Quantitative analysis of glutathione and cysteine S-conjugates of microcystin-LR in the liver, kidney and muscle of common carp (*Cyprinus carpio*) in Lake Taihu

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ABSTRACT

Tissue distribution of microcystin (MC)-LR-GSH, MC-LR-Cys and MC-LR of omnivorous fish in Lake Taihu was investigated. MC-LR and MC-LR-Cys were detected in liver, kidney and muscle. The concentration of MC-LR in liver and kidney was 0.052 μg g^{-1} DW and 0.067 μg g^{-1} DW, respectively. MC-LR-Cys appeared to be an important metabolite with average contents of 1.104 μg g^{-1} DW and 0.724 μg g^{-1} DW in liver and kidney, and the MC-LR-Cys/MC-LR ratio in liver and kidney reaching as high as 21.4 and 10.8. High MC-LR-Cys/MC-LR ratio and a significant correlation between MC-LR-Cys and MC-LR concentration in liver, suggest that liver is more active in detoxification of MC-LR by formation of MC-LR-Cys for omnivorous fish. Furthermore, there might be a balance between the accumulation and depuration/metabolism of MC-LR-Cys in kidney. The MC-LR-Cys can be formed in kidney directly, or transported from liver or other tissues, while the MC-LR-Cys in kidney might be dissociated to MC-LR or excreted. Although MC-LR and its metabolites were scarcely detected in muscle, it is necessary to investigate the distribution of toxic metabolites in edible muscle.

Key words | common carp, kidney, liver, MC-LR-Cys, MC-LR-GSH

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INTRODUCTION

The occurrence of cynobacteria bloom around the world has caused many water problems, such as deterioration of water quality, shortage of water resources and damage to the ecosystem (Carmichael 1997, Carmichael *et al.*, 2001). Microcystins (MCs) are cyclic hepatotoxic peptides produced by freshwater cyanobacteria (blue-green algae), such as *Microcystis aeruginosa, Oscillatoria, Anabaena, Nostoc,* and *Planktothrix* (Svrcek & Smith 2004). These toxins, which are reported as potent skin and liver tumor promoters since they inhibit protein phosphatases 1 and 2A (Yoshizawa *et al.* 1990; Carmichael *et al.* 2001), have been detected in various organs of animals. They can cause illnesses or even death to animals (Carmichael 1997; Codd *et al.* 2005) and human beings (Yuan *et al.* 2006). Among 80+ variants, microcystin-LR (MC-LR) is commonly detected and possesses the most toxic effect. Due to its toxicity and the fact that it is commonly detectable, the World Health Organization (WHO) has proposed a guideline value of 1.0 μg L^{-1} in drinking water and a tolerable daily intake (TDI) of 0.04 μg kg^{-1} body weight per day of MC-LR in aquatic food (Dietrich & Hoeger 2005).

Glutathione (GSH) has been verified as a key substance in the detoxification of MCs in both mammals and aquatic organisms (Kondo *et al.* 1992; Ito *et al.* 2002; Zhang *et al.* 2009). Kondo *et al.* (1992) firstly synthesized GSH and cysteine conjugation of MC-LR/MC-RR chemically and identified these conjugates in mouse liver. Then, Pflugmacher *et al.* (1998) revealed the enzymatic formation of MC-LR-GSH via soluble glutathione

