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Biochemical and Ultrastructural Changes in the Hepatopancreas of *Bellamya aeruginosa* (Gastropoda) Fed with Toxic Cyanobacteria

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This study was conducted to investigate ultrastructural alterations and biochemical responses in the hepatopancreas of the freshwater snail *Bellamya aeruginosa* after exposure to two treatments: toxic cyanobacterium (*Microcystis aeruginosa*) and toxic cyanobacterial cells mixed with a non-toxic green alga (*Scendesmus quadricauda*) for a period of 15 days of intoxication, followed by a 15-day detoxification period. The toxic algal suspension induced a very pronounced increase of the activities of acid phosphatases, alkaline phosphatases and glutathione S-transferases (ACP, ALP and GST) in the liver at the later stage of intoxication. During the depuration, enzymatic activity tended to return to the levels close to those in the control. The activity of GST displayed the most pronounced response among different algal suspensions. Severe cytoplasmic vacuolization, condensation and deformation of nucleus, dilation and myeloid-like in mitochondria, disruption of rough endoplasmic reticulum, proliferation of lysosome, telolysosomes and apoptotic body were observed in the tissues. All cellular organelles began recovery after the snails were transferred to the *S. quadricauda.* The occurrence of a large amount of activated lysosomes and heterolysosomes and augment in activity of detoxification enzyme GST might be an adaptive mechanism to eliminate or lessen cell damage caused by hepatotoxicity to *B. aeruginosa*.

KEYWORDS: Microcystins, gastropods, *Microcystis aeruginosa*, hepatopancreas, ultrastructure, acid phosphatases, alkaline phosphatases, glutathione S-transferases

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1. INTRODUCTION

Eutrophication resulting from excessive anthropogenic activities is often accompanied with the occurrence of potential toxin producing cyanobacteria in freshwater ecosystems all over the world [1]. It is well known that some species of cyanobacteria, primarily *Microcystis*, can produce cyanotoxins, especially the hepatotoxic microcystins (MCs), which are one of the most dangerous toxin groups [2]. These toxins may cause poisoning or death of wild animals [3] and even pose a potential risk for human health via the consumption of contaminated aquatic products [4].

Gastropods are important primary consumers that inhabit shallow littoral areas of temperate lakes and ponds. Toxicological studies on gastropods have mainly focused on the accumulation, distribution, and depuration of MCs [5–10]. Little work has been done on the biochemical level in the freshwater snail although the effects of toxic cyanobacteria on the activity of enzymes have been demonstrated in mussels [11, 12]. Exploration of the impact of cyanobacterial toxins on life traits and histopathology has been limited so far [13, 14]. Furthermore, previous studies have mainly been on freshwater pulmonates, which are different from prosobranchs in their feeding habits, respiration mode, and life history strategies [15]. The freshwater snail *Bellamya aeruginosa* (Gastropods: Prosobranchia) is widely distributed in aquatic ecosystems in China. They are predominant herbivores that inhabit shallow littoral areas [16]. *B. aeruginosa* is a primary food resource for black carp (*Mylopharyngodon piceus*) and is also used for human consumption. Recent studies have shown that snail may have a potential for biological management of cyanobacterial bloom [17].

Previous studies suggested that the formation of MC glutathione conjugate mediated by glutathione S-transferases (GST) is the first step in the detoxification of MCs in a wide range of aquatic organisms [18]. The family of GST enzymes is responsible for the phase II biotransformation processes, binding electrophilic substances and oxidized compounds to glutathione (GSH) [19]. This enzyme complex has been shown to react in a quantitative way with cyanobacterial toxins [18]. Several researchers have investigated the relationship between GST and MCs in aquatic organisms [11, 12, 20–23]. The activity of acid phosphatase (ACP) is a marker enzyme for lysosomal membranes and alkaline phosphatase (ALP) as an apical membrane enzyme changed in response to MCs with pathological lesions in hepatic tissues of tilapia [24, 25].

The purposes of the present study are to examine, through a laboratory experiment, the temporal changes of MC concentration and activities of the enzymes GST, ACP, and ALP, in the hepatopancreas of *B. aeruginosa* after exposure to toxic cyanobacteria. We also examine the effects these changes have on the ultrastructure of the hepatopancreas. Further, we are assessing the toxic effects of MCs on snails with suggestions on the possible mechanisms of the resistant and detoxification capacities in freshwater prosobranch snails.

2. MATERIALS AND METHODS

2.1. Biological Materials

The strains of *M. aeruginosa* (FACHB-905, concentration of MCs: $29.47 \pm 0.43\,\mu g \cdot L^{-1}$) was provided by the Institute of Hydrobiology, Chinese Academy of Science. *M. aeruginosa* was maintained in BG-11 medium under constant temperature ($25 \pm 1^{\circ}C$) and photoperiod (12-h light: 12-h dark cycle) at an irradiance of $36\,\mu E\,m^{-2}\,s^{-1}$. The green alga *Scenedesmus quadricauda* was provided by the Aquatic Ecology Laboratory of Ningbo University. *S. quadricauda* maintained in NMB3# medium [26] under constant room temperature ($20 \pm 1^{\circ}C$) and natural irradiance.

B. aeruginosa individuals with an average shell length of 25 ± 1 mm were acquired from an unpolluted pond at the Ningbo University and washcleaned. The snails were acclimatized at a constant

temperature ($25 \pm 1^{\circ}$C) and photoperiod (12-h light: 12-h dark cycle) in dechlorinated tap water and fed with *S. quadricauda* for 7 days prior to the experiment.

2.2. Experimental Setup

Prior to the experiment, the snails were starved for 2 days and placed in a temperature-controlled incubator for 24 h in order to acclimate to the experimental conditions. During the experiment, snails were fed with *S. quadricauda*, *M. aeruginosa,* or both at an algal density of 10^6 cells mL^{-1}. The snails were divided into three groups: (1) snails exposed to *S. quadricauda* (control group), (2) snails exposed to the toxic *M. aeruginosa* (cyanobacterial group), and (3) snails exposed to 50% *M. aeruginosa* and 50% *S. quadricauda* (mixed group). Each group consisted of three replicates in nine glass containers with a dimension of 30 cm × 20 cm × 20 cm. 80 snails were placed into each glass container stocked with 10 L of algal suspension. All glass containers were placed in an incubator with a constant temperature ($25 \pm 1^{\circ}$C) and photoperiod (12-h light: 12-h dark cycle) at an irradiance of 36 μE m^{-2} s^{-1}. The algal suspension was restocked once a day, and the used algal suspension was removed. After 15 days of the intoxication, the cyanobacterial and the mixed groups were fed solely with *S. quadricauda* for 15 days during the depuration period.

2.3. Determination of MC Concentration in Snail Organs and Phytoplankton

At designated exposure time, some snails were sacrificed to collect biological tissues for Enzyme Liked Immunosorbent Assay (ELISA) analyses and ultrastructural studies. Hepatopancreases from each snail were removed and recorded for weight. Tissues were homogenized in 100% methanol using a blender (IKA T8, IKA Labortechnik, Stauffen, Germany). The methanol extract was mixed with equal volumes of hexane. The upper layer was discarded, and the obtained methanolic fraction was eluted in C-18 cartridge (Supelco Inc., Bellefonte, USA) and then washed and eluted with 10 mL of 20% methanol and 10 mL of 100% methanol. The methanolic fraction was dried and redissolved in 1.0 mL of the deionized water. This suspension was filtered in nylon filter (0.45 μm) and stored at -20°C for subsequent MCs analyses. All samples were analyzed by the immunoassay method using ELISA (ENVIROLOGIX INC.). 1.0 mL from each of the three groups' algal suspensions was sampled, frozen, and thawed three times, and then centrifuged (Sorvall Biofuge Primo R Centrifuge, Thermo Electron Corp., USA) at 10000 rpm. The supernatant was also analyzed for MCs using the ELISA also procedure. MCs concentrations were expressed as MC-LR equivalents.

