Tissue distributions and seasonal dynamics of the hepatotoxic microcystins-LR and -RR in a freshwater snail (*Bellamya aeruginosa*) from a large shallow, eutrophic lake of the subtropical China

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Snails bioaccumulate microcystins in their hepatopancreas, gut and gonad, posing a risk to human consumers.

Abstract

Tissue distributions and seasonal dynamics of the hepatotoxic microcystins-LR and -RR in a freshwater snail (*Bellamya aeruginosa*) were studied monthly in a large shallow, eutrophic lake of the subtropical China during June–November, 2003. Microcystins (MCs) were quantitatively determined by High-Performance Liquid Chromatography (HPLC) with a qualitative analysis by a Finnigan LC–MS system. On the average of the study period, hepatopancreas was the highest in MC contents (mean 4.14 and range 1.06–7.42 $\mu g\,g^{-1}$ DW), followed by digestive tracts (mean 1.69 and range 0.8–4.54 $\mu g\,g^{-1}$ DW) and gonad (mean 0.715 and range 0–2.62 $\mu g\,g^{-1}$ DW), whereas foot was the least (mean 0.01 and range 0–0.06 $\mu g\,g^{-1}$ DW). There was a positive correlation in MC contents between digestive tracts and hepatopancreas. A constantly higher MC content in hepatopancreas than in digestive tracts indicates a substantial bioaccumulation of MCs in the hepatopancreas of the snail. The average ratio of MC-LR/MC-RR showed a steady increase from digestive tracts (0.44) to hepatopancreas (0.63) and to gonad (0.96), suggesting that MC-LR was more resistant to degradation in the snail. Since most MCs were present in the hepatopancreas, digestive tracts and gonad with only a very small amount in the edible foot, the risk to human health may not be significant if these toxic parts are removed prior to snail consumption. However, the possible transference of toxins along food chains should not be a negligible concern.
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1. Introduction

Eutrophication, the biological response to excess input of nutrients into a water body, is usually accompanied with frequent dominance by cyanobacteria in inland waters (Paerl et al., 2001; Havens et al., 2003).

It is of great concern to human society because of the common ability of these organisms to produce toxins (Codd, 2000). Among cyanotoxins, the hepatotoxic microcystins (MCs) are one of the most dangerous groups (Carmichael, 1997).

The presence of microcystins has been documented all over the world. The exposure to MCs was already related to incidents of acute intoxication of terrestrial animals and humans (Carmichael, 1994; Falconer, 1998; Carmichael et al., 2001; Azevedo et al., 2002). For

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chronic effects, an epidemiological survey in Haimen City (Jiangsu Province) and Fusui County (Guangxi Province) in China found a close relationship between the incidence of primary liver cancer and the MC content in drinking water (Yu, 1989, 1995; Ueno et al., 1996). MCs have the general structure cyclo-(D-Ala-L-*X*-erythro-β-methyl-D-isoAsp-L-*Y*-Adda-D-iso-Glu-*N*-Methyldehydro-Ala), where Adda refers to the β-amino acid residue of 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-idenoic acid, and *X* and *Y* represent variable L-amino acids (Carmichael, 1992; Dawson, 1998). The unusual amino acid Adda is essential for expression of biological activity (Trogen et al., 1996). Until now, more than 60 structurally different microcystins have been identified (Carmichael, 1994; Dawson, 1998). The most widely studied microcystin is MC-LR in which the variable L-amino acids are leucine and arginine.

Probably because of the general belief that the major route of human exposure to MCs is oral route through drinking water (Chorus and Bartram, 1999) and of the technical difficulty to measure MCs in animal tissues (Xie et al., 2004), little attention has been paid to bioaccumulation of microcystins in aquatic animals of natural waters. So far, there have been only occasional reports on the presence of microcystin contamination in wild aquatic animals, such as fish (Magalhàes et al., 2001; Zimba et al., 2001; Mohamed et al., 2003), mussels (Watanabe et al., 1997) and snails (Kotak et al., 1996; Zurawell et al., 1999; Ozawa et al., 2003). Such studies are still lacking in China where most freshwaters are at a high risk to toxic cyanobacterial blooms but freshwater fisheries are almost equally important as marine fisheries to Chinese consumption.

