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Effects of Temperature on the Stability of Microcystins in Muscle of Fish and Its Consequences for Food Safety

Dawen Zhang · Ping Xie · Jun Chen

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Abstract In this study, bighead carp treated with two doses, i.e. 400 and 580 μg MC-LReq (Microcystin-LR equivalent)/kg bw. After dosing bighead carp with 400 and 580 ug MC-LReq/Kg bw, the mean concentrations of microcystins (MCs) was significantly higher in boiled muscle than unboiled controls. These results indicate that the potential threat of microcystins contaminated fish to humans has been underestimated. The increase in microcystins occurs by the release of phosphatase-bound microcystins by boiling.

Keywords Microcystins · Stability · Temperature · Bighead carp

Microcystins are cyclic heptapeptides consisting of five common amino acids and two variable L-amino acids and produced by some cyaboacteria species (Botes et al. 1984). So far, more than 80 structural variants of MCs have been identified and microcystin-LR (MC-LR) is the most common and toxic among them, in which the two variable amino acids are leucine (L) and arginine (R; Babica et al. 2006). Microcystins are known to be potent tumor promoters (Nishiwaki-Matsushima et al. 1992), because they are highly specific inhibitors of protein phosphatase 1 and 2A (Mackintosh et al. 1990). The death of 52 dialysis patients in Brazil occurred because water used for dialysis was contaminated with microcystins (Dunn 1996). Epidemiological studies also have indicated that the high incidence of primary liver cancer in China is related to microcystins in drinking water (Yu 1995). A recent study pointed out that microcystins were identified for the first time in the serum of a chronically exposed human population (fishermen at Lake Chaohu, China) together with indication of hepatocellular damage (Chen et al. 2009). Moreover, some studies state that microcystins can be transferred along food chain (Smith and Haney 2006), suggesting a potential risk for human consumption of contaminated aquatic products.

Microcystins are chemically stable compounds, resistant to heat and chemical hydrolysis at near neutral pH (Harada et al. 1996). However, no study has been carried out to examine the stability of MCs accumulated in fish tissues cooking. Hence the purposes of this study were to examine the stability of MC in fish muscle after cooking, and to evaluate the potential risk of cooked MC-contaminated fish to human consumers.

Materials and Methods

MC-LR and -RR were isolated and purified from dried surface cyanobacterial blooms collected from Lake Dianchi, Yunnan Province, China. Dried alga cells were extracted three times with 25 mL 75% methanol (V/V) for 3 h at 4°C. The extract was centrifuged at 18,000 rpm and the supernatant was evaporated to dryness, and the residue was dissolved in 5 mL 100% methanol. Then the solution

D. Zhang
LED, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 510301 Guangzhou, People's Republic of China
e-mail: zdw@ihb.ac.cn

D. Zhang · P. Xie (✉) · J. Chen (✉)
Donghu Experimental Station of Lake Ecosystems, State Key Laboratory of Freshwater Ecology and Biotechnology of China Institute of Hydrobiology, Chinese Academy of Sciences, 430072 Wuhan, People's Republic of China
e-mail: xieping@ihb.ac.cn

J. Chen
e-mail: chenjun@ihb.ac.cn

was applied to a 50 cm C18 reversed-phase cartridge (100 g), which had been preconditioned by washing with methanol. Then cartridge was then washed with 50% methanol (V/V), and target compounds were colleted at different times. The purified concentration of MC-LR and -RR used in the present study were about 90% and 80%, respectively. MCs concentration was analyzed via high-performance liquid chromatography (LC-20A; Shimadzu, Kyoto, Japan). According to Gupta et al. (2003), MC-LR is most toxic variant found in cyanobacterial blooms, and the intraperitoneal (i.p.) medium lethal dose (LD_{50}) in mice for MC-RR is about five-fold that for MC-LR, hence the dose of MCs in fish was expressed as MC-LR equivalent (MC-LReq = MC-LR + MC-RR/5).

Healthy bighead carp (*Aristichthys mobilis*) with mean weights of 500 ± 10 g were purchased from a local fish hatchery in Wuhan City, China. Fish were held in the experimental 150 L aquaria for one day prior to experimentation. Water temperature was controlled at 28 ± 1°C. No food was given to the fish throughout the experiment.

