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Microcystin uptake and biochemical responses in the freshwater clam *Corbicula leana* P. exposed to toxic and non-toxic *Microcystis aeruginosa*: Evidence of tolerance to cyanotoxins

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ABSTRACT

We investigated the accumulation and adverse effects of toxic and non-toxic *Microcystis* in the edible clam *Corbicula leana*. Treated clams were exposed to toxic *Microcystis* at 100 μg of MC (microcystin)-LR_{eq} L^{-1} for 10 days. The experimental organism was then placed in toxin-free water and fed on non-toxic *Microcystis* for the following 10 days for depuration. Filtering rates (FRs) by *C. leana* of toxic and non-toxic *Microcystis* and of the green alga *Chlorella vulgaris* as a control were estimated. Adverse effects were evaluated though the activity of catalase (CAT), superoxide dismutase (SOD) and glutathione S-transferase (GST). Clam accumulated MCs (up to 12.7 ± 2.5 μg g^{-1} dry weight (DW) of free MC and 4.2 ± 0.6 μg g^{-1} DW of covalently bound MC). Our results suggest that although both toxic and non-toxic cyanobacteria caused adverse effects by inducing the detoxification and antioxidant defense system, the clam was quite resistant to cyanotoxins. The estimated MC concentration in *C. leana* was far beyond the World Health Organization's (WHO) provisional tolerable daily intake (0.04 μg kg^{-1} day^{-1}), suggesting that consuming clams harvested during cyanobacterial blooms carries a high health risk.

1. Introduction

Cyanobacterial blooms (CYBs) are a growing major problem in freshwater ecosystems. These blooms have attracted increasing public health concern because 70% of them are toxic [1]. The most widespread cyanotoxins belong to a family of cyclic heptapeptide hepatotoxins named microcystins (MCs). They are produced mainly by strains of the genera *Anabaena*, *Microcystis*, *Planktothrix*, and occasionally *Nostoc* [2].

Numerous laboratory and field studies have reported that MCs are efficiently accumulated by various aquatic

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organisms, including zooplankton, gastropods, bivalves, crustaceans and fish, and even by aquatic vertebrates such as turtles, ducks, and waterbirds [3–5]. MCs are present in diverse organs and also in muscle tissues and other edible parts [6–8]. Consuming water or food contaminated with MCs poses a risk to human health [3]. To minimize this risk, the World Health Organization (WHO) recommends a guideline value of 1 $\mu g\,L^{-1}$ of total MC-LR in drinking water and a chronic tolerable daily intake (TDI) of 0.04 $\mu g\,kg^{-1}$ body mass per day for human consumption [2].

MCs are potent and specific inhibitors of the serine threonine of protein phosphatases (PPs) PP1 and PP2A; they form a covalent linkage between the MC-N-methyldehydroalanine residue and the phosphatase's cysteine residue [9]. This inhibition leads to hyperphosphorylation of regulated proteins and disruption of numerous cellular processes, causing severe cell damage [10]. However, the toxicological effects of MCs are diverse and are understood to only a limited extent [11]. Due to the covalent linkage, studies of MCs in animal tissues have been limited to the quantification of free MC content [5]. The majority of MCs are likely covalently bound to target proteins in tissues but have not been quantified in these assessments [12]. These covalently bound MC (Co-MC) may be made bioavailable in the digestive systems of aquatic consumers through digestion of the attached PPs [13–15]. To assess the health implications, it is important to determine not only the free MC content but also the content of MC covalently bound to the PPs in animal tissues.

Both toxic cyanobacteria and MCs have pronounced negative effects on aquatic animals, including zooplankton, fish, gastropods, and bivalves [16]. In particular, oxidative stress produced by reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) may play important roles in the pathogenesis of MC toxicity [16,17]. Both ROS and MCs are removed by the activity of antioxidant and biotransformation enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) [18,19]. In addition, some freshwater bivalves have high levels of MC tolerance that may be associated with the responses of biotransformation enzymes [20]. One of the mechanisms of detoxification of MCs involves GST, which belongs to the phase II detoxification enzyme family and catalyses the conjugation of MCs with glutathione (GSH). The formation of MC–GSH conjugates reduces toxicity and accelerates the excretion of MCs [21]. These mechanisms are responsible for the defences of mussels against bioaccumulation and for their insensitivity to natural toxins [18].

The edible Asian freshwater clam *Corbicula leana* P. is an important component of freshwater macrobiota and is commonly found in eutrophic habitats [22]. It is often steamed and eaten in many countries, especially in southeast Asia [23]. During toxic CYBs, it probably accumulates MCs in its body and thus transfers it to higher trophic levels through the food chain [24]. To our knowledge, no information demonstrating toxins accumulation and elimination patterns in this species is available.

Our aims were to investigate the accumulation and elimination of free and Co-MC in the freshwater clam *C. leana* in laboratory experiments. We used an oral route in which *C. leana* was exposed to toxic *Microcystis* cells containing 100 $\mu g\,L^{-1}$ of MC-LR_{eq} for 10 days and then to non-toxic *Microcystis* cells for another 10 days, via feeding. Moreover, we also performed a filtering rate (FR) experiment to test the hypothesis that whether *C. leana* filtered toxic and non-toxic *Microcystis aeruginosa* and the green alga *C. vulgaris* at different rates. The MC content in the whole body of the clam and in the incubation water (i.e., intracellular MCs) was monitored. The adverse effects were evaluated by monitoring the activity of CAT, SOD, and GST. Finally, evidences for the clam's resistance to cyanotoxins were also discussed.

