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MICROCYSTIN KINETICS (BIOACCUMULATION AND ELIMINATION) AND BIOCHEMICAL RESPONSES IN COMMON CARP (*CYPRINUS CARPIO*) AND SILVER CARP (*HYPOPHTHALMICHTHYS MOLITRIX*) EXPOSED TO TOXIC CYANOBACTERIAL BLOOMS

ONDŘEJ ADAMOVSKÝ,† RADOVAN KOPP,‡ KLÁRA HILSCHEROVÁ,† PAVEL BABICA,† MIROSLAVA PALÍKOVÁ,§ VERONIKA PAŠKOVÁ,† STANISLAV NAVRÁTIL,§ BLAHOSLAV MARŠÁLEK,† and LUDĚK BLÁHA*†

†Centre for Cyanobacteria and Their Toxins (Institute of Botany, Czech Academy of Sciences and RECETOX, Masaryk University), Kamenice 126/3, 625 00 Brno, Czech Republic

‡Department of Fishery and Hydrobiology, Mendel University of Agriculture and Forestry, Zemědělská 1, 613 00 Brno, Czech Republic

§University of Veterinary and Pharmaceutical Sciences, Palackého 1-3, 612 42 Brno, Czech Republic

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Abstract—Two species of common edible fish, common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmichthys molitrix*), were exposed to a *Microcystis* spp.–dominated natural cyanobacterial water bloom for two months (concentrations of cyanobacterial toxin microcystin, 182–539 μg/g biomass dry wt). Toxins accumulated up to 1.4 to 29 ng/g fresh weight and 3.3 to 19 ng/g in the muscle of silver carp and common carp, respectively, as determined by enzyme-linked immunosorbent immunoassay. Concentrations an order of magnitude higher were detected in hepatopancreas (up to 226 ng/g in silver carp), with a peak after the initial four weeks. Calculated bioconcentration factors ranged from 0.6 to 1.7 for muscle and from 7.3 to 13.3 for hepatopancreas. Microcystins were completely eliminated within one to two weeks from both muscle and hepatopancreas after the transfer of fish with accumulated toxins to clean water. Mean estimated elimination half-lives ranged from 0.7 d in silver carp muscle to 8.4 d in common carp liver. The present study also showed significant modulations of several biochemical markers in hepatopancreas of fish exposed to cyanobacteria. Levels of glutathione and catalytic activities of glutathione *S*-transferase and glutathione reductase were induced in both species, indicating oxidative stress and enhanced detoxification processes. Calculation of hazard indexes using conservative U.S. Environmental Protection Agency methodology indicated rather low risks of microcystins accumulated in edible fish, but several uncertainties should be explored.

Keywords—Microcystins Bioaccumulation Toxicokinetics Biomarkers

INTRODUCTION

Hepatotoxic microcystins (MCs) are a group of peptide toxins produced by several species of freshwater cyanobacteria, such as *Microcystis* sp., *Planktothrix* sp., and so on [1]. Microcystins occurring as several structural variants are synthesized nonribosomally during the growth phase and may represent as much as 1% of the dry biomass. Although a portion of produced MCs is present extracellularly, the majority of MCs remain inside cyanobacteria, and toxins are released only after cell death [1]. Microcystins are potent inhibitors of serine/threonine protein phosphatase 1 and 2A [1], and they tend to accumulate in liver. Hepatotoxicity, liver tumor promotion, as well as other types of toxicity from MCs have been intensively studied and documented [2,3]. The World Health Organization suggested a limit for the tolerable daily intake (TDI) of 0.04 μg/kg body weight/d and corresponding provisional guideline of 1 μg/L for drinking waters for the most often studied MC variant, MC-LR [1,4].

Although the human toxicity has been studied in detail, the role of MCs in the aquatic environment remains questionable [5,6]. Some reports have described levels of MCs in fish, their metabolism, and also their toxicity [7–9], but detailed toxicokinetics and critical evaluation of human health risks from accumulated toxins remain to be resolved.

* To whom correspondence may be addressed (blaha@recetox.muni.cz).

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An important mechanism of MC toxicity documented in various laboratory animals [10], including fish [11], is oxidative stress—that is, cell damage caused by the overproduction of reactive oxygen species. Oxidative stress causes depletion of intracellular glutathione (GSH), lipid peroxidation, and oxidative damage to other biomolecules [12]. Several biomarkers of early toxic effects in fish after exposure to various stressors, including MCs, have been suggested (e.g., modulations of glutathione *S*-transferase [GST], glutathione reductase [GR], and glutathione peroxidase [GPx] [11–13]).

