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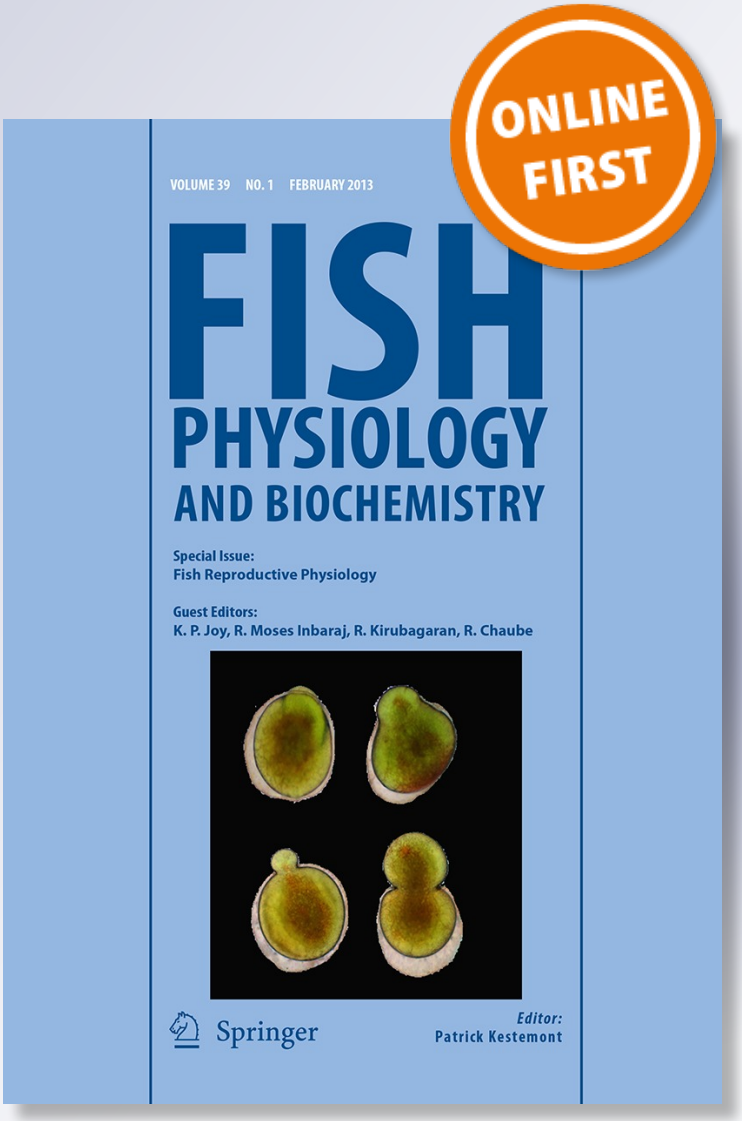

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Accumulation and biochemical effects of microcystin-LR on the Patagonian pejerrey (*Odontesthes hatcheri*) fed with the toxic cyanobacteria *Microcystis aeruginosa*

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Abstract We studied accumulation and biochemical effects of microcystin-LR (MCLR) in *Odontesthes hatcheri* after dietary administration of the cyanobacteria *Microcystis aeruginosa* (1.3 μg MCLR/g body mass, incorporated in standard fish food). After 12 h, MCLR content in liver did not differ between fish fed with crushed or intact cells, demonstrating *O. hatcheri*'s capacity to digest cyanobacteria and absorb MCLR. In the second experiment, fish received toxic cells, non-toxic cells, or control food; MCLR accumulation was monitored for 48 h. Protein phosphatase 1 (PP1), catalase (CAT), glutathione-S-transferase (GST) activities, and lipid peroxidation (as MDA) were measured in liver and intestine. Methanol-extractable MCLR was determined by PP1 inhibition assay (PPIA); extractable and protein-bound MCLR were measured by Lemieux oxidation-gas chromatography/mass spectrometry (GC/MS). MCLR accumulated rapidly up to 22.9 and 9.4 μg MCLR/g in intestine and liver, respectively, followed by a decreasing tendency. Protein-bound MCLR represented 66 to ca. 100 % of total MCLR in both tissues. PP1 activity remained unchanged in intestine but was increased in liver of MCLR treated fish.CAT and GST activities and MDA content were significantly increased by MCLR only in liver. We conclude that *O. hatcheri* is able to digest cyanobacteria, accumulating MCLR mostly bound to proteins. Our data suggest that this freshwater fish can be adversely affected by cyanobacterial blooms. However, the rapid decrease of the detectable MCLR in both tissues could imply that sublethal toxin accumulation is rapidly reversed.

Keywords *Odontesthes hatcheri* · Microcystin-LR · Digestion · Oxidative stress · Accumulation

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Introduction

Cyanobacteria are one of the main components of the phytoplankton seasonal cycle in most surface water systems and constitute an important part in the diet of many aquatic animals (such as zooplankton, mussels, and phytoplanktivorous fish) (Dionisio Pires et al. 2004; Ferraõ-Filho et al. 2002; Xie et al. 2004, 2005). Under certain conditions, cyanobacteria may become dominant, reaching massive cell densities, which are called cyanobacterial blooms (Bartram et al. 1999). The main concern with many species of cyanobacteria is that they can produce toxic secondary metabolites (cyanotoxins), which constitute a waterborne hazard

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to human and ecosystems health (Codd et al. 1999; Osswald et al. 2007; Wiegand and Pflugmacher 2005, for a review).