2.4. Transmission Electron Microscopy

For the electron microscopic studies, fresh specimens of hepatopancreas were washed in a phosphate buffer solution (pH = 7.2) and then fixed by immersing them immediately in glutaraldehyde (2.5%) fixative at 4°C for 4 h and in osmium tetroxide (1%) for 1 h. Samples were washed in the phosphate buffer solution (0.1 mol L^{-1}) for 4 times and dehydrated through a graded series of ethanol. Specimens were embedded in Epon812-araldite mixture in labeled beam capsules. Semithin sections (90 nm) from the staining with uranyl acetate and lead citrate was obtained using an ultramicrotome (PowerTomeXL, RMC, Tucson, USA). Specimens were examined under a transmission electron microscopy (H-7650, Hitachi, Tokyo, Japan).

2.5. Biochemical Analyses

At designated exposure time, some snail was sacrificed to collect biological tissues. The liver samples were perfused with ice-cold NaCl (0.9%), and then homogenized in nine-fold volumes of ice-cold NaCl (0.9%). Sample was carried out in an ice bath and performed in an ultrasonic cell disintegrator for 1 min (JY96-II,

TABLE 1: MCs concentration in hepatopancreas of snails (*B. aeruginosa*) exposed to different treatments during the experiment. Results are expressed as mean ± SD.

Time (day)		Concentration of MC (μg g^{-1} DW)		F	P
		Cyanobacteria	Mixed group		
Intoxication	1	1.53 ± 0.36	0.67 ± 0.19	13.332	0.022
	5	2.62 ± 0.35	3.06 ± 1.55	0.223	0.661
	10	5.91 ± 0.85	5.78 ± 0.07	0.066	0.810
	15	4.24 ± 0.76	6.01 ± 0.77	7.899	0.048
Detoxification	16	3.17 ± 0.61	5.25 ± 0.33	27.055	0.007
	20	1.05 ± 0.06	1.43 ± 0.17	12.795	0.023
	25	0.48 ± 0.10	0.40 ± 0.01	1.938	0.236
	30	0.56 ± 0.13	0.38 ± 0.12	2.897	0.164

Xinzhi Biotechnology and Science Inc., Ningbo, China). After centrifugation in a centrifuge at 3000 rpm for 20 min at 4°C, the resulting supernatant was separated for biochemical assay. The supernatant was assayed for ALP, ACP, and GST activities using colorimetric assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), following the manufacturer specifications.

2.6. Statistical Analysis

We used multivariate analysis of principal response curve (PRC) [27] to test temporal changes in the activities of enzymes caused by exposure to different algal suspensions as compared with those in the control (exposed to *S. quadricauda*) and also to quantify the contribution of each enzyme to separate the treatments from the control. For each sampling time, differences between treatments and control were analyzed via one-way analyses of variance (ANOVA) followed by a post hoc multiple comparisons test (Dunnett's test). The MC accumulation in liver after MCs exposure was assessed by ANOVA. Results are presented as the mean ± SD. Differences were considered significant at level $P < 0.05$. Analysis was undertaken using SPSS 13.0 for Windows.

3. RESULTS

3.1. Changes of MCs Concentrations in Hepatopancreas

The changes in MCs concentrations in hepatopancreas over the 15-day intoxication period and 15-day detoxification period were shown in Table 1. MCs concentration in the algal suspensions were 18.67 ± 2.80 μg L^{-1} in the mixed group, 37.93 ± 13.85 μg L^{-1} in the cyanobacterial group (t-test, $P < 0.05$), and undetectable in the control group. MC in the hepatopancreas was detected in both experimental groups after 24 h of feeding. Unlike the cyanobacterial group, the highest concentration of MC in the mixed group was observed at the final day of the intoxication experiment (day 15). One-way ANOVA showed that the concentration of MC differed significantly between the cyanobacterial group and the mixed group at day 1 and 15 (Table 1). There were also significant differences in MC content between the cyanobacterial group and the mixed group in the early stage of detoxification (day 16 and 20) (Table 1).

TABLE 2: The activities of enzymes ACP, ALP, and GST levels in the hepatopancreas of snails (*B. aeruginosa*) during the experiment. Results are expressed as mean ± SD. Treatments with the same superscript letter are not significantly different (Dunnett's test, $P > 0.05$).

Time (day)	Enzyme	The activities of enzymes ($U \cdot gprot^{-1}$)			F	P
		Control group	Mixed group	Cyanobacteria		
1	ACP	124.0 ± 19.2	109.6 ± 14.2	118.1 ± 3.4	0.805	0.49
5		202.4 ± 29.5^{a}	528.9 ± 59.7^{b}	276.3 ± 28.4^{a}	50.335	<0.001
10		151.9 ± 12.0^{a}	331.8 ± 39.9^{b}	210.4 ± 27.7^{c}	30.327	0.001
15		154.7 ± 40.5^{a}	383.1 ± 87.7^{b}	616.4 ± 51.7^{c}	39.989	<0.001
20		276.7 ± 29.6^{a}	322.2 ± 6.7^{ab}	458.4 ± 126.0^{b}	4.791	0.57
25		352.3 ± 42.7	274.2 ± 40.2	424.6 ± 124.4	2.692	0.146
30		258.9 ± 46.4	192.8 ± 29.0	230.71 ± 41.5	2.099	0.204
1	ALP	320.9 ± 33.1	446.6 ± 86.8	414.3 ± 141.5	1.339	0.331
5		496.7 ± 83.8	552.3 ± 108.9	400.6 ± 28.5	2.691	0.147
10		145.7 ± 37.7	176.3 ± 30.9	173.2 ± 15.9	0.971	0.431
15		124.9 ± 30.3^{a}	464.4 ± 55.8^{b}	167.3 ± 40.8^{a}	54.096	<0.001
20		333.3 ± 21.6^{a}	177.9 ± 21.2^{b}	135.6 ± 31.6^{b}	50.951	<0.001
25		184.5 ± 24.0	135.1 ± 51.4	105.3 ± 22.8	3.857	0.084
30		142.1 ± 52.2	86.3 ± 19.2	115.6 ± 12.8	2.156	0.197
1	GST	67.6 ± 15.8	58.8 ± 4.1	69.8 ± 7.5	0.942	0.441
5		70.9 ± 10.3^{a}	99.0 ± 14.8^{b}	64.9 ± 5.8^{a}	8.348	0.018
10		35.4 ± 0.9^{a}	135.4 ± 32.0^{b}	30.7 ± 5.8^{a}	29.683	0.001
15		38.8 ± 4.1^{a}	227.5 ± 58.0^{b}	44.1 ± 13.3^{a}	29.25	0.001
20		86.4 ± 4.9^{a}	99.2 ± 8.7^{ab}	120.4 ± 23.1^{b}	4.21	0.072
25		73.1 ± 4.0^{a}	97.3 ± 22.7^{ab}	107.9 ± 2.7^{b}	5.328	0.047
30		27.0 ± 0.6^{a}	28.4 ± 3.3^{a}	39.03 ± 2.8^{b}	20.357	0.002

3.2. Activities of Enzymes over Time after the Exposure to Different Algal Suspensions

The principal response curve (PRC) showed that the activities of enzymes changed due to levels of MC, allowing us to differentiate the experimental treatments from the control treatment over seven sampling dates. The Monte Carlo permutation test (499 permutations) showed that there was a significant influence of different treatments throughout the 30-day experimental period (F ratio $= 16.18$, $P = 0.002$), explaining 24.1% of total data variability. In this case, the first canonical axis accounts for 63.2% of total variability.

