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Environmental Toxicology and Chemistry, Vol. 26, No. 1, pp. 171–176, 2007

Printed in the USA
0730-7268/07 $12.00 + .00

BIOACCUMULATION OF THE HEPATOTOXIC MICROCYSTINS IN VARIOUS ORGANS OF A FRESHWATER SNAIL FROM A SUBTROPICAL CHINESE LAKE, TAIHU LAKE, WITH DENSE TOXIC *MICROCYSTIS* BLOOMS

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(*Received* 4 *May* 2006; *Accepted* 3 *August* 2006)

Abstract—In this paper, we describe the seasonal dynamics of three common microcystins (MCs; MC-RR, MC-YR, and MC-LR) in the whole body, hepatopancreas, intestine, gonad, foot, remaining tissue, and offspring of a freshwater snail, *Bellamya aeruginosa*, from Gonghu Bay of Lake Taihu, China, where dense toxic *Microcystis* blooms occur in the warm seasons. Microcystins were determined by liquid chromatography electrospray ionization mass spectrum. Microcystin (MC-RR + MC-YR + MC-LR) content of the offspring and gonad showed high positive correlation, indicating that microcystins could transfer from adult females to their young with physiological connection. This study is the first to report the presence of microcystins in the offspring of the adult snail. The majority of the toxins were present in the intestine (53.6%) and hepatopancreas (29.9%), whereas other tissues contained only 16.5%. If intestines are excluded, up to 64.3% of the toxin burden was allocated in the hepatopancreas. The microcystin content in the intestine, hepatopancreas, and gonad were correlated with the biomass of *Microcystis* and intracellular and extracellular toxins. Of the analyzed foot samples, 18.2% were above the tolerable daily microcystin intake recommended by the World Health Organization (WHO) for human consumption. This result indicates that public health warnings regarding human ingestion of snails from Taihu Lake are warranted. In addition, further studies are needed to evaluate the occurrence by *Microcystis* in relation to spatial and temporal changes in water quality.

Keywords—Microcystins *Bellamya aeruginosa* Snail Taihu Lake China

INTRODUCTION

Eutrophication resulting from excessive input of nutrients is often accompanied with the occurrence of toxic cyanobacterial blooms all over the world [1,2]. It is well known that some species of cyanobacteria, primarily *Microcystis*, can produce cyclic peptide hepatotoxins termed microcystins (MCs) [3,4]. So far, more than 70 variants of MCs have been reported [5].

Microcystins are known to be a potent hepatotoxin [6] and tumor promoter by inhibiting protein phosphatase type 1 and 2A [7,8]. These toxins cause poisoning or death of birds, domestic and wild animals, fish [9], and even pose a potential threat to human health [10]. It is reported that MCs can be transferred along the food chain [11], suggesting a potential risk for human consumption of contaminated aquatic products.

Recently, extensive studies have been made not only of the acute toxicity of MCs to laboratory mammals such as mice [12,13] and rats [14], but also on aquatic organisms, including fish [11,15,16], zooplankton [17,18], bivalves [19–21], shrimps [22], and gastropods [23–25]. However, information has been limited on tissue distribution and the seasonal dynamics of MCs in gastropods. Kotak et al. [26] and Zurawell et al. [23] described MC accumulation in the whole body of three species of gastropods. Ozawa et al. [24] analyzed the distribution of MCs in the intestine and hepatopancreas of a freshwater snail (*Sinotaia histrica*) from Lake Biwa, Japan. Chen et al. [25] described MC accumulation in the intestine, hepatopancreas, gonad, and foot of a freshwater snail (*Bellamya aeruginosa*) in Lake Chaohu during the period of toxic cyanobacterial blooms. To date, no study has been undertaken to examine MC accumulation in the offspring of snails.

Bellamya aeruginosa is widely distributed in China in habitats of lakes, rivers, ditches, and ponds and has great importance both for human consumption and for crab culture [25,27]. The snail feeds on detritus or algae on lake sediment or other substrata. The study had two objectives: to describe the dynamics of three common MCs (MC-RR, MC-YR, and MC-LR) in various tissues of *B. aeruginosa* and to provide field evidence that MCs can be transferred from adults to offspring.