The most frequent cyanotoxins in fresh and brackish waters are the cyclic peptides called microcystins (MCs), being microcystin-LR (MCLR) the most common and one of the most toxic variants (Chorus and Bartram 1999). These toxins inhibit protein phosphatases (PP) 1 and 2A, leading to increased protein phosphorylation, which is related to their cytotoxic effects and tumor-promoting activity (Carmichael 1994; Dawson 1998; Hooser 2000; MacKintosh et al. 1995). In addition, abundant evidence suggests that oxidative stress may also play a significant role in the pathogenesis of microcystin toxicity (Amado and Monserrat 2010, for a review).

Although the liver is considered as the main target organ of MCs in fish, toxic effects have also been reported to occur in other organs such as kidney, gills, gastrointestinal tract, and brain (Cazenave et al. 2005, 2006; Fischer and Dietrich 2000; Kotak et al. 1996; Molina et al. 2005; Prieto et al. 2006). Accordingly, several authors have detected the presence of these toxins in liver, intestine, kidney, muscle, gallbladder, and blood (Bury et al. 1998; Cazenave et al. 2005; Magalhães et al. 2003; Sahin et al. 1996; Smith and Haney 2006; Tencalla and Dietrich 1997; Xie et al. 2004, 2005).

In vivo and in vitro studies suggest that fish are less sensitive to MCLR than mammals. However, the mechanisms underlying this differential sensitivity are not clear yet. Intraperitoneal LD_{50} is about tenfold higher in fish than in mice and rats (Bury et al. 1998; Kuiper-Goodman et al. 1999; Råbergh et al. 1991; Tencalla et al. 1994). In addition, sensitivity to this toxin varies among fishes, for example, the common carp (*Cyprinus carpio*) appears to be more affected than the rainbow trout (*Oncorhynchus mykiss*) when the toxin is administered by oral gavage (Fischer and Dietrich 2000). These differences could be related to the feeding habits and gastrointestinal characteristics, since the long intestine of the common carp offers more surface area for toxin absorption (Bury et al. 1998; Fischer and Dietrich 2000). In contrast, Xie et al. (2004) has shown that the phytoplanktivorous silver carp (*Hypophthalmichthys molitrix*) is adapted to tolerate MCs by blocking MCLR absorption and eliminating the absorbed MCRR.

The gastrointestinal tract (GI) is considered the main site of absorption of MC (Bury et al. 1998; Tencalla et al. 1994). In fish, oral intoxication with MCs could proceed through direct consumption of cyanobacteria (in planktivorous species) or accidentally due to water drinking or along with the principal food items (consuming intact cyanobacteria and/or free toxin dissolved in water, during the late stages of blooms).

The accumulation of toxins in a given trophic level, and their effects and transport along the trophic chain could be limited by processes of detoxification/excretion. These processes have been reported for several fish species (Cazenave et al. 2006; Pflugmacher et al. 1998; Sahin et al. 1996; Smith and Haney 2006; Soares et al. 2004; Williams et al. 1997b; Xie et al. 2004). Toxin elimination is related to biotransformation reactions, resulting in products less toxic and more susceptible to be excreted (Ito et al. 2002). As a result, MCs can be found in animal tissues in different forms such as protein-bound MC and methanol-extractable forms (native MC and conjugated with GSH or Cystein) (Pflugmacher et al. 1998; Williams et al. 1997a; Yuan et al. 2006).

The Patagonian pejerrey (*Odontesthes hatcheri,* Atherinopsidae) is a planktivorous–omnivorous fish, native of inland waters of Patagonia, Argentina. It is a commercially exploited fish, and its potential for aquaculture is currently under study (Hualde et al. 2011). Among the freshwater native fish of Argentina, *O. hatcheri* and the more popular *Odontesthes bonariensis* are highly appreciated both in the local and in the international markets (Somoza et al. 2008).

O. hatcheri is abundant in hydroelectric reservoirs, such as the Exequiel Ramos Mexía reservoir, where toxic cyanobacterial blooms are frequent (Puig 1992). Hualde et al. (2011) have shown the presence of cyanobacteria in GI contents of wild and cage-reared individuals of this species from this environment. However, whether *O. hatcheri* is able to actively eat and digest cyanobacteria is still to be determined. Similarly, in individuals of a related species, *O. bonariensis*, caught in a eutrophic reservoir, Cazenave et al. (2005) have found cyanobacterial cells in GI and MCRR accumulation in liver, gills, and muscle. However, no direct evidence linking the GI cyanobacteria content with the MC detected in tissues has been provided yet.

Wenzel and Díaz (2008) have reported the presence in Patagonian aquatic environments of several potentially toxic species of cyanobacteria, which are generally known as bloom producers. However, the

natural oligotrophic conditions of these waters are usually not favorable to such events. Thus, subchronic exposure of fish to moderate concentrations of cyanobacteria seems to be a more likely scenario.

We can assume that, as many economically important fishes, *O. hatcheri* coexists with cyanobacteria and then is probably exposed to sublethal toxin concentrations, at least seasonally, even in the absence of visible cyanobacterial blooms. Accumulation of MCs under these conditions could be detrimental for the culture of this species and constitute a risk for human and environmental health. We hypothesize that *O. hatchery* is capable of digesting toxic cyanobacteria, releasing cyanotoxins into the GI lumen, which allows their absorption. On this basis, we predict that intracellular effects and accumulation of MCLR will be detected in fish orally exposed to MCLR-producing cyanobacteria.

In the present study, we evaluate the capacity of *O. hatcheri* to absorb MCLR from intact and crushed cyanobacterial cells, and the biochemical effects of this toxin on enterocytes and hepatocytes in relation with its tissue accumulation. The form (protein-bound or extractable) in which MCLR is accumulated in *O. hatcheri*'s intestine and liver is also analyzed.