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S-transferase (GST) extracted from aquatic organisms, which suggested these S-conjugates were significant metabolites in the detoxification of MC-LR. Chen *et al.* (2007) also reported the presence of MC-LR-Cys in kidney and hindgut contents of bighead carps in field work. However, few of the previous studies have discussed the relationship between free MCs and their S-metabolites for the absence of quantitative methods. Based on the quantitative method of Dai *et al.* (2008) and Wu *et al.* (2010), MC-LR/RR-GSH/Cys conjugates were subsequently quantified both in experiment and field work. Zhang *et al.* (2009) investigated the tissue distribution of MC-LR-GSH and MC-LR-Cys in the snail (*Bellamya aeruginosa*), shrimp (*Macrobrachium nipponensis*), phytoplanktivorous bighead carp (*Aristichthys nobilis*) and silver carp (*Hypophthalmichthys molitrix*), and Wu *et al.* (2013) further studied the distribution of MC-RR-GSH and MC-RR-Cys in common carp in Lake Taihu. The results obtained from Zhang *et al.* (2009) and Wu *et al.* (2013) implied that the MC-LR-Cys and MC-RR-Cys played an important role in the metabolism of MC-LR or MC-RR, since the content of MC-LR/RR-Cys are higher than their free types. To verify the role of glutathione and cysteine in the metabolism of MCs, He *et al.* (2012) quantified GSH and cysteine conjugates of MC-LR/RR in liver, kidney, intestine and muscle of bighead carp by intraperitoneal (i.p.) injection of MC-LR/RR, which also showed high ratios of MC-LR-Cys/MC-LR and MC-RR-Cys/MC-RR. Recently, MC-RR-GSH was found to be a highly reactive intermediate, which would rapidly transform to MC-RR-Cys or dissociate to MC-RR (Li *et al.* 2014).

Omnivorous fish, which live in a complex environment, are commonly farmed in Chinese lakes as the main consumed fish. Serious cyanobacterial blooms occurred at the end of May 2007 in Gonghu Bay, which resulted in a rapid deterioration of water quality (Xie 2008). The *Microcystis aeruginosa* comprised a large proportion of the phytoplankton community (>99%) in 2008 (Wang *et al.* 2010).

Therefore, common fish (*Cyprinus carpio*) samples were collected from January to December in 2008. The aims of this study were: (1) to investigate the seasonal changes of MC-LR and MC-LR-GSH/Cys metabolites in liver, kidney and muscle; and (2) to discuss the possible reasons for the distribution of glutathione and cysteine conjugates.

MATERIALS AND METHODS

Study area

Lake Taihu (30 °56′ N–31 °34′N, 119 °54′ E–120 °36′E) is situated on the ancient Yangtze River delta with an area of 2,338 km^2 and a mean depth of about 2 m. Cyanobacterial blooms often occur over wider areas in warm seasons in this lake (Qin *et al.* 2007). Gonghu Bay, is an important bay of Lake Taihu. Generally, it is utilized for water supply, flood control, fisheries, tourism, recreation, and shipping.

Synthesis of MC-LR-GSH and MC-LR-Cys

MC-LR was purchased from Wako Pure Chemical Industries, Japan. 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%. The method for the preparation of MC-LR-GSH and MC-LR-Cys is described by Kondo *et al.* (1992) and Dai *et al.* (2008). The contents of purified MC-LR-GSH and MC-LR-Cys were over 95% and confirmed by High Performance Liquid Chromatography (HPLC) (LC-20A, Shimadzu, Kyoto, Japan) and Liquid Chromatography–Mass Spectrometer (LC-MS) (Thermo Electron, Waltham, MA, USA).

Sample preparation

Stock solutions (25 μg mL^{-1}) were prepared by dissolving MC-LR, MC-LR-GSH and MC-LR-Cys in pure water. Standard solutions (0.01, 0.05, 0.1, 0.5 and 2.5 μg mL^{-1}) were prepared by serial dilutions of stock solutions using pure water. All solutions were stored at −80 °C before use.

Five *Cyprinus carpio* (body weight: 0.52 ± 0.13 kg; body length: 26.1 ± 2.7 cm) were captured from Gonghu Bay monthly from January to December 2008. The collected fish ($n = 5$) were measured and weighed, and then the liver, kidney and muscle were dissected in the field and stored frozen at −20 °C immediately. The samples were lyophilized by a freeze dryer (Martin Christ, Osterode, Germany). The lyophilized samples (0.2 g dry weight for

