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Antioxidant response in liver of the phytoplanktivorous bighead carp (*Aristichthys nobilis*) intraperitoneally-injected with extracted microcystins

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Abstract The worldwide occurrence of cyanobacterial blooms makes it necessary to perform environmental risk assessment procedures to monitor the effects of microcystins (MCs) on fish. Oxidative stress biomarkers are valuable tools in this regard. In the present study, phytoplanktivorous bighead carp (*Aristichthys nobilis*) were injected intraperitoneally (i.p.) with extracted MCs (mainly MC-RR and -LR) at two doses, 400 and 1,000 μg kg^{-1} bw, and antioxidant responses of the liver as biomarkers of oxygen-mediated toxicity were studied at 1, 3, 12, 24 and 48 h after injection. Contents of reactive oxygen species (ROS) and activities of antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD), glutathione peroxide (GPX), and glutathione reductase (GR)] as well as glutathione S-transferase (GST) in the liver in both dose groups showed a biphasic change with an increase at initial 3 h followed by a decrease after injection, owing to the roles of the antioxidant system in eliminating excessive ROS and regenerating glutathione (GSH). The increased GST was probably due to the high transcription of cytosolic GST α and ρ, suggesting the importance of MCs detoxification by GSH pathway. The stable GSH levels in liver may be explained by the high basic GSH concentration in liver, and/or an increased GSH synthesis, suggesting a high ability to detoxify MCs and to release associated high oxidative pressure in phytoplantivorous fish.

Keywords Antioxidant response · Bighead carp · Glutathione · Liver · Microcystins

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Introduction

Cyanobacteria, especially *Microcystis aeruginosa*, pose a threat to animal and human health due to their ability to produce the hepatotoxic heptapeptides, microcystins (MCs) (Codd et al. 1997; De Figereido et al. 2004). Among more than 70 microcystin isoforms, microcystin-LR (MC-LR) is the most common variant, followed by microcystin-RR (MC-RR) and microcystin-YR (MC-YR) (De Figereido et al. 2004). One of the well-studied toxic mechanisms of MCs is their inhibition of protein phosphatase 1 and 2A, leading to increased protein phosphorylation, which is directly related to their cytotoxicity and tumor-promoting activity (Codd et al. 1997; Hooser 2000). At present, there is also evidence suggesting that

oxidative stress plays an important role in the pathogenesis of MCs toxicity in many species: mice (Gehringer et al. 2004), rat (Moreno et al. 2005), and aquatic organisms such as crab (Pinho et al. 2005) and fish (Li XY et al. 2005; Jos et al. 2005; Cazenave et al. 2006; Prieto et al. 2006, 2007).

As a result of oxidative stress, fish, like many other vertebrates, try to reduce the damage using the antioxidant defense system. These antioxidant defenses comprise enzymatic and non-enzymatic mechanisms. Some of the most important antioxidant enzymes are catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione reductase (GR), while the non-enzymatic defenses include GSH and vitamins E and C (Wilhelm Filho 1996). Probably because GSH does not only act as a free radical scavenger but also conjugate with xenobiotics in the metabolic pathway leading to detoxification, more attention has been paid to the role of GSH in preserving normal cellular redox balance and protecting hepatocytes against oxidative stress than other antioxidants such as Vitamin C or E (Gehringer et al. 2004; Xu et al. 1998). Pflugmacher et al. (1998) have shown the existence of microcystin–GSH conjugate formed through GST catalysis in aquatic plants (*Ceratophyllum demersum*), invertebrates (*Dreissena polymorpha* and *Daphnia magna*) and fish (*Danio rerio*), suggesting that this is the first step to detoxify MCs. In this respect, hepatic GST activity as well as GSH concentration should be privileged biomarkers of contamination with MCs.

From recent studies, it is known that some European fish species (e.g., ciscos) are highly susceptible to MCs (Ernst et al. 2001), whereas some subtropical fish use cyanobacteria as an energy source (Xie et al. 2004). The sensitivity of a fish to MCs is probably influenced by its natural habitat and feeding mode. Species native to oligotrophic habitats are more vulnerable than species native to eutrophic ones, where MC exposure is more frequent (Snyder et al. 2002). The planktivorous fish, which feed more frequently on toxic cyanobacteria, might have developed special mechanisms to counteract MCs in their evolution. In a subchronic toxicity experiment where the phytoplanktivorous silver carp were fed with toxic fresh *Microcystis viridis* cells (MC-RR and -LR contents were 268–580 and 110–292 μg g^{-1} dry weight, respectively), Xie et al. (2004) found that MC-LR were almost not detectable in the fish liver, suggesting that silver carp may have a mechanism to degrade MC-LR actively in the intestines and are probably more resistant to MCs than other fishes. However, up to now, the mechanism implicated in high resistance to MCs in phytoplantivorous fish has not been elucidated.

