Microcystin accumulation and biochemical responses in the edible clam *Corbicula leana* P. exposed to cyanobacterial crude extract

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ABSTRACT

We investigated the accumulation and effects of cyanobacterial crude extract (CCE) containing microcystins (MCs) on the edible clam *Corbicula leana* P. Toxic effects were evaluated through the activity of antioxidant and detoxification enzymes: catalase (CAT), superoxide dismutase (SOD), and glutathione-S-transferases (GSTs) from gills, foot, mantle and remaining soft tissues. Clams were exposed to CCE containing 400 μg MC-LR$_{eq}$/L for 10 days and were then kept in toxin-free water for 5 days. Clam accumulated MCs (up to 3.41 ± 0.63 μg/g dry weight (DW) of unbound MC and 0.31 ± 0.013 μg/g DW of covalently bound MC). Detoxification and antioxidant enzymes in different organs responded differently to CCE during the experiment. The activity of SOD, CAT, and GST in the gills and mantle increased in MC-treated clams. In contrast, CAT and GST activity was significantly inhibited in the foot and mostly only slightly changed in the remaining tissues. The responses of biotransformation, antioxidant enzyme activity to CCE and the fast elimination of MCs during depuration help to explain how the clam can survive for long periods (over a week) during the decay of toxic cyanobacterial blooms in nature.

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Introduction

The occurrence of cyanobacterial blooms (CYBs) in eutrophic lakes, reservoirs, and recreational waters has become a global environmental and public health concern due to the production of a wide range of toxic secondary metabolites, so-called cyanotoxins, that once ingested are highly toxic to wildlife, livestock, and humans. Among the cyanotoxins frequently encountered, microcystins (MCs), which are cyclic hepatotoxins composed of seven amino acids with more than 80 structural variants, are the most widespread and occur in up to 75% of CYB incidents (Chorus and Bartram, 1999). MCs target liver cells, and their cellular uptake requires the activity of organic anion-transporting polypeptides (Fischer et al., 2005). Once in the cell, they can accumulate as a free form of MC or specifically interact with protein phosphatases (PP1 and PP2A) in a two-step mechanism involving a rapid and reversible binding potentially followed several hours later by covalent binding;

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they can thus accumulate as covalently bound MC (Co-MC) with hyperphosphorylation and tumor-promoting abilities (MacKintosh et al., 1990, 1995; Amado and Monserrat, 2010; Lance et al., 2010, 2014).

The cellular system of defense against MC toxicity comprises antioxidant and detoxification enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST). SOD and CAT are antioxidant enzymes. SOD catalyzes the dismutation of superoxide anion (O_2^-) into oxygen and H_2O_2, whereas CAT catalyzes the conversion of two molecules of H_2O_2 into two molecules of water and one of oxygen (Lushchak, 2011). The mechanism of MC detoxification in aquatic organisms involves GSTs, members of the phase II detoxification enzyme family that catalyze the conjugation of MCs with glutathione (GSH) (Pflugmacher et al., 1998). This conjugation is generally considered the primarily route of MC detoxification in aquatic organisms; it results in the formation of compounds that are more polar and thus more easily excreted (Pflugmacher et al., 1998; Wiegand et al., 1999; Beattie et al., 2003).

The toxicology and ecotoxicology of MCs have been investigated in detail (Duy et al., 2000; Wiegand and Pflugmacher, 2005). However, toxicologists have focused only on isolating MCs (Beattie et al., 2003; Li et al., 2003; Kist et al., 2012) or using purified MCs (Cazenave et al., 2006; Contardo-Jara et al., 2008; Pavagadhi et al., 2012, Sun et al., 2012) in toxicity studies; the toxicity of complex cyanobacterial crude extract (CCE) has not been evaluated to the same extent. Several recent findings indicate that water from CYBs contains not only MCs but also a mixture of hazardous substances that can evoke more pronounced toxic effects than can MCs or other well-recognized cyanotoxins alone (Pietsch et al., 2001; Burýšková et al., 2006; Falconer, 2007; Palíková et al., 2007; Smutná et al., 2014). It would therefore be valuable to evaluate the effects of these complex cyanobacterial biomasses on aquatic organisms.