each sample) were extracted three times with 5 mL of 0.01 mol L^{-1} EDTA-Na_2-5% acetic acid by sonicating for 3 min (30% amplitude, 60 W, 20 kHz, Sonics VC130 PB, Newtown, CT, USA) at 0 °C and then centrifuged at 15,000 × g (BR4, Jouan, Winchester, VA, France) at 4 °C. Extraction and enrichment of MC-LR-GSH, MC-LR-Cys and MC-LR were performed according to the methods of Dai *et al.* (2008). Solid phase extraction employing Oasis HLB cartridges (500 mg, Waters, Milford, MA, USA) was used for clean-up and preconcentration of the supernatant. Firstly, the cartridges were preconditioned with 10 mL of methanol followed by 10 mL of water, then washed with 20 mL of water followed by 20 mL of 20% MeOH. The elution was performed with 20 mL of MeOH. The eluent was evaporated to dryness and the residue was dissolved in 100 μL 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 then eluted with 20 mL of 70% MeOH. This fraction was evaporated to dryness and re-dissolved in 100 μL of the mobile phase.

Analysis of MCs and their metabolites

Analyses were performed using a Finnigan LC-MS system comprising a Thermo Surveyor autosampler, a Surveyor mass spectrum (MS) pump, a Surveyor photo diode array system, and a Finnigan LCQ-Advantage MAX ion trap mass spectrometer (Thermo Electron, Waltham, MA, USA) equipped with atmospheric pressure ionization fitted with an electrospray ionization (ESI) source (Thermo Electron). The instrument control, data processing, and analysis were conducted by using Xcalibur software (Thermo Electron). Separation was carried out using a Waters XBridge C18 column (2.1 × 100 mm, dp 3.5 μm, Waters Corporation, USA) with a C18 guard column (2.1 × 10 mm, dp 5 μm, Thermo Electron Corporation, USA).

The mobile phase consisted of solvent A [water + 0.05% (v/v) formic acid]/solvent B [acetonitrile + 0.05% formic acid]. MC-LR, MC-LR-GSH and MC-LR-Cys were separated by the following linear gradient program: 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 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.

Statistics

Values were shown by means ± standard error (SE). Values below the detection limit were set to half of the detection limit, while the values for the target compounds that were not detected in the samples were set to zero. Spearman correlation analysis was conducted to determine the relationship between MC-LR-Cys or MC-LR-GSH concentration and MC-LR content in tissues of common carp using SPSS for Windows (version 13.0; SPSS, Chicago, IL, USA).

RESULTS

The selected reaction monitoring (SRM) chromatograms of MC-LR and their metabolites are presented in Figure 1(a). For quantification purposes, mass spectra of the product ions were monitored at *m/z* 599.3 and 977.6 from the parent ion at *m/z* 995.5 for MC-LR, and product ions at *m/z* 599.3, 995.4, and 1,029.4 were from the parent ion at *m/z* 1,116.5 of for MC-LR-Cys, while for MC-LR-GSH, the mother ion at *m/z* 652.0 of $[M + H]^{2+}$ was cleaved to *m/z* 587.3 and 1,168.4. The retention times for MC-LR-GSH, MC-LR-Cys and MC-LR were 10.34 min, 10.72 min and 11.67 min, respectively (Figure 1(b)).

Figure 2 summarizes the monthly changes of MC-LR and its glutathione and cysteine S-conjugates in the liver, kidney and muscle of the common carp. Values below the limit of detection (LOD) (0.004 μg g^{-1}) were taken as zero. MC-LR and MC-LR-Cys were observed in most liver and kidney samples. The content of MC-LR in liver ranged from 0.017 to 0.141 μg g^{-1} DW, and showed the highest peak in June with an average of 0.052 μg g^{-1} DW. MC-LR-Cys content in liver peaked in February, and varied between 0.029 and 4.050 μg g^{-1} (with an average of 1.104 μg g^{-1} DW).