The bighead carp (*Aristichthys nobilis*) is an important phytoplanktivorous fish because of its roles in aquatic ecosystems as a direct consumer of zooplankton and phytoplankton, its potential for biological management of cyanobacterial blooms, and its value as a food fish (Xie and Liu 2001). Preliminary studies in our laboratory have demonstrated that extracted MCs from cyanobacterial cells are able to induce time-dose dependent ultrastructural changes in the liver of bighead carp after i.p. administration of MCs within 24 h post-injection, whereas the recovery of liver was observed at 48 h post-injection. In light of the above, this study aims to evaluate antioxidant systems through the assay of CAT, SOD, GPX, GR, GST and GSH, as well as reactive oxygen species (ROS), in the liver of the phytoplantivorous bighead carp after intraperitoneal injection with extracted toxins of MCs for further understanding of the mechanisms of hepatic antioxidation and detoxification in fish.

Materials and methods

Toxin

The cyanobacterial materials used in the experiment were collected from surface blooms (phytoplankton cells) of Lake Dianchi, Yunnan, China. According to microscopic examination, the predominant species was *Microcystis aeruginosa.* The microcystins were extracted by the methods of Park et al. (1998) with some improvements. Briefly, the materials were extracted three times with 10 ml of 75% methanol (v/v) for 3 h at 4°C. The extract was centrifuged at 10,000*g* for 30 min, and the supernatants were pooled and applied to a C_{18} cartridge (Dalian Institute of Chemical and Physical, China). The cartridge containing the microcystin was rinsed with 10 ml of water and MCs were finally eluted from the C_{18} cartridge with 10 ml of methanol. The elution was evaporated and then the residue was dissolved in water. This solution was used for the toxic experiment. Prior to use, the toxin-containing solution were analyzed by a reverse-phase high-performance liquid chromatograph (HPLC) (Shimadzu, LC-10A) equipped with an ODS

column (Cosmosil 5C18-AR, 4.6 × 150 mm; Nacalai, Japan) and a SPA-10A UV–vis spectrophotometer set at 238 nm. The sample was separated by using linear gradient elution of 50–70% aqueous methanol containing 0.05% trifluoroacetyl (TFA) in 25 min at a flow rate of 1 ml/min. MCs were identified by their UV spectra and retention times, and by using commercial microcystin-LR RR and YR (Wako Pure Chemical Industries, Japan) as standards. The obtained microcystin was MC-RR + MC-LR + MC-YR with purity >80%. The microcystin content in the cyanobacterial material was 1.41 mg g^{-1} dry weight (DW), among which MC-RR, -LR and -YR were 0.84, 0.50 and 0.07 mg g^{-1} DW, respectively.

Fish, treatments and sample preparation

Juvenile bighead carp with a mean weight of 51 ± 2 g were purchased from a local fish hatchery (Wuhan, China). Fish were acclimated for 3 days prior to experimentation in 100-l aquaria containing dechlorinated tap water. Water temperature was 20 ± 1°C, pH was maintained at 7.4 and dissolved oxygen values were 7.5 mg/l. No food was given to the fish in the course of the experiment.

The treated fish were injected i.p. with the doses of approximately 0.5 ml extracted solution of MCs, amounting to 400 and 1,000 μg MC-LR + MC-RR per kg body weight (bw), respectively. The control fish were injected i.p. with equal volumes of distilled water. In the experiment, sampling points were set during a period of 48 h (1, 3, 12, 24 and 48 h post-treatment). At each sampling point, three MCs-treated fish for each dose group were anesthetized with 0.02% tricaine methane sulfonate (MS-222) solution for 5–10 min before they were killed by transsection of the spinal cord. Three control fish were anesthetized and killed at both 0 and 48 h. Liver were excised, freed of attached tissue, and weighed. Experiments were performed according to the guidelines of the Ethical Committee for Animal Experiments at Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan of China.