2. Materials and methods

2.1. Culture of toxic and non-toxic Microcystis and of green alga

The toxic cyanobacterium *M. aeruginosa* (strain NIES-1086), non-toxic *M. aeruginosa* (strain NIES-101), and the green alga *Chlorella vulgaris* (strain NIES-2170) were ordered from the National Institute for Environmental Studies (NIES) collection (Tsukuba, Ibaraki, Japan). The toxic strain NIES-1086 produced MCs at a concentration of 15.21 $fg\,cell^{-1}$, as determined by high-performance liquid chromatography (HPLC). Toxic strain was cultured in MA medium and non-toxic strain in CB medium [25,26]. The green alga was grown in soil extract medium (SEM) [27]. All cultures were maintained at 24 °C under a 12:12 light:dark photoperiod with white fluorescent lights at a photosynthetic photon flux density (PPFD) of 30 $\mu mol\,m^{-2}\,s^{-1}$. When the culture had reached the stationary phase, cells were harvested by centrifugation at 4200 × *g* at 4 °C for 30 min (Avanti HP-26XP, Beckman Coulter, Brea, CA, USA). Both cyanobacteria and green algae were cultured as single cells.

2.2. Collection and maintenance of clam

Freshwater clam *C. leana* (Fig. 1) was collected at a freshwater fisheries experimental station in Oita Prefecture, Japan, and transported alive to the laboratory. The clams were introduced into 50-L aquatic aquariums containing dechlorinated tap water and a 5-cm sand layer as a substrate, with sufficient aeration. Before the experiments, clams were kept at a density of below 100 individuals/50 L and acclimatized for 1 month at a PPFD of 20 μmol photons $m^{-2}\,s^{-1}$ under a 12:12 light:dark photoperiod. The water temperature was 22 ± 1 °C, the pH 7.5 ± 0.3, and the dissolved oxygen concentration 7.9 ± 0.6 $mg\,L^{-1}$. The incubation water was totally renewed every 3 days. The clams were fed daily with *C. vulgaris* at a concentration of 2×10^3 $cell\,mL^{-1}$. The wet weight of the clams was 5.14 ± 0.72 g and the shell length was 2.12 ± 0.53 cm.

2.3. Filtering rate

Nine clams of the same shell size were gently scrubbed under tap water and allocated randomly to three groups of three. They were then incubated individually in 500-mL beakers with 300 mL of dechlorinated tap water, with

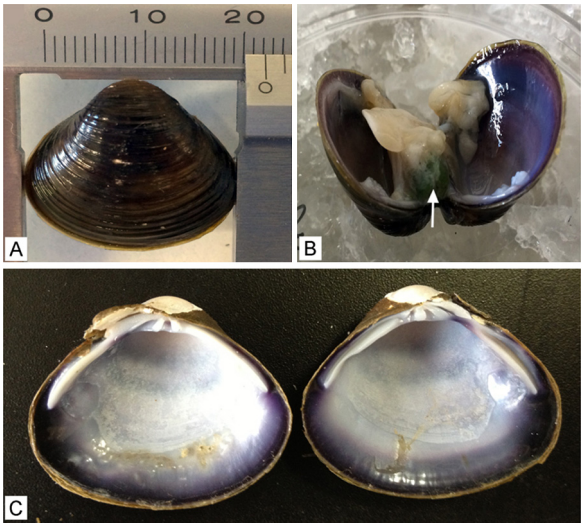

Fig. 1. Photos of the clam *Corbicula leana*. (A) Whole external shell; (B) soft tissues, arrow indicates cyanobacterial cells accumulated in soft tissues; and (C) inner shell-side.

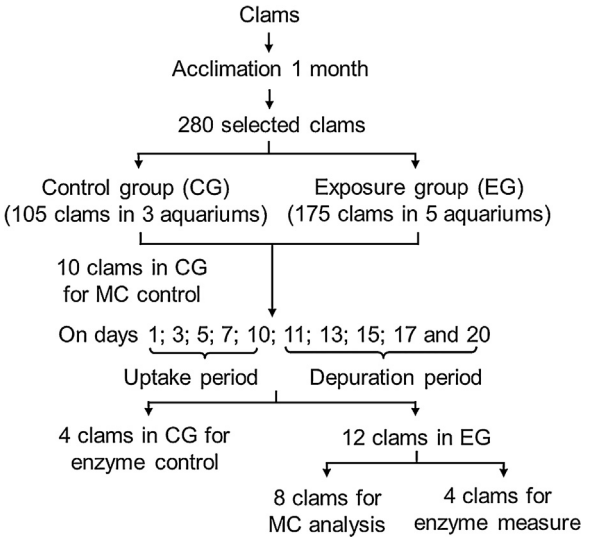

Fig. 2. Experimental design of the feeding experiment.

constant aeration at a temperature of $22 \pm 1\,^{\circ}C$. Beakers with phytoplankton but no clams were used as controls. After 12 h of acclimation with no food, when each individual had its valves open, toxic *M. aeruginosa*, non-toxic *M. aeruginosa*, or *C. vulgaris* was added to the water to a final concentration of 2×10^6 cells mL^{-1}. This density is often found in water bodies [28,29]. Water samples were taken at 1-h intervals for 6 h. Cell density was estimated directly by using a Speirs-Levy Eosinophil counting slide under an Olympus light microscope, as described by Andersen [30]. FR, defined as the volume of water (in mL) cleared with suspended particles per unit time (h) by each individual, was calculated according to the method of Coughlan [31].