The present research was conducted on a freshwater snail (*Bellamya aeruginosa*) in a large shallow, eutrophic subtropical freshwater lake (Lake Chaohu) where heavy cyanobacterial blooms outbreak in the warm seasons of every year. This snail is high in production and used both for direct human consumption and for the diary food of crab culture. The purposes of this study are to examine distributions and seasonal changes of microcystins-LR and -RR in various organs (digestive tracts, hepatopancreas, gonad and foot) of *B. aeruginosa*, and to evaluate the relative importance of different organs in the accumulation of MCs with comments on the potential risk to human consumption.

2. Materials and methods

Lake Chaohu ((31°25′–31°43′)N, (117°16′–117°51′)E), located in Anhui Province of southeastern China, is among the five largest freshwater lakes in China. It has a surface area of 760 km², a mean depth of 3.06 m, and a mean retention time of 136 days. Since the

1950s, eutrophication of the lake water has increased steadily and heavy cyanobacterial blooms (mainly composed of *Microcystis* spp. and *Anabaena* sp.) have occurred in the warm seasons of each year in recent decades.

The freshwater snail *B. aeruginosa* belongs to the family of Viviparidae. It is widely distributed in China with habitats of lakes, rivers, ditches and ponds (Liu et al., 1993). In Lake Chaohu, the production of *B. aeruginosa* reached 28 084t in 2002, and is of great commercial importance both for human consumption and for crab culture (personal communication of Dr. Deng D W). During June–November 2003, snails were collected monthly near Zhongmiao where surface cyanobacterial blooms (mainly *Microcystis aeruginosa* and *Anabaena spiroides*) frequently accumulated densely by wind. The snail had a shell width of 13.36 ± 4.87 mm and a shell height of 21.64 ± 3.85 mm. The collected snails were immediately frozen at −20 °C, and then dissected into four parts (digestive tracts including stomach, hepatopancreas, gonad and foot) in the laboratory. The collected organs were frozen at −80 °C prior to microcystin analysis. Since there was insufficient toxins in the digestive tracts, hepatopancreas, gonad and foot to allow for individual analysis, we pooled, respectively, all digestive tracts, hepatopancreas, gonad and feet of 60 dissected snails. Thus, each value represents an average amount of microcystins in the organs of 60 individuals.

Extraction and analysis of the microcystins in the digestive tracts, hepatopancreas, gonad and foot of the snail basically followed the method of Xie et al. (2004): lyophilized samples (ca. 0.5 g DW for each organ) were homogenized and extracted three times with 10 ml of BuOH:MeOH:H_2O (1:4:15) for 24 h while stirring. The extract was centrifuged at 18 000 rpm and the supernatant was diluted with water. This diluted extract was directly applied to 5 g of a reversed phase ODS cartridge, which had been preconditioned by washing with 50 ml of 100% MeOH and 50 ml of H_2O. The column was washed with water (50 ml), followed by water–MeOH (4:1, 100 ml). Elution from the column with 90% MeOH (100 ml) yielded the toxin-containing fraction. The toxin-containing fraction was evaporated to dryness. Then the residue was dissolved with 100% MeOH (5 ml) and then eluted with 70% MeOH (20 ml), the toxin-containing fraction was also evaporated to dryness. This fraction was dissolved with 100% MeOH and the methanol solution was subjected to a reverse-phase high-performance liquid chromatography (HPLC) equipped with an ODS column (Cosmosil 5C18-AR, 4.6 × 150 mm, Nacalai, Japan) and an SPD-10A UV–vis spectrophotometer set at 238 nm. A gradient starting at 50% (v/v) aqueous methanol with 0.05% trifluoroacetyl (TFA) was increased to 70% (v/v) in 25 min at a flow rate of 1 ml/min. MC concentrations

were determined by comparing the peak areas of the test samples with those of the standards available (MC-LR and MC-RR, Wako Pure Chemical Industries, Japan).