Major aims of the present study were to investigate kinetics of accumulation and elimination of MCs in the tissues of two cyprinid freshwater species, common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmichthys molitrix*). Both fish species are among the most widespread fish in Europe and Asia, and they often are cultured as important edible fish. In addition, the present study examined profiles of biochemical markers in hepatopancreas after cyanobacterial exposure and evaluated the health risks of MCs accumulated in fish tissues.

MATERIALS AND METHODS

Experimental design

Experiments simulated the natural situation in the environment. Fish (*C. carpio* and *H. molitrix*; average age, two years) were obtained from Pohořelice Fisheries (Pohořelice, Czech Republic). Uptake and accumulation of MCs was studied in the outdoor pond during two-month (nine-week) exposures of

Table 1. Kinetics of microcystin (MC) concentrations in the muscle and liver (ng MC/g tissue fresh wt) of common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmichthys molitrix*)[a]

Week	Water MCs[b]	Biomass MCs[c]	Silver carp			Common carp		
			Fish weight (g)	Muscle MCs	Liver MCs	Fish weight (g)	Muscle MCs	Liver MCs
Accumulation								
0	22.7	539	202 ± 46 (10)	0[d] (3)	0[d] (3)	125 ± 28 (10)	0[d] (4)	0[d] (4)
4	13.8	425	319 ± 78 (10)	10.6 ± 9.9 (10)	93.2 ± 50.7 (10)	127 ± 42 (10)	9.8 ± 6.4 (7)	132 ± 59 (7)
9	14.2	182	324 ± 78 (10)	5.2 ± 3.4 (7)	124 ± 56 (7)	128 ± 37 (10)	7.3 ± 4.6 (7)	68.7 ± 42 (7)
BCF[e] (mean/maximum)				0.62/1.7	7.3/13.3		0.57/1.1	7.8/12.8
Elimination								
0		—	421 ± 92 (10)	0.9 ± 0.3 (5)	21.0 ± 14.8 (5)	46 ± 9 (10)	1.2 ± 0.3 (5)	17.2 ± 7.0 (5)
1		—	380 ± 102 (10)	0[d] (5)	9.3 ± 3.7 (5)	47 ± 16 (10)	0.2 ± 0.1 (5)	13.7 ± 2.7 (5)
2		—	435 ± 86 (10)	0[d] (5)	0.9 ± 0.8 (5)	40 ± 10 (10)	0[d] (5)	2.3 ± 0.4 (5)

[a] Values represent the mean ± standard error, with the number of investigated fish given in parentheses.
[b] Water concentrations of total MCs (sum of MC-LR, -RR, and -YR; μg/L).
[c] Biomass MCs concentrations (μg/g dry wt).
[d] Less than the limit of detection (liver, 0.31 ng/g fresh wt; muscle, 0.13 ng/g).
[e] Bioconcentration factors (ratio between the mean/maximum tissue concentration and the average water concentration 17 μg/L).

fish to a complex cyanobacterial bloom dominated by *Microcystis aeruginosa* (45%), *Microcystis ichthyoblabe* (45%), and *Anabaena flos-aquae* (5%). Kinetics of MC elimination (after the transfer to clean water) was studied in fish that naturally accumulated MCs in the pond with *Microcystis* spp. Fish were not externally fed during experiments, and no mortalities were recorded. Fish (n = 3–10 individuals/treatment) were collected, weighed, and measured on weeks 4 and 9 (accumulation) and on weeks 1, 2, 4, 6, and 8 (during elimination) (Table 1). The tissue samples were immediately frozen and stored at −80°C for analyses of MCs and biomarkers. Parameters of water in the exposure/elimination experiments were as follows (given for the accumulation and elimination experiments, respectively; mean ± standard error): temperature, 18.9 ± 3.8 and 19.6 ± 1.3°C; dissolved oxygen, 18.2 ± 2.0 and 11.1 ± 3.2 mg/L; and pH, 9.4 ± 0.4 and 9.1 ± 0.2.