2.4. Feeding experiment

After acclimation, clams were placed in eight aquariums (35 clams in each) containing 2 L of dechlorinated tap water and a 2-cm sand layer as a substrate, with constant aeration. The clams were allocated randomly to an exposure group (175 clams) and a control group (105 clams).

For MC uptake, toxic *M. aeruginosa* cells were added to each aquarium to a final concentration of 100 μg MC-LR_{eq} L^{-1} on days 0, 3, 5, and 7 of the uptake period, which lasted for 10 days. This MC concentration is often found in the natural environment during blooms [2,28]. The clams were then collected and relocated into aquariums containing dechlorinated tap water as a toxin-free water. They were kept in these aquariums for another 10 days as a depuration period. During the depuration, the toxic *M. aeruginosa* were completely replaced by non-toxic *M. aeruginosa* at a concentration of 6×10^6 cells mL^{-1}. The non-toxic *Microcystis* cells were completely renewed on days 0, 3, 5, and 7 of the depuration period, which corresponded to days 11, 13, 15, 17 and 20 of the experiment. The water was also completely replaced at the same times. The control group was incubated in dechlorinated tap water and fed with *C. vulgaris* at a concentration of 6×10^6 cells mL^{-1} at the same times with the exposure group. Dead clams were removed and counted daily.

On days 1, 3, 5, 7, and 10 (in the uptake period) and days 11, 13, 15, 17, and 20 (in the depuration period) we sampled 12 clams from assigned aquarium. For MC quantification eight clams were rinsed gently under dechlorinated tap water. The shell was removed immediately and the remaining tissues were freeze-dried completely and kept at $-30\,^{\circ}C$ until MC extraction. Ten clams sampled before the start of the experiment were used as controls. To measure enzyme activity, in both groups we first dissected the gills, foot, and mantle of four clams (pooled) and the remaining tissues (kept individually) on ice. The samples were immediately frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ until enzyme extraction. The feeding experimental setup is presented in Fig. 2.

2.5. Extraction and analysis of MCs in incubation water

MCs (intracellular content) in the incubation water were monitored every day during uptake and on days 11, 13, 15, 17, and 20 in the depuration period. The incubation water (10–100 mL) was filtered through Whatman glass-fiber filter (GF/C). The filters were then dried completely at $45\,^{\circ}C$ and kept at $-30\,^{\circ}C$. MC extraction and analysis were performed by using a reversed-phase HPLC as described by Pham et al. [32].

2.6. Extraction and analysis of free MC in clams

Free MC was extracted as previously reported by Xie and Park [33], with minor modifications. Briefly, freeze-dried tissues (about 100–150 mg) were homogenized in 3 mL of BuOH:MeOH:H_2O (5:20:75, v/v/v) by using a homogenizer (Polytron, Kinematica AG, Luzern, Switzerland) and extracted three times with 5 mL of the same solution, each for 24 h with shaking in darkness. After sonication for 1 min (Taitec Ultrasonic, Saitama, Japan), the samples were centrifuged at $2000 \times g$ at $4\,^{\circ}C$ for 30 min. The supernatants

were then combined, evaporated to 10 mL, diluted three times with ultrapure water, and applied to an Oasis HLB cartridge (60 mg, Waters Corp., Milford, MA, USA) that had been preconditioned with 3 mL MeOH 100% and 10 mL ultrapure water. The column was first washed with 3 mL MeOH 20% and then eluted with 3 mL MeOH 100%. This eluate was evaporated to dryness under reduced pressure at below 40 °C. The MC fraction was suspended in 500 μL MeOH 100% and then kept at −20 °C before reversed-phase HPLC analysis as described above. Duplicate samples with duplicate analysis (yielded 4 measurements, $n = 4$) were used in this determination.

2.7. Extraction and analysis of total MC in clams

Total MC (free and Co-MC) was extracted as previously reported by Neffling et al. [34], with minor modifications. Briefly, freeze-dried tissues were homogenized and trypsinated with 3 mL of 500 μg mL^{-1} trypsin in Sorensen's phosphate buffer (pH 7.5) at 37 °C for 3 h. This was followed by oxidation with 0.1 M $KMnO_4$ and 0.1 M $NaIO_3$ (pH 9.0) for 3 h at room temperature. The reaction was quenched with sodium bisulphite solution (40%, w/v) until colorless at pH 2 with 10% sulphuric acid. After centrifugation (2000 × *g*, 4 °C, 30 min) of the sample, the supernatant was collected and diluted five times with ultrapure water. It was then applied to an Oasis HLB cartridge (60 mg; Waters Corp.) that had been preconditioned with 3 mL MeOH 100% and 10 mL ultrapure water. The column was first washed with 3 mL MeOH 20%, and then the 2-methyl-3-methoxy-4-phenylbutanoic acid (MMPB) fraction, which is the product of MC oxidation, was eluted with 3 mL MeOH 80%. This eluate was evaporated to dryness and then re-dissolved in 500 μL MeOH 100%. MMPB was converted to its methyl ester (me) by using a 10% BF_3–methanol kit (Sigma-Aldrich, Tokyo, Japan). The samples were then dissolved in *n*-hexane and kept at −30 °C before gas chromatography–mass spectrometry (GC–MS) analysis. The Co-MC content was estimated by subtracting the free MC content from the total content. 4-Phenylbutylric acid (4-PB) (Sigma-Aldrich, Tokyo, Japan) was used as an internal standard [35]. MMPB-d_3 and MC-LR purchased from Wako (Osaka, Japan) were used as external standards.