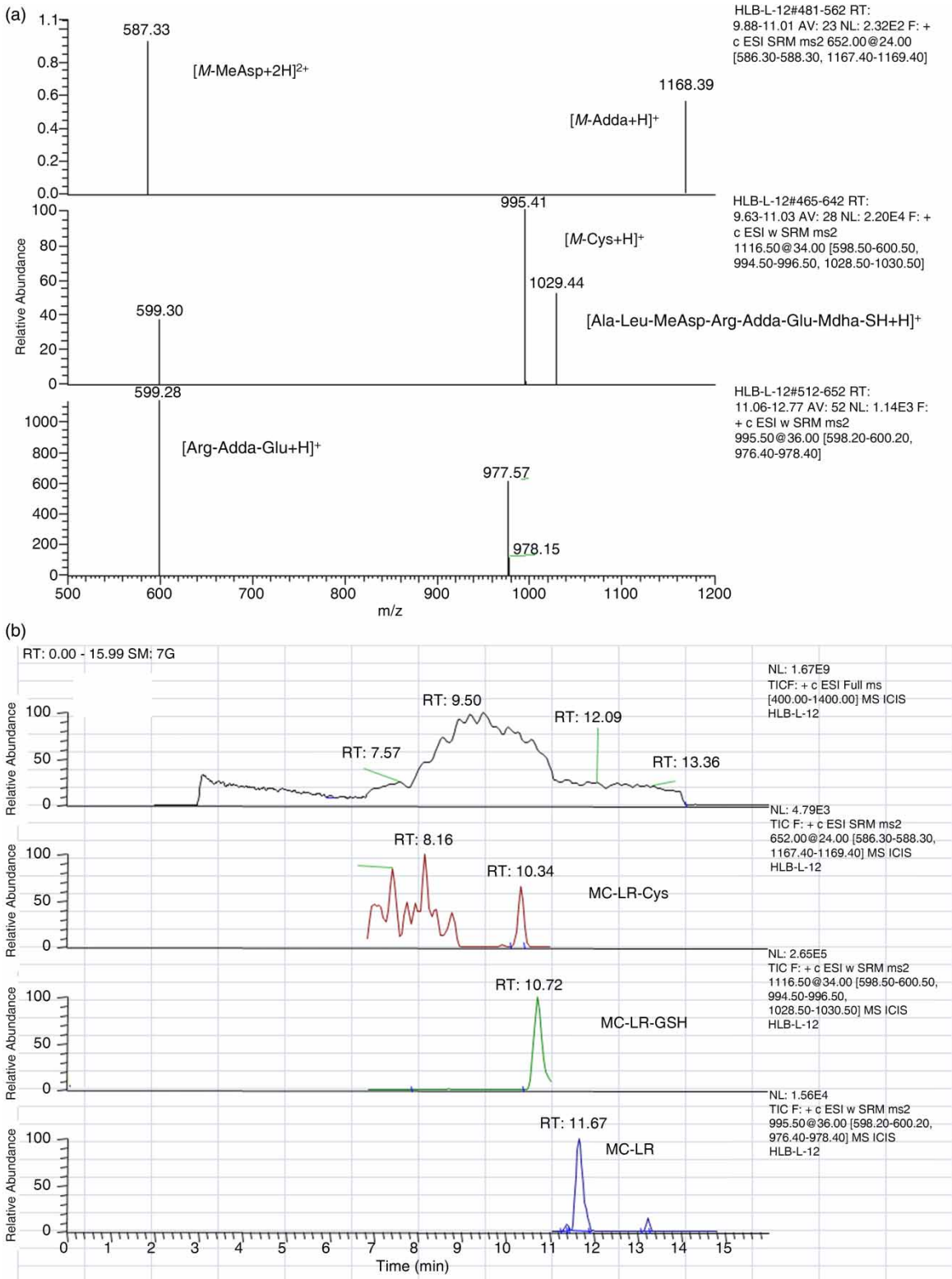

Figure 1 | ESI-LC/MS/MS in SRM chromatograms and product ion MS for MC-LR and its metabolites (MC-LR-GSH and MC-LR-Cys) in the liver of common carp from December 2008, Lake Taihu, China. Shown are: (a) total ion and SRM chromatograms for MC-LR and its two metabolites (MC-LR-GSH and MC-LR-Cys); (b) product ion MS for MC-LR-GSH, MC-LR-Cys and MC-LR.