Materials and methods

Chemicals

MCLR standard solution, 3 mg L^{-1} was obtained from DAOS, Argentina. Reduced glutathione (GSH), 1-chloro-2, 4-dinitrobenzene (CDNB), bovine seroalbumine (BSA), phenylmethylsulfonyl fluoride (PMSF), thiobarbituric acid (TBA), KIO_4, Na_2SO_4, trichloroacetic acid (TCA), Commassie brillant blue G 250, and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St Louis, MO). Para-nitrophenyl phosphate disodium salt (*p*NPP), hexane, diethyl ether, trifluoroacetic acid (TFA), $MgCl_2$, and HCl were from Merck (Germany). Na_2CO_3 and KCl were from Anedra (Argentina). $MnCl_2$ and $KMnO_4$ were from Mallinckrodt Baker (Phillipsburg, NJ) and tris (hydroxymethyl) aminomethane (Tris) from Serva (Germany).

Acclimation of fish

Odontesthes hatcheri, $n = 120$, mean weight 13.5 ± 3.5 g, obtained from the Centro de Ecología Aplicada del Neuquén (CEAN), Junín de los Andes, Argentina (39° 54′ 58.84″ S; 71° 06′ 22.79″ W), were transferred to the laboratory where they were held in aquaria (15 individuals/aquarium) with 40 L of freshwater obtained from the Chimehuin river. The aquaria were set up with a continuous system of water filtration and aeration. Temperature was kept at 13 ± 3 °C. Fish were acclimated for 1 week before the beginning of the experiments and received 1.0 % body mass (bm) ratio of experimental pelletized pejerrey food per day (Hualde et al. 2011).

Microcystis aeruginosa

The two *Microcystis aeruginosa* strains used in the experiments were kindly provided by Dr. Raquel Soares from the Federal University of Río de Janeiro, Brazil. NPDC1 is a nontoxic strain, and NPJB1 is a toxic strain that produces mostly microcystin-LR, in a concentration of 230 ± 27 μg MCLR equivalents/10^6 cells (Azevedo et al. 1994; Brena et al. 2006). *M. aeruginosa* was cultured in 50 mL flasks containing 20 mL of ASM-1 medium (Gorham et al. 1964), maintained at 20 ± 2 °C, with 12 h light–dark cycle with cool-white fluorescent light illumination. Before preparing the fish food for each experiment, we measured the MCLR content in our cultures by protein phosphatase inhibition assay (PPIA) (see below). The result obtained from five independent samples, measured in triplicate, was 245 ± 67 μg MCLR equivalents/10^6 cells. Additionally, we confirmed qualitatively the presence of only MCLR in these cultures by MALDI-TOF analysis.

Experimental design

The treatments consisted of acute exposure to a sublethal dose of MCLR trough a unique administration of cyanobacterial cells added in the food. Toxic treated groups received 1.3 μg MCLR/g bm. This dose was selected in order to compare our results with previous studies on fish (e.g. Williams et al. 1997b; Jos et al. 2005) and was based on a previous work of our laboratory in which a similar treatment produced biochemical effects with no mortality (Ferrada 2010). In all treatments, food was prepared by crushing the pellets in a mortar. The obtained powder was mixed with distilled water (control group) or with the corresponding volume of *M. aeruginosa* culture,

resulting in small sticky pellets. The cells of *M. aeruginosa* utilized in each experiment were previously washed in distilled water and separated by centrifugation (10,000×*g*) for eliminating free MCLR.

For all treatments, the food ratio was reduced to 0.5 % bm to ensure that no uneaten food remained in the aquaria. Due to the shoal behavior of this fish at feeding, food was administered to each group instead of feeding each fish independently. This administration way implies difficulties at detecting the exact quantity of MCLR intake but ensures that fish accept the food. We checked visually that all fish were fed and that all food was consumed.

Experiment 1

The first experiment consisted of two treatments ($n = 3$ fish/treatment), defined as follows: (1) Intact toxic cells, individuals fed with pejerrey food mixed with cells *M. aeruginosa* strain NPJB1; (2) Crushed toxic cells, individuals fed with pejerrey food mixed with crushed cells of the same strain, previously disrupted by 3 cycles of freeze-thawing and homogenization (Lawton and Edwards 2001, for a review). Additionally, we checked visually the presence of crushed cells with a light microscope (1,000×).

Taking into account that pejerrey digestion process takes about 12 h (Hualde pers. com.), all the individuals from each treatment were killed 12 h after feeding. Intestine (without gut content) and liver were removed, weighed and thoroughly washed with ice-cold homogenization buffer (40 mM Tris–HCl, 20 mM KCl, 30 mM $MgCl_2$, pH 8.6 with 2 mM PMSF) and were homogenized (Omni 1,000 motorized homogenizer at 20,000 rpm) (1:4 w/v). Homogenates were centrifuged at 11,000×*g* for 15 min, and the supernatants were subject to Lemieux oxidation and gas chromatography/mass spectrometry (GC/MS) for measuring total MCLR (see below for detailed explanation).

Experiment 2

The second experiment consisted of the following treatments: (1) Control group, fed with pejerrey food; (2) Cyanobacterial control, fed with pejerrey food mixed with crushed cells of *M. aeruginosa* strain NPDC1 (nontoxic); (3) Crushed toxic cells, fed with fish food mixed with crushed cells of *M. aeruginosa* strain NPJB1.

Six individuals from each treatment were killed 3, 6, 12, 24, and 48 h after feeding. Intestine and liver were removed, washed, and weighed, and homogenates were prepared as described for experiment 1. After centrifugation, supernatants were used for enzymatic assays, MDA, and protein quantitation. For treatment 3, approximately half of each tissue sample from three fish/time was separated and subject to methanol extraction of MCLR as described below. Hepatosomatic index (HSI) was calculated as liver mass/(total body mass−liver mass) * 100.