Toxin analyses by high-performance liquid chromatography

Concentrations of MCs in the cyanobacterial biomass and water (Table 1) were measured by high-performance liquid chromatography (HPLC) as described by Lawton et al. [14] with methods previously used in our laboratory [15]. Briefly, extracts of lyophilized biomass (50% v/v methanol) or water samples (MCs concentrated by solid-phase extraction using SepPack C18 cartridges [Waters, Millford, MA, USA]) were analyzed with a HPLC Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany) on a Supelcosil ABZ+ Plus (length, 150 mm; inner diameter, 4.6 mm; film thickness, 5 μm; Supelco, Bellefonte, PA, USA) at 30°C. The binary gradient of mobile phase (flow rate, 1 ml/min) consisted of H_2O plus 0.1% trifluoroacetic acid and acetonitrile plus 0.1% trifluoroacetic acid (linear increase during 0–30 min from 20–59% of acetonitrile). Chromatograms at 238 nm were recorded with an Agilent 1100 Series photodiode-array detector, and MCs were identified by the retention time and characteristic absorption spectra (200–300 nm). Quantification was based on external calibrations of three MC variants (MC-LR, -RR, and -YR).

Tissue extractions

Tissue extractions were performed according to the method described by Magalhaes et al. [16]. The frozen sample (0.4 g fresh wt) was homogenized with methanol (3 ml), sonicated in an ultrasonic bath for 30 min, and centrifuged at 4,000 *g* for 10 min. Supernatant was collected and the pellet re-extracted three times using the same procedure. Obtained methanol fractions were pooled and repeatedly extracted (three times) with 1 ml of hexane to remove lipids (hexane layers discarded). Methanol extract was evaporated at 50°C, and the residue was dissolved in 1 ml of water and analyzed for MCs using enzyme-linked immunosorbent immunoassay (ELISA). Recovery of the method (~25%; data not shown) was not considered during calculations to remain consistent with values previously reported in the literature [16–21].

ELISA for MCs

Concentrations of MCs in the fish tissues were analyzed by direct competitive ELISA according to the method described by Zeck et al. [22] using a modification described previously in detail [15]. Briefly, high-protein-binding, 96-well microplates (Nunc, Wiesbaden, Germany) were incubated overnight with the anti-mouse immunoglobulin (ICN MP Biomedicals, Solon, OH, USA). After a wash, plates were incubated for 1 h with mouse monoclonal IgG MC10E7 developed against MC-LR (5,000-fold dilution; ALEXIS, Lausen, Switzerland). The reaction was based on the competition of MCs in the sample with the conjugate of MC-LR–horseradish peroxidase [22]. The activity of horseradish peroxidase was determined using the 3,3′,5,5′-tetramethylbenzidine (absorbance, 420 nm; reference, 660 nm) with a microplate reader (GENios Spectra Fluor Plus; Tecan Group, Männedorf, Switzerland). Each sample was analyzed in three replicates and the results compared with the 0.125 to 2 μg/L calibration curve of MC-LR con-

structed for each individual ELISA plate. Samples from both exposed and control fish were analyzed, and no significant nonspecific interferences of the tissue extracts with ELISA were observed. The antibody used in the present study (MC10E7) has been shown to have 100 and 96% cross-reactivity with MC-LR and MC-RR, respectively [22]. Because these two MC variants were dominant in the present study, detected concentrations were considered to be a sum of MCs. We cannot exclude that the ELISA also detected MC fragments in fish tissues, such as glutathione-MC conjugates. This was not studied in detail, however, our approach was comparable with those in previous studies [16–21].

Biomarker analyses

Hepatopancreas samples (1 g) were homogenized on ice with 1 ml of phosphate buffer saline (pH 7.2), and supernatant was collected after centrifugation (5 min, 2,500 *g*, 4°C) and stored at −80°C before analyses. Protein concentrations were determined according to the method of Lowry et al. [23] using bovine serum albumin as a standard.

Concentration of glutathione was determined according to the method described by Ellmann [24] using 5,5′-dithiobis-2-nitrobenzoic acid as a substrate. Before analyses, the samples were treated with trichloroacetic acid (25% w/v) and centrifuged (6,000 *g*, 10 min). Supernatant was mixed with 0.6 μM 5,5′-dithiobis-2-nitrobenzoic acid in Tris-HCl/ethylenediaminetetra-acetic acid (EDTA) buffer (0.5 M tris[hydroxymethyl]-aminomethane–hydrochloric acid, 0.5 M Tris, and 12.5 mM EDTA; pH 8.9) and incubated for 5 min at room temperature. Absorbance was measured at 420/680 nm, and the concentrations (nmol GSH/mg protein) were calculated from the calibration of standard reduced GSH.