The activities of enzymes were stimulated, a behavior observed in both treatments during the intoxication period (Figure 1). Dunnett's test showed that the exposure to toxic cyanobacteria produced the dynamic of enzymatic activity in treatments that were different from that in the control during the peak of accumulation of MC in the hepatopancreas (10, 15 days) (Table 2). During the 15-day intoxication, PRC and Dunnett's test showed that enzymatic activity tended to return to levels close to those in the control. However, after 25 days, effects of the exposure to the cyanobacteria were still detectable in the GST activities in the treatments (Table 2). Treatments that exposed to toxic cyanobacteria (mixed and cyanobacteria group) showed different levels of ALP and ACP activity from the control in the initial part of the detoxification period (Table 2). However, at the end of the experiment (25, 30 days), the activities of ALP and ACP in both treatments did not differ from that in the control treatment.

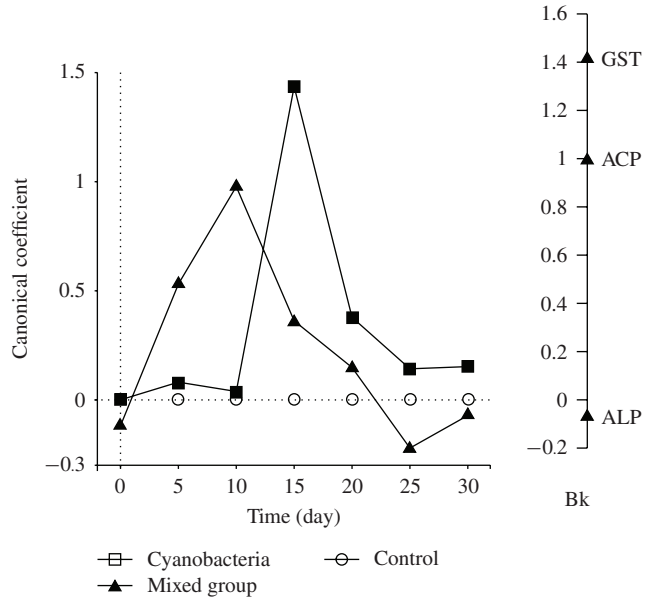

FIGURE 1: Principal response curve (PRC) of enzyme activity in the snails under different treatments (control, mixed group, cyanobacteria group) at seven sampling events (0, 5, 10, 15, 20, 25, and 30 days after the beginning of the experiment). Bk indicates the weight of biochemical parameters measured: glutathione S-transferases (GST), acid phosphatase (ACP), and alkaline phosphatase (ALP).

In Figure 1, the weight values (Bk) represent the weight of each enzyme in the PRC, which helps data interpretation (enzymes with Bk values farther from zero are the ones most affected by changes observed among treatments over time). Thus, the activity of GST, which showed the highest Bk values in the module, was that most influenced by different algal suspensions. Meanwhile, ALP, and ACP showed lower values and are consequently less affected by the MCs.

3.3. Ultrastructural Observation

Transmission electron microscopy of hepatopancreas from all the control group showed distinct cell junction (Figure 2(a)), a round nucleus with a prominent central nucleolus (Figure 3(a)), rough ER arranged in parallel layers (Figure 5(a)) or in association with mitochondria with dense matrices (Figure 4(a)), and several lipid droplets and glycogen granules (Figure 2(d)). Lysosomes and vacuoles could only rarely be observed. After 5 days, widening of intercellular spaces was first noticed (Figure 2(b)). Membrane blebbing occurred with a conspicuous separation of hepatocytes shrunk in the treatment group during the detoxification (Figure 2(c)). Morphologic alterations in nuclei became most prominent in both experimental groups. The toxic algae induced a progressive deformation of the nuclear outline (Figure 3). After 5 days, there was a prominent decrease in the amount of homochromatic with compaction of heterochromatin in the cyanobacterial group (Figure 3(e)), eventually resulting in the disappearing of nucleolus after 10 days (Figure 3(f)). However, in the mixed group, nuclei were condensed after 5 days (Figure 3(b)), the nucleolus could no longer be discerned in some hepatocytes after 15 days (Figure 3(c)). In addition, some binucleate cells were observed (Figure 3(d)). Nuclei have recovered to the level of control in the mixed group after 15 days of detoxification (Figure 3(g)). Nucleolus could be discerned at the end of the experiment in the cyanobacterial group (Figure 3(h)) although the shape of hepatocytes was still not round enough. In the mixed group, mitochondria did not change evidently but temporarily increased within 5-day postexposure

