Determination of microcystin-LR and its metabolites in snail (*Bellamya aeruginosa*), shrimp (*Macrobrachium nipponensis*) and silver carp (*Hypophthalmichthys molitrix*) from Lake Taihu, China

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ABSTRACT

This paper describes seasonal changes of microcystin-LR (MC-LR) and its glutathione (MC-LR-GSH) and cysteine conjugates (MC-LR-Cys) in three aquatic animals – snail (*Bellamya aeruginosa*), shrimp (*Macrobrachium nipponensis*) and silver carp (*Hypophthalmichthys molitrix*) collected from Lake Taihu, China. MC-LR, MC-LR-GSH, and MC-LR-Cys were determined by liquid chromatography electrospray ionization mass spectrum (LC–ESI-MS). The mean MC-LR concentrations in the hepatopancreas of snail and shrimp and liver of silver carp were 6.61, 0.24, and 0.027 $\mu g\,g^{-1}$ dry weight (DW), respectively; while the average MC-LR-Cys concentrations were 0.50, 0.97, and 5.72 $\mu g\,g^{-1}$ DW, respectively. MC-LR-GSH was usually not detectable in these samples. The above results suggest that: (1) in aquatic animals, especially fish, the main excretion form of MC-LR could be MC-LR-Cys, but not MC-LR-GSH, whereas MC-LR-Cys might play an important role in detoxication of MC-LR and (2) that efficiency of MC-LR-Cys formation differs among species. The main detoxication pathway of MC-LR in aquatic animals is suggested as follows: when MC-LR enters into liver/hepatopancreas, it firstly conjugates with polypeptide or protein (including GSH, PP-1 and 2A) containing Cys residues, perhaps also some free cysteine; subsequently, MC-LR-Cys is degraded from these polypeptide or protein; and finally is excreted from animals by the compound of MC-LR-Cys.

1. Introduction

Microcystins (MCs) are a family of cyclic hepatotoxic heptapeptides produced by some species of freshwater cyanobacteria including several strains from the genera *Microcystis*, *Oscillotoria*, *Anabaena*, *Nostoc*, and *Planktothrix* (Svrcek and Smith, 2004). Microcystins have the general structure of cyclo (-D-Ala-X-D-MeAsp-Y-Adda-D-Glu-Mdha), where X and Y are two variable amino acids, D-MeAsp, Mdha, Adda are abbreviations of D-methylaspartic acid, N-methyldehydroalanine and (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid, respectively (Carmichael et al., 1988). To date, more than 80 variants have been isolated and identified, differing primarily in the two L-amino acids at positions 2 and 4 and methylation/demethylation on MeAsp and Mdha (Dietrich and Hoeger, 2005). Among these microcystins isoforms, MC-LR is the most common and the most toxic variant (Gupta et al., 2003).

It is well known that microcystins can cause poisoning or death of fish, birds, domestic and wild animals (Carmichael, 2001), as well as illnesses and mortality in human (Azevedo et al., 2002). In 1996, a serious accident of MC-toxication resulting in more than 50 human deaths occurred in Brazil due to the use of microcystins contaminated hemodialysis waters (Carmichael, 2001; Azevedo et al., 2002). Some studies demonstrated that the high incidence of primary liver cancer in eastern China was related to the presence of microcystins in the drinking water (Yu, 1989). Recently, microcystins were identified for the first time in the serum (average 0.228 ng MC-LReq mL^{-1}) of a chronically exposed human population (fishermen at Lake Chaohu, China) together with indication of hepatocellular damage (Chen et al., 2009).

Until to now, extensive field investigations have been conducted to document bioaccumulation and distribution of MCs in various aquatic organisms (snail, mussel, and various fish) in Lake Taihu, China (Chen and Xie, 2005; Chen et al., 2006, 2007; Zhang et al., 2007, 2009a). These studies reported that microcystins can accumulate in various organs of aquatic animals and also can be transferred along the food chain, hence pose potential threats to human health (Chen et al., 2009).

Previous studies reveal that glutathione plays an important role in the metabolic pathway of MCs in both mammals and a wide

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range of aquatic organisms (Runnegar et al., 1987; Hermansky et al., 1991; Kondo et al., 1992, 1996; Pflugmacher et al., 1998; Ito et al., 2002). Hermansky et al. (1991) reported that pretreatment of mice with GSH protected them against microcystin-LR lethality. The conjugation of microcystins to glutathione and cysteine has been tested under chemical conditions and the resulting conjugates were firstly identified by the Frit-FAB LC/MS (Kondo et al., 1992). Subsequently, Kondo et al. (1996) identified the presence of microcystins glutathione and cysteine conjugates in liver of mouse and rat treated with microcystins. In vitro formation of microcystin-LR conjugate can take place enzymatically via soluble glutathione S-transferase (GST) in aquatic macrophyte, invertebrates, fish eggs, and fish (Pflugmacher et al., 1998). Ito et al. (2002) studied the distribution of MC-LR and its glutathione and cysteine conjugates in different tissues in mice by immunostaining method. However, all the above studies just provided indirect evidence for the detoxication of MCs in organisms, since no study has made quantitative determinations of microcystin glutathione and cysteine conjugates simultaneously in organisms. Hence, it is an imperative need to understand the detoxication mechanisms of microcystins in animals through quantitative determinations of MC-LR and its two metabolites (MC-LR-GSH and MC-LR-Cys).

Lake Taihu (30°5′–32°8′N and 119°8′–121°55′E), the third largest freshwater lakes in China, is located in the east part of China. It has the total surface area of approximately 2338 km², with a mean water depth of 1.9 m and a maximum depth of about 2.6 m. During the past decades, the lake has undergone a steady increase in eutrophication, and with a regular occurrence of cyanobacterial surface blooms in the warm seasons each year (Xie, 2008). Zhang et al. (2009b) pointed out that the toxic Microcystis aeruginosa was the absolute dominant species in the water column of Lake Taihu during the bloom period.