Glutathione *S*-transferase activity was measured spectrophotometrically using 1 mM 1-chloro-2,4-dinitrobenzene and 2 mM GSH as substrates according to the method described by Habig et al. [25]. Specific activity was expressed as nanomoles of formed product per minute per milligram of protein.

Activity of GPx was determined from the rate of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation, recorded as the decrease in absorbance at 340 nm [26]. The reaction mixtures contained 3 mM GSH, 1.2 mM butylhydroperoxide, 1 U of GR (1 U of GR reduces 1.0 mmol of oxidized glutathione per minute at pH 7.6 at 25°C), and 0.15 mM NADPH in 0.1 M potassium phosphate/1 mM EDTA buffer (pH 7.0). Also, the activity of GR in fish was determined by spectrophotometric measurement of NADPH oxidation in microplates [27]. The reaction mixtures contained 0.05 M potassium phosphate/1 mM EDTA buffer (pH 7.0), 1 mM glutathione-oxidized disodium salt, 0.1 mM NADPH, and the tissue extract (0.25% v/v). Specific activities of both GPx and GR were expressed as nanomoles of NADPH oxidized per minute per milligram protein.

Statistical calculations

Significant differences were determined using Student's *t* test or analysis of variance followed by Dunnett's post-hoc tests. Data normality was checked with the Kolmogorov-Smirnov test, and homogeneity of variances was assessed with the Levene's test. The *p* values less than 0.05 were considered to be statistically significant for all tests. Calculations were performed using the Statistica for Windows® 7.0 software package (StatSoft, Tulsa, OK, USA). Elimination kinetic curves and MC half-lives were calculated using the one-phase exponential decay equation incorporated in the GraphPad Prism 4 software (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

The present study describes toxicokinetics (accumulation and elimination) of MCs in the tissues of common carp and silver carp. Although several authors reported MC concentrations in zooplankton, shellfish, or fish [28–32], the kinetics of MC accumulation and elimination in fish have not been investigated in detail.

A summary of our results is given in Table 1 and in Figures 1 and 2. Microcystins accumulated in the muscle of common carp and silver carp up to 9.8 and 10.6 ng/g fresh weight, respectively. Concentrations approximately an order of magnitude higher were determined in the hepatopancreas, which is the target organ for MCs [33,34]. The muscle to liver concentration ratio in the present study (1:10) corresponded to that in the previous study with Atlantic salmon [33], but a higher ratio (1:20) was found in common carp compared with that in the study by Li et al. [18].

Average MC concentrations in both studied species generally were comparable (Table 1), but slightly higher levels were found in the liver of common carp in comparison to those in the liver of silver carp (compare, e.g., week 4 of the accumulation experiment) (Table 1). This may be related to possible resistance of phytophagous silver carp to MCs in comparison with the benthophagous common carp (as also suggested by Snyder et al. [19]). Calculated bioconcentration factors (BCFs; average and maximum tissue concentrations divided by the average water concentration of 17 μg/L) ranged from 0.6 to 1.7 in the muscle and from 7.3 to 13.3 in the liver of both species. To our knowledge, the BCFs for MCs in fish were not previously reported, but our results generally correspond to previously reported values for aquatic macrophytes (MC BCF ~0.1–5.9 [35]). Higher BCFs (range, 12–22) were reported for structurally related peptide cyanotoxin nodularin in various zooplankton species [36].

Kinetics of MC accumulation in hepatopancreas seems to be species-specific. In common carp, a peak in MC concentrations occurred after four weeks, followed by an apparent decrease after nine weeks (a trend that is comparable to the changes in muscle of both species) (Table 1). On the other hand, continuous accumulation of MCs was recorded in hepatopancreas of silver carp during the entire exposure period (up to 124 ng/g fresh wt) (Table 1). Differences may be explained, for example, by phytoplanktivorous feeding of silver carp, which actively ingests cyanobacterial cells, whereas only passive MC intake can be expected in omnivorous and benthophagous common carp [19].