Filter feeders, such as bivalves, are highly affected during toxic CYBs or after bloom decay because they usually insert themselves into sediments on the beds or shores of lakes or rivers and filter small particles via their gills. These sessile filter feeders are therefore seriously affected by the presence of toxic cyanobacterial colonies during CYBs or after blooms have begun to decay. Increased attention is being paid to the accumulation and effects of MCs in bivalves, because humans consume these organisms (Ibelings and Chorus, 2007). Unlike the case in fish and mammals, there have been relatively few studies of the accumulation and biological effects of cyanotoxins in bivalve mollusks (Gérard et al., 2009; Sabatini et al., 2011).

The edible Asian freshwater clam Corbicula leana is commonly found in eutrophic habitats (Byrne et al., 2000). It is easily collected and maintained in the laboratory. Although its living area is easy to be polluted by contaminants, people in many countries often steam and eat whole (Hwang et al., 2004). During toxic CYBs, it may probably accumulate MCs and transfer to higher trophic levels through the food chain (Poste and Ozersky, 2013). To our knowledge, little or no information is available that demonstrate MCs from aqueous extracts accumulate and eliminate from this species.

In the present study, we examined the effects of a crude extract of CYBs containing MCs on the freshwater edible clam

C. leana P., as well as the accumulation and depuration of MCs by this species. Our aims were to understand how dissolved MCs in water column from cyanobacterial cell lysis (often occur at the end of a bloom), effect on aquatic life and to reveal the clam's system of defense against MCs via the activity of the antioxidant or detoxification enzymes CAT, SOD, and GST in various organs (gills, foot, mantle, and remaining tissues).

1. Materials and methods

1.1. Rearing the organisms

Freshwater clams were collected at a freshwater fishery experimental station in Oita Prefecture, Japan, and transported alive to the laboratory. The clams were introduced into sufficient aerated 50-L aquatic aquariums containing dechlorinated tap water and with a 5-cm sand layer as the substrate. Before the experiments, clams were kept at a density of below 100 individuals per 50 L and acclimatized for 1 month at a photosynthetic photon flux density of 20 μmol photons/(m^2·sec) under a 12:12 light:dark photoperiod. The water temperature was 22°C ± 1°C, pH 7.5 ± 0.3, and the dissolved oxygen concentration 7.9 ± 0.6 mg/L. All of the incubation water was renewed every 3 days. The clams were fed daily with the green alga Chlorella sp. at a concentration of 2×10^3 cell/mL, the alga was grown in SEM medium (Kong et al., 2012). The wet weight of individual clams was 5.22 ± 0.79 g and the shell length was 2.46 ± 0.57 cm.

1.2. Preparation of cyanobacterial crude extract

CCE was prepared as previously reported by Pietsch et al. (2001), with minor modifications. Briefly, 4 kg wet weight of bloom material (mainly Microcystis spp., collected from Lake Kasumigaura, Japan, by using a plankton net) was frozen at −30°C for 2 days and then thawed at room temperature. After the material had thawed completely, it was ice-cooled and sonicated for 1 min. This freeze–thaw–sonicate cycle was repeated four times. The samples were then centrifuged at 3000 g at 4°C for 30 min to remove cell debris. The CCE supernatant was collected and kept at −30°C until use.

Subsamples of CCE were used for MC analysis. Briefly, CCE was centrifuged at 6000 g at 4°C for 15 min. The supernatant was collected, dried completely, and redissolved in 500 μL of 100% MeOH. The samples were analyzed by HPLC for MC quantification. MC-RR, MC-LR, and MC-YR (Wako, Osaka, Japan) were used as standards. The HPLC analysis showed that the CCE contained three MC congeners, namely MC-RR (53%) and MC-LR (45%) and the minor congener MC-YR (2%), at a total concentration of 7892 μg/L.

1.3. Experimental set-up

Clams (240 individuals) were placed in eight aquariums (30 clams per aquarium) containing 2 L distilled water and a 2-cm sand layer as a substrate, with constant aeration. These aquariums were kept at a photosynthetic photon flux density of 20 μmol photons m^{-2} sec^{-1} under a 12:12 light:dark

photoperiod. The water temperature was 22 ± 1°C. Clams were allocated randomly to an exposure group (120 clams) and a control group (120 clams).