Measurement of biochemical parameters in liver

Liver samples were homogenized (1:10, w/v) in a cold (4°C) buffer solution containing Tris base (20 mM), EDTA (1 mM), dithiothreitol (DTT, 1 mM; Sigma), sucrose (0.5 mM) and KCl (150 mM) and phenylmethylsulfonyl fluoride (PMSF, 1 mM; Sigma), with pH adjusted to 7.6. Homogenates were centrifuged at 9,500*g* (4°C) for 20 min and the supernatants used as the enzyme source. The activity of CAT was measured according to Aebi (1984) with slight modifications. The reaction mixture in a total volume of 3 ml contained 67 mM sodium phosphate buffer (pH 7.0) and 15 mM H_2O_2. The reaction was initiated by the addition of 0.01 ml of enzyme extract. CAT activity was determined by measuring the rate of disappearance of H_2O_2 at 240 nm for 3 min. The assay of SOD was based on a method described by Bayer and Fridovich (1987). The enzyme activity was defined as the amount of enzyme required to result in 50% inhibition of the rate of the nitroblue tetrazolium (NBT) reduction measured at 560 nm. The activities of GPX, GR, GST and the contents of GSH and ROS were assayed by the kits supplied by the Nanjing Bioengineering Institute, China. To determine GPX activity, we used GSH as a substrate and measured the conjugation of GSH and Ellman's reagent, 5,5′-dithiobis-(2-nitrobenzoic)-acid (DTNB). GR activity was determined by measuring NADPH oxidation at 340 nm. GST activity was detected by evaluating the conjugation of GSH with the standard model substrate 1-chloro-2,4-dinitrobenzene (CDNB). For the GSH assay, liver samples were treated with trichloroacetic acid as clarifier according to the instruction of the manufacturers. Then, GSH was determined using DTNB as substrate to measure the absorbency change at 412 nm. ROS formation (mainly hydroxyl radical) was determined spectrophotometrically by measuring the rate of color intensity change of gress agent at 550 nm based on the Fenton reaction. All enzymatic activities are expressed in nkat/mg prot, where 1 kat is the conversion of 1 mol of substrate per second.

Protein contents were determined by the Coomassie blue method using bovine serum albumin as a standard (Bradford 1976). All the experiments were repeated three times.

Statistical analysis

Analysis was undertaken using SPSS 11.5 for Windows. As we did not find any significant differences in the major biochemical parameters between 0 and 48 h of the control fish using an unpaired two-tailed Student's *t*-test ($P > 0.05$); we took the mean values

of 0 and 48 h as controls. The effect of time after MCs exposure was assessed by one-way analyses of variance (ANOVA 1) followed by a post hoc multiple comparisons test (Bonferroni's test). Results are presented as the mean ± SE. Differences were considered significant at the level of $P < 0.05$.

Results

The temporal alternations of ROS contents and antioxidant enzymes CAT, SOD, GPX and GR activities in both dose groups are presented in Fig. 1, which shows a biphasic change with an increase at initial 3 h followed by a progressive decrease. There was no significant difference in ROS contents between the two dose groups throughout the experiment, while there was a significant increase ($P < 0.05$) at 3 h post-injection before progressively returning to control values at 48 h post-injection (Fig. 1a). Similarly, CAT activity in the liver of bighead carp in both dose groups increased significantly ($P < 0.05$) at 3 h post-injection compared with the control (Fig. 1b). There was also a significant increase of SOD activity ($P < 0.05$) at 3 h in both dose groups when compared to the control (Fig. 1c). GPX activity in the 1,000 μg kg^{-1} dose group was significantly higher ($P < 0.05$) at 3 h than in the control, but the difference was not significant ($P > 0.05$) in the 400 μg kg^{-1} dose group (Fig. 1d). For the GR activity, significant increases were observed at 1 h ($P < 0.01$ in the 400 μg kg^{-1} dose group, $P < 0.05$ in the 1,000 μg kg^{-1} dose group) and 3 h post-injection ($P < 0.01$) in both dose groups. And at 24 h post-injection, there was also a significant increase ($P < 0.05$) in GR activity in the 1,000 μg kg^{-1} dose group compared to the control group (Fig. 1e).