The aims of the present study were: (1) to examine seasonal dynamics of MC-LR and its conjugates (MC-LR-GSH, MC-LR-Cys) in various organisms (snails (Bellamya aeruginosa (Reeve, 1862)), shrimp (Macrobrachium nipponensis) and silver carp (Hypophthalmichthys molitrix)) collected from Lake Taihu and (2) to compare the difference of the detoxication pathway among the aquatic animals.

2. Materials and methods

2.1. Preparation of MC-LR and its glutathione and cysteine conjugates

2.1.1. Preparation of MC-LR

MC-LR was isolated and purified from surface blooms collected from Lake Dianchi, China, as described in a recent study (Dai et al., 2008). The content of purified MC-LR was over 95% and its identity was confirmed and determined with LC–MS.

2.1.2. Formation of MC-LR-GSH and MC-LRCys

L-Glutathione (L-GSH) and L-Cysteine (L-Cys) were purchased from Acros Organics (Geel, Belgium) and the purity of GSH and Cys was greater than 99%. MC-LR-GSH and MC-LR-Cys were prepared by the method of Dai et al. (2008) and Dai (2008). Briefly, MC-LR (4 mg) reacted with L-GSH (124 mg) in 10 mL 5% potassium carbonate aqueous solution while stirring for 2 h at room temperature. The reaction mixture was neutralized with about 15 mL 0.2 M hydrochloric acid and applied to an ODS C_{18} cartridge (5 g, Waters, Milford, MA, USA). The cartridge was rinsed with 10 mL water and eluted by 20 mL methanol to give the reaction product. The reaction product was purified further by a semipreparative reversed phase liquid chromatography (Waters 600, USA; flow rate 1 mL/min; detection, UV (238 nm)) with an ODS C18 reversed phase semipreparative column (7.8 × 300 mm, 10 μm, Waters, Milford, MA, USA) to yield 3.2 mg of the purified GSH conjugate

of microcystins. The mobile phase was the mixture of methanol and water. MC-LR-Cys was formed and purified similarly. The reaction of microcystin-LR (4 mg) with L-Cys (48 mg) yielded 3 mg of the purified Cys conjugate of microcystin-LR. The content of purified glutathione and cysteine conjugates of MC-LR were over 95% and confirmed by HPLC (LC-20A, Shimadze, Kyoto, Japan) and LC–MS (Thermo Electron, Waltham, MA, USA).

2.2. Study area

Meiliang Bay, a part of Lake Taihu, with water surface area of 125 km², accommodates municipal and industry wastewater from Wuxi City, and acts as principal water source for the city. It is the most eutrophic part of the lake, characteristic of extremely dense accumulation of toxic Microcystis blooms in the summer (Xie, 2008). Gonghu Bay (31°31′–23′N, 120°16′–35′E) is located at the northeast portion of Taihu Lake (Fig. 1).

2.3. Sample preparation

Snails (B. aeruginosa; body weights: 4.68 ± 1.19 g; shell height: 2.80 ± 0.28 cm) and shrimp (Macrobrachium nipponensis; body weights: 2.3 ± 1.5 g; body weight: 5.9 ± 1.3 cm) were collected monthly from Gonghu Bay and Meiliang Bay, respectively, from November to December 2004 and from July to October 2005. The collected snails and shrimps were immediately frozen at −20 °C, and the hepatopancreas of snails and shrimps were dissected in the laboratory. To insure adequate amount of samples, we pooled separate hepatopancreas of 20 snails and shrimps into two composite samples, respectively.

Silver carp (H. molitrix; body weight: 1021 ± 325 g; body length: 34.5 ± 4.8 cm) was artificially stocked into a pen (Meiliang Bay, Taihu Lake, China) with a total area of 1.08 km² in January 2005. Two fish were collected from this pen monthly from July to December 2005. The collected fish were measured, weighed, and sacrificed immediately, and then dissected in the field into liver, kidney, and intestinal content, finally frozen at −20 °C immediately.
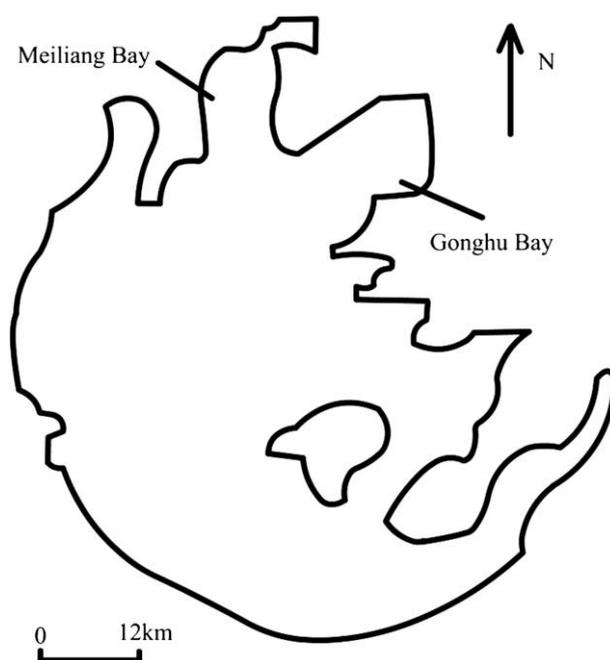

Fig. 1. Sampling locations in Lake Taihu (30°5′–32°8′N and 119°8′–121°55′E), China.