Qualitative analysis of MCs was performed using a Finnigan LC–MS system comprising a thermo surveyor auto sampler, a surveyor MS pump, a surveyor PDA system, and a Finnigan LCQ-Advantage MAX ion trap mass spectrometer equipped with an atmospheric pressure ionization fitted with an electrospray ionization source (ESI). The instrument control, data processing, and analysis were conducted by using Xcalibur software. Separation was carried out under the reversed phase on Hypersil GOLD 5 μm column (2.1 mm i.d. × 150 mm). The isocratic mobile phase consisted of solvent A [water + 0.05% (v/v) trifluoroacetic acid (TFA)] / solvent B [acetonitrile + 0.05% TFA]. The gradient went from 15% B (stand 2 min) to 95% B (stand 7 min) over the first 13 min, followed by a decrease to 15% B (stand 5 min) over the subsequent 3 min. Sample injection volumes were typically 10 μl. LC–MS conditions were as follows: ESI spray voltage 4.54 kV, sheath gas flow rate 30 unit, auxiliary gas flow rate 0 unit, capillary voltage 45.67 V, capillary temperature 230 °C, and multiplier voltage −801.62 V. Data acquisition was in the positive ionization centroid mode with full mass mode. MS tuning and optimization were achieved by infusing microcystin-RR and monitoring the $[M + H]^+$ ion at m/z 520.

3. Results

The presence of MC-LR and -RR in tissues of the snail is shown in Figs. 1 and 2. The chromatograms of the MC-LR and -RR standards, the extracts of digestive tracts, hepatopancreas and gonad of *B. aeruginosa* are compared. It is clear that the toxins were taken up and a part was extractable. Our microscopic examinations found that algae in the food items of the gut of *B. aeruginosa* in June were almost all *Microcystis* cells, confirming the ingestion of large amounts of *Microcystis*.

MCs in digestive tracts varied between 0.8 and 4.54 μg g^{-1} DW with an average of 1.693 μg g^{-1} DW (Fig. 3a). MC contents in the hepatopancreas varied between 1.06 and 7.42 μg g^{-1} DW with an average of 4.14 μg g^{-1} DW (Fig. 3b). In gonad, MC contents were between 0 and 2.62 μg g^{-1} DW with an average of 0.715 μg g^{-1} DW (Fig. 3c). In foot, MC was present only in the sample of October, and thus the average was only 0.01 μg g^{-1} DW (Fig. 3d). Both digestive tracts and hepatopancreas had their maximum MC contents in August, while the maximum MC content in gonad was in November. The mean MC contents were significantly higher in hepatopancreas than in digestive tracts (paired *t*-test, $P = 0.026$) or than in gonad (paired *t*-test, $P = 0.03$). There was a positive correlation between

MCs of digestive tracts and MCs of hepatopancreas of *B. aeruginosa* ($r = 0.6399$, $P = 0.171$). However, such correlation was absent between hepatopancreas and gonad ($r = -0.117$, $P = 0.825$).

The ratio of MC-LR/MC-RR in the digestive tracts varied from 0.22 to 0.88 with an average of 0.44. In hepatopancreas, this ratio changed from 0.33 to 1.33 with an average of 0.63. In gonad, the average ratio increased to 0.96 (range 0–2.07).

4. Discussion

In the present study, there was a positive correlation between MC of digestive tracts and MC of hepatopancreas of *B. aeruginosa* ($r = 0.6399$), although statistically not significant ($P = 0.171$), suggesting that to some extent accumulation of MC in hepatopancreas depended on MC level in the food resources. Zurawell et al. (1999) report that for three gastropods (*Lymnaea stagnalis*, *Helisoma trivolvis* and *Physa gyrina*), the concentration of MC-LR in the tissue (whole body excluding shell) was correlated with toxin in the phytoplankton of the water column based on log–log transformed data in seven Canadian lakes.

In the present study, MC content was significantly higher in the hepatopancreas (mean ± SD: 4.14 ± 2.49) than in the digestive tract of *B. aeruginosa* (mean ± SD: 1.69 ± 1.43), indicating a substantial bioaccumulation of MC in the hepatopancreas of the snail. This is contradictory to the results for a resident snail (*Sinotaia histrica*) in a Japanese lake (Lake Biwa) where MC content of the hepatopancreas was only 1/6 that of intestine in October with trace or undetectable amount when MC content of intestine was between 2.59 and 4.82 μg g^{-1} DW during July–September (Ozawa et al., 2003). On the other hand, in Lake Suwa, Watanabe et al. (1997) reported a slightly higher MC content in the hepatopancreas (1.57 μg g^{-1}) than in the gut (1.31 μg g^{-1}) for a freshwater mussel (*Unio douglasiae*) although the measurement was conducted only for one sample. In spite of the fact that the measurement of MC in intestinal tracts may be occasionally affected by various factors (e.g., sampling procedures, degree of food digestion), the constantly lower MC content in the digestive tract of *B. aeruginosa* in our study lake was most likely a real reflection of their food resources on the sediment where a high sedimentation rate of sand materials probably led to a relatively low MC content in the food of the snails.