2.8. GC–MS analysis

GC–MS analysis was performed on a GCMS-QP2010 Plus mass spectrometer linked to a GC-2010 gas chromatography system (Shimadzu, Kyoto, Japan) equipped with an Rxi-5ms column (30 m × 0.25 mm ID, phase thickness 0.25 mm; Restek, Bellefonte, PA, USA). Helium was used as the carrier gas, at a flow rate of 1.5 mL min^{-1} in splitless mode. The program used for the analysis was 80 °C for 1 min, followed by an increase to 280 °C at 8 °C min^{-1}. The other conditions were as follows: ion source temperature 200 °C, injection port temperature 230 °C, detector temperature 250 °C and interface temperature 280 °C. Methyl 4-PB (me4-PB) and meMMPB were detected by using selected-ion monitoring (SIM) mode. Ions at 91 and 104 *m*/*z* were selected for me4-PB, and those at 75, 78, 91, 131, and 134 *m*/*z* were selected for meMMPB [36]. GC–MS solution software was used for the quantitative calculation. Duplicate samples with duplicate analysis (yielded 4 measurements, $n = 4$) were used in this determination.

2.9. Enzyme extraction and measurement

Enzymes were extracted as previously reported by Wiegand et al. [37], with minor modifications. Briefly, samples (gills, foot, mantle, and remaining soft tissues) were homogenized in ice-cool 0.1 M sodium phosphate buffer (pH 6.5) (1:5, w/v) containing 20% (v/v) glycerol, 1 mM ethylenediaminetetraacetic acid, and 1.4 mM dithioerythritol. The homogenate was centrifuged at 10,000 × *g* at 4 °C for 15 min to eliminate cell debris, and the resulting supernatant was used for enzyme measurements. GST (EC 2.5.1.18), SOD (EC 1.15.1.1), and CAT (EC 1.11.1.6) activity was detected with GST, SOD, and CAT assay kits (Cayman, Ann Arbor, MI, USA) at wavelengths of 340, 460, and 540 nm, respectively, by using a Fluoroskan Ascent fluorometer (Thermo Electron Corp., Milford, MA, USA). All enzyme activities were calculated in terms of protein content, as measured with a protein assay kit purchased from Bio-Rad Laboratories (Hercules, CA, USA). Each enzymatic assay was performed in triplicate.

2.10. Statistical analyses

Data on CAT, SOD, GST, and MCs are presented as means ± SD. Differences between the exposure and control groups were tested for significance by using one-way analysis of variance (ANOVA). When the ANOVAs were significant, we used pairwise comparison by Tukey's honest significant difference (HSD) post hoc test to detect significant differences between the exposure concentrations and the control. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Filtering rate

In the control beakers the mean cell concentrations of all strains were almost constant over the course of the experiment. The FR of 2×10^6 cells mL^{-1} of toxic *Microcystis* by *C. leana* was 16.0 ± 3.3 mL g^{-1} wet weight (WW) h^{-1}; that of non-toxic *Microcystis* was 15.3 ± 3.2 mL g^{-1} WW h^{-1}, and that of *C. vulgaris* was 7.0 ± 1.1 mL g^{-1} WW h^{-1} (Fig. 3).

One-way ANOVA showed that the *Microcystis* FRs were significantly higher than that of green alga. There was no significant difference in FRs between the toxic and non-toxic *Microcystis*. We observed that larger amounts of pseudo-feces were expelled in the beakers containing the toxic or non-toxic *Microcystis* than in the *C. vulgaris* beakers.

3.2. MC concentrations in incubation water

Concentrations of MCs in the control incubation water were under the detection limit (data not shown).

At the starting and renewal points, the concentration of MCs in the incubation water was 105 ± 4.7 μg $MC\text{-}LR_{eq}$ L^{-1}. The MC concentration in the incubation water slowly and

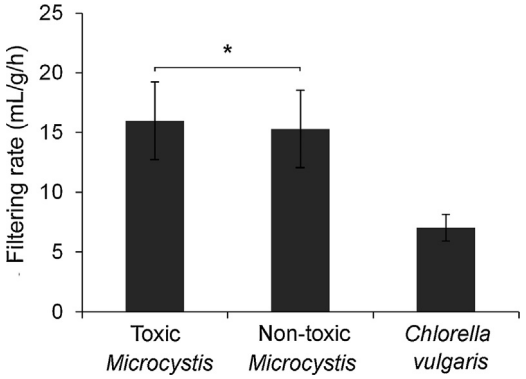

Fig. 3. Mean (±SD) filtering rates (FRs) [in mL g^{-1} wet weight (WW) h^{-1} over 6 h] after feeding of *Corbicula leana* with toxic *Microcystis* (NIES-1086), non-toxic *Microcystis* (NIES-101), or the green alga *Chlorella vulgaris* (NIES-2170). Asterisk indicates significant difference ($P < 0.05$).

continuously declined after each renewal. After 1 day the MC concentration had decreased to $96 \pm 3.8\ \mu g\ L^{-1}$, after 2 days to $89 \pm 4.1\ \mu g\ L^{-1}$, and after 3 days to $83 \pm 1.5\ \mu g\ L^{-1}$ (Fig. 4).