In the exposure group, CCE containing MCs was added to each aquarium to a final concentration of 400 μg MC-LR$_{eq}$ L^{-1} on day 0. The water and MCs were completely replaced on day 5 of the 10-day exposure period. The clams were then collected and relocated into aquariums containing distilled, toxin-free water; they stayed in these aquariums for 5 days of depuration. The experiment therefore lasted a total of 15 days (10 days of MCs exposure following by 5 days of toxin depuration). In the control group only the water was replaced on day 5. No food was provided during the uptake and depuration periods. Dead clams were removed and counted daily. Six hours after the start of exposure and again on days 1, 3, 5, and 10 of the exposure period and days 11, 13, and 15 of the depuration period, 15 clams were sampled for MC quantification and enzyme measurements. For MC quantification, the shells were immediately removed; the whole soft tissues were then freeze-dried for 48 hr and keep at −30°C until MC analysis. Ten clams sampled before the start of the experiment were used as controls. For measurement of enzyme activity, the clams in both groups were rinsed gently under dechlorinated tap water. The gills, mantle, and foot of five clams (pooled) and the remaining tissues (kept individually) were dissected on ice. The samples were then immediately frozen in liquid nitrogen and stored at −80°C until enzyme extraction.

1.4. Extraction and analysis of MCs in incubation water

We measured MC concentrations in the incubation water immediately after the start of exposure and depuration period, at 6 hr after the start of exposure and again on days 1, 3, and 5 of the exposure period and on days 11, 13, and 15 of the depuration period. The incubation water (about 10 to 100 mL) was collected and filtered through GF/C filters. The filtrate was then passed through PresepC18 (ODS) cartridge (Wako Pure Chemical Industries Ltd., Osaka, Japan) that had been preconditioned with 5 mL MeOH 100% and 10 mL ultrapure water; it was then subjected to final elution with 3 mL MeOH 100% and dried completely. The MC fraction was then redissolved in 500 μL MeOH 100% and kept at −30°C until HPLC analysis. MCs (-RR, -LR, -YR) were analyzed with an reversed-phase HPLC system equipped with a UV detector (Shimadzu 10 A series, Shimadzu Corporation, Kyoto, Japan) by using the methods of Wang et al. (2013).

1.5. Extraction and analysis of unbound MC

Free MC was extracted as previously reported by Xie and Park (2007), with minor modifications. Briefly, freeze-dried tissues (about 100 to 150 mg per sample) were homogenized in 3 mL of BuOH:MeOH:H$_2$O (5:20:75, $v/v/v$) by using a homogenizer (Polytron, Kinematica AG, Littau-Luzern, Switzerland) and extracted three times with 5 mL of the same solution, each time for 24 hr with shaking in darkness. After sonication for 1 min, the samples were centrifuged at 2000 g at 4°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 elution fraction was evaporated to dryness under reduced pressure at below 40°C. MCs were suspended in 300 μL MeOH 100%; they were then kept at −20°C before reversed-phase HPLC analysis. Duplicate samples with duplicate analyses were used in this determination ($n = 4$).

1.6. Extraction of total MCs

Total MC (free- and Co-MC) was extracted as previously reported by Neffling et al. (2010), with minor modifications. Briefly, freeze-dried tissues were homogenized and trypsinated with 3 mL of 500 μg/mL trypsin in Sorensen's phosphate buffer (pH 7.5) at 37°C for 3 hr; this was followed by oxidation with 0.1 mol KMnO$_4$ and 0.1 mol NaIO$_3$ (pH 9.0) for 3 hr at room temperature. The reaction was quenched with sodium bisulfite solution (40% w/v) until colorless at pH 2 with 10% sulfuric acid. After sample centrifugation (2000 g, 30 min, 4°C), the supernatant was collected, diluted five times with ultrapure water, and 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%. The eluate fraction was evaporated to dryness and redissolved in 500 μL MeOH 100%. The MMPB was converted to its methyl ester (meMMPB) by using a 10% BF$_3$-methanol kit (Sigma-Aldrich, Tokyo, Japan) (Fig. 1). The derivatized samples were dissolved in n-hexane and kept at −20°C before GC–MS analysis. The Co-MC content was thus estimated by subtracting the free MC content from the total MC content. 4-Phenylbutyric acid (4-PB) was used as an internal standard (Sano et al., 1992). MMPB-d$_3$ and MC-LR purchased from Wako Pure Chemical Industries were used as external standards.

1.7. GC–MS analysis

We used a DSQ II mass spectrometer linked to a Trace GC Ultra gas chromatograph system (Thermo Scientific, Waltham, MA, USA) equipped with an Rxi-5 ms 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 (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. 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. Methylated 4-PB (me4-PB) and meMMPB were detected by using 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 for meMMPB (Suchy and Berry, 2012). Xcalibur software was used for quantitative analysis of these analytes. Duplicate samples with duplicate analyses were used ($n = 4$).