Four bighead carp were divided randomly into two groups. The fish were administered a single i.p. injection of microcystins at doses of either 400 (RR:LR = 10:1) or 580 (RR:LR = 1:1) μg MC-LReq/kg bw. And then these fish were sacrificed at 3 and 6 h post-injection. Fish was dissected and each muscle sample was divided into two parts: the first part was lyophilized directly as control; the second part was boiled for 5 min (boiled muscle). All treated samples were lyophilized for MCs determination. Additionally, we also analyzed MCs concentration in the water in which the sample was boiled, and expressed as μg/g fish muscle (dry weight; the MCs dissolved in the water divided by the total weight of the fish muscle that was boiled in water). Hence the actual MCs concentration in the treated muscle samples was the total of MCs in muscle sample and water.

MCs in the water were analyzed quantitatively according to Park and Lwami (1998).

The lyophilized muscle samples were extracted and analyzed according to the method of Zhang et al. (2007) with minor modification as follows: lyophilized samples (~0.3 g dry wt for each tissue) were homogenized in a mortar and extracted three times with 20 mL of butanol:methanol:water (1:4:15) for 24 h while stirring.

Qualitative and quantitive analysis of MCs were performed using a Finnigan LC-MS system. An Agilent ODS 3.5-μm column (2.1 mm i.d. × 100 mm) was used for LC separation. The column oven temperature was 45°C. 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 voltage 3.36 V; capillary temperature 250°C; And multiplier voltage −853.19 V; and tube lens offset, 55 V. Data acquisition was in the positive ionization centroid mode. The mass spectrometer was set to electrospray ionization positive-ion mode, and mass spectrometer tuning and optimization were achieved by infusing MC-RR with ion of $[M + 2H]^{2+}$ at *m/z* 520. Quantification of MCs was achieved through the total signal of tandem MS (MS/MS). The precursor ion was $[M + 2H]^{2+}$ at *m/z* 520 for MC-RR, whereas the precursor ion was $[M + H] +$ at *m/z* 995.5 for MC-LR. Collision energy was 37% for both MC-RR and MC-LR. All the values presented in the text were measured by LC-MS/MS.

Standards of MCs (-LR and -RR) used for LC-ESI-MS analysis were purchased from the Pure Chemical Industries, Osaka, Japan. The limit of detection for the MCs in the tissues of fish was 0.0033 μg/g DW.

The SPSS® (Chicago, IL, USA) for windows (ver. 13.0) statistical software was used for all analyses. A two tailed *t*-test was used to test for significant differences of MCs concentration between boiled muscle and control.

Results and Discussion

Microcystins in muscle of bighead carp were determined by LC-ESI-MS. Based on total ion chromatogram of ESI $LC\text{-}MS^2$ measurement of microcystins in muscle of fish, mass chromatograms monitored at *m/z* 520, and the presence of $[M + H]^+$ ion at *m/z* 452 and 887, it is confirmed that peak obtained at 8.24 min was derived from MC-RR. Similarly, peaks obtained at 11.07 min were derived from MC-LR, as the peaks were detected by monitoring with *m/z* 995.5, and the mass chromatogram showed $[M + H]^+$ ion at *m/z* 967 and 599 for MC-LR (Appendix Fig. 3).

It is well known that microcystins can bioaccumulate in aquatic animals (Smith and Haney 2006; Zhang et al. 2007), and these toxins can be transferred along the food web to high trophic levels, even to human beings (Smith and Haney 2006), hence consumption of aquatic animals containing MCs represents potential risk to human health. Moreover, Wannemacher (1989) reported that MC-LR is stable even at temperatures up to 300°C in laboratory conditions. These results suggest that cooking can not remove microcystins accumulated in aquatic animals.

In our study, a significant difference was found in MCs concentration between the lyophilized and boiled muscle (including the samples collected from 400 to 580 μg MC-LReq/kg bw groups) from MC-treated bighead carp (Independent-samples *T*-test, $p < 0.01$, n = 8). After exposure of bighead carp to 400 μg MC-LReq/kg bw, MCs concentration in boiled muscle (0.11 and 0.18 μg/g DW for 3 and 6 h post-injection, respectively) was higher than that in lyophilized muscle (0.038 and 0.057 μg/g DW for 3 and 6 h post-injection, respectively) (Fig. 1a, b). MCs was also detected in the water in which the muscle was boiled, hence the actual MCs concentration in both the boiled muscle (total of MC concentration in boiled muscle and water) were much higher than