All experimental protocols were approved by the Bioethics Committee of the Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, Argentina (6060/116).

Endogen PP1 activity

PP1 activity in liver and intestine samples was measured according to Carmichael and An (1999). Briefly, the reaction solution was prepared by dissolving 1 mM DTT, 0.5 mg/mL BSA, and 0.5 mM $MnCl_2$ in homogenization buffer. The substrate solution was made by dissolving 40 mM *p*NPP in reaction solution. 90 µL of reaction solution plus 10 µL supernatant (sample) and 100 µL substrate solution were added to each well of a microtiter plate. After 10 min of incubation, absorbance at 405 nm was read every 5 min during 40 min at 25 °C. Each sample was measured in triplicate. PP1 activity was expressed as nkatal/mg protein.

Glutathione-S-transferase (GST, EC1.11.1.9)

Activity of GST was measured according to Habig et al. (1974), using 100 mM GSH (in phosphate buffer 100 mM, pH 6.5) as substrate. The sample was mixed with phosphate buffer and GSH solution. The reaction was started by adding CDNB (in ethanol). The absorbance at 340 nm was followed for 5 min. Each sample was measured in duplicate. GST activity was expressed as nkatal/mg protein.

Catalase activity (CAT, EC1.11.1.6)

Enzyme activity was calculated using an extinction coefficient of 40 M^{-1} cm^{-1} (Aebi 1984). The decay of hydrogen peroxide was monitored during 30 s at

240 nm, in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7) and 10 mM H_2O_2. Each sample was measured in duplicate. Results were expressed as nkatal/mg protein.

Lipid peroxidation

Lipid peroxidation was estimated by measuring thiobarbituric acid reactive substances (TBARS), using a modified protocol form of Beuge and Aust (1978). Briefly, each supernatant was mixed with thiobarbituric acid (TBA) solution followed by incubation at 100 °C for 15 min. After cooling, the reaction mixture was centrifuged, and the supernatant absorbance was read at 535 nm. TBARS concentration was estimated using the extinction coefficient of MDA-TBA complex (156 mM^{-1} cm^{-1}). Results were expressed as ng MDA/mg protein. Each sample was measured in triplicate.

Protein content

Total soluble protein content was measured by the method of Bradford (1976), using BSA as standard. Results were expressed as mg protein/mL.

Methanol extraction and C18 clean-up

Liver and intestine samples from treatment 3 were homogenized in 100 % methanol, in a proportion of 0.1 g/mL, centrifuged at 11,000×*g* for 10 min, set-aside supernatant and extracted the tissue twice again repeating the previous steps (Carmichael and An 1999). This solvent extraction procedure allows to purify all the MCLR fractions not bound to protein (extractable MCLR) and to remove interfering substances. The combined methanolic supernatants obtained were evaporated with N_2, and the resultant oily residues were diluted in Milli-Q water with 0.1 % TFA. Aliquots of these samples were cleaned through a ZipTip C_{18} micro-column (Millipore, USA) to purify and concentrate MCLR. The concentrated toxin was eluted with 5 µL of 80 % methanol. Methanol was evaporated, and then the toxin redissolved in Milli-Q water for analysis by PPIA according to Carmichael and An (1999). This procedure yielded a recovery of 72 ± 6 % (3 samples measured by triplicate). A calibration curve was built using a MCLR standard. Pellets and the remaining of the methanolic supernatants were saved for subsequent analysis of total MCLR.

Lemieux oxidation

Extractable and protein-bound MCLR were determined by GC/MS after Lemieux oxidation to 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) (Sano et al. 1992) according to Williams et al. (1997b). Supernatants of liver and intestine homogenates (in buffer) from the experiment 1 were subject to exhaustive Lemieux oxidation. Supernatants and pellets from methanolic extraction (pooled samples from 3 individuals per time) from the experiment 2 were processed in the same way.

Initially, for 1 g of tissue, 8 mL of stock oxidant solution (5.7 mg of $KMnO_4$ and 318.6 mg KIO_4 in 16 mL distilled water) and 23 mg Na_2CO_3 were used. The reaction was stirred overnight, pH was kept at 9, and color was kept purple by adding additional K_2CO_3 and $KMnO_4$/$NaIO_4$ when necessary. Excess oxidant was reduced with 20 % sodium metabisulfite, and the weakly alkaline reaction mixture was acidified with drops of 10 % H_2SO_4, diluted with additional H_2O, and then extracted with diethyl ether (4 × 15 mL). The ether extracts were combined, respectively, and dried over anhydrous Na_2SO_4. These extracts were subsequently analyzed by GC/MS, and the MMPB present was quantitated using a calibration curve based on dilutions of a MCLR standard subject to Lemieux oxidation.

Gas chromatography–mass spectrometry (GC/MS) analysis

Capillary GC/MS analyses were carried out on an Agilent 6890 series chromatograph linked to an Agilent 5973 series mass spectrometer with electron impact ionization in SIM mode. A HP-5 MS column (0.25 µm coating, 0.25 mm i.d. × 30 m; Agilent 19091S-433) was used with helium as carrier gas (flow rate, 24.4 cm/s), constant pressure, and 12ψ. The program rate for PTV inlet was 80 °C (0.00 min) then 720 °C/min to 250 °C (2.00 min) splitless mode. The program rate for the analyses was 75 °C (0.00 min) initial time, 5 °C/min to 200 °C (0.00 min) total time 35 min. The other conditions were as follows: ion source, 230 °C; quadrupole temperature, 150 °C; interface, 280 °C; injection volume, 3 µL.