As seen in Fig. 2a, the activity of GST in the liver of bighead carp in both dose groups increased

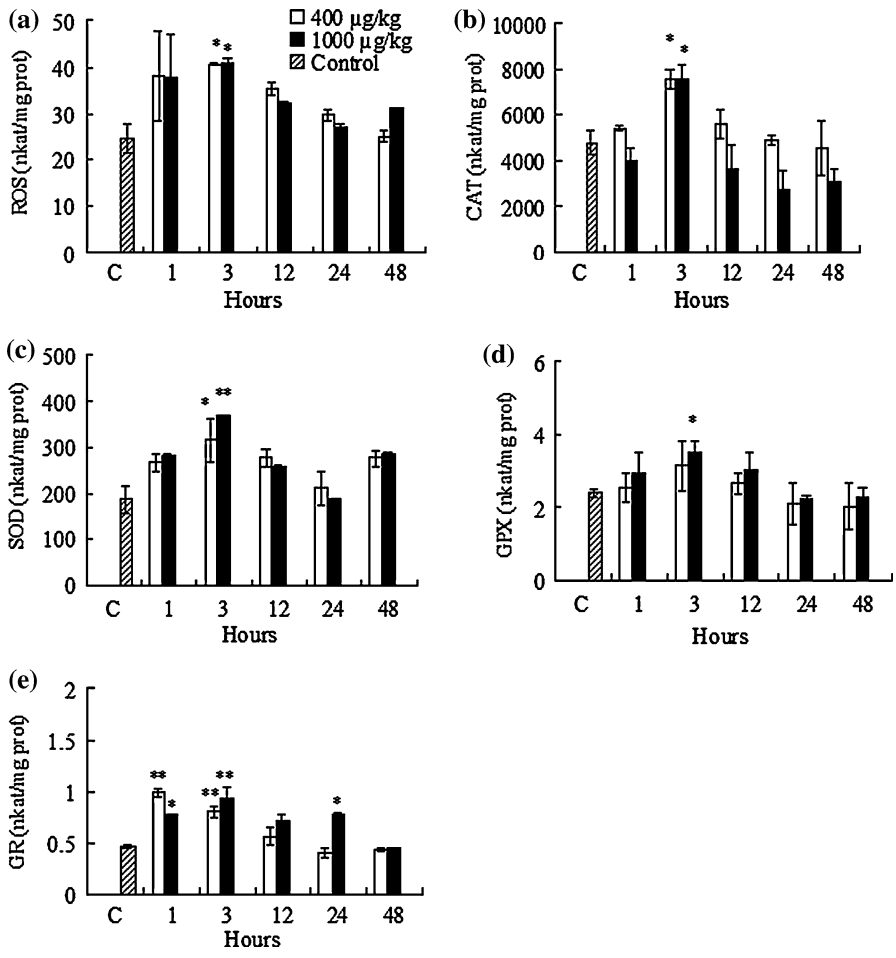

Fig. 1 Temporal alternations of ROS contents (**a**) and the activities of CAT (**b**), SOD (**c**), GPX (**d**), GR (**e**) in the liver of bighead carp (*Aristichthys nobilis*) injected i.p. with 400 and 1,000 μg MCs kg^{-1} bw, respectively. Data are represented as means ± SD ($n = 3$). The mean values of 0 and 48 h used as controls. The significance levels observed are * $P < 0.05$ and ** $P < 0.01$ in comparison to control group values

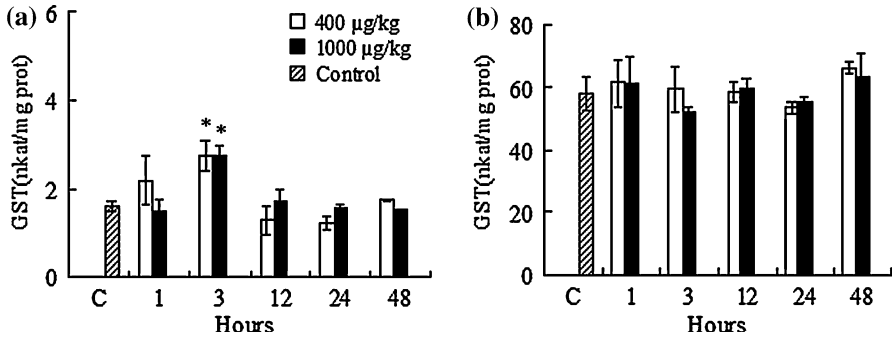

Fig. 2 Temporal alternations of GST activity (**a**) and GSH level (**b**) in the liver of bighead carp injected i.p. with 400 and 1,000 μg MCs kg^{-1} bw, respectively. Data are represented as means ± SD ($n = 3$). The mean values of 0 and 48 h used as controls. The significance level observed is * $P < 0.05$ in comparison to control group values

significantly ($P < 0.05$) at 3 h post-injection, followed by complete recovery at 48 h, but no obvious depletion in GSH levels in both dose groups was observed after injection with acute doses of MCs (Fig. 2b).