MATERIALS AND METHODS

Study areas

Lake Taihu (30°5′–32°8′N, 119°8′–121°55′E) is located in the eastern part of China. It is the third largest freshwater lake in China and has a surface area of 2,428 km^2, a mean water depth of 1.9 m, and a maximum depth of about 3.5 m. From 1981 to now, the lake has witnessed a steady increase in eutrophication, characteristic of a regular occurrence of cyanobacterial surface blooms in the warm seasons of each year [28–30]. Gonghu Bay (31°31′–23′N, 120°16′–35′E) is located at the northeastern portion of Taihu Lake with abundant submerged plants. Three sampling sites were chosen in this study (Fig. 1).

Water quality

Water samples were collected monthly from the three sites of Gonghu Bay with Tygon tubing fitted with a one-way valve from November 2004 to October 2005. Each integrated water sample was a mixture of two subsamples: one from 0.5 m

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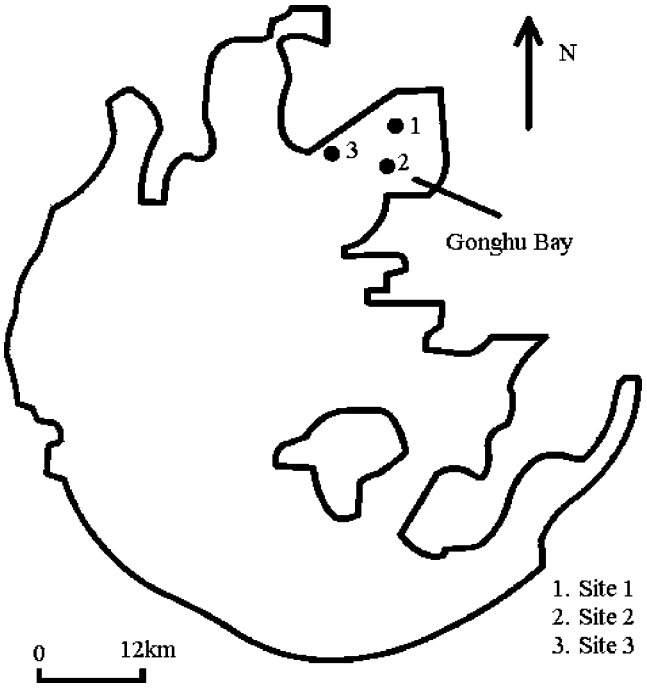

Fig. 1. Sketch of Taihu Lake showing the sampling sites in Gonghu Bay, China, from November 2004 to October 2005.

below the surface and one from 0.5 m above the bottom. Transparency was measured by a Secchi disk. Total phosphorus (TP) and total dissolved phosphorus were measured by colorimetry after digestion with H_2SO_4 [31]. Total nitrogen (TN) and total dissolved nitrogen were determined by the Kjeldahl method. Ammonium (NH_4-N) was determined by the Nessler method and nitrite (NO_2-N) by the Kjeldahl method. Ammonium (NH_4-N) was determined by the Nessler method and nitrite (NO_2-N) by the α-naphthylamine method [32]. One liter of lake water was fixed with 5% formalin, and *Microcystis* cells were separated ultrasonically before microscopic examination. *Microcystis aeruginosa* biomass (mg fresh weight/L) was estimated from its geometric cell volume, assuming a mean density of 1 mg/mm.

Microcystin in the lake was fractionated to intracellular and extracellular toxins. The intracellular toxins were extracted from cyanobacterial cells filtered from 1 L of lake water on the GF/C glass fiber filter (Whatman, Brentford, UK). The filtrate (1 L) was used to detect the extracellular toxins.