All above samples were lyophilized by a Christ® Alpha 2–4 freeze dryer (Martin Christ, Osterode, Germany). These lyophilized samples were extracted and purified following the methods of Dai et al. (2008) and Dai (2008) with minor modification: the lyophilized samples (∼0.1 g dry wt for each sample) were extracted three times with 5 mL of 0.01 M EDTA-Na$_2$-5% acetic acid by sonicating 3 min (30% amplitude, 60 W, 20 KHz, Sonics VC130 PB, Newtown, CT, USA) at 0 °C and then centrifuged at 14 000 r/min (BR4, Jouan, Winchester, VA, France) at 4 °C. The supernatant was first applied to an Oasis HLB cartridge (500 mg, waters, Milford, MA, USA), which had been preconditioned by washing with 10 mL 100% MeOH and 10 mL distilled water. The column containing sample was washed with 20 mL water followed by 20 mL 20% MeOH, and then eluted with 20 mL 100% MeOH. The eluant was evaporated to dryness and the residue was dissolved in 100% MeOH. This solution was applied to a Sep-Pak silica gel cartridge (2 g, waters, Milford, MA, USA) which had been preconditioned by 100% MeOH. The column was washed with 20 mL 100% MeOH and analytes, and then eluted with 20 mL of 70% MeOH. This fraction was evaporated to dryness and redissolved in 100 μL of the LC mobile phase.

2.4. Analysis of MC-LR and its metabolites

Qualitative and quantitative analysis of MC-LR, MC-LR-GSH, MC-LR-Cys were performed using a Finnigan LC–MS system comprising a Thermo Surveyor auto sampler, a Surveyor mass spectrum (MS) pump, a Surveyor photo diode array (PDA) system, and a Finnigan LCQ-Advantage MAX ion trap mass spectrometer (Thermo Electron, Waltham, MA, USA) equipped with an atmospheric pressure ionization fitted with an electrospray ionization source (ESI) (Thermo Electron). The instrument control, data processing, and analysis were conducted by using Xcalibur software (Thermo Electron). Separation was carried out using an Agilent ZOEBAX SB-C18 Column (2.1 mm i.d. × 150 mm, 3.5 μm, Agilent Corporation, Santa Clara, CA, USA). The mobile phase consisted of solvent A [water + 0.05% (v/v) formic acid]/solvent B [acetonitrile + 0.05% formic acid]. The following linear gradient programme was used: 0 min (75% A, 25% B), 8 min (45% A, 55% B), 13 min (40% A, 60% B), 14 min (30% A, 70% B), 15 min (75% A, 25% B), 20 min (75% A, 25% B). The total flow rate was held at 0.2 mL/min at analysis stage. After the analysis stage, the percentage of solution B was adjusted to 25% and the flow rate was increased to 0.3 mL/min for 5 min before the next injection to renew the initial condition rapidly. Sample injection volume was typically 10 μL. Mass spectrum tuning and optimization were achieved by infusing microcystin-LR and monitoring the [M+H]$^{2+}$ ion at m/z 995.5. The MS analytical conditions were as follows: ESI spray voltage 4.54 kV; sheath gas flow rate 20 unit; auxiliary gas flow rate 0 unit; capillary temperature 250 °C and multiplier voltage −853.19 V. The tube lens voltage was 55 V for MC-LR and 50 V for MC-LR-GSH and MC-LR-Cys. Quantification of MC-LR, MC-LR-Cys, and MC-LR-GSH was performed using the selected reaction monitoring (SRM) mode. For quantification purposes, mass chromatograms monitored the product ions at m/z 599.2 and 977.4 from the parent ion at m/z 995.5 of [M+H]$^+$ for MC-LR, and the product ions at m/z 599.2, 995.3, and 1029.3 from the parent ion at m/z 1116.5 of [M+H]$^+$ for MC-LR-Cys, while the product ions at m/z 587.3 and 1168.4 from the product ion at m/z 652.0 of [M+H]$^{2+}$ for MC-LR-GSH. Collision energy was 24%, 38% and 36% for MC-LR-GSH, MC-LR-Cys, and MC-LR, respectively. The limit of detection (LOD) of MC-LR and its metabolites (MC-LR-GSH and MC-LR-Cys) are 0.005 μg g^{-1} DW.

2.5. Statistical analysis

Pearson correlation analysis was conducted to determine the relationship between MC-LR-Cys concentration and MC-LR content

in the tissues of aquatic animals using SPSS for Windows (Ver 13.0; SPSS, Chicago, IL, USA). The relationships were considered significant at $p < 0.05$.

2.6. Recovery experiment

Recovery experiments were carried out in quadruplicate spiking 100 mg of freeze–dried fish liver samples with MC-LR, MC-LR-Cys, and MC-LR-GSH solution at 0.5 μg g^{-1} DW. The extraction and analysis were performed as described above, and the recovery and the relative standard deviation of the analytical method were calculated.

3. Results

3.1. Characterization and relationship of MC-LR and its two conjugates

Fig. 2 shows the ESI LC/MS/MS selected reaction monitoring (SRM) chromatograms for MC-LR and its two metabolites (MC-LR-GSH and MC-LR-Cys) in the intestinal content of silver carp from August 2005, Lake Taihu, China. Based on SRM chromatogram monitored ion at m/z 652.0, and the presence of [M+H]$^+$ ions at m/z 587.3 and 1168.3, it was confirmed that peak obtained at 9.48 min was derived from MC-LR-GSH. Similarly, peaks obtained at 9.67 min and 11.50 min were derived from MC-LR-Cys and MC-LR, respectively, as the peaks were detected by monitoring ions at m/z 1116.5 and m/z 995.5, respectively, and the selected ion chromatogram showed [M+H]$^+$ ions at m/z 599, 995, and 1029 for MC-LR-Cys and m/z 599 and 977 for MC-LR, respectively. The MC-LR-Cys to MC-LR ratios in hepatopancreas of snail and shrimp, and liver of silver carp were 1/12, 4 and 94, respectively. The ratios of MC-LR concentration in hepatopancreas of snail or shrimp to that in liver of silver carp were 108 and 4, respectively.