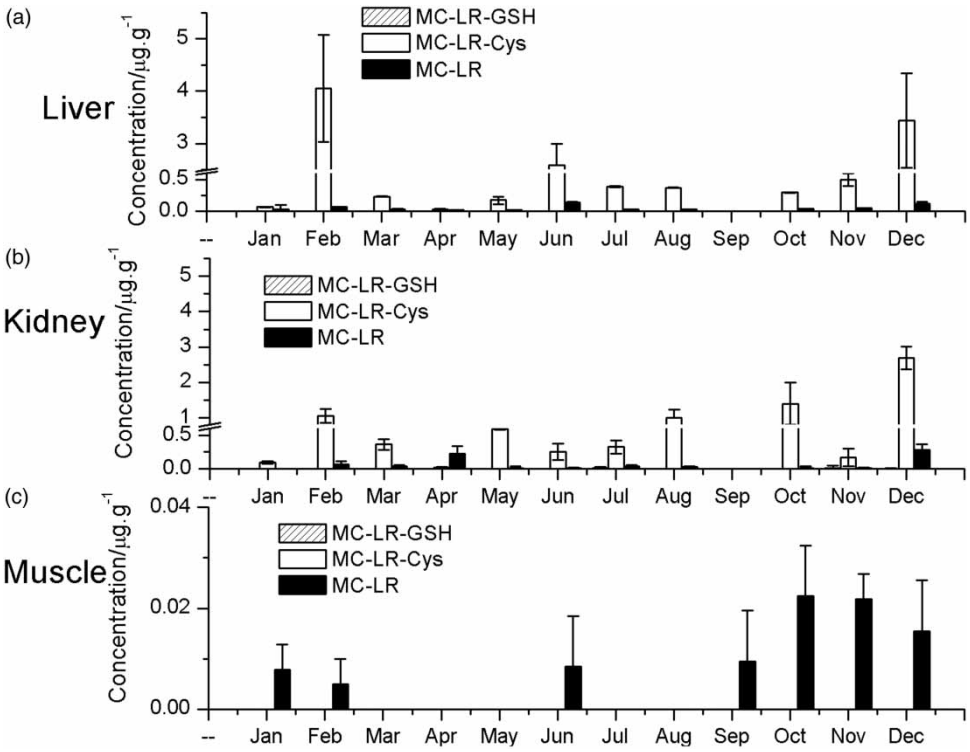

Figure 2 | Seasonal changes in MC-LR, MC-LR-GSH, and MC-LR-Cys concentrations of (a) liver, (b) kidney and (c) muscle of common carp (*Cyprinus carpio*) in Gonghu Bay, Lake Taihu, China, from January to December 2008. LOD = 0.004 μg g^{-1} DW.

Seasonal changes of MC-LR (0.011 – 0.288 μg g^{-1} DW, average 0.067 μg g^{-1} DW) and MC-LR-Cys (0.023 – 2.686 μg g^{-1} DW, average 0.724 μg g^{-1} DW) in the kidney of common carp are summarized in Figure 2(b). MC-LR and MC-LR-Cys both peaked in December. The lowest level of MC-LR-GSH (<0.015 μg g^{-1} DW) was detected in kidney of July and November.

MC-LR contents in muscle were obviously lower than that in liver and kidney (LR <0.022 μg g^{-1} DW) (Figure 2(c)), and no -GSH and -Cys S-conjugates of MC-LR were detected.

During the study period, except for samples from September, MC-LR and MC-LR-Cys were detected in all of the liver and kidney. The highest concentration of MC-LR-Cys was detected in liver, followed by kidney, and the average content of MC-LR was in the order of kidney > liver > muscle. MC-LR-Cys content showed a slight decrease from liver (1.104 μg g^{-1}) to kidney (0.724 μg g^{-1}). A highly significant correlation was found between MC-LR and MC-LR-Cys concentrations ($r = 0.866$, $p < 0.01$; $n = 12$) in liver of common carp.