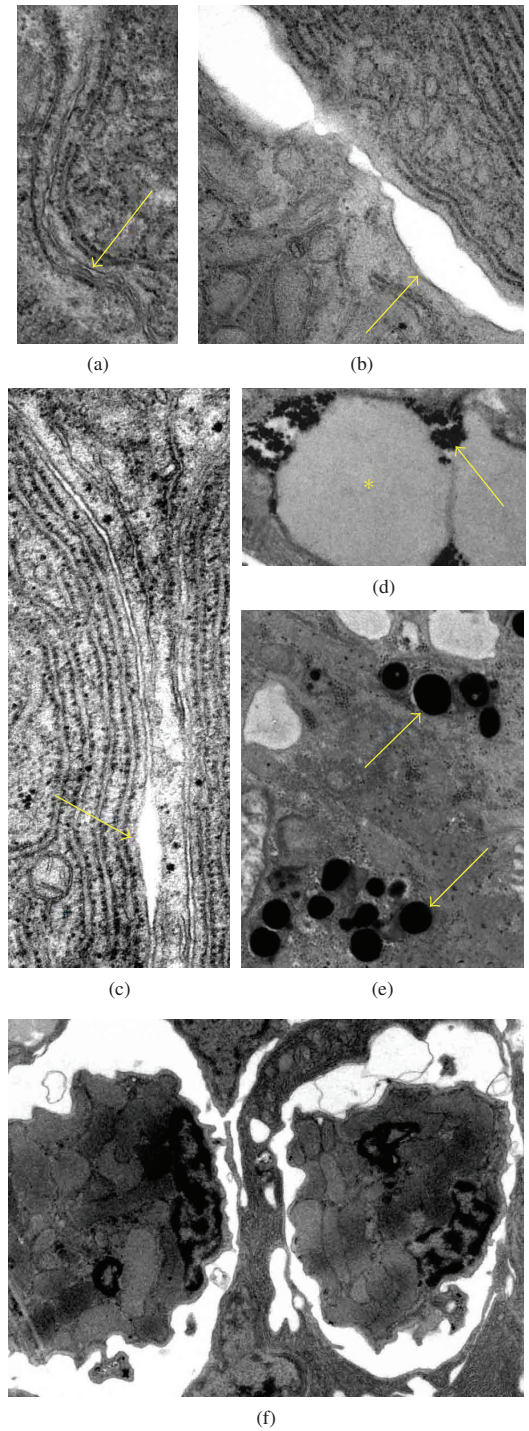

FIGURE 2: Ultrastructural changes in the hepatopancreas of *B. aeruginosa* exposed to the suspension of *M. aeruginosa* and mixed algae, respectively. (a) Cellular junctions of the control group (yellow arrow), 60000x. (b) The dilation of intercellular space in the cyanobacterial group at 10 days postexposure (yellow arrow), 60000x. (c) The recovery hepatocyte in the mixed group after 10 days of detoxification (yellow arrow), 60000x. (d) Lipids (yellow star) and glycogen (yellow arrow) of control snail, 50000x. (e) The presence of lipofuscins in the cyanobacterial group after the detoxification (yellow arrow), 15000x. (f) The presence of apoptotic body in the cyanobacterial group after the intoxication, 15000x.

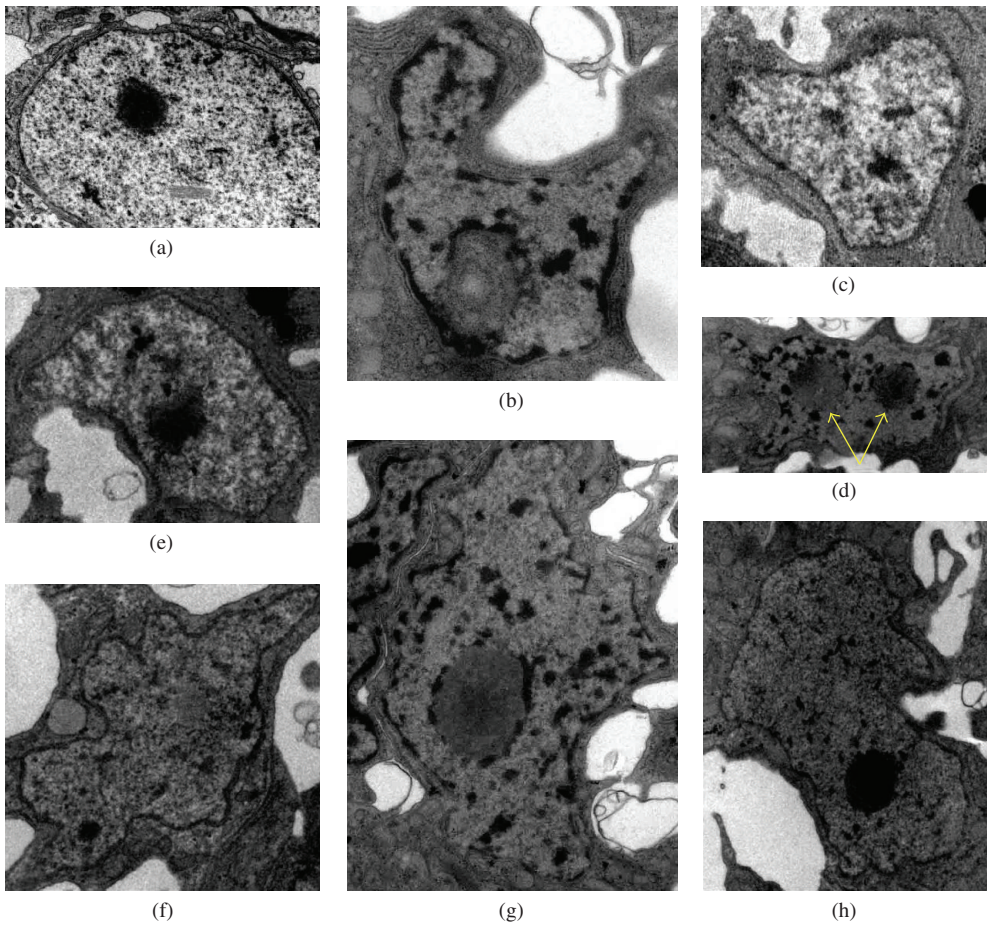

FIGURE 3: Ultrastructural changes in the hepatopancreas of *B. aeruginosa* exposed to the suspension of *M. aeruginosa* and mixed algae, respectively. (a) Nucleus of control snail, 12000x. (b) and (c) The deformation and fading of nucleus in the mixed group at 5 days and 15 days postexposure, respectively, 30000x and 20000x. (d) The binucleate cell (yellow arrow), 12000x. (e) The condensation of chromatin in the cyanobacterial group at 5 days postexposure, 20000x. (f) The fading of nucleus in the cyanobacterial group at 10 days postexposure, 20000x. (g) The recovery of nucleus in the mixed group after 15 days of detoxification, 22000x. (h) The recovery of nucleus at the end of the experiment in the cyanobacterial group, 16000x.

by rough estimation of the number, and, only at 10 day, the densely stained mitochondria presented dilated cristae (Figure 4(b)). However, in the cyanobacterial group, the mitochondria proceeded to lose cristae and matrix (Figure 4(c)), and the induction of myelinated structures within the matrix was found after 15 days of intoxication (Figure 4(e)). After 15 days of depuration, most mitochondria showed considerable recovery with only slightly swollen forms present in both treatment groups (Figures 4(d) and 4(f)). In the mixed group, modification of rough endoplasmic reticulum (rER) was significant since whirling of rER at the periphery of plasma membrane was present at day 10 (Figure 5(d)), while, at day 15, hepatocytes displayed fragmentation of the rER (Figure 5(c)), partial or total loss of ribosomes of rER was observed (Figure 5(b)). However, these were observed after 5 days after exposure in the cyanobacterial group. rER showed substantial recovery in both experiment groups during the detoxification (Figure 5(e)). The snail treated with mixed algae showed the proliferation of lysosomes and heterolysosomes after day 5 (Figure 6(a)). However, in the cyanobacterial group, the cytoplasm was highly vacuolized as well as increasing of heterolysosomes (Figure 6(b)). During the depuration, hepatocytes displayed proliferation of lysosomes as the time went on