In the present study, MC contents in digestive tracts of *B. aeruginosa* averaged 1.693 μg g^{-1} DW, while those in the collected surface cyanobacterial blooms in our study lake reached 240 μg g^{-1} DW (a difference of 141.8 times). This suggests that the food sources of *B. aeruginosa* in Lake Chaohu were quite low in MC
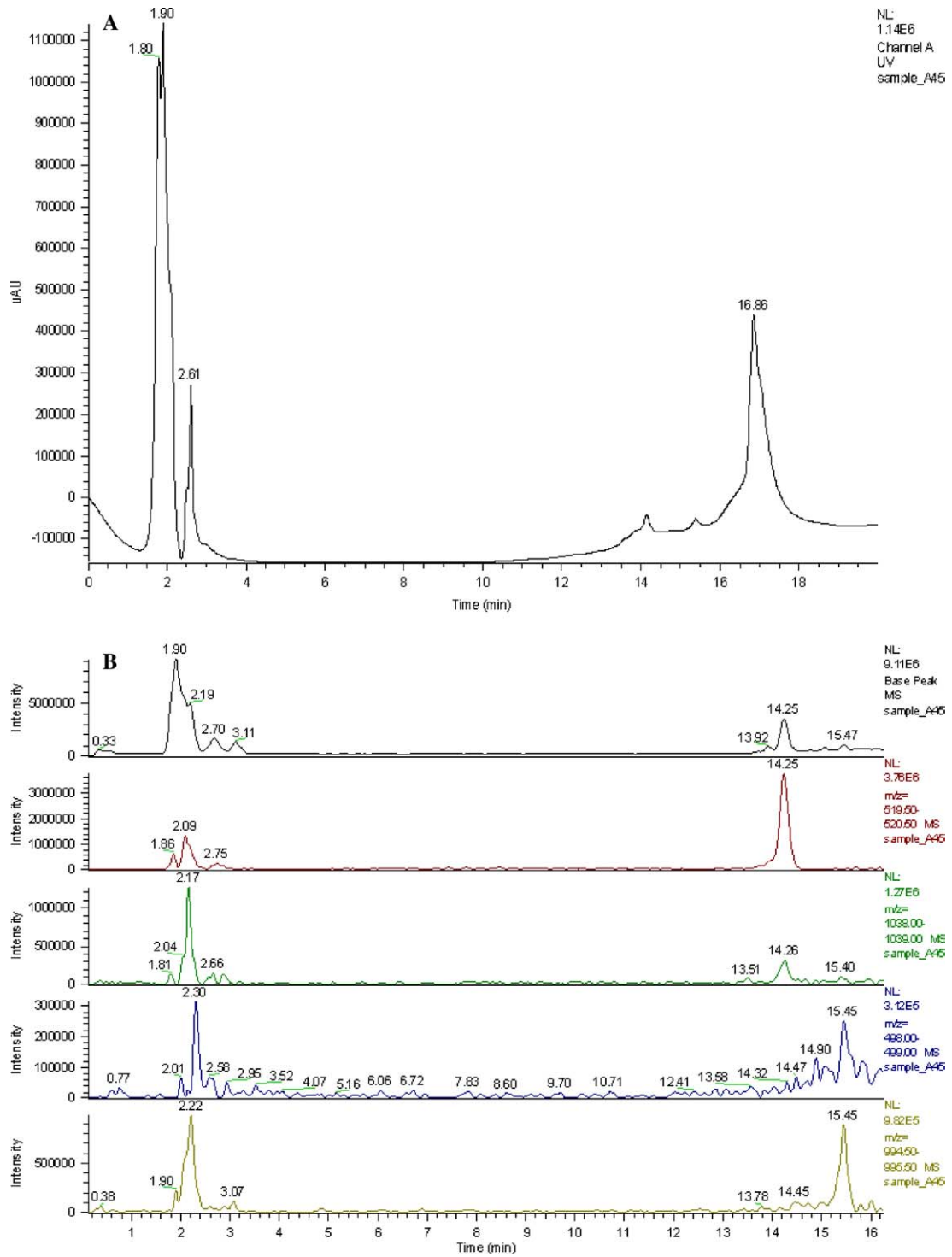
Fig. 1. High-performance liquid chromatogram monitored at 238 nm (a), mass chromatograms monitored at m/z 520, 1038, 498 and 995 (b), ESI mass spectrum at 14.25 min (microcystin-RR) (c), and ESI mass spectrum at 15.45 min (microcystin-LR) (d).
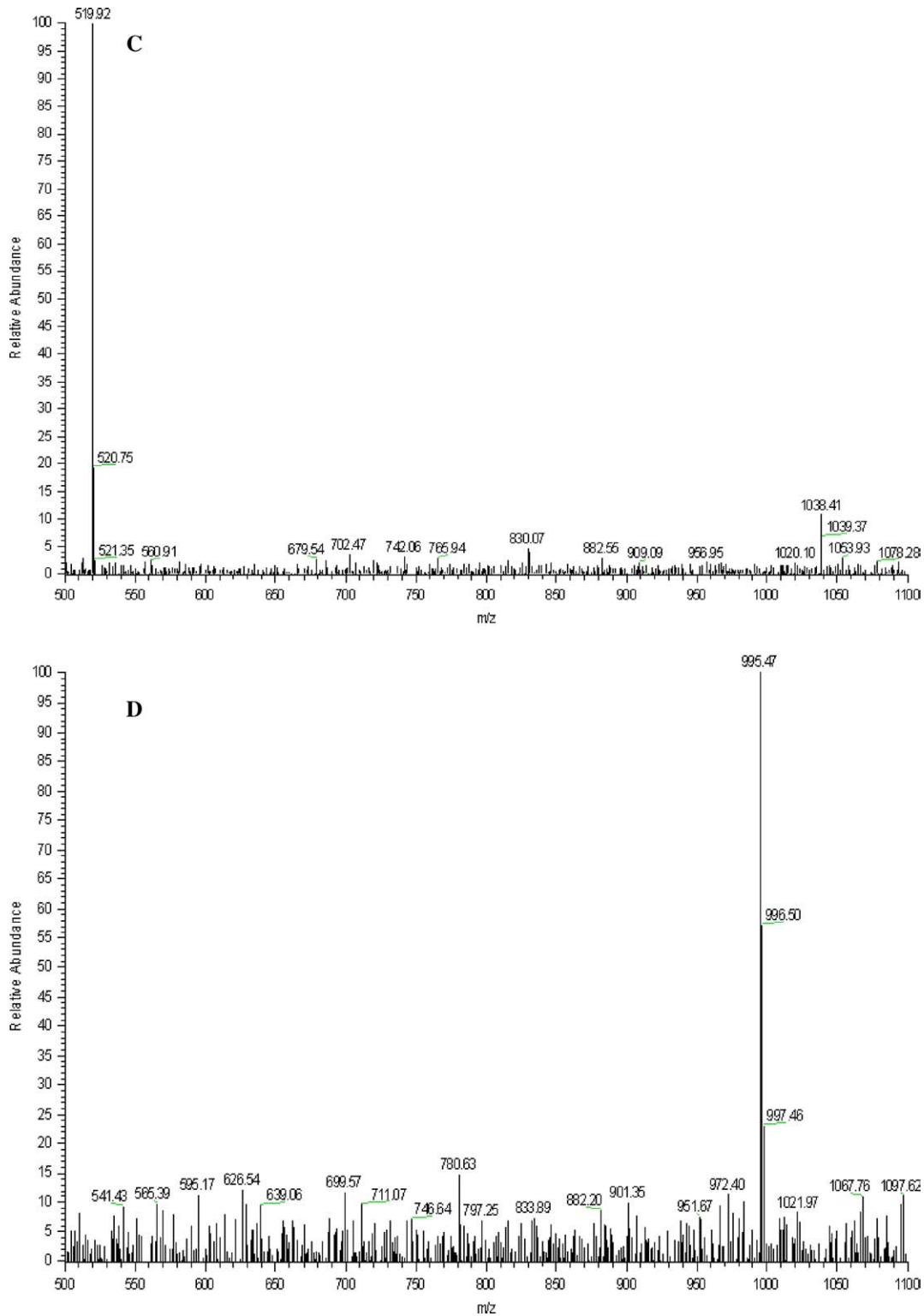
Fig. 1. (*continued*)