1.8. Enzyme extraction

Enzymes were extracted as previously reported by Wiegand et al. (2000), with minor modifications. Briefly, samples (gill, foot, mantle, remaining soft tissues) were homogenized in 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
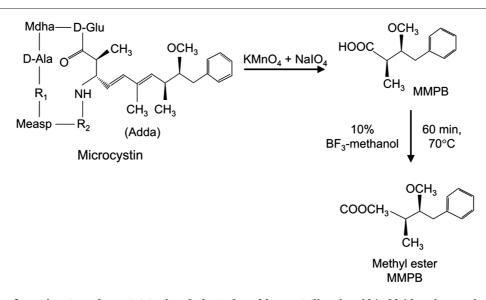
Fig. 1 – Oxidation of 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) in microcystins to the carboxylic acid 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) and its methyl ester.

dithioerythritolin in ice. The homogenate was centrifuged at 10,000 g at 4°C for 15 min to eliminate cell debris and other fragments. The supernatant was used for enzyme activity measurement. We used a Fluoroskan Ascent fluorometer (Thermo Electron Corp., Milford, MA, USA) to detect the activities of GST (EC 2.5.1.18), SOD (EC 1.15.1.1), and CAT (EC 1.11.1.6) at wavelengths of 340, 460, and 540 nm, respectively, with GST, SOD, and CAT assay kits purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All enzyme activities were calculated in terms of the protein content, as measured with a Quick Start Bradford protein assay kit purchased from Bio-Rad Laboratories (Hercules, CA, USA). Each enzymatic assay was performed in triplicate.

1.9. 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 pair wise comparison by using Tukey's 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.

2. Results

2.1. Microcystin concentrations in incubation water

MC concentrations in the control incubation water were under the detection limit (data not shown). We monitored MC concentrations in the incubation water during the first 5 days of uptake and during the depuration period. In the uptake experiment, MCs were immediately and continuously cleared from the incubation water. After only 6 hr, the MC concentration in the water had decreased to 326.3 ± 13.5 μg/L; after 1 day it was 262.2 ± 12.9 μg/L, after 3 days 185.8 ± 10.7 μg/L, and after 5 days 121.1 ± 3.1 μg/L (Fig. 2).

There was no release of the unmetabolized parent compound into the toxin-free water during the depuration period.

2.2. Uptake and depuration of free and Co-MC

There were no deaths in either of the groups of animals during the experiments. The control samples contained no MCs at detectable concentrations (data not shown).

Extractable free MC accumulated in the clams during the uptake and depuration periods was shown in Fig. 2. Typically, the free MC concentration in the whole clams increased rapidly after the start of exposure and peaked (at 3.4 ± 0.63 μg/g DW) after about 1 day. It then gradually declined over the rest of the exposure period. The free MC content was well correlated with the concentration of MCs in the incubation water ($r = 0.65$, $P < 0.01$). The Co-MC concentration increased slowly during the uptake and depuration periods, peaking (at 0.31 ± 0.013 μg/g DW) on day 11. It gradually declined thereafter.

During the depuration period, free MC was quickly eliminated from the clam tissues and below the limit of detection by HPLC. In contrast, the Co-MC concentration was enhanced on the first day of depuration and then gradually declined, although Co-MC was still detectable at the end of the depuration period (Fig. 2).

2.3. Biotransformation enzyme activity

We measured GST activity in various tissues of both the exposure and the control groups (Fig. 3). GST activity in the gills was significantly greater in the exposure group than in the control group, but only on days 0.25, 1, 3, and 11. Significant elevation of GST was also observed at days 10 and 11 in mantle. In contrast, GST activity in the foot was significantly lower in the exposure group than in the control group on days 3, 5, 10, and 13, although it had returned to the control level by the end of the experiment. GST activity in the remaining tissues did not differ significantly over time between the two groups.
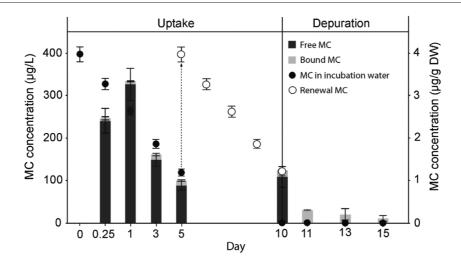
Fig. 2 – Concentrations of free microcystins (MC), covalently bound MC in *Corbicula leana*, and of MC in incubation water, during the uptake and depuration periods. Arrow indicates the time of renewal of the MC concentration during the uptake period.