Discussion

In the present study, the initial significant increases in ROS contents (mainly hydroxyl radical) in the liver of bighead carp in both dose groups at 3 h post-injection suggest that MCs exposure induced oxidative stress. Oxidative stress is usually defined as an adverse reaction resulting from the exposure of molecules, cells, or tissues to excess levels of free radical oxidants, especially ROS (Ding et al. 1998). A continuous production of ROS in cells or tissues could attack lipid polyunsaturated fatty acids (PUFAs) and lead to PUFA oxidation. PUFA oxidation is believed to be highly deleterious to cells by damaging cell membranes, inactivating cellular enzymes, and damaging DNA. It has been found that cyanobacterial extracts can enhance intracellular production of ROS in cultured rat hepatocytes (Ding et al. 1998). An increase of ROS was also observed in isolated hepatocytes of common carp (*Cyprinus carpio*) when exposed to MC-LR, responsible for apoptosis and even necrosis of hepatocytes. In the present study, the activities of CAT, SOD, GPX and GR in the liver of bighead carp significantly increased at 3 h post-injection, which indirectly verified that oxidative stress was occurring in the liver. This result also indicated that toxin-induced oxidative stress was involved in the mechanism of hepatotoxicity of MCs.

Effects of MCs on antioxidant systems of fish have been studied in isolated carp hepatocytes (Li et al. 2003), liver of loach (*Misgurnus mizolepis*) (Li XY et al. 2005) and tilapia (*Oreochromis* sp.) either injected i.p. with MC-LR or exposed to a cyanobacterial blooms (Jos et al. 2005; Pireto et al, 2006, 2007). Li et al. (2003) observed enhanced CAT and SOD activities in isolated hepatocytes of common carp when exposed to a dose of 10 μg/l for 6 h. Prieto et al. (2006) showed that i.p.-administered pure MC-LR (500 μg/kg) induced a significant increase in the activities of CAT, SOD and GR in the liver of tilapia. Regarding the oral route, Jos et al. (2005) found that cyanobacterial cells at a dose of 60.0 μg MC-LReq/fish/day can also induce significant increases in the activity of CAT, SOD and GPX in the liver of tilapia for 21 days. A decrease in the activities of CAT, SOD, GPX and GR was observed in tilapia when they were orally exposed to a single dose of cyanobacterial cells containing 120 μg/fish MC-LR for 24 and 72 h (Pireto et al. 2007). However, in the present study, the antioxidant enzymes CAT, SOD, GPX and GR showed a biphasic change with an increase at the initial 3 h followed by a decrease after injection. This might be due to the roles of the antioxidant system in eliminating excessive ROS. In our study, although ROS (mainly hydroxyl radical) had an initial significant increase in the liver of bighead carp in both dose groups, they could not prevail over the antioxidant enzyme elimination. As a consequence, ROS (mainly hydroxyl radical) induced by MCs was overwhelmed by antioxidant enzymes in the liver of bighead carp, as evidenced by a progressive decline of antioxidant enzymatic activities in both dose groups.

Apart from the oxidative stress related effects, i.p. injection with the extracted MCs in bighead carp also produced a time-dose dependent hepatic apoptosis and necrosis in both dose groups within 24 h post-injection, followed by a progressive histological recovery at 48 h post-injection (Li L et al. 2005), which agrees with the recovery of antioxidant enzymes CAT, SOD, GPX and GR at 48 h post-injection. Malbrouck et al. (2004) found recovery of liver structure and regeneration of hepatocytes 96 h post-injection in goldfish as well as reconstruction of the tissue structure 21 days post-injection (of 125 μg MC-LR/kg bw). It seems that lesions caused by MCs can recover in fish. Thus, bighead carp in acute exposure to MCs showed a progressive recovery of structure and function in terms of histological and biochemical features.