contents probably due to either massive presence of decomposed cyanobacteria or ingestion of massive inorganic particles from the surface sediments, or to both. In Lake Biwa, on October 11, 2000, MC contents were $284 \, \mu g \, g^{-1}$ DW in seston and $19.5 \, \mu g \, g^{-1}$ DW in

the intestines of the Japanese snail *Sinotaia histrica*, indicating a difference of 14.6 times (Ozawa et al., 2003). In an experimental tank (Tank A), MC contents were $206.9 \, \mu g \, g^{-1}$ DW in seston and $78.8 \, \mu g \, g^{-1}$ DW in the gut of the phytoplanktivorous silver carp, indicating
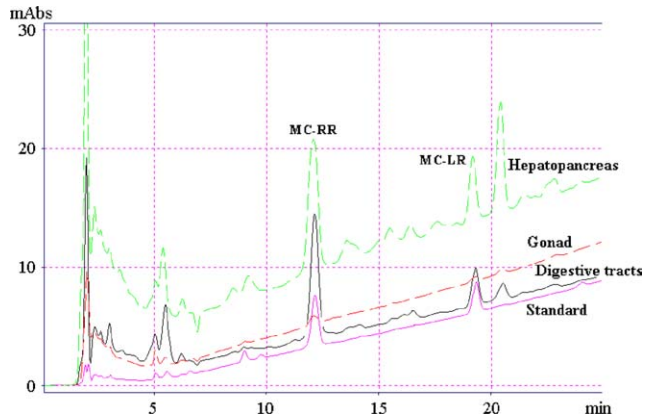
Fig. 2. A comparison of the chromatograms (monitored at 238 nm) of the standard MC-LR and -RR, the extracts of digestive tracts, hepatopancreas and gonad of *B. aeruginosa* collected from Lake Chaohu in June 2003.

a difference of only 2.6 times (Xie et al., 2004). In an Egyptian fish farm, MC contents were 1120 µg g^{-1} DW in *Microcystis* blooms, and 8.21 µg g^{-1} DW (the maximum) in the gut of the phytoplanktivorous tilapia, indicating a difference of 136.4 times. It should be noted, however, that tilapia have a stomach with pH value as low as 1.25 or even 1.0 in the stomach fluids during digestion (Payne, 1978), suggesting the possibility of active degradation of MCs in the stomach and thus the presence of low MC contents in the guts.

The relationship between MC content of the intestinal tracts and that of hepatopancreas/liver was also variable for other aquatic animals. In an Egyptian fish farm containing toxic *Microcystis* blooms, MC content in organs of tilapia was examined in June, and it was found that the ratio of MC in liver to MC in gut was >1 when MC in gut was relatively low (ca. 0.17 µg MC g^{-1} WW), but <1 when MC in gut reached the highest (0.82 µg MC g^{-1} WW) (Mohamed et al., 2003). In an 80-day subchronic toxic experiment for silver carp fed with fresh toxic *Microcystis*, MC content was significantly higher in the intestines (mean 78.8 with a range of 49.2–115.3 µg MC g^{-1} DW) than in the liver (mean 12.0 with a range of 7.84 – 17.8 µg MC g^{-1} DW) (Xie et al., 2004). In a laboratory experiment, the crayfish *Procambarus clarkii* were fed with toxic *Microcystis aeruginosa* (ca. 2.3 mg MC g^{-1} DW) for 23 days, and percentages of MC in the hepatopancreas and intestine were 38% and 53%, respectively (Vasconcelos et al., 2001).