Snail samplings

Bellamya aeruginosa were collected monthly from the three sites of Gonghu Bay from November 2004 to October 2005. Average body weight of the snail was 4.544 ± 1.23 g, with a shell height of 2.758 ± 0.295 cm and a shell width of 1.639 ± 0.199 cm (n = 1,980). We could not collect the snail samples from all three sites in January 2005 because the snails moved to a deeper depth of sediment. The collected snails were immediately frozen at −20°C and then dissected in the laboratory into five parts: intestine (including stomach), hepatopancreas, gonad, foot, and remaining tissue, which are the tissues excluding intestine, stomach, hepatopancreas, gonad, and foot. Most of the remaining tissue was composed of muscle. At the same time, we collected offspring (young snails) from the gonads of adult females if there were enough individuals for toxin analysis during the reproductive period. The offspring were washed carefully with distilled water to avoid possible MC attachment from the adults. The collected organs and offspring were frozen at −80°C before MC analysis. To ensure adequate sample, we pooled separate snail organ material (n = 60) into one composite sample.

Microcystin analysis

All the frozen tissues and offspring of the snail were lyophilized before MC analysis. We extracted MCs from the snail tissues following the method of Chen and Xie [21]: lyophilized samples (~0.5 g dry weight for each tissue) were homogenized and extracted three times with 25 ml of butanol-methanol-water (1:4:15) for 24 h while stirring. The extract was centrifuged at 36,290 *g*, and the supernatant was diluted with water.

Qualitative and quantitive analysis of MCs were performed with a Finnigan liquid chromatography mass spectrum (LC-MS) system comprising a Thermo Surveyor auto sampler, a Surveyor mass spectrum 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 (LC-ESI-MS; Thermo Electron). The instrument control, data processing, and analysis were conducted with Xcalibur software (Thermo Electron). Separation was carried out under the reversed phase on Hypersil GOLD (Thermo Electron) 5-μm column (2.1 mm i.d. × 150 mm). The isocratic mobile phase consisted of solvent A (water + 0.05% [v/v] formic acid)/solvent B (acetonitrile + 0.05% formic acid). We used the following linear gradient program: 0 min 30% B, 2 min 30% B, 7 min 50% B, 11 min 100% B, 14 min 100% B, 15 min 30% B, 25 min 30% B. Sample injection volume was typically 10 μl. Mass spectrum tuning and optimization were achieved by infusing MC-RR and monitoring the $[M + 2H]^{2+}$ ion at m/z 520. The MS analytical conditions were as follows: ESI spray voltage 4.54 kV; sheath gas flow rate 20 units; auxiliary gas flow rate 0 units; capillary voltage 3.36 V; capillary temperature 250°C; multiplier voltage 853.19 V; and tube lens offset 55 V. Data acquisition was in the positive ionization centroid mode. The MS detection was operated in four segments: first, a full-scan mode with a mass range between 400 and 1,400 for 4.2 min; second, two scan events with a full-scan mode as in segment 1 and an MS^2 mode with a mass range between 140 and 1,100 (parent ion, 520; isolation width, 1; normalized collision energy, 37%; 4.8 min duration); third, three scan events with a full-scan mode as in segment 1 and MS^2 modes with mass ranges between 270 and 1,100 and 285 and 1,100, respectively (parent ion, 995.5 and 1,045.5, respectively; isolation width, equal for both; normalized collision energy, equal for both, 35%; 4.8 min duration); and fourth, a full-scan mode as in segment 1.

Standards of MCs (-LR, -YR, and -RR) used for LC-ESI-MS analysis were purchased from the Pure Chemical Industries (Osaka, Japan). The limit of detection for the MCs was 0.01 μg/ml.

Statistical analysis

The SPSS® (Chicago, IL, USA) for Windows (Ver 13.0) statistical software was used for correlation and analysis of variance.

Table 1. The annual means (range) of physicochemical parameters at the three sites in Gonghu Bay of Lake Taihu, China[a]

	Site 1	Site 2	Site 3
TN (mg/L)	1.91 (0.29–3.98)	2.87 (0.20–5.31)	2.73 (0.72–4.56)
TDN (mg/L)	1.4 (0.12–3.12)	1.96 (0.01–4.83)	1.62 (0.20–4.57)
TP (mg/L)	0.067 (0.005–0.137)	0.098 (0.01–0.3)	0.12 (0.007–0.31)
TDP (mg/L)	0.023 (0.014–0.032)	0.029 (0.015–0.05)	0.034 (0.004–0.066)
NH_4-N (mg/L)	0.43 (0.042–1.03)	0.68 (0.042–2.16)	0.52 (0.068–1.27)
NO_2-N (mg/L)	0.012 (0.0001–0.050)	0.030 (0.001–0.12)	0.024 (0.004–0.110)
Temperature (°C)	18.9 (1.8–32)	19.0 (1.9–32)	19.0 (1.8–32)
Water depth (cm)	220 (175–275)	224 (190–280)	159 (131–180)
Transparency (cm)	114 (37–220)	50 (33–70)	46 (32–70)

[a] TN = total nitrogen; TDN = total dissolved nitrogen; TP = total phosphorous; TDP = total dissolved phosphorous.