The detoxification of MCs in liver is known to occur firstly via conjugation to GSH by GST (Pflugmacher et al. 1998). Augmented GST activity is utilized to indicate MCs detoxification in several aquatic organisms like microalgae, plants, invertebrates and fish (Gehringer et al. 2004). Nevertheless, the responses of GST activity to MCs in fish have been so far quite variable (Wiegand et al. 1999; Best et al. 2002; Li et al. 2003; Malbrouck et al. 2004; Cazenave et al. 2006). Either induced or inhibited activity of GST after exposure to MCs was reported in different fish species. Inhibition of GST activity was reported in zebrafish embryos exposed for 24 h to a mixture of MC-LR at 0.5 μg/l and cyanobacterial lipopolysacharides at 0.5 μg/l (Best et al. 2002) and in diverse organs of *Corydoras paleatus* exposed for 24 h to microcystin-RR at concentrations of 0.5 2, 5, and 10 μg/l, respectively (Cazenave et al. 2006). However, GST activity was either elevated in the early life stages of the zebrafish (Wiegand et al. 1999) or unaffected in hepatocytes of common carp exposed for 2, 4 and 6 h to MC-LR at a dose of 10 μg/l and in juvenile goldfish exposed for 96 h to microcystin-LR at a dose of 125 μg/kg bw throughout the experiment (Li et al. 2003; Malbrouck et al. 2004). It appears that different experimental conditions and/or interspecies differences in susceptibility to MCs influences on the responses of GST in fish. In the present study, the significant increase in GST activity in the liver of bighead carp in both dose groups at 3 h post-injection could be due to enhanced biotransformation reactions and higher rates of MCs conjugation, suggesting the importance of MCs detoxification by GSH pathway. Actually, the glutathione S-transferases are a multiple gene family of dimeric enzymes. Fu and Xie (2006) reported five cytosolic GSTs (ρ, μ, θ, α and π), mitochondrial GST (κ) and microsomal GSTs (mGST1, mGST2 and mGST3) from the liver of common carp exposed to MC-LR at 50 μg/kg, in which increased levels of transcription of hepatic GST α, ρ and mGST3 isoforms were detected at 6 h post stimulation, while the transcription of μ, θ and mGST isoforms were relatively stable and all the GST isoforms except GST κ and ρ recovered at 72 h to original levels compared with controls. Compared with the result of Fu and Xie (2006), the increased GST in our experiment was probably due to the high transcription of cytosolic GST α and ρ. Furthermore, the accurate influence of MCs exposure on the phase II detoxification systems need to be quantificated at variations in gene transcription of GST isoforms.

The content of intracellular GSH is a function of the balance between its use and synthesis. Initially, GSH is consumed by conjugation via GST or by glutathione disulfide formation when GSH reacts with ROS. It is then replaced either by enzymatic reduction catalyzed by GR or by de novo synthesis. In primary cultured rat hepatocytes exposed to cyanobacterial extract, Ding et al. (2000) suggested that the use of GSH by the initial conjugation to MC-LR may trigger an increase in GSH synthesis, possibly by activating the transcription of γ-glutamylcysteine synthetase, the rate limiting enzyme in GSH synthesis. Gehringer et al. (2004) reported an increase in GSH levels overall in the livers of mice treated with MC as well as an increase in the transcription of the enzymes involved in the synthesis of glutathione (Gehringer et al. 2004). Nevertheless, response of intracellular GSH content to MCs exposure in fish has been so far quite different, probably due to different exposure dose, time and routes, or different composition of MCs or different physiological characters of the selected tissue. Xu et al. (1998) observed a decrease in GSH concentration in the liver of goldfish following an i.p. injection of MC-LR (10 μg MC-LR/kg and higher). A similar decrease was also demonstrated by Li et al. (2003) on cultured hepatocytes of common carp exposed to MC-LR. The reduction of GSH content after MC-LR exposure may be explained by its increased utilization in the detoxification process, by a reduced GSH synthesis, or by both mechanisms. On the other hand, a significant increase in GSH level was reported in the liver of silver carp