3.2. MC-LR and its conjugates in snail

The monthly changes of MC-LR (Mean ± SD; 3.73 ± 0.166–8.87 ± 0.633 μg g^{-1} DW, average 6.61 ± 2.17 μg g^{-1} DW), MC-LR-Cys (Mean ± SD; 0.115 ± 0.020–0.83 ± 0.10 μg g^{-1} DW, average 0.500 ± 0.285 μg g^{-1} DW), and MC-LR-GSH (below detection limit) in the hepatopancreas of snails are shown in Fig. 3a. MC-LR-GSH was detected only in the hepatopancreas of snails collected in July and September, 2005. There was a clear positive correlation between MC-LR and MC-LR-Cys concentrations in the hepatopancreas of snails ($r = 0.976$, $p < 0.01$; $n = 10$).

3.3. MC-LR and its conjugates in shrimp

Fig. 3b shows the seasonal variations of MC-LR (Mean ± SD; 0.031 ± 0.004–0.605 ± 0.179 μg g^{-1} DW, average 0.244 ± 0.220 μg g^{-1} DW), MC-LR-Cys (Mean ± SD; 0.306 ± 0.011–2.19 ± 0.039 μg g^{-1} DW, average 0.969 ± 0.729 μg g^{-1} DW), and MC-LR-GSH (Mean ± SD; below detection limit to 0.009 ± 0.004 μg g^{-1} DW, average, 0.002 ± 0.004 μg g^{-1} DW). A significant correlation was found between MC-LR-Cys and MC-LR concentrations in the hepatopancreas of shrimps ($r = 0.886$, $p < 0.01$; $n = 12$).