DISCUSSION

In the present study, MC-LR was detected in 90% of liver and kidney samples. Liver has been considered as the target organ for detoxification of MC-LR, and most of the studies found MC-LR prefer to accumulate in this tissue (Williams *et al.* 1997). However, it appeared that MC-LR may also intensively distribute to kidney for excretion in fish exposed to long-term cyanobacterial blooms. Milutinovic *et al.* (2003) observed that kidneys were far more affected than the liver, and suggested kidney may be another key tissue for detoxication of MC-LR in chronic exposure. It is well known that MCs can be transported via the blood stream and distributed to blood-irrigated organs, including liver, intestine, kidney, lung, etc. In this study, MC-LR was less detected in liver than kidney, which indicated that MC-LR may be effectively eliminated from liver and distributed to kidney when exposed to a long-term bloom.

The importance of GSH conjugates in detoxification of xenobiotics has been underlined in much of the literature

(Ketterer *et al.* 1983). Several studies have identified the glutathione and cysteine S-conjugates of MC-LR in various tissue both in field or conditional works (Pflugmacher *et al.* 1998, 2001; Chen *et al.* 2007). Pflugmacher *et al.* (1998, 2001) suggests the synthesis of MC glutathione conjugate via GST is the first step to detoxify MCs for a wide range of aquatic plants, and MC-GSH and MC-Cys were also detected in mice and rats after acute exposure to MCs in laboratory experiments (Ito *et al.* 2002) and bighead carp from Lake Chaohu in field work (Chen *et al.* 2007). Up to now, Xie's group have discussed the role of tissue distribution of these conjugates in aquatic animals and mammals (Zhang *et al.* 2009; He *et al.* 2012; Li *et al.* 2014; Guo *et al.* 2015). Zhang *et al.* (2009, 2012) detected the MC-LR and its metabolites in liver of bighead carp, and found the content of MC-LR-Cys was several times that of MC-LR, but GSH conjugates were much lower than those of Cys conjugates. The glutathione conjugates of MC-LR and MC-RR were always below the LOD in the bighead carp which were i.p. injected (He *et al.* 2012). MC-RR-GSH was reported as an intermediate and converted to MC-RR-Cys rapidly by i.p. injection of MC-RR-GSH in bighead carp (Li *et al.* 2014). Similar results were obtained in our study, MC-LR-GSH could be occasionally detected in kidney ($0.015\ \mu g\ g^{-1}$ DW in July and November). MC-LR-GSH might have followed a similar pathway and biotransformed to MC-LR-Cys or disassociated to MC-LR.

It should be noted that the content of MC-LR-Cys in liver was extremely high. Previous reports suggested that covalently bound MCs might be the dominant form of MCs. Tencalla and Dietrich found cellular protein bounded toxin was the main type identified in liver after 3 h i.p. injection (Tencalla & Dietrich 1997). Williams *et al.* (1997) indicated less than 0.1% of the total MC was burden in the mussel's tissue when fed by *M. aeruginosa*. These cysteine conjugates were also an important covalently bound type of MC-LR which was quantitatively detected (Zhang *et al.* 2009; He *et al.* 2012; Wu *et al.* 2013). In this study, a higher content of MC-LR-Cys and lower content of MC-LR were detected in the liver, and the mean ratio of MC-LR-Cys/MC-LR reached as high as 21.4, suggesting the liver exhibits strong activity in removal of MC-LR via an MC-LR-Cys biotransformation pathway. There are several possible routes for MC-LR-Cys in liver. Firstly, MC-LR-Cys here might be dissociated from the covalently bounded conjugates of polypeptide and protein, which are abundant in Cys residues, such as protein phosphatases 1 and 2A (Bagu *et al.* 1997) and oatp/OATP superfamily (Fischer *et al.* 2005). Secondly, MC-LR-Cys was supposed to convert from MC-LR-GSH, since a decrement of GSH levels were observed in liver of goldfish and *Cyprinus carpio* (Xu *et al.* 1998; Jiang *et al.* 2011). Meanwhile, malondialdehyde (MDA) and GSH-related enzymes (GPX, GR, and GST) were also significantly changed in rat liver, and MC-LR induced time-dependent alterations of GSH levels in rat liver (Li *et al.* 2015; Chen *et al.* 2016). Additionally, transportation from other tissue through blood circulation was also a reasonable route. The water solubility of MC-LR was enhanced by adding thiols of GSH and Cys (Ito *et al.* 2002), which makes these conjugates facile to transport and clean-up through tissues.