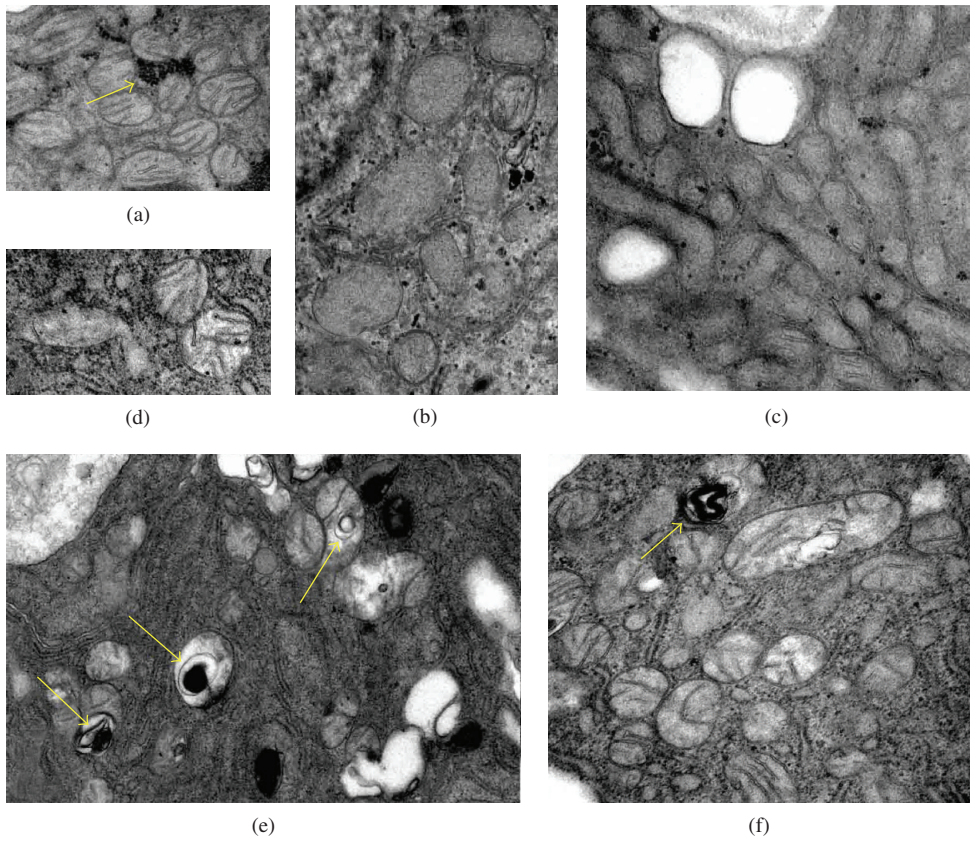

FIGURE 4: Ultrastructural changes in the hepatopancreas of *B. aeruginosa* exposed to the suspension of *M. aeruginosa* and mixed algae, respectively. (a) Mitochondria and glycogen (yellow arrow) of control snail, 50000x. (b) The swelling and vesiculation of mitochondria in the mixed group at 10 days postexposure, 50000x. (c) The loss of cristae and matrix in mitochondria in the cyanobacterial group at 15 days postexposure, 50000x. (d) Most mitochondria recovered considerably at the end of the experiment in the mixed group, 50000x. (e) The occurrence of myelinated structures (yellow arrow) in matrix of mitochondria in the cyanobacterial group at 15 days postexposure, 32000x. (f) The recovery of mitochondria and myelinated structures (yellow arrow) at the end of the experiment in the cyanobacterial group, 32000x.

(Figure 6(c)). A large amount of telolysosomes was shown in both experimental groups (Figures 6(d) and 6(e)).

4. DISCUSSION

During the intoxication period, MC accumulated in the hepatopancreas rapidly over time, with the highest concentration of 6.01 μg g^{-1} DW, indicating strong enrichment of MC in the snail's hepatopancreas. These results agree with the findings of the previous laboratory studies on the MC accumulation in freshwater snails [6, 8, 9]. However, in the present study, the MC level in the mixed group was higher than that in the cyanobacterial group. A possible explanation for these results is that the MCs that could not be detoxified were covalently bound to protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) in the cyanobacterial group, which is considered as the toxic mechanism of MC [28]. After penetration in the cytoplasm of the cell, MCs can be excreted after conjugation with glutathione [18] or interact with the catalytic subunit of Ppases such as PP1 and PP2A via a reversible or a covalent binding [28]. Significant increase in GST activity in the liver of snails in mixed group during intoxication period could result in

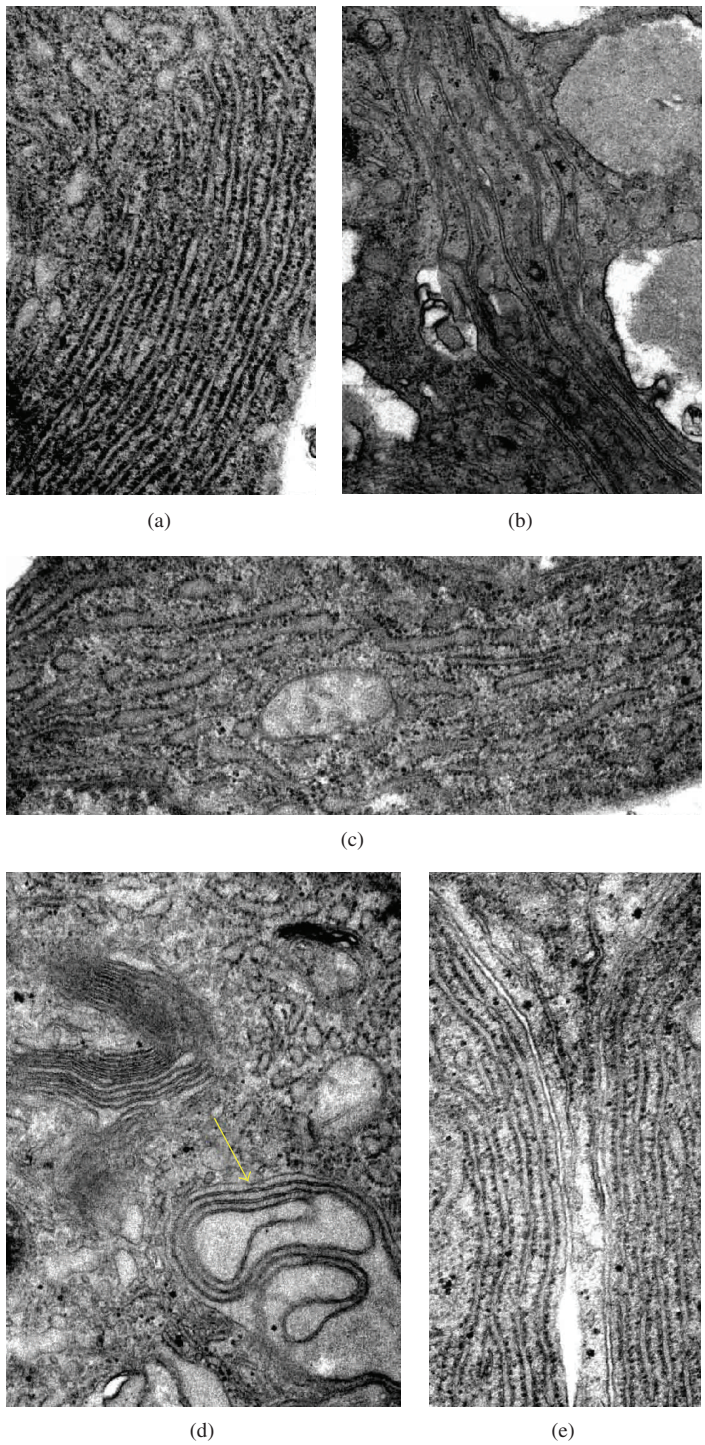

FIGURE 5: Ultrastructural changes in the hepatopancreas of *B. aeruginosa* exposed to the suspension of *M. aeruginosa* and mixed algae, respectively. (a) rER of control snail, 60000x. (b) The swelling and degranulation of rER in the mixed group at 15 days postexposure, 40000x. (c) The fragmentation of rER in the mixed group at 15 days postexposure, 40000x. (d) The whirling of rER (yellow arrow) in the mixed group at 10 days postexposure, 40000x. (e) The recovery of rER in the cyanobacterial group after 10 days of detoxification, 50000x.