2.4. Antioxidant enzyme activities

We examined the effects of CCE containing MCs on SOD activity in the various clam tissues (Fig. 4). SOD activity in the gills was significantly greater in the exposure group than in the control group on all measurement days in the exposure period except day 10. In the mantle this was also true for all measurement days in the exposure period except day 3. In contrast, in the foot there were no significant differences in SOD activity between the two groups at any time. In the remaining tissues SOD activity was significantly greater in the exposure group than in the control group, but only on days 0.25 and 5. Unlike the case with GST, during the depuration period there were no differences in SOD activity between the two groups in any of the tissues.

We then examined changes in CAT activity (Fig. 5). CAT activity in the gills was significantly greater in the exposure group than in the control group, but only on days 0.25, 1, and
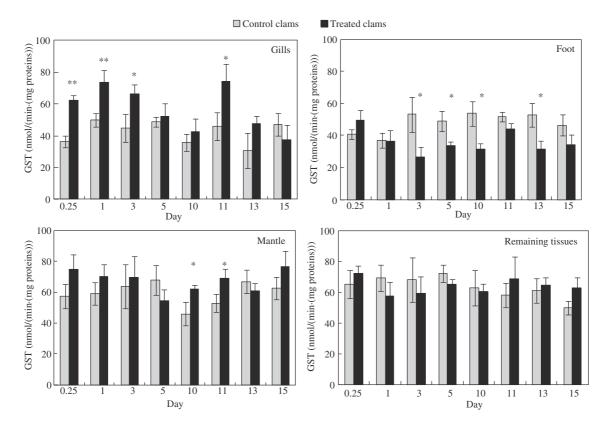

Fig. 3 – Production of glutathione S-transferase (GST) in various tissues of *Corbicula leana* exposed to toxic cyanobacterial bloom crude extract. Asterisks indicate significant differences compared with controls at the respective time points (*$p < 0.05$, **$p < 0.01$).
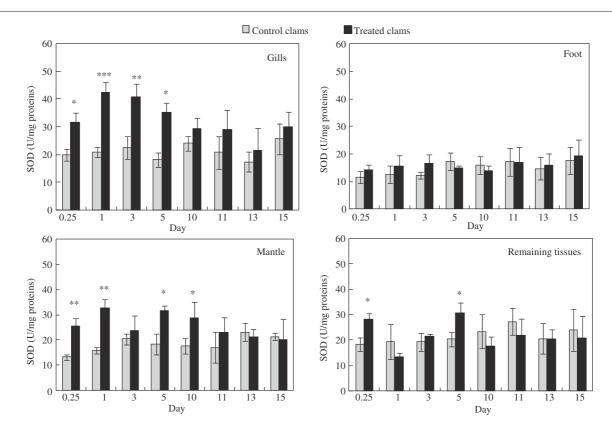
Fig. 4 – Production of the antioxidant enzyme superoxide dismutase (SOD) in various tissues of *Corbicula leana* exposed to toxic cyano bacterial bloom crude extract. Asterisks indicate significant differences compared with controls at the respective time points (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
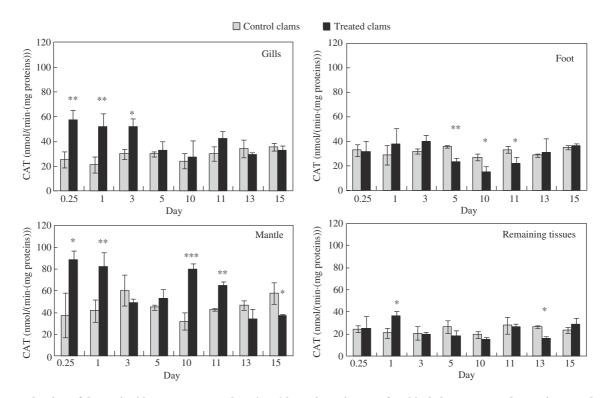

Fig. 5 – Production of the antioxidant enzyme catalase (CAT) in various tissues of *Corbicula leana* exposed to toxic cyano bacterial bloom crude extract. Asterisks indicate significant differences compared with controls at the respective time points (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3. In contrast, CAT activity in the foot was significantly lower in the exposure group than in the control group on days 5, 10, and 11 but thereafter returned to control levels. There were no detectable trends in CAT activity in the mantle and the remaining tissues. CAT activity in the mantle was significantly greater in the exposure group than in the control group on days 0.25, 1, 10, and 11 but significantly lower at the end of the experiment, on day 15. In the remaining tissues, CAT activity was significantly greater in the exposure group than in the control group on day 1 but significantly lower than in the control on day 13.