Calibration curve was established for the MMPB obtained from MCLR standard subject to Lemieux oxidation. To estimate the percentage recovery of MMPB, 3 liver tissue homogenates (47.3 mg ± 2)

were spiked with 100 ng of MCLR, homogenized and subject to Lemieux oxidation. Each sample was run in triplicate.

Statistical analysis

Results were subject to two-way analysis of variance (ANOVA) and presented as mean ± SEM. The effects of treatments were assessed by LSD planned comparisons or by post hoc Newman-Keuls comparisons, when appropriate. We considered a value of $p < 0.05$ as statistically significant (Zar 1999).

Results

No mortality was observed at any time during experimental periods. No changes in the external aspect or in swimming behavior were visually detected in treated fish respect to the control group.

Gas chromatography–mass spectrometry (GC/MS) analysis

MMPB was detected at 15.659 min. The authenticity of this compound was confirmed by the analysis of characteristic fragment ions according to Williams et al. (1997b). The spikes showed a recovery of 67.25 ± 26 %. The detection limit that gave a consistent calibration was lower than 0.2 ng of MMPB. Calibration curve is shown in Fig. 1.

Experiment 1

Lemieux oxidation–GC/MS showed that supernatants from liver homogenates from fish fed with intact toxic cells had a quantity of MCLR (334 ± 134 ng MC/g) that is very similar to that found in fish fed with crushed toxic cells (349 ± 139 ng MC/g). This suggests that *O. hatcheri* is capable of digesting cyanobacteria cells. Fish that received intact toxic cells accumulated 174 ± 95 ng MC/g in intestine tissue, but we could not detect properly MCLR in intestine of fish fed with crushed toxic cells in this experiment.

Experiment 2

No significant changes were observed in the hepatosomatic index of MCLR exposed fish in comparison with control individuals (data not shown). Livers from fish of all the treatments presented a general healthy aspect (pinkish coloration with no visible signs of disintegration).

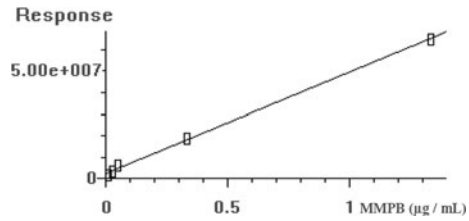

Fig. 1 GC/MS calibration curve (response vs. concentration, in µg/mL) for MMPB obtained from MCLR standard subject to Lemieux oxidation

Microcystin accumulation

The results of methanol extraction-PPIA applied to detect extractable MCLR in liver and intestine tissues are summarized in Table 1 along with the results obtained for extractable and protein-bound MCLR obtained by Lemieux oxidation–GC/MS. The total MCLR detected by Lemieux oxidation–GC/MS accounted for 1–31.7 % and 0.1–11.11 % of the administered dose, in intestine and liver, respectively. The analysis of the extractable MCLR fraction showed important differences according to the method of analysis applied. PPIA showed very low levels of MCLR in comparison with the corresponding GC/MS measurements, suggesting that not all the MCLR forms present in this fraction are capable of inhibiting PP1 activity.

The major proportion of MCLR was found in the methanolic extraction pellets from both tissues analyzed by Lemieux oxidation and GC/MS, which represents protein-bound MCLR. Intestine seems to accumulate more MCLR than liver, both as protein-bound and as extractable toxin. Protein-bound and extractable MCLR measured by GC/MS are high in both tissues at the beginning of the experiment and then decrease along time. However, this tendency is not so clear in the extractable MCLR detected by PPIA (Table 1).

Endogen PP1 activity

Liver PP1 activity showed a decreasing tendency along time in all treatments (ANOVA, $p < 0.001$). No

Table 1 MCLR accumulated in *O. hatcheri* liver and intestine, measured by Lemieux oxidation-GC/MS and Protein phosphatase inhibition assay

Sample	Pellet–GC/MS		Supernatant–GC/MS		Supernatant–PPIA	
	ng MCLR/g tissue[a]	% Of dose detected	ng MCLR/g tissue	% Of dose detected	ng MCLR/g tissue[b]	% Of dose detected
Intestine 3 h	22,881	31.7	–	–	13.63 ± 6	0.019
6 h	4,658	6.5	105	0.15	5.47 ± 2	0.008
12 h	3,385	4.7	79	0.11	12.55 ± 5	0.017
24 h	710	1.0	–	–	7.50	0.010
48 h	–	–	84	0.12	4.90	0.007
Liver 3 h	1,943	2.2	1,021	1.18	3.3	0.004
6 h	9,400	10.9	186	0.21	1.3	0.002
12 h	1,284	1.5	107	0.12	2.62 ± 1	0.003
24 h	384	0.4	66	0.08	5.95 ± 5	0.007
48 h	75	0.1	–	–	2.62 ± 1	0.003

A dash indicates points were MCLR could not be detected

[a] Values correspond to pools of 3 individuals

[b] Values are mean ± SE obtained from 3 individuals

significant difference among treatments was detected ($p > 0.05$). However, when we performed an a priori LSD comparison between treatment 3 (fish fed with toxic cells) and treatments 1 and 2 (control groups) combined, treatment 3 had significantly higher PP1 activity ($p < 0.001$) (Fig. 2a).

GST

Two-way ANOVA for liver GST activity showed significant results both for time and treatment ($p < 0.001$), with no significant interaction. *Post hoc* comparisons yielded significant differences among all the treatments. Both cyanobacterial treatments (toxic and nontoxic cells) induced an increase of GST activity in comparison with fish that received control food ($p < 0.001$), indicating a possible effect of the cyanobacterial cells. However, GST activity was significantly higher in treatment 3 than in treatment 2 ($p < 0.05$), suggesting specific effects of MCLR (Fig. 3a).