RESULTS

Total nitrogen and TP concentrations and transparency of lake water were lower at site 1 than at sites 2 and 3 (Table 1). Correlation between TN and extracellular toxins was significantly negative ($r = -0.409$, $p < 0.05$) and TN and *Microcystis* biomass ($r = -0.408$, $p < 0.05$), and correlation was negative between TN and intracellular toxins, but not significant ($r = -0.303$, $p = 0.072$). The correlation between TP and *Microcystis* biomass and intracellular or extracellular toxins was not significant ($p > 0.05$; Table 2). Microscopic examinations on the intestine contents of *B. aeruginosa* showed many *Microcystis* cells in July, indicating massive ingestion of *Microcystis* by the snail.

Microcystins in various organs (hepatopancreas, intestines, gonad, foot, remaining tissue, and offspring) of *B. aeruginosa* were determined by LC-ESI-MS. On the basis of total ion chromatogram of LC-ESI-MS^2 measurement of MCs in the tissues of *B. aeruginosa*, mass chromatograms monitored at *m/z* 520, and the presence of $[M + H]^+$ ion at *m/z* 452 and 887, it is confirmed that the peak obtained at 5.81 min was derived from MC-RR. Similarly, peaks obtained at 11.38 and 11.72 min were derived from MC-YR and MC-LR, respectively, inasmuch as the peaks were detected by monitoring with *m/z* 1,045.5 and 995.5, respectively, and the mass chromatogram showed $[M + H]^+$ ion at *m/z* 1,045.5 and 599 for MC-YR and *m/z* 995.5 and 599 for MC-LR, respectively.

The MC content in snail tissues varied significantly. Hepatopancreas had the highest average content (2.33 μg/g), followed by intestine (1.56 μg/g), gonad (0.38 μg/g), remaining tissue (0.15 μg/g), and foot (0.10 μg/g). In terms of toxin burden, the majority of the toxins were present in the intestine (53.6%) and hepatopancreas (29.9%), whereas gonad, foot, and the remaining tissue contained only 16.5%. If intestines are excluded, up to 64.3% of the toxin burden was allocated in the hepatopancreas (Table 3). The mean MC content was significantly higher in hepatopancreas than in gonad (ANVOA, $p < 0.01$), but the variance of the MC content in hepatopancreas and intestine was not significant (ANVOA, $p = 0.108$). Detectable MCs also accumulated in the offspring, and there was a good positive correlation in MC content between the offspring and gonad ($r = 0.845$, $p < 0.01$; Table 4).

There were great seasonal variations in MC content in various tissues of the snail. The highest concentrations in the hepatopancreas (7.67 μg/g), intestine (5.31 μg/g), and gonad (1.96 μg/g) were all observed in July when *Microcystis* biomass in the lake water was the highest (Figs. 2 and 3). The biomass of *Microcystis* in the water column correlated significantly with the MC concentrations in the intestine ($r = 0.783$, $p < 0.01$), hepatopancreas ($r = 0.638$, $p < 0.01$), and gonad ($r = 0.775$, $p < 0.01$; Table 2).

Good positive correlation was demonstrated between MC content in the intestine and that in the hepatopancreas ($r = 0.789$, $p < 0.01$). A significant positive correlation was also found between MC concentration in the gonad and that in the intestine ($r = 0.858$, $p < 0.01$) or hepatopancreas ($r = 0.670$, $p < 0.01$). However, correlation in MC content between intestines and foot or remaining tissue was not significant ($p > 0.05$; Table 4). The extracellular or intracellular MC content in the water was significantly correlated with the MC content in most tissues (except foot and remaining tissue) or the offspring of *B. aeruginosa* ($r = 0.478$–0.783, $p < 0.05$; Table 2).