3. Discussion

In natural environments and under experimental conditions, concentration of dissolved MCs in water can be expected to be decreased by such processes as adsorption onto particulate materials, attachment to substrates, and degradation by intracellular organic matter and bacteria (Harada and Tsuji, 1998; Grützmacher et al., 2009; Wörmer et al., 2010; Ma et al., 2012; Shimizu et al., 2011, 2012). In our experiment, the concentration of MCs in the incubation water had decreased by about 69% after 5 days of incubation. This result agreed well with the finding in another study that after 3 days of incubation the concentration of dissolved MC-LR had decreased by more than 50% (Contardo-Jara et al., 2008). However, we still understand little about the natural degradation of MCs in complex CYB extracts. Our results may suggest that the degradation of MCs in CCE is a result of the combined effects of physical, chemical, and biological factors, including uptake by aquatic animals. However, the main contributors to toxin degradation remain unknown and need further investigation.

Exposed to dissolved MC may resulted in low accumulation in animal tissues. We revealed here that toxin uptake by *C. leana* was lower than that by most other mussels and snails exposed to living cells. The maximum levels of free MC measured in *C. leana* (3.4 ± 0.63 μg/g DW) were similar to the MC content in the mussel *Anodonta* sp. collected from Lake Kastoria, in Greece (Gkelis et al., 2006), but they were much lower than those in other bivalve species *e.g.*, 16 μ/g DW in *Mytilus galloprovincialis* (Amorim and Vasconcelos, 1999, exposed to living cells of *Microcystis*), 16.3 μg/g DW in *Dreissena polymorpha* (Pires et al., 2004; exposed to living cells of *Microcystis*) and 70 μg/g DW in *Anodonta cygnea* (Eriksson et al., 1989, exposed to living cells of *Oscillatoria*) during laboratory exposure. However, even when data on MC accumulation in other bivalves are presented (Yokoyama and Park, 2003; Chen and Xie, 2005; Vareli et al., 2012) they are not suitable for comparison with ours, because, most were obtained from measurements in individual tissues and not the whole body. In general, different species no doubt have different capacities for toxin accumulation, uptake, and tolerance and 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 (Galanti et al., 2013).

Because MCs covalently bind to (protein phosphatases) PPs and cannot be extracted from the covalent complex by using organic solvents, detection of MCs in animal tissues has been limited to free MC (for reviews see Ibelings and Chorus, 2007; Martins and Vasconcelos, 2009). By using an oxidation procedure adapted from previously developed methods (Sano et al., 1992; Neffling et al., 2010; Suchy and Berry, 2012), we provided evidence for the existence and accumulation of Co-MC in *C. leana* tissues (Fig. 2). On average, 0.5% dissolved MCs from incubation water was bound in *C. leana* during the 15-day experiment (data not shown). However, the clam rapidly eliminated the MCs when cultured in toxin-free water. Williams et al. (1997) reported that the total MC content in the mussel *Mytilus edulis* transferred to untreated saltwater dropped from 337 μg to 11 μg/g FW in 4 days, after which time it was undetectable. Prepas et al. (1997) have also shown that MC concentrations significantly decrease within 6 days of depuration in the clam *Anodonta grandis simpsoniana*. Also, immediate uptake and rapid release of MCs have been observed in *D. polymorpha* (Pires et al., 2004; Contardo-Jara et al., 2008) and *M. galloprovincialis* (Amorim and Vasconcelos, 1999). We found here that free MC rapidly began to be released when the clam was transferred to toxin-free water, but the percentage of bound MC increased (and reached 100% of the total MC content) during the depuration period (Fig. 2). This increase may have occurred due to the enhancement of the free MC binding to PPs. At the end of the 5-day depuration period, *C. leana* tissues still contained 0.15 ± 0.01 μg/g DW of Co-MC. Although depuration is commonly judged to be rapid in mussel species, it is equally clear that depuration is incomplete, even after a considerable period of time (Wiegand et al., 1999; Ibelings and Chorus, 2007). Therefore, Co-MC levels should be considered in predictions of risk to higher trophic organisms and humans.