CAT

CAT activity in liver also showed a significant decrease along time although less clear than that of PP1 ($p < 0.05$). There was no significant overall difference among treatments analyzed by ANOVA ($p > 0.05$), but treatment 3 resulted significantly higher than the combination of treatments 1 and 2 in the LSD a priori comparison ($p < 0.05$) (Fig. 3c).

Lipid peroxidation

The concentration of MDA in liver showed a general decreasing tendency along the experiment. ANOVA yielded significant differences for both time and treatment ($p < 0.001$) with no interaction between factors (Fig. 3e). Treatment 3 (fish fed with crushed toxic cells) had a higher concentration of MDA than control groups ($p < 0.001$); no significant difference was observed between treatments 1 and 2. A tendency to compensate lipid peroxidation is evident along time since treatment 3 reached control levels at 24 h.

Effects on intestine

As for intestine, none of the biochemical variables described above suffered discernible changes that could be associated to MCLR or other cyanobacterial compounds. Significant differences were observed only along time (ANOVA $p < 0.05$ for PP1 and $p < 0.001$ for CAT, GST and MDA) (Figs. 2 and 3).

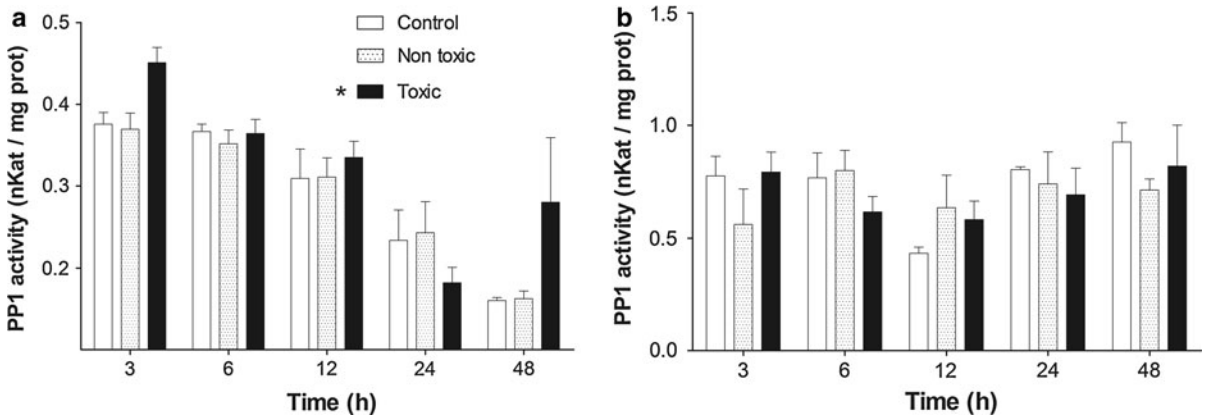

Fig. 2 Endogen activity of protein phosphatase 1 (PP1) in liver (**a**) and intestine (**b**) of *O. hatcheri* orally exposed to toxic and nontoxic *M. aeruginosa*. Values are expressed as mean ± SEM ($n = 6$). Asterisk in the legend indicates significant differences between the toxic treatment and the combination of nontoxic and control treatments in liver (a priori LSD comparison, $p < 0.001$). No significant differences were detected in intestine

Discussion

In the present work, *O. hatcheri* were fed with cyanobacterial cells of a MCLR-producing *M. aeruginosa* strain. This study is focused on the ability of *O. hatcheri* to digest cyanobacteria, the accumulation kinetics, and the toxic effects of a unique oral administration of MCLR.

The risk of incorporating MCs for planktivorous and omnivorous fish depends primarily on their capacity to digest cyanobacteria and absorb toxins, unless MCs are dissolved in water at high concentration as it occurs in the late stage of a bloom. Although, it is not clear whether *O. hatcheri* actively grazes on cyanobacteria, the results of the present work, in which fish fed with intact or crushed toxic cells have accumulated similar quantities of MCLR in liver, suggest that once inside the GI lumen cyanobacteria are effectively digested, and MCLR is released and taken up.

We have measured MCLR by two different techniques, Lemieux oxidation–GC/MS and PPIA in order to elucidate the kinetics of accumulation of MCLR in liver and intestine. At 3 h post ingestion, roughly 32 and 2 % of the administered dose (1.3 μg/g bm) are detectable as total MCLR in intestine and liver, respectively. These proportions change to 7 and 11 % at 6 h, suggesting transference of part of the toxin from intestine to liver and probably direct excretion to the intestinal lumen. This would mean that the intestine of *O. hatcheri* acts as a barrier for the uptake of this toxin. At longer periods, MCLR content tends to be very low or undetectable in both organs.

Protein-bound MCLR is the predominant form detected in both tissues; this fraction represents from 98 to c.a. 100 % and 66 to ca. 100 % of the total burden, in intestine and liver, respectively. In a pioneer work, Williams et al. (1997b) have measured total and extractable MCLR in liver of salmons intraperitoneally injected with 2 μg MCLR/g bm, by Lemieux oxidation–GC/MS and PPIA. As in our experiment, these authors report that most of the MC accumulated is covalently bound to proteins. At 7 h post injection, total MCLR in salmon liver accounts for about 33 % of the injected dose (Williams et al. 1997b). These values are similar to the 32 % that we detect at 3 h in intestine of *O. hatcheri*. In contrast, we find only 2 and 11 % in liver at 3 and 6 h, respectively. These differences in the accumulation pattern between both works are likely related to the route of intoxication since the peak in liver MCLR in *O. hatcheri* follows that of intestine. Although most MCLR in *O. hatcheri*'s intestine at 3 h appears as protein-bound, an important portion of it seems to be present in liver later. Since, to our knowledge, there are no previous reports on MCLR bound to proteins in fish intestine, we cannot discuss the metabolism, transference, and excretion of MCLR further.