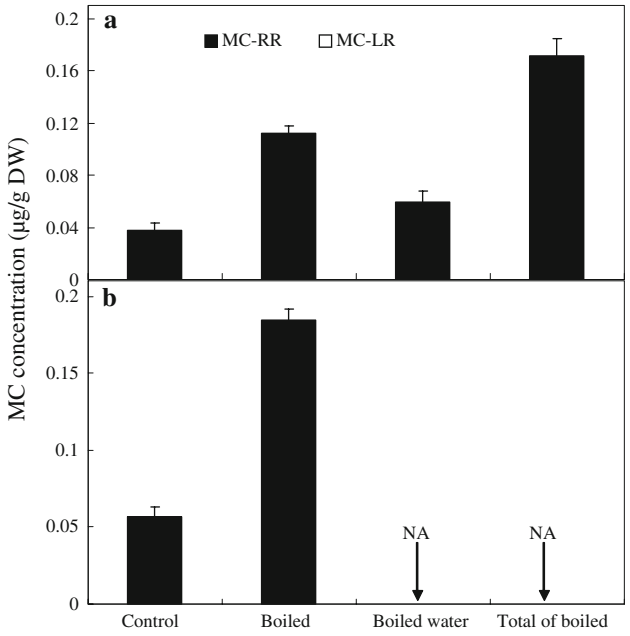

Fig. 1 MC concentration in muscle collected at 3 h **(a)** and 6 h **(b)** post-injection from bighead carp dosed i.p. with 400 μg/kg bw MC-LReq. Control: sample was lyophilized directly; Boiled: sample was put into cool water and heat to boiling and continued to be boiling for 5 min; Boiled water: the water in which the muscle was boiled; Total of Boiled: Total of MCs concentration in muscle and boiled water; Values are expressed as means ± standard deviations for two replicates; NA = Not analyzed

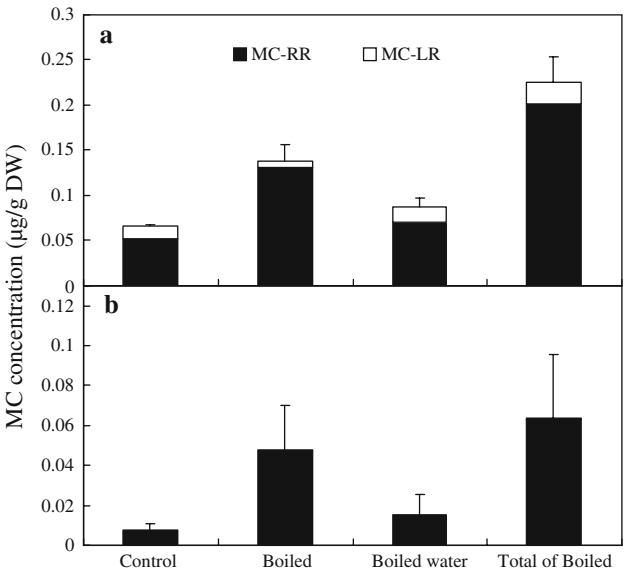

Fig. 2 MC concentration in muscle collected at 3 h **(a)** and 6 h **(b)** post-injection from bighead carp dosed i.p. with 580 μg/kg bw MC-LReq. Control: sample was lyophilized directly; Boiled: sample was put into cool water and heat to boiling and continued to be boiling for 5 min; Boiled water: the water in which the muscle was boiled; Total of Boiled: Total of MCs concentration in muscle and boiled water; Values are expressed as means ± standard deviations for two replicates

that in lyophilized muscle. Similar trends were observed in the muscle of bighead carp treated with the dose of 580 (RR:LR = 1:1) μg MC-LReq/kg bw (Control: 0.052 and 0.0079 μg/g DW for 3 and 6 h post-injection, respectively; Boiled: 0.132 and 0.048 μg/g DW for 3 and 6 h post-injection, respectively) (Fig. 2a, b). It is necessary to point out that microcystins extracted with MeOH is only a part of total MCs in aquatic animals (ca. 0.1%–26%) (Williams et al. 1997a, b), and large amounts of remaining MCs are covalently bound to the target molecules (e.g. protein phosphatases, GSH, Cys). Furthermore, previous studies demonstrated that the interaction of microcystins with protein phosphatases consists of a two-step mechanism: the first step involves a rapid but reversible binding with protein phosphatases, and the second step involves formation of a covalent bond between microcystins and phosphatases, leading to irreversible inactivation, and this step occurs over a period of hours (Craig et al. 1996).