The present study indicates that for *B. aeruginosa*, MC of hepatopancreas (mean 4.14 µg g^{-1} DW) was much higher than those of gonad (0.715 µg g^{-1} DW) and foot (0.01 µg g^{-1} DW). To our knowledge, this is the first study to report the accumulation of MC in gonad of snails and to compare the accumulation of MC between hepatopancreas and foot of snails. Previous studies have only reported MC in the whole bodies of three Canadian snails (Kotak et al., 1996; Zurawell
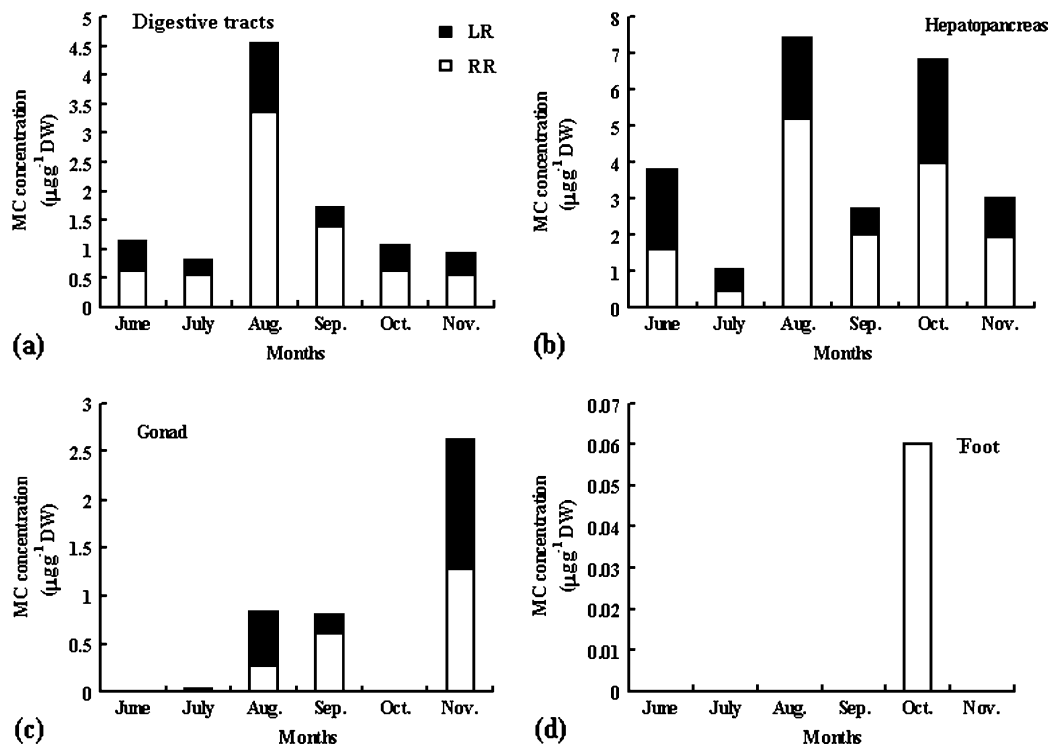

Fig. 3. The seasonal changes of MC-LR and -RR concentrations (µg g^{-1} DW) in (a) digestive tracts, (b) hepatopancreas, (c) gonad and (d) foot of *Bellamya aeruginosa* in Lake Chaohu during June and November, 2003.

et al., 1999) or MC in intestine and hepatopancreas of a Japanese snail (Ozawa et al., 2003).

In the present study, although the target organ of MC was hepatopancreas, a substantial amount of MC accumulated in the gonad of *B. aeruginosa*. In Lake Suwa (Japan), a freshwater mussel (*Unio douglasiae*) accumulated 1.57 and 1.19 $\mu g \, MC \, g^{-1}$ DW in hepatopancreas and gonad, respectively (Watanabe et al., 1997). Our results indicate that the average ratio of MC-LR/MC-RR showed a steady increase from digestive tracts (0.44) to hepatopancreas (0.63) and to gonad (0.96), suggesting that MC-LR might be more resistant to degradation in the snail. Since MC-LR is much more toxic than MC-RR, accumulation of MC-LR is more harmful in terms of human health.

In the present study, the majority of MC was present in the hepatopancreas, digestive tracts and gonad, whereas only a very small amount of MC was in the foot that is usually the edible part. The risk to human health may not be significant if these toxic parts are removed prior to snail consumption. However, in China, one of the most popular ways of cooking snails is to put the whole body in boiling water, and thus it will be a risk to drink the soup of snails contaminated by MC. In North America and Japan, few people directly consume freshwater snails, and most concerns are on the possible transference of toxins along food chains (Kotak et al., 1996; Zurawell et al., 1999; Ozawa et al., 2003), since snails are not only an important food source for fish, but also waterfowl, crayfish, and amphibians (Stein et al., 1984; Lodge, 1986). In fact, substantial presence of MCs has been identified in organs of various fishes including the snail-eating common carp (personal communication by Xie LQ). Therefore, the role of snails as a vector of MC in the food web should be clarified in our future study.

Acknowledgements

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