The long-term effects and accumulation of MCs have been studied on mussel (Pires et al., 2004), fish (Magalhaes et al., 2001; Palíková et al., 2003) and other zooplanktonic species (DeMott, 1999; Hulot et al., 2012). These studies all showed that MCs had an inhibitory effect, mostly on growth, feeding and generally survival of the experimental animals. Continual oral exposures to low doses of MCs have also shown chronic liver injury, but more important is the possibility of carcinogenesis and tumor growth promotion (Chorus and Bartram, 1999). The results raise concerns that long-term exposure to even very low levels of MCs may be significant, and could ultimately result in liver cancer and other liver diseases in humans. The current study revealed that the toxin uptake by *C. leana* from dissolved MCs is possible. Despite these relatively low levels, however, our results raise concerns about chronic toxicity from a human health perspective, because humans may be consuming clams contaminated with MCs, and consumption of food contaminated with MCs could promote cancer (Duy et al., 2000). We used a coefficient of 100 to convert dry weight to wet weight in the case of this clam; our results showed that the total MC content of the clams exceeded the tolerable daily intake of 0.04 μg/kg^{-1} of body mass per day (Fig. 6). Our results therefore suggest that *C. leana* represents a health risk to consumers when aquatic MC concentrations are high.

It is well known that the family of GST enzymes is the most important group for MC detoxification (Burmester et al., 2012). We found an elevation in GST activity in the gills during the first 3 days of exposure, suggesting that there was an immediate response by the tissue to the CCE. This response can be due either to an increase in MC conjugation with GSH or to the detoxification of endogenous molecules such as membrane peroxides (Pinho et al., 2005). The higher GST activity in the exposure group
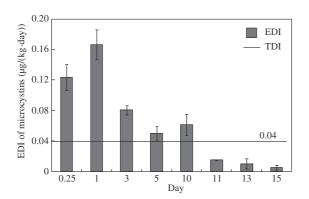
Fig. 6 – Estimated daily intake (EDI) of microcystins by a person (60 kg) consuming 300 g (fresh weight) of *Corbicula leana*. Horizontal line indicates the maximum tolerable daily intake (TDI) for humans (0.04 μg/(kg·day)), as proposed by the World Health Organization (Chorus and Bartram, 1999).

suggested that there was increased MC conjugation capability in the gills of these animals. The same defense system against MC toxicity has been reported in the gut and gills of *M. galloprovincialis* mussel when exposed to *Microcystis* extracts but no change was found when exposed to living *Microcystis* cells or pure toxins (Vasconcelos et al., 2007), in the gills of crabs exposed to an aqueous extract of the toxic cyanobacterium *Microcystis aeruginosa* (Vinagre et al., 2003; Pinho et al., 2005), and in the gills and intestines of catfish *Corydoras paleatus* exposed to high concentrations of MC-RR (Cazenave et al., 2006). Probably, aqueous extracts of the cyanobacterium cause stronger effects on GST activity than living cells or pure toxins. The further increase in GST activity in the gills on day 11 may have been the result of excessive GST synthesis, which was thereafter regulated in response to the lack of MCs in the depuration period. Contrastingly to the gills, in the foot, mantle, and remaining tissues GST expression was inhibited or did not change after exposure. Decreased GST activity in these tissues may be related to GSH depletion in response to MC toxicity (Amado et al., 2011); it may also result in altered biochemical effects in organisms exposed to MCs (Malbrouck et al., 2003).

Toxic cyanobacterium, pure toxins or CCE containing MCs all induce ROS production, resulting in oxidative stress to organisms. These ROS activate the expression of several antioxidant enzymes, including SOD and CAT, which constitute the major defensive system against ROS (Amado et al., 2011; Lushchak, 2011; Paskerová et al., 2012). Exposure of the freshwater clam *Diplodon chilensis patagonicus* to toxic *Microcystis* leads to an increase in oxidative stress, as indicated by enhanced CAT and SOD activity (Sabatini et al., 2011). Similarly, the exposure of the mussel *M. edulis* to an extract of the cyanobacterial toxin nodularin leads to an increase in CAT activity (Kankaanpää et al., 2007). The same observations were also reported in the mussel *D. polymorpha* exposed to pure MC-LR (Contardo-Jara et al., 2008). Here, we found significant changes in both CAT and SOD enzyme activity in various tissues of *C. leana*. These findings indicate that there was an activation of the antioxidant defensive system as a direct or indirect response to ROS generation after exposure to CCE containing MCs. More specifically, the alterations that we found in antioxidant enzyme

activity were likely caused mainly by the presence of MCs and partly by the presence of other compounds in the CCE (Dao et al., 2013). Also, our results are consistent with the observations of Burmester et al. (2012), who found that SOD activity in two bivalves, *D. polymorpha* and *Unio tumidus*, was elevated in various tissues after exposure with purified MC-LR or CCE.