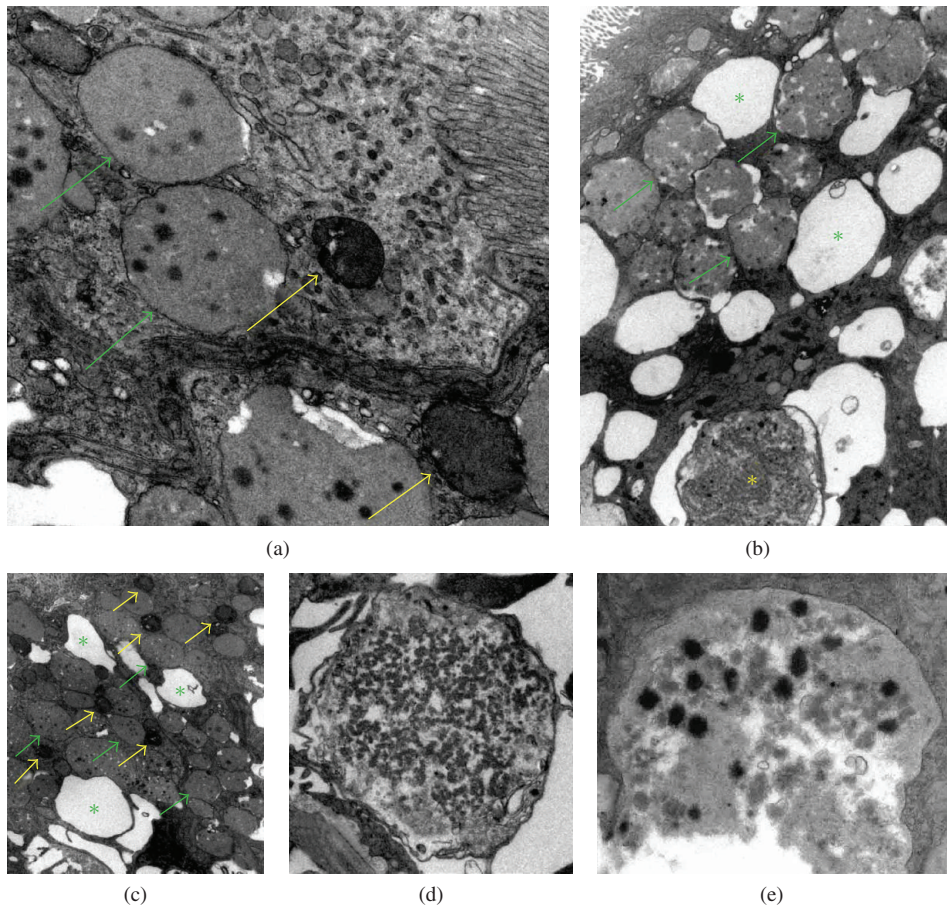

FIGURE 6: Ultrastructural changes in the hepatopancreas of *B. aeruginosa* exposed to the suspension of *M. aeruginosa* and mixed algae, respectively. (a) The proliferation of lysosomes (yellow arrow) and heterolysosomes (green arrow) in the mixed group at 5 days postexposure, 40000x. (b) The vacuoles (green star), telolysosomes (yellow star), and heterolysosomes (green arrow) in the cyanobacterial group at 10 days postexposure, 18000x. (c) A large amount of lysosomes (yellow arrow), heterolysosomes (green arrow), and the vacuoles (green star) in the cyanobacterial group during the depuration, 8000x. (d) and (e) The telolysosome in the mixed and cyanobacterial group during the depuration, respectively, 25000x.

enhanced biotransformation reactions and higher rates of MCs conjugation [23]. The ELISA method cannot detect the covalent MCs [10] although they may detect MCs metabolites such as MC-glutathione conjugates [29]. In addition, a large amount of pseudofaeces rich in mucus, which is energetically expensive to produce [30], was observed in the cyanobacterial treatment. Hence, the energy of detoxification was no longer available, and the energy balance can thus be altered by the stressful effect of MCs, as shown by Juhel et al. [31] for the zebra mussel exposed to toxic *M. aeruginosa.* It is a likely explanation for why there were no significant differences between the control and cyanobacterial group in the GST activity.

During the depuration period, the GST activity increased in cyanobacterial group, attaining higher values than those observed in control. This is because of the fact that the toxic algae were no longer present in ambient. MC concentrations in the hepatopancreas decreased quickly, indicating that *B. aeruginosa* possessed high depuration efficiency. Because the elimination of toxins requires high energy inputs [32], we speculate that the nontoxic *S. quadricauda* might have supplied energy to the snails and that could be used in the protection of the snails from tissue damage. Therefore, the group that also fed with a nontoxic diet had less pathological damage, which is evident from electron microscopic examinations.

The hepatopancreas of molluscs is a large digestive gland which is involved in several functions including the extracellular and intracellular digestion of food, storage of lipids, glycogen, and minerals; it is also the main site of nutrient absorption and plays a major role in detoxification [33]. The lysosomes of mollusc hepatopancreas may suffer morphological and functional changes caused by pollutants and may be valuable as bioindicators of environmental pollution [34]. The lysosomal proliferation observed in the present study is in accordance with the results obtained from fish were exposed to toxic *Microcystis* [22, 25], and this lysosome activation could be an adaptative mechanism to eliminate or reduce cell damage caused by MC. Likewise, a large amount of heterolysosomes and telolysosomes, which are also called secondary lysosomes and residual bodies, respectively, in both experimental groups, indicated that the cells secreted actively. Among the various specialised compartments that comprise the endolysosomal system that the digestive cells of molluscs possess [35], heterolysosomes, which occupy the majority of the mid-cytoplasmic portion in digestive cells, are reactive for marker hydrolases such as ACP [36]. In our case, ACP showed a similar response pattern of GST and was induced during the peak of accumulation of MC in the hepatopancreas (Table 2). ALP is basically a membrane-bound enzyme, and any perturbation in the membrane properties caused by interaction with MCs could alter the ALP activity. This result is in agreement with previous studies reporting ALP changes due to the accumulation of MC in the liver of exposed tilapia [24, 25]. As previously observed in the prawn hepatopancreas, because of exposure to environmental stressors, MCs increase ACP activity by interacting with lysosomes [37]. This agrees with the ultrastructural changes obtained in our study and also with previous in vitro investigations carried out on fish cell lines (RTG-2 and PHLC-1), which found a very potent concentration-dependent stimulation of the lysosomal function with MC-LR [38]. This may be related to the cytoskeletal modifications and the induction of oxidative stress that have been pointed out by several authors [39]. Ultrastructural observation showed that at the beginning of the depuration stage, all organelles were in recovery; lysosomes were hyperactive and fused with endocytic vesicles to form heterolysosomes. The material in the heterolysosomes increased, and phagocytosis took place. Braunbeck et al. [40] investigated the effects of microcystin on fish. He observed lysosome hyperplasia and thought that it was the compensation mechanism to renew the cellular component under the pressure of microcystin. Li et al. [22] observed the occurrence of a large amount of activated heterolysosomes, which might be an adaptive mechanism to eliminate or lessen cell damage caused by MCs through lysosome activation. When heterolysosomes no longer contain any usable substances, they turn into telolysosomes. In present study, the number of heterolysosome and telolysosome in depuration progress was greater than that in the accumulation phase. Our findings suggest that during the depuration process, the snails had a strong capacity for digestion and self-renewal that might be an adaptation mechanism to toxic depuration.