The above results may suggest that boiling seems to make the toxins in animal tissues easier to extract. The possible reason is that the interaction of microcystins with protein phosphatases was still in the second step during the study period, and boiling may accelerate the revisable MCs release from phosphatases. However, there has been no study on extraction efficiency of MCs in aquatic animals by hot water. So the extraction efficiency of MCs by hot water is needed in future study.

Microcystins are rarely ingested by human in amounts high enough for a lethal acute dose, but chronic toxic effects from exposure through food are probable, especially if there is long-term frequent exposure. The World Health Organization (WHO) has set a provisional guideline for microcystin-LR in drinking water as 1 μg/L (Falconer et al. 1994). Based on this limit, WHO established a provisional tolerable daily intake (TDI) of 0.04 μg/kg body weight per day for MC-LR in the aquatic products (Chorus and Bartram 1999). Presently, the safety assessment on microcystins in aquatic products was according to the free MCs concentration in the edible part of them. However, in the present study, the free MCs concentration in the muscle that was boiled was significantly higher than that in control fish. Hence, we can conclude that the potential threat of aquatic animals contaminated with microcystins to human health was underestimated. Finally, a substantial amount of MCs in the boiling water suggests that eating soup of MC-contaminated fish also poses a potential hazard to humans. So much attention should be paid to the combined risks from both MC-contaminated fish and soup to human health in the future.

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Appendix

See Fig. 3.