Table 2. Correlation analyses for the extracellular toxins, intracellular toxins, and *Microcystis* biomass versus the concentrations in different tissues and offspring of *Bellamya aeruginosa*[a]

	Extracellular toxins (μg/L)	Intracellular toxins (μg/L)	*Microcystic* biomass (mg/L)
Hepatopancreas	0.478**	0.638**	0.652**
Intestine	0.575**	0.783**	0.777**
Gonad	0.619**	0.775**	0.859**
Foot	0.254	−0.165	−0.131
Remaining tissue	0.248	0.002	0.130
Offspring	0.646*	0.684**	0.838**
TN[b]	−0.303	−0.293	−0.408*
TP[b]	0.160	0.150	−0.034

[a] Significant at the ** $p < 0.01$ and * $p < 0.05$ levels.
[b] TN = total nitrogen; TP = total phosphorous.

DISCUSSION

In this study, TN and extracellular toxins and TN and *Microcystis* biomass were significantly and negatively correlated;

Table 3. Dry weight of the different tissues of *Bellamya aeruginosa* as a percentage of total weight, microcystin (MC) content, and percentage of toxins present in the different tissues of the snail collected from Gonghu Bay of Lake Taihu, China, from November 2004 to October 2005

			Toxins (%)	
Tissue	Dry wt (%)	Mean MC (μg/g)	Including intestine	Excluding intestine
Intestine	23.06	1.56 (0.023–13.76)	53.6	
Hepatopancreas	8.58	2.33 (0.003–12.11)	29.9	64.30
Gonad	9.78	0.38 (0.008–4.3)	5.5	11.9
Foot	28.6	0.10 (0.003–0.86)	4.2	9.1
Remaining tissue	29.98	0.15 (0.004–1.07)	6.8	14.7

Table 4. Correlation between toxin concentrations in the tissues of *Bellamya aeruginosa*[a]

Tissue	Intestine	Hepatopancreas	Gonad
Hepatopancreas	0.789**		
Gonad	0.858**	0.670**	
Foot	0.020	0.069	0.099
Remaining tissue	0.205	0.132	0.283
Offspring	0.835**	0.829**	0.845**

[a] Significant at the ** $p < 0.01$ and * $p < 0.05$ levels.

no significant correlation was present between TP and intracellular toxins or *Microcystis* biomass, and negative, but not statistically significant, correlation was present between TN and intracellular toxins. These results are in disagreement with previous studies in other waterbodies [33,34], perhaps because of the strong effects of wind action that frequently accumulated dense cyanobacterial blooms in Gonghu Bay from other parts of the lake, which also might have weakened the correlation between extra- and intracellular toxins and major nutrients.

Previous studies have indicated that substantial MCs accumulate in the gonads of snails and mussels and in the eggs of shrimps [21,22,25]. We are the first, in this study, to report the presence of MCs in the offspring of adult snails. Toxin content in the gonad and in the offspring correlated well, suggesting that MCs were likely transfered from adult females to their young with physiological connection. Gérard et al. [35] reported that when the pond snail *Lymnaea stagnalis* was exposed to 33 μg/L MC-LR for a period of six weeks, survival, growth, and locomotion of the snail were not affected, but their fecundity decreased to half. Baganz et al. [36] reported that when zebrafish (*Danio rerio*) were exposed to 50 μg/g MC-LR, their spawning activity and success were reduced. Wang et al. [37] reported that microinjection of MC-LR into developing zebrafish embryos induced lethality of embryos in a dose- and time-dependent manner and caused embryo abnormalities. In Lake Taihu, the snail has a breeding season