3.4. MC-LR and its conjugates in silver carp

The seasonal changes of MC-LR, MC-LR-GSH, and MC-LR-Cys concentrations in the liver, kidney, and intestinal content of silver carp are summarized in Fig. 4. In liver, MC-LR shows the highest peak in July. MC-LR-Cys peaked in August, and MC-LR-GSH was detected in only one sample from August. No significant relationship was found between MC-LR and MC-LR-Cys concentration in liver of
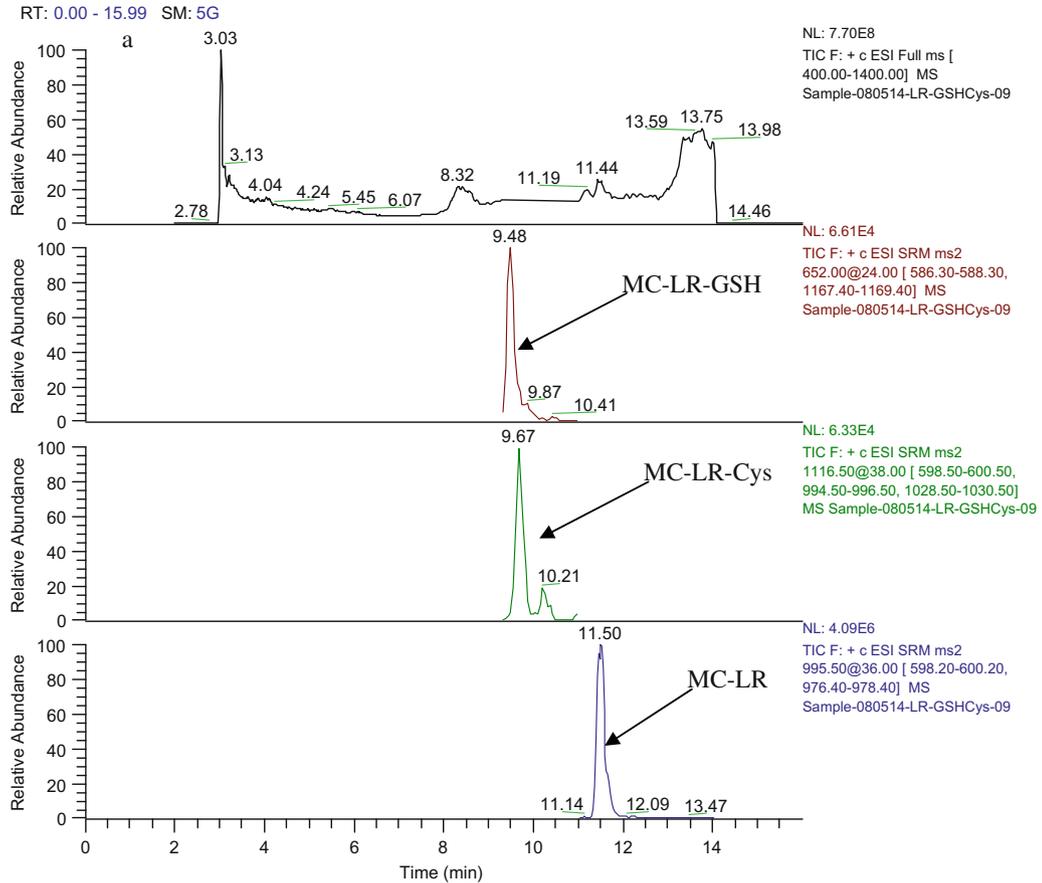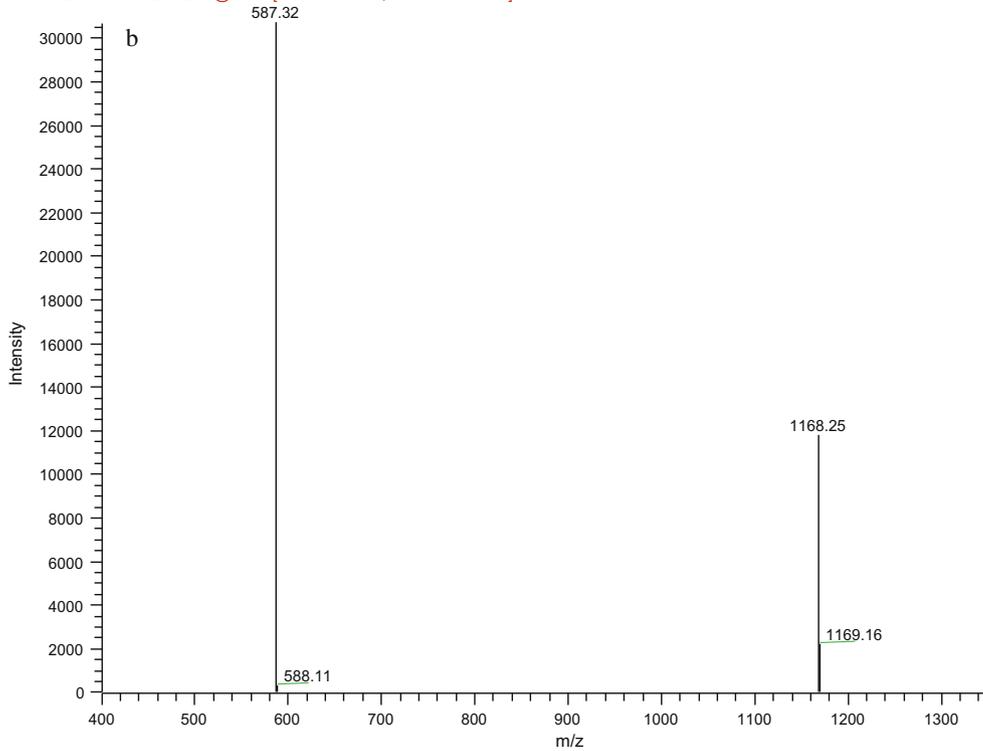