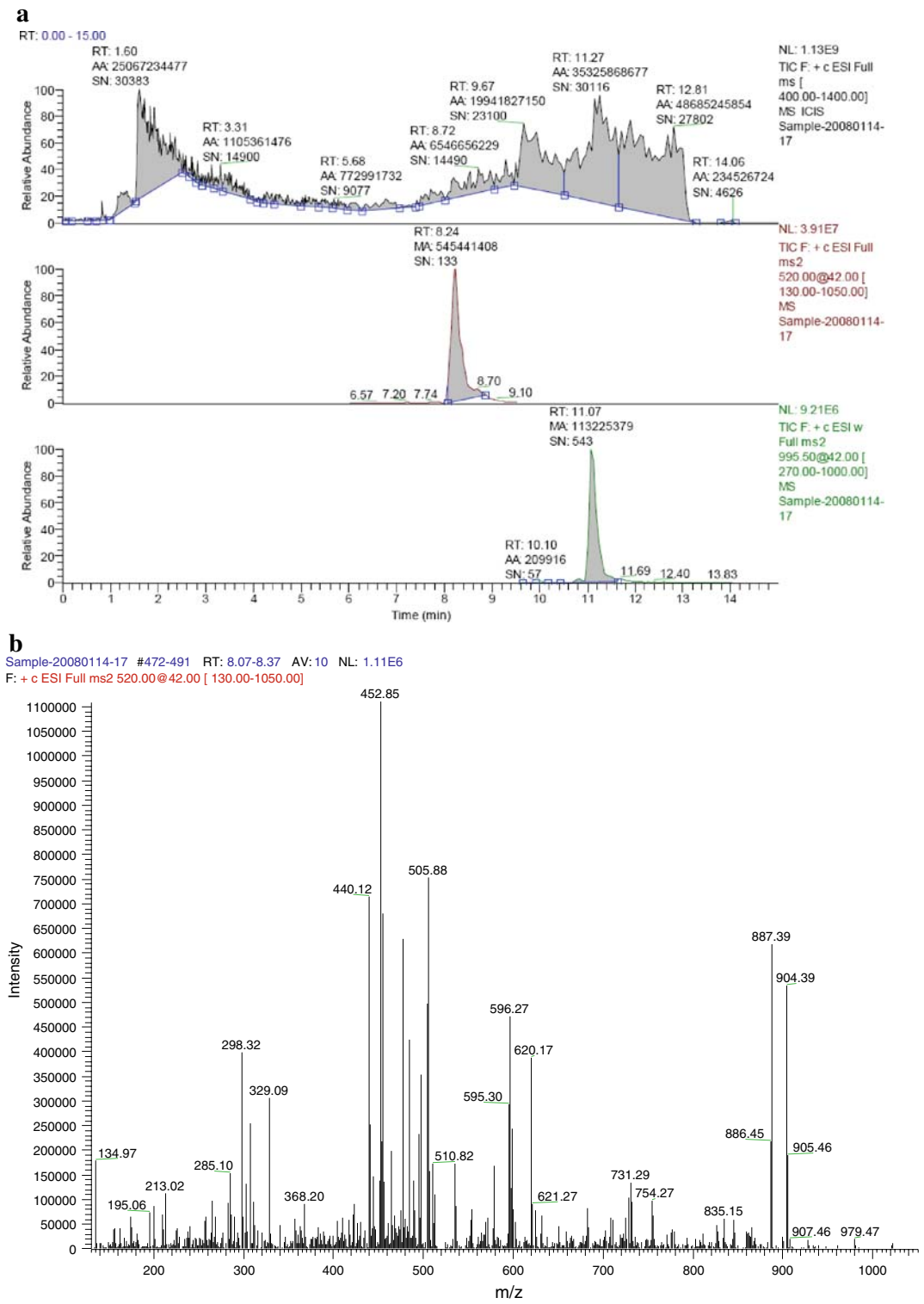

Fig. 3 **(a–c)**ESI LC/MS analysis of microcystins in muscle collected at 3 h post-injection from bighead carp dosed i.p. with 580 μg/kg bw MC-LReq. **a** ESI LC-MS^2 chromatograms monitored at m/z 520,995. **b** ESI LC-MS^2 spectrum at 8.24 min (MC-RR). **c** ESI LC-MS^2 spectrum at 11.07 min (MC-LR)

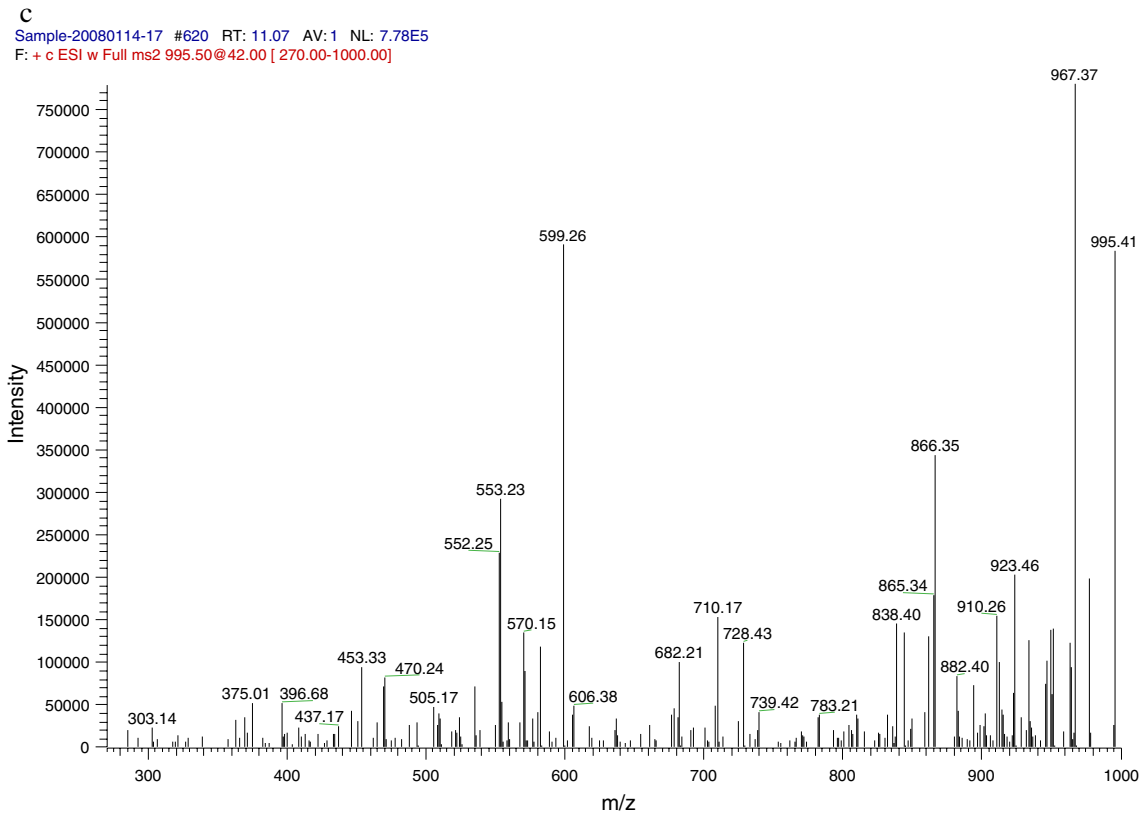

Fig. 3 continued

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