In addition, Williams et al. (1997b) report a similar rate of elimination of the accumulated MCLR form the liver, to that described herein for *O. hatcheri*. This suggests that fish can degrade the proteins bound to the

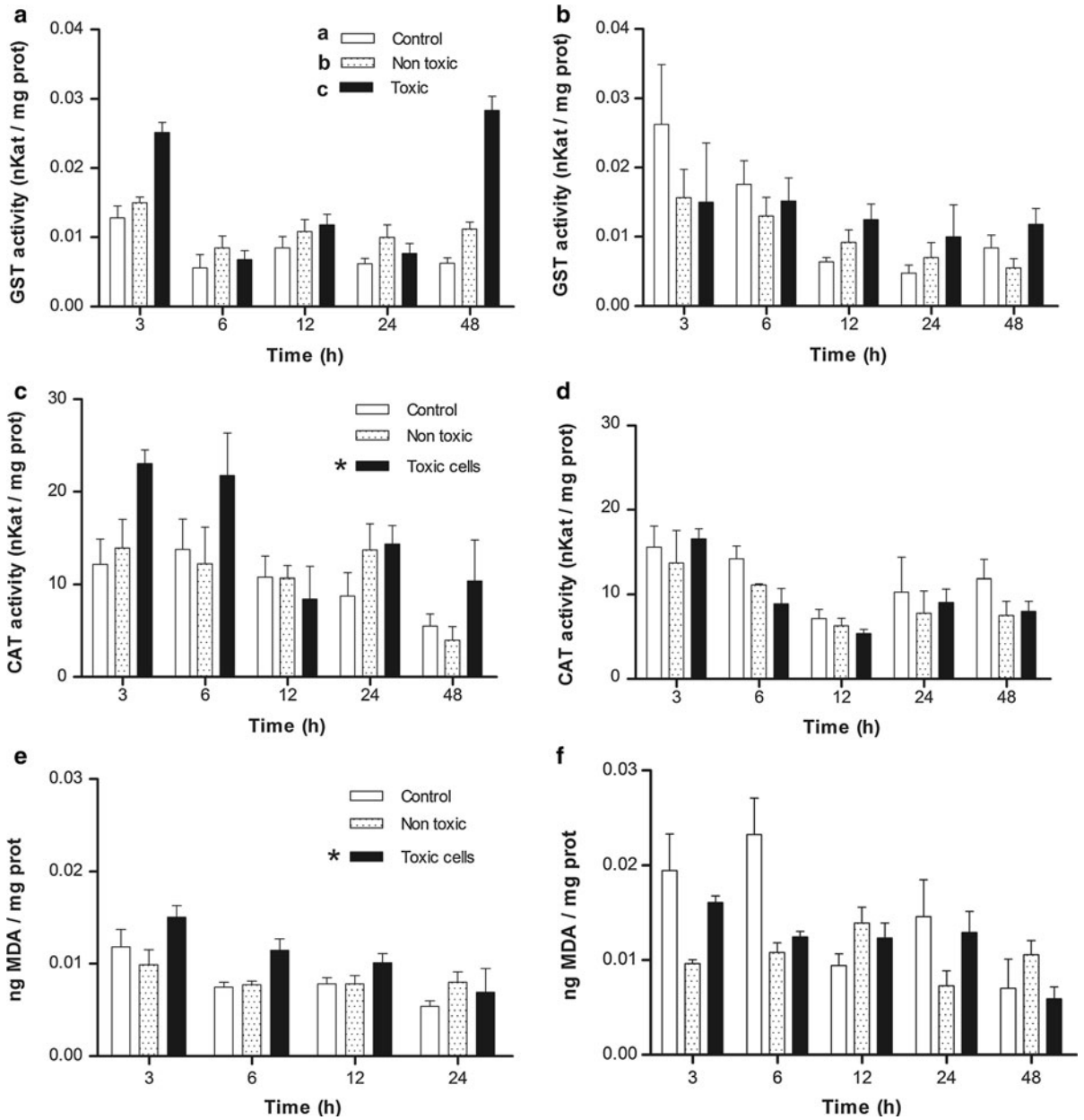

Fig. 3 Glutathione-S-transferase activity (**a**, **b**), catalase activity (**c**, **d**), and lipid peroxidation (**e**, **f**) in liver and intestine of *O. hatcheri* orally exposed to toxic and nontoxic *M. aeruginosa* cells. Values are expressed as mean ± SEM ($n = 6$). Different letters in the legends indicate significant differences among treatments in liver ($p < 0.05$ or $p < 0.001$). *Asterisk* in the legend indicates significant differences between the toxic treatment and the combination of nontoxic and control treatments (a priori LSD comparison, $p < 0.05$). No significant differences were detected in intestine

toxin to smaller compounds susceptible to be transported and excreted in the term of hours.

In regard to extractable MCLR, there are important differences (1–2 orders of magnitude) between the results obtained by the methods of analysis applied. By Lemieux oxidation–GC/MS, we detect much higher concentrations than by PPIA. This suggests that part of the MCLR present in the methanolic extract is not capable of inhibiting PP1. In this fraction, we expect to find all the non-protein-bound MCLR forms

(Pflugmacher et al. 1998; Williams et al. 1997a; Yuan et al. 2006), namely native MCLR and its conjugates with GSH and Cys. Although these conjugates have been reported as less toxic than free MCLR (Kondo et al. 1992), Ito et al. (2002) have shown that these are not much less capable of inhibiting PP1 and PP2A than parent MCLR.