Fig. 2. ESI LC/MS/MS selected reaction monitoring (SRM) chromatograms and product ion mass spectrum for MC-LR and its two metabolites (MC-LR-GSH and MC-LR-Cys) in the intestinal content of silver carp from August 2005, Lake Taihu, China. Shown are: (a) total ion and SRM chromatograms for MC-LR and its two metabolites (MC-LR-GSH and MC-LR-Cys); (b) product ion mass spectrum for MC-LR-GSH; (c) product ion mass spectrum for MC-LR-Cys; (d) product ion mass spectrum for MC-LR.
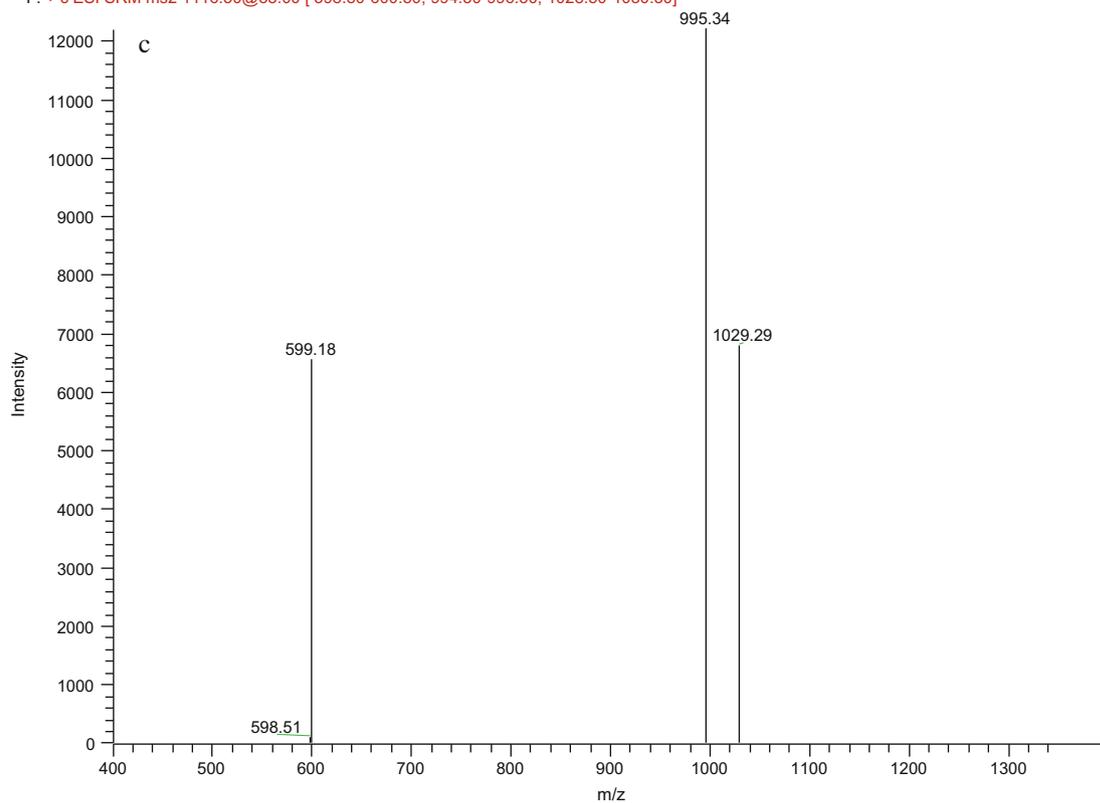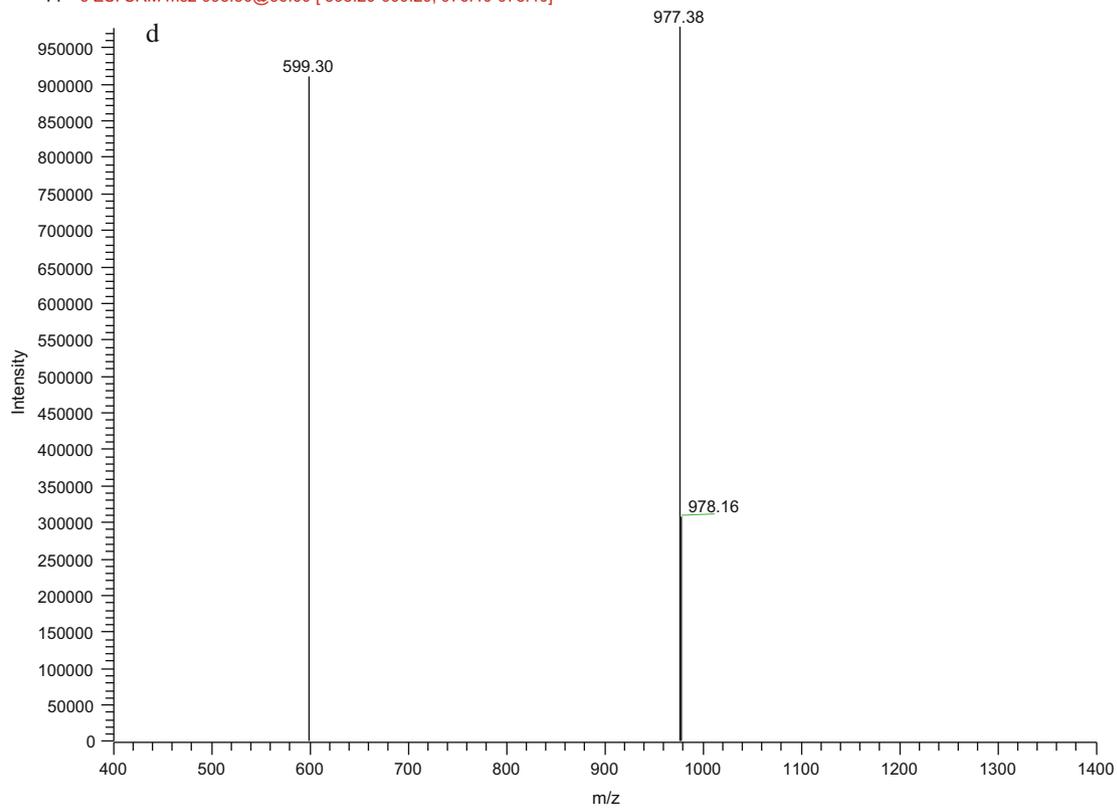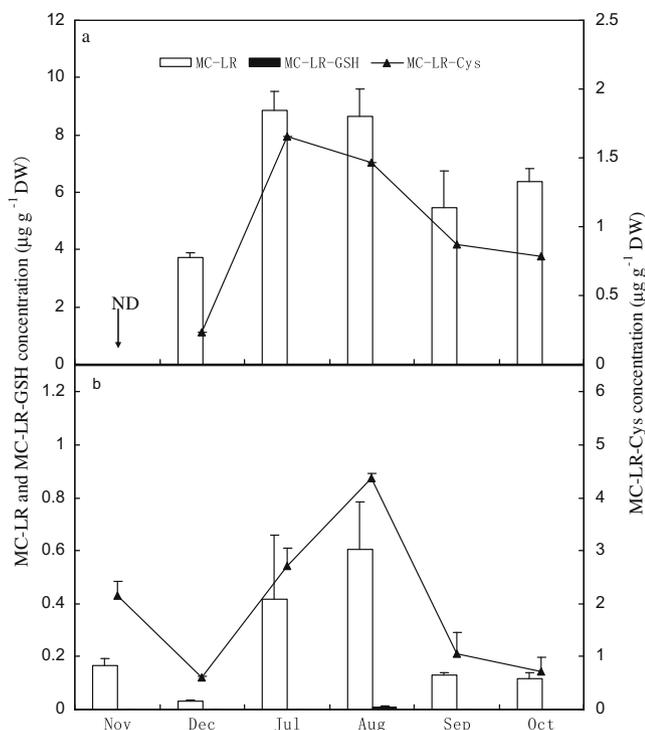
Fig. 2 (*continued*)