3.3. Uptake and depuration of free and Co-MC

There were no deaths in the exposure or control groups during the experiments. The control samples contained no detectable MCs (data not shown), whereas exposed clams showed substantially enhanced toxin accumulation. We monitored the changes in MC accumulation by the clams during the uptake and depuration periods (Fig. 4). Typically, free MC concentrations rose rapidly after the start of exposure and peaked (at $12.7 \pm 2.5\ \mu g\ g^{-1}$ dry weight (DW) after about 3 days. The free MC content was relatively constant at the different time points during the exposure period. In contrast, the Co-MC concentration slowly increased during the uptake period, peaking at $4.2 \pm 0.6\ \mu g\ g^{-1}$ DW on day 10. Free MC was quickly eliminated from the clam tissues in the depuration period and declined from 12.3 ± 1.4 to $5.7 \pm 0.3\ \mu g\ L^{-1}$ after 24 h (day 11), then to $1.3 \pm 0.3\ \mu g\ L^{-1}$ after 72 h (day 13). It was under the detection limit after 7 days of depuration, which corresponded to day 17 of the experiment. In contrast, Co-MC level was almost unchanged during the first 5 days of depuration. They then gradually declined but were still detectable at the end of the depuration period (Fig. 4).

3.4. Biotransformation enzyme

We examined the effects of toxic and non-toxic *Microcystis* on GST activity in different organs in the exposure and control groups (Fig. 5). Inhibition of GST activity in the gills was observed after exposure of the clams to toxic *Microcystis* for 5 days, and there was a significant difference compared with the controls after 7 days and at the end of the experiment. A decrease in GST activity was also observed on days 10 and 15 in the foot and days 10 and 20 in the remaining tissues. In contrast, in the mantle, GST activity was significantly higher than in the controls on day 11, but there were no significant differences at other time points.

3.5. Antioxidant enzyme activity

We examined the effects of toxic and non-toxic cyanobacteria on SOD activity in the different clam tissues (Fig. 6). In the gills, SOD activity was significantly higher in the exposed clams than in the controls on days 5 and 10 (exposure period) and on days 11 and 15 (depuration period). There was also a significant elevation of SOD activity on days 1 and 5 in the foot and on days 10 and 17 in the mantle. However, SOD activity in the gills, foot, and mantle eventually returned to control levels at the end of the depuration, exposure to non-toxic *Microcystis*. In the remaining tissues, SOD activity did not differ significantly from that in the controls, with the exception of a significant inhibition at the end of the experiment (day 20).

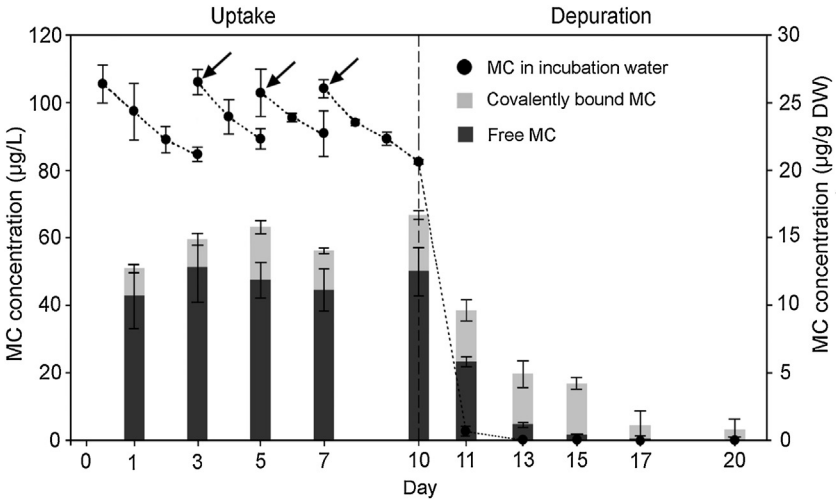

Fig. 4. Concentrations of microcystins (MCs) in incubation water, and of free MC and covalently bound MC (Co-MC) accumulated in clams during the uptake and depuration periods. Arrows indicate points at which the MC concentration and the water were renewed during the uptake period.

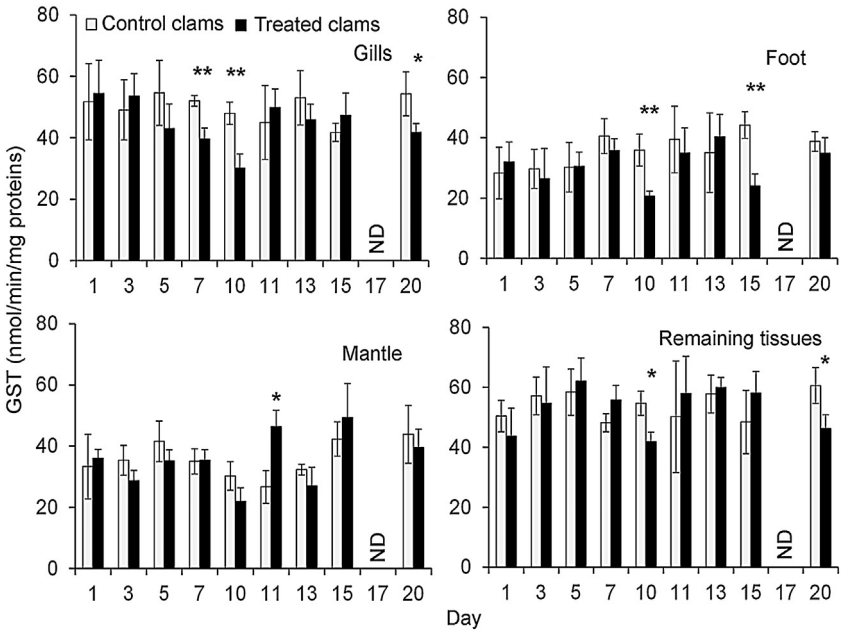

Fig. 5. Changes in glutathione S-transferase (GST) activity (nmol min^{-1} mg^{-1} protein) in various tissues of clams fed with toxic *Microcystis* (days 0–10) or non-toxic *Microcystis* (days 11–20). Asterisks indicate significant differences compared with controls (fed with *Chlorella vulgaris*) at the respective time points (*$P < 0.05$, **$P < 0.01$). ND, not detected.