(*Hypophthalmichthys molitrix*) exposed to a natural population of cyanobacterial bloom for 25 days, indicating an increased GSH demand as a result of oxidative stress and/or induction of detoxification enzymes in MCs-exposed fish (Blaha et al. 2004). Decrease in GSH content in the liver of juvenile goldfish was not significant after an i.p. injection of MC-LR (125 μg kg^{-1}) for 96 h (Malbrouck et al. 2004), which was attributed to its important basic GSH concentration. Furthermore, GSH depletion or formation limits the ability of an organism to detoxify MC-LR or prevent oxidative damage (Wiegand and Pflugmacher 2005). Previous studies on toxicity of MC-LR on animal hepatocytic antioxidant systems also directly demonstrate that antioxidant systems, mainly GSH, could be relevant indices in explaining the sensitivity of some vertebral species to MCs (Takenaka and Otsu 1999). The fact that bighead carp seem less affected by high doses of MCs was reported in previous studies performed in our laboratory in which a cystein conjugate of MC-LR (MCLR-Cys) was detected in most months (July 2004–March 2005) in kidney sample. This indicated that GSH detoxification pathway was an important route for the detoxification and excretion of MC-LR in bighead carp (Chen et al. 2007). So the stable GSH levels in bighead liver in our study may be explained by the high basic GSH concentration in bighead carp liver, and/or an increased GSH synthesis, suggesting a high ability to detoxify MCs and to release associated high oxidative pressure in phytoplantivorous fish.

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References

Aebi H (1984) Catalase in vitro. In: Packer L (ed) Methods in enzymology, vol 105. Academic Press, Orlando, pp 121–126

Bayer WF, Fridovich JL (1987) Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal Biochem 161:559–566

Best JH, Pflugmacher S, Wiegand C, Eddy FB, Metcalf JS, Codd GA (2002) Effects of enteric bacterial and cyanobacterial lipopolysaccharides, and of microcystin-LR, on glutathione S-transferase activities in zebrafish (*Danio rerio*). Aquat Toxicol 60:223–231

Blaha L, Kopp R, Simkova K, Mares J (2004) Oxidative stress biomarkers are modulated in silver carp (*Hypophthalmichthys molitrix* Val) exposed to microcystin-producing cyanobacterial water bloom. Acta Vet Brno 73:477–482

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein dye-binding. Anal Biochem 72:248–254

Cazenave J, Bistoni MA, Pesce SF, Wunderlin DA (2006) Differential detoxification and antioxidant response in diverse organs of Corydoras paleatus exposed to Microcystin-RR. Aquat Toxicol 76:1–12

Chen J, Xie P, Zhang DW, Lei HH (2007) *In situ* studies on the distribution patterns and dynamics of microcystins in biomanipulation fish-bighead carp (*Aristichthys nobilis*). Environ Pollut 147:150–157

Codd GA, Ward CJ, Bell SG (1997) Cyanobacterial toxins: occurrence, modes of action health effects and exposure routes. In: Seiler JP, Vilanova E (eds) Applied toxicology. Springer, Berlin, pp 399–410

De Figereido DR, Azeiteiro UM, Esteves SM, Goncalves FJM, Pereira JM (2004) Microcystin-producing blooms—a serious global public health issue. Ecotoxicol Environ Saf 59:151–163

Ding W-X, Shen H-M, Zhu HG, Ong C-N (1998) Studies on oxidative damage induced by cyanobacteria extract in primary cultured rat hepatocytes. Environ Res 78:12–18

Ding W-X, Shen H-M, Ong C-N (2000) Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induced rapid apoptosis in rat hepatocytes. J Toxicol Environ Health A 64:507–519

Ernst B, Hitzfeld BC, Dietrich DR (2001) Presence of *Planktothrix* sp. and cyanobacterial toxins in Lake Ammersee, Germany and their impact on whitefish (*Coregonus lavaretus* L.). Environ Toxicol 16:483–488

Fu J, Xie P (2006) The acute effects of microcystin LR on the transcription of nine glutathione S-transferase genes in common carp *Cyprinus carpio* L. Aquat Toxicol 80(3):261–266

Gehringer MM, Shephard EG, Downing TG, Wiegand C, Neilan BA (2004) An investigation into the detoxification of microcystin-LR by the glutathione pathway in Balb/c mice. Int J Biochem Cell Biol 36:931–941

Hooser SB (2000) Fulminant hepatocyte apoptosis in vivo following microcystin-LR administration to rats. Toxicol Pathol 28:726–733