The elimination experiment demonstrated that MC is rapidly removed from the tissues after the transfer of fish to clean water (Table 1). In both species, calculated elimination half-lives were shorter for muscle (0.7–2.8 d) than for liver (3.5–8.4) (Fig. 1). To our knowledge, information regarding MC depuration from the fish is rare [20,37,38]; however, studies of MC elimination from some invertebrates also suggest fast elimination of MCs. For example, a half-life of 8 d was reported for freshwater snail [39], and half-lives from 3.0 to 4.8 d were observed in bivalves [40]. In contrast to the rapid elimination observed in our manipulated experiments (Fig. 1), slower MC removal from silver carp and Nile tilapia has been reported in natural lakes (elevated MCs during the period 15–40 d after the end of the accumulation period [20,37]).

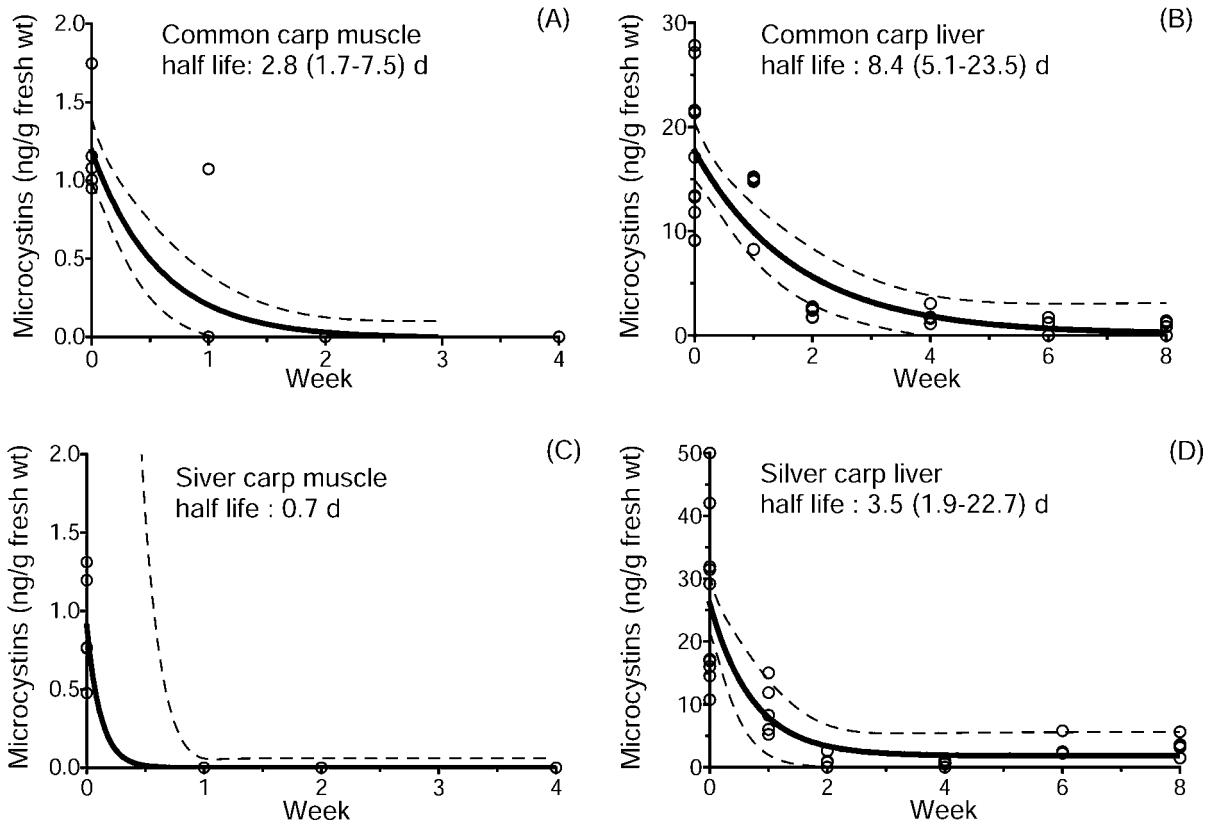

Fig. 1. Microcystin elimination from the tissues of common carp (*Cyprinus carpio*) (**A** and **B**) and silver carp (*Hypophthalmichthys molitrix*) (**C** and **D**). Presented are individual tissue concentrations, elimination curves (solid lines) with 95% confidence intervals (dashed lines), and half-lives in days (mean values with 95% confidence intervals in parentheses).