In the present study, MC-LR-Cys was also distributed effectively in kidney. He *et al.* (2012) pointed out that MC-LR-Cys were successfully biotransformed in kidney only in the low dose group of bighead carp at 48 h post injection, and the MC-LR-Cys/MC-LR ratio was 9.04. Therefore, for the MC-LR-Cys/MC-LR ratio of 10.8 in this work, as well as the highest activity of γ-glutamyltransferase in kidney (Anders 1980), it appears that MC-LR-Cys in kidney may be formed directly or indirectly (He *et al.* 2012; Zhang *et al.* 2012; Li *et al.* 2014; Guo *et al.* 2015). Briefly, there was a balance between accumulation and removal of MC-LR-Cys in kidney. On the one hand, MC-LR-Cys formed in liver or other tissues were subsequently transported to kidney, since MC-LR-Cys with high hydrophilicity was easier to transport. On the other hand, MC-LR-Cys can be formed directly in kidney. During this process, MC-LR-Cys could be dissociated to MC-LR or excreted, resulting in the high content of MC-LR in kidney. All the explanations above might be responsible for the observed distribution of MC-LR-Cys in kidney.

Both tissues showed high distribution of MC-LR-Cys, and formation efficiency of MC-LR-Cys in liver was 2-fold of that in kidney, and there is a significant correlation between MC-LR and MC-LR-Cys concentrations in liver ($r = 0.866$, $p < 0.01$; $n = 12$). It should be noted that liver showed a higher distribution of MC-LR-Cys with the depletion of MC-LR, this is consistent with the

lower content of MC-LR in liver, and this suggests that MC-LR was effectively metabolized in liver, where there was a high MC-LR-Cys/MC-LR ratio. It might be thus speculated that in the conditions of chronic exposure, MC-LR-Cys could be formed and dissociated both in liver and kidney, and further transported to kidney for excretion.

Kondo *et al.* (1992) indicated the Cys-S-conjugates as low toxicity conjugates, but covalently bound MCs as the dominant type in the tissues (Kondo *et al.* 1992). Therefore, the MC-LR and MC-LR-Cys in muscle were quantified. Luckily, MC-LR contents were obviously lower than those in liver and kidney (LR < 0.022 $\mu g\, g^{-1}$ DW) (Figure 2(c)), and no -GSH and -Cys S-conjugates of MC-LR were detected in muscle of common carp exposed to toxic bloom. However, what about other covalently bound MCs unidentified in the muscle? It is possible that the risk associated with consuming aquatic animals contaminated with MCs may have been underestimated in the definition of the TDI of MC-LR proposed by WHO (Dietrich & Hoeger 2005). In our future studies, a reasonable assessment on human consumption of aquatic animals is needed to evaluate the potential hazard of the other types of covalently bound MCs measured.

CONCLUSIONS

Distribution of MC-LR in kidney was higher than that in liver, while MC-LR-Cys was more distributed in liver. The probable reasons for the lower distribution of MC-LR and high MC-LR-Cys in liver may lie with the effective metabolism of MC-LR by dissociation of covalently bound metabolites or formation of MC-LR-Cys directly. The MC-LR-Cys also can be formed directly in kidney, and another source of MC-LR-Cys in kidney may be more attributed to transportation from other tissue. The high content of MC-LR in kidney may be attributed to the transportation from other tissue and dissociation of MC-LR-Cys. Although none of the metabolites of MC-LR were detected, GSH/Cys S-conjugates remain toxic, and it is reasonable to take these S-conjugates or other covalently bound MCs into consideration in human risk assessment of fish consumption in future work.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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