Jos Á, Pichardo S, Prieto AI, Repetto G, Vázquez CM, Moreno I, Cameán AM (2005) Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis* sp.) under laboratory conditions. Aquat Toxicol 72:261–271

Li XY, Liu YD, Song LR, Liu J (2003) Responses of antioxidant systems in the hepatocytes of common carp (*Cyprinus carpio* L.) to the toxicity of microcystin-LR. Toxicon 42:85–89

Li XY, Chung I, Kim J, Lee J (2005) Oral exposure to *Microcystis* increases activity-augmented antioxidant enzymes in the liver of loach (*Misgurnus mizolepis*) and

has no effects on lipid peroxidation. Comp Biochem Physiol C 141:292–296

Li L, Xie P, Chen J (2005) In vivo studies on toxin accumulation in liver and ultrastructural changes of hepatocytes of the phytoplantivorous bighead carp i.p.-injected with extracted microcystins. Toxicon 46:533–546

Malbrouck C, Trausch G, Devos P, Kestemont P (2004) Effect of microcystin-LR on protein phosphatase activity in fed and fasted juvenile goldfish *Carassius auratus* L. Toxicon 43:295–301

Moreno I, Pichardo S, Jos Á, Gomez-Amores L, Mate A, Vázquez CM, Cameán AM (2005) Antioxidant enzyme activity and lipid peroxidation in liver and kidney of rats exposed to microcystin-LR administered intraperitoneally. Toxicon 45:395–402

Park HD, Kim B, Kim E, Okino T (1998) Hepatotoxic microcystins and neurotoxic anatoxin-a in cyanobacterial blooms from Korean lakes. Environ Toxicol Water Qual 13:225–234

Pflugmacher S, Wiegand C, Oberemm A, Beattie KA, Krause E, Codd GA, Steinberg CEW (1998) Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication. Biochim Biophys Acta 1425:527–533

Pinho GLL, Rosa MC, Maciel FE, Bianchini A, Yunes JS, Proenca LAO, Monserrat JM (2005) Antioxidant responses and oxidative stress after microcystin exposure in the hepatopancreas of an estuarine crab species. Ecotoxicol Environ Saf 61:353–360

Pireo AI, Jos Á, Pichardo S, Moreno IM, Cameán AM (2006) Differential oxidative stress responses to microcystins LR and RR in intraperitoneally exposed tilapia fish (*Oreochromis* sp.). Aquat Toxicol 77:314–321

Prieto AI, Pichardo S, Jos Á, Moreno I, Cameán AM (2007) Time-dependent oxidative stress response after acute exposure to toxic cyanobacterial cells containing microcystins in tilapia fish (Oreochromis niloticus) under laboratory conditions. Aquat Toxicol 84:337–345

Snyder GS, Goodwin AE, Freeman DW (2002) Evidence that channel catfish, *Ictalurus punctatus* (Rafinesque) mortality is not linked to ingestion of the hepatotoxin MC-LR. J Fish Dis 25:275–285

Takenaka S, Otsu R (1999) Effects of L-cysteine and reduced glutathione on the toxicities of microcystin-LR: the effect for acute liver failure and inhibition of protein phosphatase activity. Aquat Toxicol 48:65–68

Wiegand C, Pflugmacher S (2005) Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. Toxicol Appl Pharmacol 203:201–218

Wiegand C, Pflugmacher S, Oberemm A, Meems N, Beattie KA, Steinberg C, Cood GA (1999) Uptake and effects of microcystin-LR on detoxification enzymes during the ontogenesis of the zebrafish (*Danio rerio*). Int Rev Hydrobiol 85:413–422

Wilhelm Filho D (1996) Fish antioxidant defenses. A comparative approach. Braz J Med Biol Res 29:1735–1742

Xie P, Liu JK (2001) Practical success of biomanipulation using filter-feeding fish to control cyanobacteria blooms: synthesis of decades of research and application in a subtropical hypereutrophic lake. J Sci World 1:337–356

Xie LQ, Xie P, Ozawa K, Honma T, Yokoyama A, Park HD (2004) Dynamics of microcystins-LR and -RR in the phytoplanktivorous silver carp in a sub-chronic toxicity experiment. Environ Pollut 127:431–439

Xu LH, Chen GS, Chen JP, Xu JM, Zhang YY (1998) Toxic effects of microcystin on fish liver. Acta Hydrobiol Sin 22:378–379 (in Chinese)