Taken together, bioaccumulation of MCs in the fish is a dynamic process depending on both uptake and metabolization/elimination [6]. Interspecies variability in MC metabolism and elimination, however, as well as environmental factors (e.g., temperature [40]) that may affect MC toxicokinetics will require further research.

We also investigated a set of glutathione-related biomarkers in the hepatopancreas of both fish species (Fig. 2). Activity of GR (significantly elevated in a majority of experimental variants, especially in common carp) was the most sensitive biomarker of cyanobacterial exposure (Fig. 2). On the other hand, changes in GPx activity were less sensitive in our experiments.

Inductions of GST seem to correspond to detoxification of MCs by GST-mediated conjugation with GSH [9,41,42]. Elevated GSH concentrations and activities of the GR (the enzyme regenerating GSH from its oxidized form [13]) further reveal increased demands for reduced GSH because of enhanced detoxification and/or oxidative stress induced by toxic cyanobacteria [11,12,43]. Our present study, however, demonstrates that biochemical adaptations are only temporary and that prolonged exposures may result in signs of general toxicity—that is, suppression of GSH levels and inhibition of GR activity (compare the four- and nine-week exposures for silver carp, as shown in Fig. 2).

Apparent time-, species-, and MC variant–dependent variability exists in biochemical responses of organisms to MCs [44]. Inductions of GST are among the most often reported responses [9,42] (present study), but other authors also have reported rapid, 24-h inhibitions of GST in *Corydoras paleatus* exposed to purified MC-RR [45]. Modulations of biomarkers in the present study confirm an important role for oxidative stress in the toxicity of complex cyanobacterial bloom, and it also demonstrates that biochemical parameters (especially GR, GST, and GSH) may serve as sensitive early markers of adverse effects in fish. Direct interpretation of biomarker responses remains complicated, however, and further research will be needed to characterize both natural variability and temporal changes in responses to toxicants.

It has been suggested that accumulated MCs in edible fish may represent a risk to human health, and it has been demonstrated that MCs are stable and not degraded by heat during cooking [46]. We have calculated the hazard index (HI), a ratio between the estimated daily intake (EDI) and chronic TDI, based on our results using an U.S. Environmental Protection Agency methodology [47]. To derive the EDI, we have considered a one-year exposure, 48 fish meals per year (100% contaminated), ingestion rate of 132 g per serving of meat, human body weight of 70 kg, and maximum concentration of MCs in fish fillet observed in the present study (29.3 ng/g fresh wt in silver carp). Using this worst-case scenario and considering a chronic TDI (0.04 μg/kg/d for MC-LR [1]), a calculated HI of 0.19 indicates a nonsignificant risk from MCs accumulated in fish meat (HI $<$ 1 [47]). Interestingly, relatively high HIs, ranging from 2.35 to 3.66, which correspond to realistically edible critical amounts of fish food (82–545 g/serving) were reported previously by Magalhaes et al. [16]. Those authors, however, compared the single-day intake of MCs with the chronic (i.e., year-round derived) TDI value, which could overestimate the total risk. Another factor that may affect total risk is relatively low recovery of MCs from animal tissues (reported values range from 3% [48] to 25% [present study]), which usually is not considered during cal-