Fig. 3. Seasonal changes of MC-LR, MC-LR-GSH, and MC-LR-Cys concentrations in hepatopancreas of *Bellamya aeruginosa* (a) and *Macrobrachium nipponensis* (b) in Gonghu and Meiliang Bay, respectively, Lake Taihu, China, from November to December 2004 and from July to October 2005. Values are expressed as means ± standard deviations for two replicates; DW = dry weight; ND = not detected; LOD (limit of detection) = 0.005 µg g^{-1} DW.

Fig. 4. Seasonal changes of MC-LR, MC-LR-GSH, and MC-LR-Cys concentrations in liver, kidney, and intestinal content of silver carp (*Hypophthalmichthys molitrix*) in Meiliang Bay, Lake Taihu, China, from July to December 2005. Values are expressed as means ± standard deviations for two replicates; DW = dry weight; ND = not detected; LOD (limit of detection) = 0.005 µg g^{-1} DW.
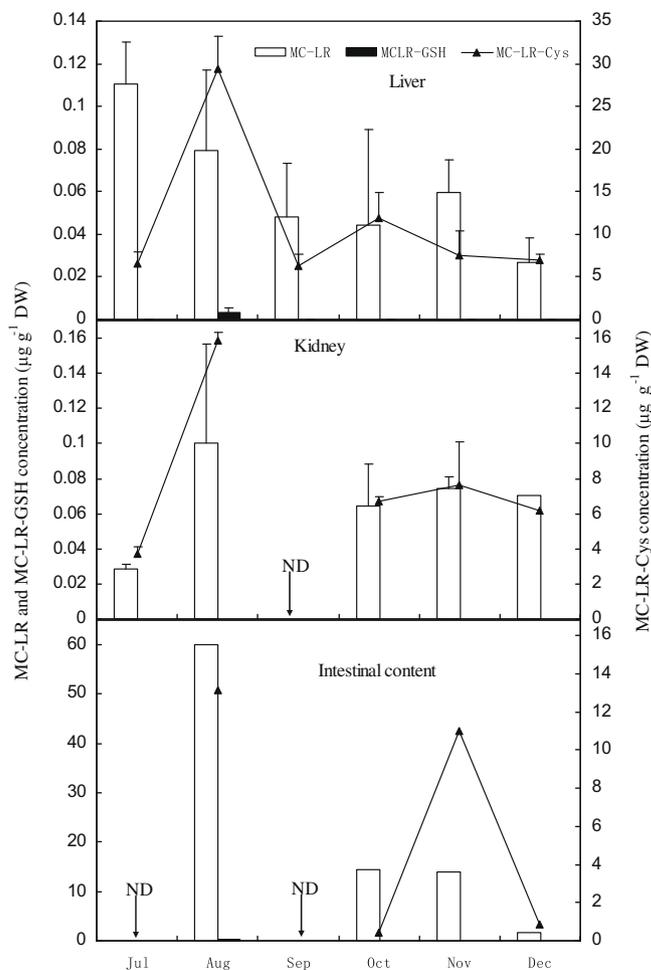
silver carp ($r = 0.558$, $p = 0.188$; $n = 12$). MC-LR concentration in liver of silver carp varied between 0.027 and 0.11 µg g^{-1} DW with an average of 0.061 µg g^{-1} DW, and MC-LR-Cys concentration ranged from 3.17 to 14.7 µg g^{-1} DW with an average of 5.72 µg g^{-1} DW. In the kidney, MC-LR concentration ranged from 0.029 to 0.100 µg g^{-1} DW with an average of 0.068 µg g^{-1} DW and MC-LR-Cys content varied from 1.85 to 7.93 µg g^{-1} DW with an average of 4.01 µg g^{-1} DW. No MC-LR-GSH was detected in kidney during the study period. A significant correlation was found between MC-LR and MC-LR-Cys concentrations in intestinal content of silver carp ($r = 0.674$, $p = 0.046$; $n = 10$). In the intestinal contents, the average concentrations of MC-LR-GSH, MC-LR-Cys, and MC-LR were 0.105, 3.17, 22.5 µg g^{-1} DW, respectively. No significant correlation was found between concentrations of MC-LR and MC-LR-Cys ($p > 0.05$) or between concentrations of MC-LR and MC-LR-GSH ($p > 0.05$) in intestinal contents.

3.5. Recovery experiment

The average recoveries from fish ($n = 4$) liver were 62.9% (ranging from 59.7% to 67.2%), 83.5% (ranging from 81.6% to 85.0%), and 87.0% (ranging from 80.5% to 94.1%) for MC-LR-GSH, MC-LR-Cys, and MC-LR, respectively. The relative standard deviations (RSDs) of MC-GSH, MC-LR-Cys, and MC-LR were 6%, 2%, and 6%, respectively.

4. Discussion

There have been several experimental studies on the qualitative analyses of microcystins metabolites in various organisms (Kondo et al., 1996; Pflugmacher et al., 1998; Ito et al., 2002). The microcystins metabolites (microcystins glutathione and cysteine

conjugates) were firstly detected and identified in vivo in mouse and rat livers by Kondo et al. (1996). Then Pflugmacher et al. (1998) reported microcystin-LR glutathione conjugate formed in vitro enzymatically via soluble glutathione S-transferase. And only one experimental study examined MC-LR-GSH concentrations in the liver of crucian carp after the intraperitoneal injection of single dose of microcystins (100 µg MC-LR equivalent kg^{-1} body weight) (Dai et al., 2008). Here, for the first time, seasonal changes in MC-LR and its glutathione and cysteine conjugates were detected in various aquatic animals from natural conditions.

It is well known that microcystins in tissues of animals are including free microcystins and covalently bound microcystins (Mackintosh et al., 1990; Craig et al., 1996; Williams et al., 1997a; Pflugmacher et al., 1998). In the present study, MC-LR-Cys concentration in silver carp, shrimp, and snail was as 93, 4, and 1/12 times as that of MC-LR content, respectively; while MC-LR-GSH concentration was above the detection limit (0.009 µg g^{-1} DW) only in two shrimp samples collected from August, and the average MC-LR-Cys to MC-LR-GSH ratio in these two samples was over 250. The above results suggest that (1) MC-LR-Cys could be the main covalently bound MC-LR in aquatic animals, and that (2) dominant form of MC-LR differs among species, i.e., in silver carp and shrimp, the dominant form of MC-LR was MC-LR-Cys, while the free MC-LR was the dominant form of MC-LR in snails. When the saltwater mussels (*Mytilus edulis*) were fed *M. aerugin-*

osa, less than 0.1% of the total microcystin burden in the mussel tissue was found to be extractable with MeOH (Williams et al., 1997a). Williams et al. (1997b,c) reported that less than 40% of the total toxin burden in livers of salmon via i.p. injection was extracted with MeOH. When rainbow trout (*Oncorhynchus mykiss*) were gavaged with freeze–dried toxic cells of *M. aeruginosa*, about 63% of the toxin present in liver at 3 h was covalently bound to cellular proteins (Tencalla and Dietrich, 1997).