We propose that the non-PP1-inhibiting part of the methanolic fraction obtained in this experiment corresponds to MCLR bound to cellular compounds different from GSH and Cys and/or to products of degradation of proteins previously bound to MCLR. Considering that the protein-bound MCLR accumulated in both tissues decreases along the experiment, the latter option seems more likely. Additional evidence of metabolization of proteins bound to MCLR in aquatic animals has been provided by Amorim and Vasconcelos (1999) and Soares et al. (2004), for mussels and fish, respectively. In both studies, the high MCLR concentrations found in depuration periods after intoxication are explained as a consequence of protein turnover that would lead to the release of toxins, making them detectable by ELISA.

Although abundant evidence from in vitro studies (Honkanen et al. 1990; Ito et al. 2002, MacKintosh et al. 1990, 1995; among others) points to endogen PP1 inhibition by MCLR, we detect increased activity of this enzyme in the liver of toxin treated *O. hatcheri*. This unexpected response could involve rapid post-translational modulation (Bollen 2001; Oliver and Shenolikar 1998; Therien and Blostein 2000; Wang et al. 2010a, b) and/or *de novo* synthesis of enzyme molecules. To our knowledge, the in vivo effects of MCLR on fish endogen PP activity have been measured in a few studies (Malbrouck et al. 2004; Runnegar et al. 1999; Tencalla and Dietrich 1997; Wang et al. 2010a, b), and only the latter two reports show PP activation in liver and brain of Zebra fish chronically treated with MCLR. However, the results presented by Runnegar et al. (1999) suggest that intravenous injection of 0.005 μg MCLR/g bm increased PP activity in the liver of *Raja erinacea.* At higher doses, these authors have reported no effect and 85 % inhibition, respectively. For rainbow trout liver, Tencalla and Dietrich (1997) have reported strong inhibition of PP, 1 and 3 h after oral gavage with 5.7 μg MCLR/g bm (a dose which is fourfold higher than that of the present study), followed by a rapid recovery of enzyme activity.

In accordance with Wang et al. (2010a), we suggest that, depending on the species and the applied doses, posttranslational stimulation and/or *de novo* synthesis can compensate or even overcome the inhibitory effect of MCLR. However, this should be tested in further dose–response studies including modulation of PP activity and gene expression by qPCR.

In the present work, in spite of the important accumulation of MCLR in intestine, the liver is the only organ, in which significant enzymatic responses and oxidative damage have been detected. Liver GST activity increases after exposure to both toxic and nontoxic cyanobacterial cells, being the increase more pronounced in the toxic treatment. GST catalyzes the conjugation of GSH with diverse electrophilic substrates including MCLR. This conjugation is thought to be the initial step in the detoxification of this compound (Pflugmacher et al. 1998), and this process is an important event for preventing MC bioaccumulation and toxicity.

Different responses of GST have been described in fishes exposed to MCs: inhibition (Cazenave et al. 2006), induction (Li et al. 2005), or even no effect (Li et al. 2003; Malbrouck et al. 2003). The increased GST activity observed in *O. hatcheri* could reflect the conjugation of MCLR with GSH, which would favor the biliary excretion of the toxin. On the other hand, the small but significant increment in GST activity observed in fish fed with nontoxic cells could be due to other cyanobacterial components apart from MCLR such as lipopolysaccharides (LPS). However, as we have demonstrated that *O. hatcheri* is able to digest *M. aeruginosa* cells and, besides, we fed fish with crushed nontoxic cells, we cannot discriminate between the effects of intracellular or cell wall compounds like LPS.

Liver CAT activity is increased in the toxic treatment coinciding with an increase in oxidative damage to lipids (MDA). Interestingly, the highest values of CAT and MDA are detected at 3 and 6 h, coinciding with the highest concentrations of total MCLR accumulated in this organ. This suggests a probable pro-oxidant effect of MCLR in liver, which is partially compensated by induction of CAT. Increased liver CAT activity after exposure to MCs has also been reported for tilapia (Jos et al. 2005; Prieto et al. 2006) for *Coridoras paleatus* (Cazenave et al. 2005) and for isolated hepatocytes of *C. carpio* (Li et al. 2003). In the two former papers, MDA is also increased. On the other hand, Atencio et al. (2008) have shown increased

MDA with decreased CAT activity in liver of *Tinca tinca*.

Considering the relationship between alimentary habits and sensitivity to MCs discussed by Fischer and Dietrich (2000), *O. hatcheri* is a planktivorous–omnivorous fish, whose diet is similar to that of *C. carpio*. However, the former species appears to be less sensitive to MCLR, at a similar dose. This suggests inter-specific differences in susceptibility to MCLR which go beyond the alimentary strategies and probably imply different mechanisms of detoxification and excretion. However, further studies on these mechanisms and on MCLR accumulation after more prolonged exposure are needed.

Conclusion

The results of this study show that *O. hatcheri* is able to digest toxic cyanobacteria and rapidly accumulate MCLR in intestine and liver (mainly protein-bound). This process is followed by toxin elimination from these tissues. The dose applied (1.3 μg MCLR/g bm) alters only slightly the oxidative balance of the liver but not that of the intestine. The enzymatic responses detected in liver and the elimination of MCLR from intestine and liver are evidences of detoxification–excretion capacity at least under these experimental conditions. This is important for aquaculture and environmental health, since most of the toxin absorbed by *O. hatcheri* would not be available for humans or predators a few hours to 1 day after exposure to cyanobacteria.

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