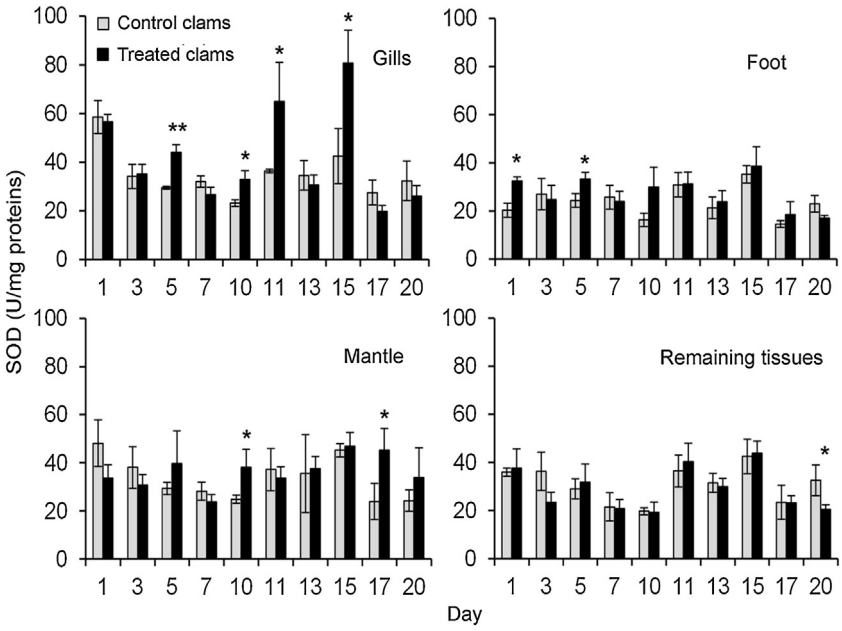

Fig. 6. Changes in superoxide dismutase (SOD) activity (U mg^{-1} proteins) in various tissues of clams fed with toxic *Microcystis* (days 0–10) or non-toxic *Microcystis* (days 11–20). Asterisks indicate significant differences compared with controls (fed with *Chlorella vulgaris*) at the respective time points (*$P < 0.05$, **$P < 0.01$).

We examined changes in CAT activity in the different tissues (Fig. 7). There were no definable trends. In the gills, CAT activity in the exposure group was significantly higher than in the controls on days 5, 7, and 15 but was significantly lower on day 20. Significant elevation of CAT activity in the exposure group was also observed on day 13 in the foot and on days 5 and 11 in the mantle. In the remaining tissues there were no differences in CAT activity between the control and exposure groups over the course of the experiment.

4. Discussion

This study is the first to describe MCs uptake and depuration in the edible clam *C. leana* grazing on toxic and non-toxic cyanobacteria *Microcystis* and on the green alga

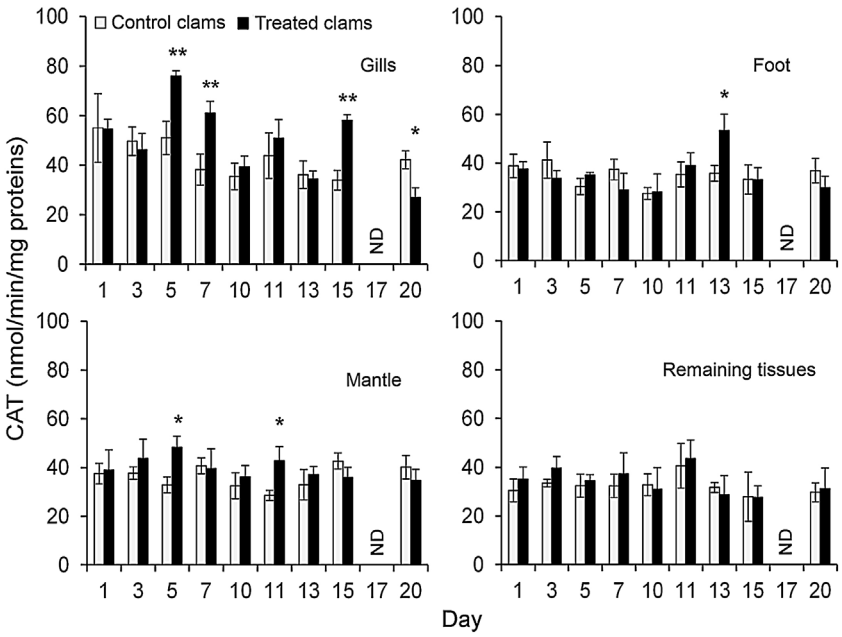

Fig. 7. Changes in catalase (CAT) activity (nmol min^{-1} mg^{-1} protein) in various tissues of clams fed with toxic *Microcystis* (days 0–10) or non-toxic *Microcystis* (days 11–20). Asterisks indicate significant differences compared with controls (fed with *Chlorella vulgaris*) at the respective time points (*$P < 0.05$, **$P < 0.01$). ND, not detected.