GSH is known to be present in high concentration in most living cells and can bind spontaneously with electrophiles. GSH conjugation with xenobiotics has been traditionally viewed as a metabolic pathway leading to detoxication (Chasseaud, 1976; Ketterer et al., 1983). As for microcystins, MC-LR is known to conjugate with glutathione (GSH) and ulteriorly degrades to MC-LR-Cys (Ito et al., 2002), thereby increasing water solubility, reducing the toxicity and enhancing excretion of MC-LR (Kondo et al., 1992, 1996). In the present study, MC-LR-GSH was observed occasionally in the silver carp liver and snail hepatopancreas with concentration near the detection limit; and only the MC-LR-GSH concentration in samples from August was above the detection limit, though the MC-LR-GSH was detected in all samples of shrimps. However, the MC-LR-Cys concentrations in hepatopancreas of snail (max. $0.826 \, \mu g \, g^{-1}$ DW; mean: $0.500 \, \mu g \, g^{-1}$ DW) and shrimp (max. $2.189 \, \mu g \, g^{-1}$ DW; mean: $0.969 \, \mu g \, g^{-1}$ DW) and liver of silver carp (max. $14.7 \, \mu g \, g^{-1}$ DW; mean: $5.72 \, \mu g \, g^{-1}$ DW) were relatively high. Meanwhile, Kondo et al. (1992) confirmed that thiols of GSH and Cys add unclophilically to the α,β-unsaturated carbonyl of the Mdha moiety in microcystins. Microcystins bind covalently with the Cys-273 of the PP-1 and 2A (Bagu et al., 1997). The above results suggest that the main detoxication pathway of microcystins in aquatic animals is as follows: when MC-LR enters into the liver/hepatopancreas, it firstly conjugates with polypeptide or protein (including GSH, PP-1 and 2A) containing Cys residues, maybe also some free cysteine; subsequently, the MC-LR-Cys is degraded from these polypeptide or protein, and finally is excreted from animals by the compound of MC-LR-Cys. Hence we conclude that in aquatic animals, especially fish, the main excretion form of MC-LR is MC-LR-Cys, but not MC-LR-GSH, whereas MC-LR-Cys plays an important role in detoxication of MC-LR. Qiu et al. (2007) reported that no discernable difference in GSH level was found in the liver of silver carp from Lake Taihu during their study periods (from January to December 2005). After exposure of crucian carp to a single dose of $100 \, \mu g$ MC-LR equivalent kg^{-1} body weight, the MC-LR-GSH concentration in all the liver samples was near $0.05 \, \mu g \, g^{-1}$ DW, and the maximum concentration of MC-LR-GSH was just $0.083 \, \mu g \, g^{-1}$ DW (Dai et al., 2008).

In the present study, the varying MC-LR-Cys to MC-LR ratios (1/12–94) in the studied animals and the ratios of MC-LR concentration in snail and shrimp hepatopancreas to that in silver carp liver (108 and 4, respectively) suggest that (1) different animals have different metabolic pathways of MC-LR, and that (2) the efficiency of MC-LR-Cys formation differs among the species examined. It is likely that in silver carp, most part of microcystins conjugated with polypeptide and/or protein containing cysteine residues leading to detoxication through formation of cysteine conjugates could be the main metabolic pathway of microcystins. While in snail, besides the conjugation of MCs with various protein or polypeptides, other more important metabolic pathways of microcystins (e.g. direct excretion of the free microcystins) might exist. Our previous study pointed out that the MC-LR concentration in heopatopancreas ($2.87 \, \mu g \, g^{-1}$ DW) was higher than that in intestine ($1.05 \, \mu g \, g^{-1}$ DW) of the snails used in the present experiment, and average MC-LR concentration in hepatopancreas decreased to a low level ($0.038 \, \mu g \, g^{-1}$ DW) after the cyanobacterial blooms disappeared (Zhang et al., 2007). Furthermore, few dead snails were found during the study period. Hence we can conclude that snails may have a special mechanism to tolerate high amount of free MC-LR in their hepatopancreas.

In the present study, the MC-LR concentration in liver, kidney and intestinal contents of silver carp was largely lower than that of MC-LR-Cys, meanwhile, MC-LR-GSH was occasionally detectable in the liver sample from August and no MC-LR-GSH was detected in the kidney samples, suggesting that (1) MC-LR-Cys formed in silver carp liver is actively transported to kidney and intestine for excretion, and that (2) only a few fraction of MC-LR in liver examined conjugates with GSH leading to detoxication. Since Qiu et al. (2007) examined annual changes of GSH level in the liver of silver carp (*H. molitrix*) used in the present study, and no discernable difference in GSH level of the liver of silver carp was found during the study period. Ito et al. (2002) indicated that MC-LR-Cys conjugates formed in the livers were actively transported and excreted from kidneys and intestines of mice. Chen et al. (2006, 2007) reported that MC-LR-Cys was detected in the kidney and hind-gut content samples of silver carp and bighead carp.

Microcystins are known to be potential risks to human health (Yu, 1989; Carmichael, 2001; Azevedo et al., 2002; Chen et al., 2009). To avoid potential health risk, the World Health Organization (WHO) established the tolerable daily intake (TDI) of $0.04 \, \mu g \, kg^{-1}$ body weight per day for MC-LR (Chorus and Bartram, 1999). Now, free microcystins in aquatic animals are considered as the safety assessment standards of aquatic animals. However, in the present study, considerable amounts of MC-LR-Cys were found in the liver and hepatopancrease in aquatic animals. The potential threat to human health of aquatic animals contaminated with microcystins may be underestimated, since the toxicity of MC-LR-Cys is still significant (LD_{50} of MC-LR-Cys was 7-fold that of MC-LR to mice; Kondo et al., 1992). Hence, a reasonable safety assessment method of human consumption of aquatic animals should take the covalently bound microcystins concentration into consideration.

5. Conclusions

(1) The main detoxication pathway of microcystins in aquatic animals is suggested as follows: when MC-LR enters into liver/hepatopancreas, it firstly conjugates with polypeptide or protein (including GSH, PP-1 and 2A) containing Cys residues, perhaps also some free cysteine; subsequently, MC-LR-Cys is degraded from these polypeptide or protein; and finally is excreted from animals by the compounds of MC-LR-Cys.

(2) In aquatic animals, especially fish, the main excretion of MC-LR could be MC-LR-Cys, but not MC-LR-GSH, whereas MC-LR-Cys might play an important role in detoxication of MC-LR.

(3) The efficiency of MC-LR-Cys formation differs among species.

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