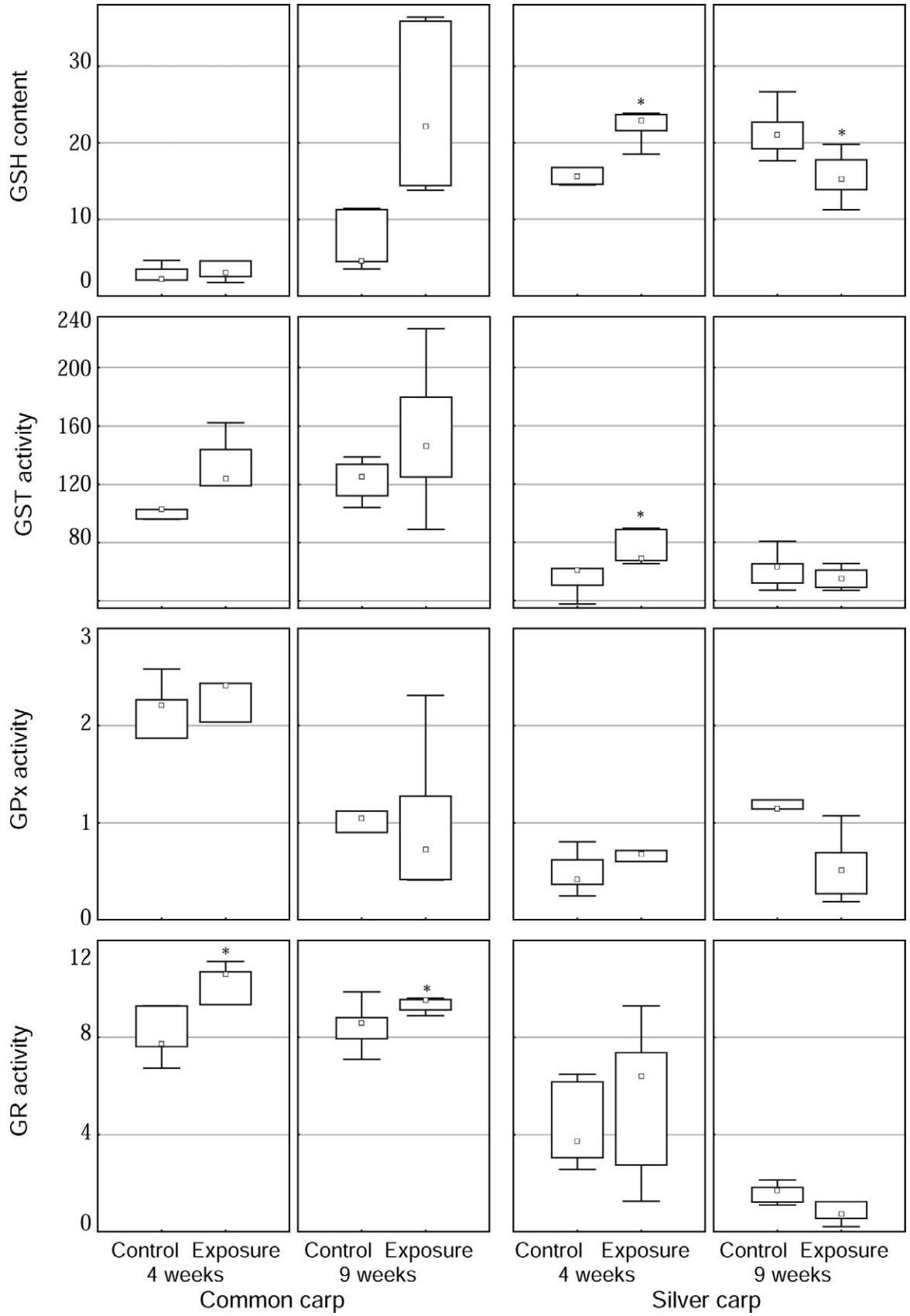

Fig. 2. Modulations of biochemical parameters in fish hepatopancreas after four and nine weeks of exposure to cyanobacterial biomass. Level of glutathione (GSH; nmol/mg protein), activity of glutathione *S*-transferase (GST; nmol/min/mg protein), and activities of glutathione peroxidase (GPx; nmol nicotinamide adenine dinucleotide phosphate [NADPH]/min/mg protein) and glutathione reductase (GR; nmol NADPH/min/mg protein). Box includes the 25th to 75th percentiles, with the middle point representing the median and the whiskers showing the extremes. An asterisk indicates a statistically significant difference from control ($p < 0.05$, Student's t test).

culations but may lead to possible underestimation of EDI. Taken together, MCs accumulated in edible fish tissues eventually may pose a risk to certain groups of people (e.g., fishermen consuming large amounts of contaminated fish), but uncertainties remain in both analytical approaches and risk assessment calculations.

CONCLUSIONS

Our results demonstrate kinetics of MC accumulation and elimination in two common Eurasian freshwater fish species, common carp and silver carp. We found that in most cases, maximum MC concentrations accumulated within the first four weeks of exposure, and prolonged periods (nine weeks) resulted in a less significant increase. Our results suggest rapid elimination of MCs from the fish tissues (half-life in days), but further research should focus on interspecies differences in metabolization and natural factors affecting MC toxicokinetics. The role of oxidative stress and changes of detoxification capacity in response of the fish on cyanobacterial exposure was confirmed by modulations of several biochemical parameters (e.g., GR, GSH, and GST in both species). Calculation of hazard indexes using conservative U.S. Environmental Protection Agency methodology indicates a rather low risk of accumulated MCs in edible fish, but several uncertainties should be explored.

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