C. vulgaris. Although aquatic animals have been shown to select and ingest only non-toxic food in laboratory experiments [38,39], our results rejected the hypothesis that the clams would graze less efficiently on toxic strains than on non-toxic ones, because no significant differences in FRs were observed. On the other hand, our observations were well correspondence with the results of Pires and Donk [40], Pires et al. [41] and Gazulha et al. [42], who reported no significant difference in FR between mussels grazing on toxic and on non-toxic *Microcystis*. The second hypothesis—that clams would graze less efficiently on toxic strains than on green algae—was rejected as well. In contrast, the clams had a higher FR on toxic *Microcystis* than on the green alga (Fig. 3). We observed more pseudo-feces were expelled by clams fed cyanobacteria than by those fed the green alga (data not shown); clams are able to sort captured material, ingesting the nutritious particles (*C. vulgaris*) and rejecting the unpalatable ones (*Microcystis*) as pseudo-feces. Their pumping rates may therefore be enhanced when there is a relative abundance of unpalatable food. Similar observations have been reported by Pires et al. [13] namely that mussels filter toxic *Microcystis* faster than the green algae *Nannochloropsis* and *Scenedesmus*. However, our observations contradict those of Liu et al. [43] and Pires et al. [44], in which the FR did not change significantly in the three mussels *Anodonta anatina*, *Dreissena polymorpha* and *Unio douglasiae* when they were provided with varying concentrations of cyanobacteria and green algae. Possibly, FR may depend not only on the food type (including the MC content) and the mussel species but also the food concentration and physical parameters such as temperature, salinity, pH, and season [45–47].

Field and experimental studies have reported the accumulation and distribution of MCs in different bivalve species fed on toxic cyanobacterial cells or exposed to purified MCs. Commonly, the highest MC content is found in the hepatopancreas [6,7,13,48–50]. Despite intensive studies, the accumulation and distribution of free MC and Co-MC in bivalves gavaged with toxic cyanobacteria at environmentally relevant concentrations are not yet fully understood. Our laboratory experiments revealed that free MC was accumulated by *C. leana* by 1 day after the start of exposure. The peak levels of free MC measured in *C. leana* (12.7 $\mu g\, g^{-1}$ DW) were similar to the MC concentration measured in the zebra mussel *D. polymorpha* (11 $\mu g\, g^{-1}$ DW; [13]) and the freshwater mussel *Mytilus galloprovincialis* (10.5 $\mu g\, g^{-1}$ DW; [51]) during laboratory exposure. However, even when data on accumulation in other bivalves are available [1,6,49,52] they are not suitable for comparison with ours, because, unlike in our study, most were obtained from measurements in individual tissues and not the whole body. In general, MC accumulation in aquatic animals is likely to be affected by a number of factors, such as the exposure route, exposure duration and exposure dose, target tissues as well as by the mussel species [53].

Because of the difficulties of extracting covalently linked MC, analysis of MCs in animal tissues has until now been limited to quantification of the free MC content. By using an oxidation procedure adapted from previously developed methods [34,36] we were able to detect Co-MC in *C. leana* tissues (Fig. 4). On average, 3.2% (the number was calculated from mean percentages of the Co-MC contents from the MC concentrations in incubation water on days 1, 3, 5, 7 and 10) of MC content in incubation water was bound in *C. leana* during the 10-day exposure period (data not shown). Fortunately, our observations showed that mussels cultured in toxin-free water rapidly eliminate the free MC content within several days, this has been reported

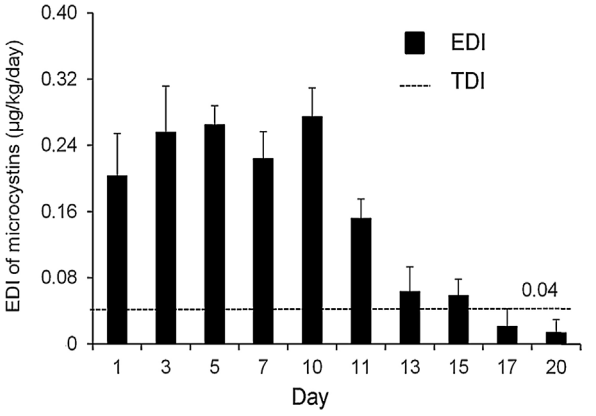

Fig. 8. Estimated daily intake (EDI) of microcystins by a person (60 kg) consuming 100 g of clam (fresh weight) per day [3]. Horizontal line indicates the maximum tolerable daily intake (TDI) of 0.04 μg kg^{-1} day^{-1}, as proposed by the WHO [2].

elsewhere [12,48] and this study. Similar results have also been reported in *D. polymorpha* [13,20] and in *M. galloprovincialis* [54]. In contrast, the rate of elimination of Co-MC from *C. leana* was lower than that of free MC, and the clam's tissues still contained a detectable amount of Co-MC after 10 days in toxin-free water. Therefore, to accurately assess the risks to humans, Co-MC in edible aquatic animals need to be quantified. Meanwhile, their toxicity and potential transfer to higher trophic levels remain unknown.

Freshwater mussels are among the most numerous macro-invertebrates in the world's streams, rivers, ponds, and lakes [22], where cyanobacteria frequently bloom. Consumption of bivalve molluscs is a potential route of human exposure to hepatotoxic MCs, especially in the case of molluscs harvested from lakes and reservoirs that routinely experience toxic CYBs [50,55]. We used a linear equation, $y = 0.0116x$ ($r^2 = 0.94$, $n = 30$), to convert the clam's whole-body wet weight to dry weight. Assuming that a 60-kg person consumes 100 g WW (~1.16 g DW) of the clam per day [3], the maximum estimated daily intake during the accumulation period would be more than seven times the TDI guideline value (Fig. 8). Based on this laboratory experiment, our results suggest that *C. leana* poses a high risk to human consumers. Consumption of this clam should be limited when there are high concentrations of toxic cyanobacteria present in the water from which it is harvested. Our estimation was made for a healthy adult, and the risks to children, the elderly, and sensitive individuals are likely to be higher.