At the beginning of exposure, all cell organelles could still protect the snails from the environmental toxins. It is noteworthy that one of the early changes was the increase of amount in mitochondria, but other cell organelles did not appear to be affected. The increase in mitochondria indicated that *B. aeruginosa* tried to save energy in order to process toxins [39]. Mitochondria are one of the production sites for oxygen-free radicals. When the structure and function are affected, the reduction capacity of superoxide dismutase is also impaired, leading to the increases in oxygen-free radicals and the occurrence in apoptosis and cell injury [41]. Ding and Ong [39] reported that the induction of free radical formation and mitochondrial alterations were two major events found in microcystin-treated cultured rat hepatocytes. The mitochondrial alterations, that is, loss of mitochondrial membrane potential and mitochondria permeability transition are recognized as key steps in apoptosis [39]. However, when the ribosomes fell off from the ER, protein transport immediately ceased. The lysosome vesicles first increased, and then the small vesicles were integrated into large vesicles. After exposure to MC, vacuoles and myelin-like residual bodies were observed in the hepatocytes, indicating that the snail was clearing apoptotic bodies, and the process of apoptosis took place. With the accumulation of toxins, cellular organelles were damaged, resulting in pathological changes and impaired detoxification ability. The pathological damages in the cyanobacterial group were more severe than those in the mixed group.

In this study, first nucleoli were pyknosised, and chromatin was condensed. Some studies have reported that the genetic material is the target molecule of microcystin [42]. Microcystin could change the structure and function of the genetic material and therefore is genotoxic [43]. Since microcystin affects genetic material, great attention should be taken when snails are used for human consumption. Triebskorn [44] reported that the changes in nucleus did not necessarily cause body death but could result in cell death. When overloaded, the nuclear envelope disintegrated, and nucleus was broken. The injured hepatocytes could recover after the diet of the experimental animals was replaced with a nontoxic one, which is demonstrated in our depuration experiment.

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REFERENCES

[1] K. E. Havens, R. T. James, T. L. East, and V. H. Smith, "N:P ratios, light limitation, and cyanobacterial dominance in a subtropical lake impacted by non-point source nutrient pollution," *Environmental Pollution*, vol. 122, no. 3, pp. 379–390, 2003.

[2] W. W. Carmichael, "The cyanotoxins," in *Advances in Botanical Research*, J. A. Callow, Ed., pp. 211–256, Academic Press, London, UK, 1997.

[3] I. Stewart, A. A. Seawright, and G. R. Shaw, "Cyanobacterial poisoning in livestock, wild mammals and birds–an overview," in *Advances in Experimental Medicine and Biology*, H. K. Hudnell, Ed., pp. 613–618, Springer Press, New York, NY, USA, 2008.

[4] J. Chen, P. Xie, L. Li, and J. Xu, "First identification of the hepatotoxic microcystins in the serum of a chronically exposed human population together with indication of hepatocellular damage," *Toxicological Sciences*, vol. 108, no. 1, pp. 81–89, 2009.

[5] R. W. Zurawell, B. G. Kotak, and E. E. Prepas, "Influence of lake trophic status on the occurrence of microcystin-LR in the tissue of pulmonate snails," *Freshwater Biology*, vol. 42, no. 4, pp. 707–718, 1999.

[6] K. Ozawa, A. Yokoyama, K. Ishikawa, M. Kumagai, M. F. Watanabe, and H. D. Park, "Accumulation and depuration of microcystin produced by the cyanobacterium Microcystis in a freshwater snail," *Limnology*, vol. 4, no. 3, pp. 131–138, 2003.

[7] J. Chen, P. Xie, L. Guo, L. Zheng, and L. Ni, "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," *Environmental Pollution*, vol. 134, no. 3, pp. 423–430, 2005.

[8] E. Lance, L. Brient, M. Bormans, and C. Gérard, "Interactions between cyanobacteria and Gastropods. I. Ingestion of toxic *Planktothrix agardhii* by *Lymnaea stagnalis* and the kinetics of microcystin bioaccumulation and detoxification," *Aquatic Toxicology*, vol. 79, no. 2, pp. 140–148, 2006.

[9] E. Lance, E. Bugajny, M. Bormans, and C. Gérard, "Consumption of toxic cyanobacteria by *Potamopyrgus antipodarum* (Gastropoda, Prosobranchia) and consequences on life traits and microcystin accumulation," *Harmful Algae*, vol. 7, no. 4, pp. 464–472, 2008.

[10] E. Lance, M. R. Neffling, C. Gérard, J. Meriluoto, and M. Bormans, "Accumulation of free and covalently bound microcystins in tissues of *Lymnaea stagnalis* (Gastropoda) following toxic cyanobacteria or dissolved microcystin-LR exposure," *Environmental Pollution*, vol. 158, no. 3, pp. 674–680, 2010.

[11] V. M. Vasconcelos, C. Wiegand, and S. Pflugmacher, "Dynamics of glutathione-S-transferases in *Mytilus galloprovincialis* exposed to toxic *Microcystis aeruginosa* cells, extracts and pure toxins," *Toxicon*, vol. 50, no. 6, pp. 740–745, 2007.

[12] S. Fernandes, M. Welker, and V. M. Vasconcelos, "Changes in the GST activity of the mussel *Mytilus galloprovincialis* during exposure and depuration of microcystins," *Journal of Experimental Zoology Part A*, vol. 311, no. 3, pp. 226–230, 2009.

[13] C. Gérard, L. Brient, and B. Le Rouzic, "Variation in the response of juvenile and adult gastropods (*Lymnaea stagnalis*) to cyanobacterial toxin (microcystin-LR)," *Environmental Toxicology*, vol. 20, no. 6, pp. 592–596, 2005.

[14] E. Lance, C. Paty, M. Bormans, L. Brient, and C. Gérard, "Interactions between cyanobacteria and gastropods. II. Impact of toxic *Planktothrix agardhii* on the life-history traits of *Lymnaea stagnalis*," *Aquatic Toxicology*, vol. 81, no. 4, pp. 389–396, 2007.

[15] C. Gérard, V. Poullain, E. Lance, A. Acou, L. Brient, and A. Carpentier, "Influence of toxic cyanobacteria on community structure and microcystin accumulation of freshwater molluscs," *Environmental Pollution*, vol. 157, no. 2, pp. 609–617, 2009.

[16] C. Y. Chen, Y. L. Liang, K. P. Sung, and S. T. Wang, "On ecological distributions and population densities of mollusca in lake tunghu, Wuchang," *Acta Hydrobiologica Sinica*, vol. 5, pp. 371–379, 1975.

[17] S. Han, S. Yan, K. Chen et al., "15N isotope fractionation in an aquatic food chain: *Bellamya aeruginosa* (Reeve) as an algal control agent," *Journal of Environmental Sciences*, vol. 22, no. 2, pp. 242–247, 2010.

[18] S. Pflugmacher, C. Wiegand, A. L. Oberemm et al., "Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication," *Biochimica et Biophysica Acta*, vol. 1425, pp. 527–533, 1998.