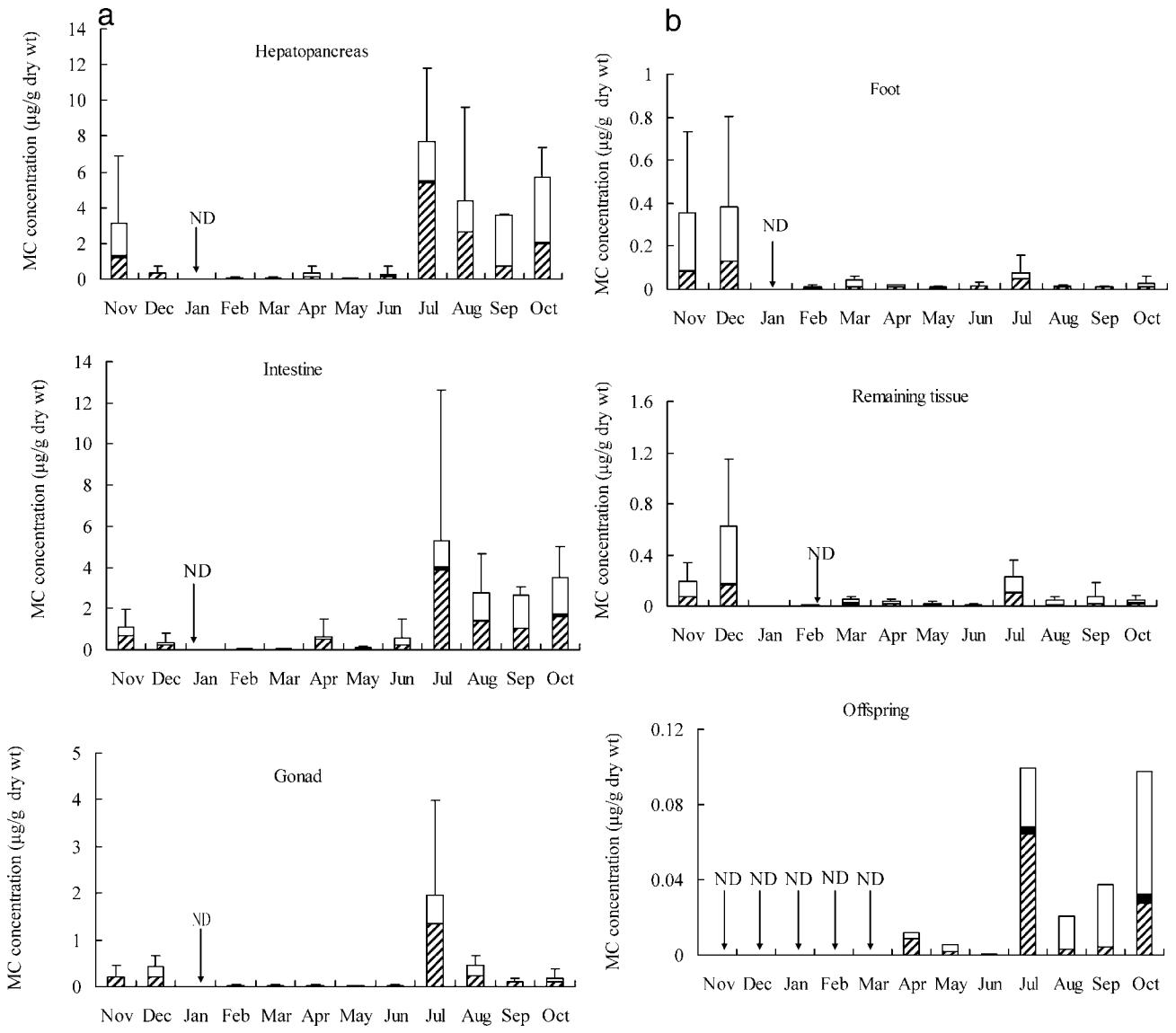

Fig. 2. The temporal changes of microcystin (MC)-LR, -RR, and -YR in the hepatopancreas, intestine, gonad, foot, remaining tissue, and offspring of *Bellamya aeruginosa* in Gonghu Bay, China, from November 2004 to October 2005. MC = microcystin; -LR = MC-LR; -RR = MC-RR, -YR = MC-YR; ND = not detectable. —□— -LR; —■— -YR; —▨— -RR.