Detoxification of MCs by aquatic animals occurs first via conjugation to GSH, a reaction catalyzed by GSTs [21]. Nevertheless, the responses of GST activity to MCs in mussels and other aquatic animals are variable [16,56]. It appears that the exposure time, route, composition of MCs, and target tissue all influence the GST response after exposure to MCs or toxic cyanobacteria. An increase in the activity of these enzymes can arise from activation of existing enzymes or from de novo enzyme synthesis, whereas decreased activity can be a sign of saturation due to substrate inhibition caused by the presence of high toxin concentrations [57–59]. Inhibition of GST activity has been observed in the mussel *Unio tumidus* exposed to MC-LR or cyanobacterial crude extract (CCE) [59]. Similar results have been reported in the gills of the freshwater mussel *D. polymorpha* after exposure to MC-LR at 100 μg L^{-1} for 1 h; the highest MC content in whole mussel tissues was detected at this time point [20]. We found significant inhibitions of GST activity in the gills and foot at several time points. This response of the detoxification system was synchronous with the increase in the rate of MC accumulation by the clam (Figs. 4 and 5). Depression of GST activity may result from inhibition of GST synthesis as a result of high levels of accumulation of MCs during the exposure period [58]. Decreased GST activity in these tissues may be also related to GSH depletion in response to MC toxicity [16]. Gavrilović et al. [60] reported that GST activity in the gills of three cyprinid fishes, *Rutilus rutilus* (roach), *Blicca bjoerkna* (white bream), and *Carassius gibelio* (Prussian carp), was inhibited during a CYB with high concentration of MCs. The same observations have been reported in the gills of zebrafish (*Danio rerio*) upon sub-chronic exposure to MC-LR [61] and in the liver, gills, intestine, and brain of the catfish *Corydoras paleatus* exposed to dissolved MC-RR at concentrations from 0.5 to 10 μg L^{-1} [57]. Probably, CYBs or MCs, or both, can inhibit GST activity in a range of aquatic animals.

Toxic cyanobacteria and MCs could alter antioxidant systems and induce ROS production, resulting in the oxidative stress that has been well documented in aquatic species [16,17]. SOD, CAT, and other molecules such as lipoic and dihydrolipoic acid are essential components of antioxidative defence systems [16,19], and increased SOD and CAT activity in animal tissues plays an important role in eliminating excessive ROS [62,63]. In a recent study, Burmester et al. [59] reported that SOD activity in *D. polymorpha* was elevated in most mussel tissues after exposure to MC-LR or CCE, or both, but CAT activity was barely affected. Increased SOD and CAT activity has been observed in the freshwater clam *Diplodon chilensis patagonicus*, but only at 5 and 6 weeks after exposure to toxic *Microcystis* [64]. We found here that SOD and CAT activity in *C. leana* was elevated only at some points and in some tissues during the exposure and depuration periods (Figs. 6 and 7). Our results were partly in line with those of Burmester et al. [59], who observed that SOD in *U. tumidus* was slightly induced during 7 days of exposure to MC-LR or CCE, or both. In contrast, studies performed on crustaceans such as shrimp (*Palaemonetes argentinus*), on bighead carp (*Aristichthys nobilis*), and on loach (*Misgurnus mizolepis*) given intraperitoneal MC-LR injections or exposed to toxic cyanobacteria have shown significant elevations of antioxidant enzymes, especially in the liver [53,65,66]. Probably, intraperitoneal injection of MC caused stronger effects than MC accumulated via oral ingestions.

Although no MC was present in the incubation water during the depuration period, SOD, CAT, and GST activity was still induced by the non-toxic *Microcystis*. This is evidence that both toxic and non-toxic *Microcystis* have caused oxidative stress in the clam and that other toxic, unidentified compounds might have elicited the changes in enzymes induced by non-toxic *Microcystis*. Probably, other components of the complex cyanobacteria biomass

contribute to these oxidative stresses. However, these effects are likely temporary in the case of both toxic and non-toxic *Microcystis*, and prolonged exposure may lead to adaptation. We found here that although some enzyme activities were induced they returned to control levels at later time points. In other words, clams are temporarily affected by toxic and non-toxic cyanobacteria but are overall quite resistant. This resistance may occur because of an ability to expel toxic cyanobacterial cells without metabolizing them [59,67] and an efficiently eliminate cyanotoxins or toxic cyanobacteria cells without metabolizing them. In addition, recent evidence indicates that multixenobiotic resistance (MXR) mechanisms represent a general biological defence of many marine and freshwater mussels against environmental toxicants [20,68]. Animal cells can trigger an MXR mechanism that serves as an efflux transporter against a broad spectrum of natural and man-made toxicants under stress conditions. This mechanism needs further investigation in *C. leana*.

5. Conclusions

Our experiments confirmed the transient bioaccumulation of free MC and Co-MC in *C. leana* after exposure to toxic cyanobacteria. Although MC was partially eliminated from the clams after the clams were transferred to MC-free medium, the Co-MC content would have still been potentially toxic to animals and humans. Both toxic and non-toxic *Microcystis* caused oxidative stress, as shown by the induction of antioxidant and detoxification systems. However, our laboratory experiments suggested that GST activity did not play a major role in the elimination of MC. The clam may be able to efficiently eliminate cyanotoxins or toxic cyanobacteria cells without metabolizing them. Alternative mechanisms, such as MXR, could be responsible for MC removal and resistance during exposure. These mechanisms need to be studied further.

Transparency document

The Transparency document associated with this article can be found in the online version.

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