[19] W. H. Habig, M. J. Pabst, and W. B. Jakoby, "Glutathione S transferases. The first enzymatic step in mercapturic acid formation," *Journal of Biological Chemistry*, vol. 249, no. 22, pp. 7130–7139, 1974.

[20] X. Li, Y. Liu, L. Song, and J. Liu, "Responses of antioxidant systems in the hepatocytes of common carp (*Cyprinus carpio* L.) to the toxicity of microcystin-LR," *Toxicon*, vol. 42, no. 1, pp. 85–89, 2003.

[21] T. Qiu, P. Xie, Z. Ke, L. Li, and L. Guo, "In situ studies on physiological and biochemical responses of four fishes with different trophic levels to toxic cyanobacterial blooms in a large Chinese lake," *Toxicon*, vol. 50, no. 3, pp. 365–376, 2007.

[22] L. Li, P. Xie, S. Li, T. Qiu, and L. Guo, "Sequential ultrastructural and biochemical changes induced in vivo by the hepatotoxic microcystins in liver of the phytoplanktivorous silver carp *Hypophthalmichthys molitrix*," *Comparative Biochemistry and Physiology C*, vol. 146, no. 3, pp. 357–367, 2007.

[23] L. Li, P. Xie, and L. Guo, "Antioxidant response in liver of the phytoplanktivorous bighead carp (*Aristichthys nobilis*) intraperitoneally-injected with extracted microcystins," *Fish Physiology and Biochemistry*, vol. 36, no. 2, pp. 165–172, 2010.

[24] R. Molina, I. Moreno, S. Pichardo et al., "Acid and alkaline phosphatase activities and pathological changes induced in Tilapia fish (*Oreochromis sp.*) exposed subchronically to microcystins from toxic cyanobacterial blooms under laboratory conditions," *Toxicon*, vol. 46, no. 7, pp. 725–735, 2005.

[25] L. Atencio, I. Moreno, A. I. Prieto, R. Moyano, A. M. Molina, and A. M. Cameań, "Acute effects of microcystins MC-LR and MC-RR on acid and alkaline phosphatase activities and pathological changes in intraperitoneally exposed Tilapia fish (*Oreochromis sp.*)," *Toxicologic Pathology*, vol. 36, pp. 449–458, 2008.

[26] C. X. Zhou, B. Ma, F. X. Wang, B. Xu, and X. J. Yan, "The population growth and variation of nitrate-N and phosphate-P in the mix-culture of *Phaeodactylum tricornutum* Bohl. and *Prorocentrum micans*," *Marine Sciences*, vol. 30, pp. 58–61, 2006.

[27] P. J. Van Den Brink and C. J. F. Ter Braak, "Principal response curves: analysis of time-dependent multivariate responses of biological community to stress," *Environmental Toxicology and Chemistry*, vol. 18, no. 2, pp. 138–148, 1999.

[28] J. T. Maynes, H. A. Luu, M. M. Cherney et al., "Crystal structures of protein phosphatase-1 bound to motuporin and dihydromicrocystin-LA: elucidation of the mechanism of enzyme inhibition by cyanobacterial toxins," *Journal of Molecular Biology*, vol. 356, no. 1, pp. 111–120, 2006.

[29] J. S. Metcalf, K. A. Beattie, S. Pflugmacher, and G. A. Codd, "Immuno-crossreactivity and toxicity assessment of conjugation products of the cyanobacterial toxin, microcystin-LR," *FEMS Microbiology Letters*, vol. 189, no. 2, pp. 155–158, 2000.

[30] M. S. Davies and S. J. Hawkins, "Mucus from marine molluscs," *Advances in Marine Biology*, no. 34, pp. 1–71, 1998.

[31] G. Juhel, J. Davenport, J. O'Halloran et al., "Pseudodiarrhoea in zebra mussels *Dreissena polymorpha* (Pallas) exposed to microcystins," *Journal of Experimental Biology*, vol. 209, no. 5, pp. 810–816, 2006.

[32] C. Wiegand, S. Pflugmacher, A. Oberemm et al., "Uptake and effects of microcystin-LR on detoxication enzymes of early life stages of the zebra fish (*Danio rerio*)," *Environmental Toxicology*, vol. 14, no. 1, pp. 89–95, 1999.

[33] A. Beeby and L. Richmond, "Calcium metabolism in two populations of the snail *Helix aspersa* on a high lead diet," *Archives of Environmental Contamination and Toxicology*, vol. 17, pp. 507–511, 1988.

[34] G. P. Domouhtsidou and V. K. Dimitriadis, "Lysosomal and lipid alterations in the digestive gland of mussels, *Mytilus galloprovincialis* (L.) as biomarkers of environmental stress," *Environmental Pollution*, vol. 115, no. 1, pp. 123–137, 2001.

[35] I. Marigómez and L. Baybay-Villacorta, "Pollutant-specific and general lysosomal responses in digestive cells of mussels exposed to model organic chemicals," *Aquatic Toxicology*, vol. 64, no. 3, pp. 235–257, 2003.

[36] Y. Robledo, I. Marigómez, E. Angulo, and M. P. Cajaraville, "Glycosylation and sorting pathways of lysosomal enzymes in mussel digestive cells," *Cell and Tissue Research*, vol. 324, no. 2, pp. 319–333, 2006.

[37] P. S. Bhavan and P. Geraldine, "Profiles of acid and alkaline phosphatases in the prawn *Macrobrachium malcolmsonii* exposed to endosulfan," *Journal of Environmental Biology*, vol. 25, no. 2, pp. 213–219, 2004.

[38] S. Pichardo, A. Jos, J. L. Zurita, M. Salguero, A. M. Camean, and G. Repetto, "The use of the fish cell lines RTG-2 and PLHC-1 to compare the toxic effects produced by microcystins LR and RR," *Toxicology in Vitro*, vol. 19, no. 7, pp. 865–873, 2005.

[39] W. X. Ding and C. N. Ong, "Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity," *FEMS Microbiology Letters*, vol. 220, no. 1, pp. 1–7, 2003.

[40] T. Braunbeck, D. E. Hinto, and B. Streit, "Cytological alterations in fish hepatocytes following in vivo and in vitro sublethal exposure to xenobiotics-structural biomarkers of environmental contamination," in *Fish Ecotoxicology*, pp. 61–141, Birkhäuser, Berlin, Germany, 1998.

[41] I. V. Klyubin, K. M. Kirpichnikova, A. M. Ischenko, A. V. Zhakhov, and I. A. Gamaley, "The role of reactive oxygen species in membrane potential changes in macrophages and astrocytes," *Membrane and Cell Biology*, vol. 13, no. 4, pp. 557–566, 2000.

[42] W. X. Ding, H. M. Shen, H. G. Zhu, B. L. Lee, and C. N. Ong, "Genotoxicity of microcystic cyanobacteria extract of a water source in China," *Mutation Research—Genetic Toxicology and Environmental Mutagenesis*, vol. 442, no. 2, pp. 69–77, 1999.

[43] B. Žegura, B. Sedmak, and M. Filipič, "Microcystin-LR induces oxidative DNA damage in human hepatoma cell line HepG2," *Toxicon*, vol. 41, no. 1, pp. 41–48, 2003.

[44] R. Triebskorn, "Ultrastructural changes in the digestive tract of *Deroceras reticulatum* (Müller) induced by a carbamate mollusicide and by metaldehyde," *Malacologia*, vol. 31, pp. 141–156, 1989.

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