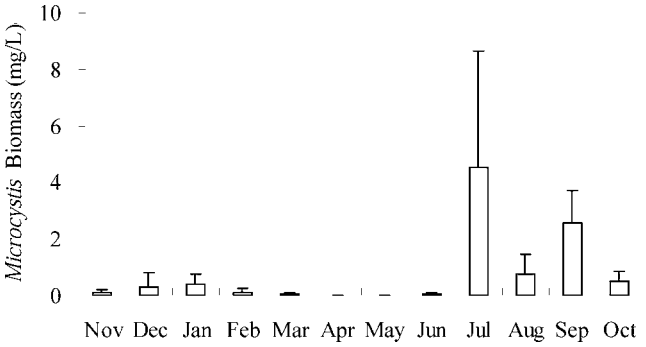

Fig. 3. The variation of the mean biomass of *Microcystis* (mg/L) at the three sites of Gonghu Bay, China, from November 2004 to October 2005.

coinciding with the period of the toxic cyanobacterial blooms; therefore, the effects of MCs on the survival and population dynamics of the snail need to be evaluated in future studies.

In this study, a significant positive correlation existed between the MCs in the intestine and those in the hepatopancreas or gonad. This indicates that the accumulation of MCs in the snails depends on the food resources. Similar results were also found for the same snail in Lake Chaohu of China and for four mussels in Lake Taihu [21,25]. However, MCs in the stomach were not correlated with the MCs in the hepatopancreas of two shrimps in Lake Chaohu [22]. This could be because of the differences of diet and food uptake between the stationary (muscles or snails) and mobile invertebrates (shrimps). It is likely that MC amounts in the hepatopancreas and gonad are generally more dependent on the ingested food in the digestive tracts of stationary invertebrates than of mobile invertebrates.

In this study, the MC content in the intestine, hepatopancreas, and gonad were correlated with the biomass of *Microcystis* and intracellular toxins in the lake water. Zurawell et al. [23] report that MC-LR content in the three snails (whole body excluding shell) were correlated with the toxin content in the phytoplankton of the water columns from seven Canadian lakes on the basis of log-log–transformed data. Yokoyama and Park [20] report that the MC content in the hepatopancreas of *Unio douglasiae* was linearly correlated with intracellular MCs in phytoplankton in the water column of in Lake Suwa. Similar correlation was also found for zooplankton [26]. These indicate that MC content in the tissues of mollusks depends on the toxin level in the food resource. In this study, the extracellular MCs were highly correlated with the MC concentration in the hepatopancreas, intestine, gonad. This is different from the results Zurawell et al. [23] and Yokoyama and Park [20] report for snails and mussels, respectively.

The World Health Organization (WHO) has determined a tolerable daily intake (TDI) of 0.04 μg/kg body weight per day for MC-LR (http://www.who.int/water_sanitation_health/resourcesquality/toxicyanbact/en/) [38]. It is necessary to estimate the critical amount (g wet weight) of the snails ingested to reach the TDI for MC. As is described by Gupta et al. [39], the intraperitoneal (i.p.) medium lethal dose (LD50) in mice for MC-RR and MC-YR is as about fivefold and 2.5-fold that for MC-LR, respectively, and the coefficients used to convert MC-RR and MC-YR into the MC-LR equivalent (MC-LReq) were 0.2 and 0.4, respectively. In this study, a coefficient of 3.75 was used to convert dry weight to wet weight of foot. If we assume a 60-kg person eats 100 g of snail feet per day, then 18.2% of the foot samples were above the tolerable daily intake recommended by WHO for human consumption. The maximum of MC-LReq in the foot samplings reached 0.674 μg/g dry weight, equal to a daily intake of 0.30 μg MC-LReq/kg body weight, which is 7.5 times higher than the TDI value suggested by WHO. People living around Lake Taihu regard the feet, or even the whole snail, as delicious food, so it is potentially dangerous for human health. These data indicate that health warnings for snail from Taihu Lake are warranted. Further studies regarding the relationship between water quality, *Microcystis* biomass, and toxin formation in Taihu Lake of locations outside of Gonghu Bay are needed. Because snails are an important food source for waterfowl, birds, and fish, the risk of MC transfer along the food chain should be evaluated in our future studies.

Acknowledgement—Sincere thanks are given to Zhou Qiong, Qiu Tong, and Wen Zhourui for their help in the fieldwork. This research was supported by a key project of CAS (grant KSCX2-SW-129) and a fund of the National Natural Science Foundation of China (grant 30530170).

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