5.5; and 55 µg of microcystins kg^{-1} during 96 h (b). Data are expressed as mean + 1 standard deviation (n = 4). Similar letters means absence of statistical differences ($p > 0.05$).

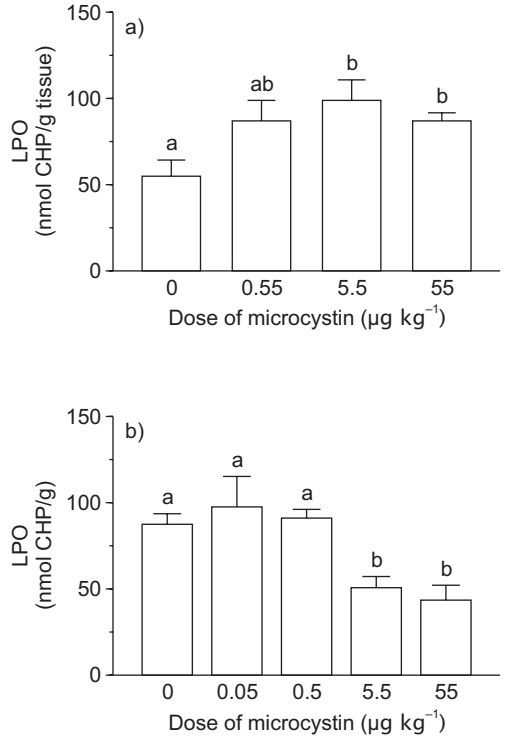

Figure 3 – Lipid peroxides content (nmol of CHP.g tissue^{-1}) in hepatopancreas of the estuarine crab *Neohelice granulata* exposed to 0; 0.55; 5.5; and 55 µg of microcystins kg^{-1} during 48 h (a) or after exposure to 0; 0.05; 0.55; 5.5; and 55 µg of microcystins kg^{-1} during 96 h (b). Data are expressed as mean + 1 standard deviation (n = 5). Similar letters means absence of statistical differences ($p > 0.05$). CHP: cumene hydroperoxide, employed as standard.

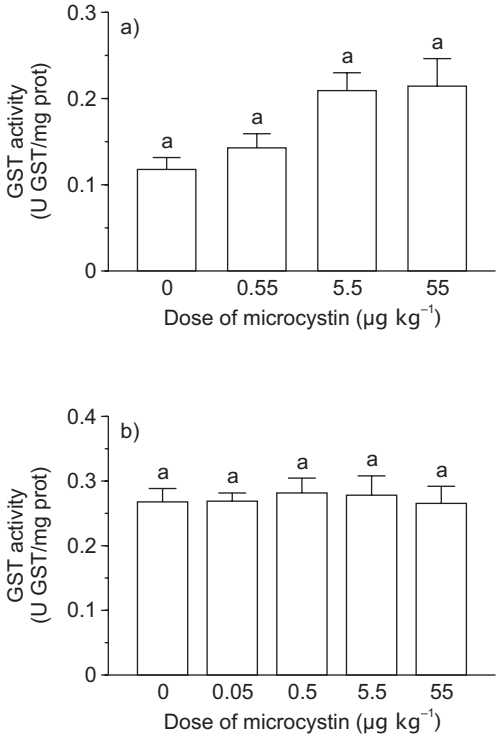

Figure 4 – Glutathione *S*-transferase (GST) activity (GST units) in hepatopancreas of the estuarine crab *Neohelice granulata* exposed to 0; 0.55; 5.5; and 55 µg of microcystins kg^{-1} during 48 h (a) or after exposure to 0; 0,05; 0,55; 5,5; and 55 µg of microcystins kg^{-1} during 96 h (b). Data are expressed as mean + 1 standard deviation (n = 5). Similar letters means absence of statistical differences ($p > 0.05$).

The elimination rates of MC are very variable for different aquatic crustacean species, ranging from hours to approximately 3 weeks in the case of the shrimp *Penaeus monodon* (Kankaanpää *et al.*, 2005) and the prawn *Procambarus clakii* (Vasconcelos *et al.*, 2001), respectively. The results of present study show that the elimination rate of MC in *N. granulata* is absent or very low, since the accumulated MC in its hepatopancreas remained almost unaltered after one week of depuration. Another hypothesis that could explain low rate of MC elimination found in this study is that most of MC is bound to protein phosphatases, since these are its main target in most organisms. ELISA is not able to detect bound microcystin, so most of detected MC is free MC, which is a minor part of the total MC in the tissue.

The results obtained in the bioassays showed that MC induced oxidative stress in crabs orally exposed to these toxins, since it was observed augmented levels of LPO after 48 h exposure. Diminished levels of LPO after 96 h of exposure in parallel with no GST response indicate that the oxidative damage is attenuated by some mechanism(s) of antioxidant defenses other than GST. This mechanism could be the action of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) or glutathione peroxidase (GPx). Some previous studies described augmented activity of these enzymes after exposure to microcystin (Li *et al.*, 2003; Pinho *et al.*, 2003), moreover Almeida *al.* (2004) described a protective role of phospholipid hydroperoxide glutathione peroxidase (PHGPx) against lipid peroxidation in mussels *Perna perna*. Another hypothesis to explain the lower LPO levels in MC exposed crabs after 96 h is through hepatocytes renovation, taking account the high renovation rate of hepatopancreas epithelium (Al-Mohanna & Nott, 1989).

Future avenues should consider the dynamics of MC depuration in *N. granulata* and to analyze the role of antioxidant mechanisms to lower the levels of oxidative products such as LPO. The measurement of reactive oxygen species in the hepatopancreas of this species should be equally important to characterize the oxidative stress generation that MC directly or indirectly promote.

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