A far more controversial question concerns the adverse effects of pure cyanotoxins, toxic living cells or CCE contains MCs on CAT activity. Elevation of CAT activity and other antioxidant enzymes has been observed in the crab hepatopancreas after 48 hr of exposure to MCs from CCE (Pinho et al., 2005) or in shrimp (*Litopenaeus vannamei*) injected with MCs (Gonçalves-Soares et al., 2012). In contrast, CAT activity was significantly reduced, and SOD activity unchanged, in the crab hepatopancreas after a 7-day exposure to a high-dose *M. aeruginosa* aqueous extract (Pinho et al., 2005). Likewise, CAT activity in larvae of the bighead carp *Hypophthalmichthys nobilis* is significantly reduced upon MC-LR exposure, suggesting that CAT activity is inhibited by MC-LR (Sun et al., 2012). In our clam, CAT activity in the mantle was significantly lower in the exposure group than in the control group at the end of the experiment, possibly because at that point the mantle was less efficient than the gills and foot at neutralizing the impact of oxidative stress. In contrast, the reduction in CAT activity in the foot toward the end of the exposure period could have been due to the generation of superoxide radicals during oxidative stress; these molecules have been reported to inhibit CAT activity (Kono and Fridovich, 1982). Therefore, toxic effects depend not only on the dose and kind of toxin, the route of exposure, and the duration of exposure, but also on the target organ, the state of the organism, and the species (Malbrouck and Kestemont, 2006; Pavagadhi et al., 2012; Sun et al., 2012).

Contrastingly, multixenobiotic resistance (MXR) in the freshwater mussel *D. polymorpha* is evidence of the insensitivity of bivalves to purified cyanobacterial toxins (Contardo-Jara et al., 2008). Our results also correspond to those of Fischer and Dietrich (2000), who observed no deaths, malformations, or growth inhibition in *Xenopus laevis* embryos exposed to purified MCs at up to 2000 ug/L for 96 hr. Similarly, no developmental toxicity of MCs (at up to 20,000 μg/L) has been observed in the toad *Bufo arenarum* (Chernoff et al., 2002). Antioxidant enzyme levels may be elevated in response to cellular oxidative stress in animal cells (Dias et al., 2009; Turja et al., 2014), and the increased rate of synthesis of these antioxidant enzymes could be a plausible explanation for the insensitivity following MC exposure in some experimental groups (Pavagadhi et al., 2012). Our results demonstrate that biochemical toxic effects are only temporary and that prolonged exposure can lead to adaptation to cope with deleterious effects. The significant changes in GST, SOD, and CAT activity that we found in *C. leana* probably reflect adaptation to oxidative conditions. However, in toxin-free water, both of the antioxidant enzymes and detoxification enzyme showed adaptive responses at several time points whereby enzyme activity was induced and then returned to control levels. The responses of antioxidant and detoxification enzymes might thus contribute to the MC and cyanotoxin tolerance of *C. leana*.

Many aquatic organisms live and reproduce in contaminated waters, suggesting that they have ways to resist or tolerate

contaminants (xenobiotics) in their environments (Cornwall et al., 1995). Exposure to toxins can trigger the MXR mechanism, which serves as a first line of defense against a broad spectrum of natural and man-made toxicants in the cells (Bard, 2000; Faria et al., 2011). Contardo-Jara et al. (2008) point out that the interactions of various defense mechanisms against MC toxicity in the freshwater mussel *D. polymorpha* are due to high constitutive levels of P-glycoprotein and the reaction of MXR mechanisms; this explains the clams' survival success, even when they are exposed to high MC concentrations. Further, studies are needed to give us an integrated view of toxin insensitivity and the MXR mechanism in the clam *C. leana*. To our knowledge, this is the first report of MC uptake by, and the biochemical responses of, this edible clam in the context of safe food production.

4. Conclusions

Our findings provide insights into the uptake of CCE containing MCs at high concentrations by *C. leana* and the consequent biochemical responses of the clam under laboratory conditions. We highlight the involvement of antioxidant and biotransformation systems in detoxification of MCs. It explains the possible tolerance of *C. leana* continuously exposed to high levels of MCs. In addition, it reveals that MCs are accumulated by the clam via the uptake of dissolved MCs in water bodies. Our findings should also improve our understanding of the impacts of MC-containing cyanobacteria dissolved in the water column on aquatic life under natural conditions. However, further research is required to deepen our understanding of the fate and transfer of MCs and the toxicity of other hazardous substances from CCE.

Acknowledgments

R E F E R E N C E S

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