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Multi-xenobiotic-resistance a possible explanation for the insensitivity of bivalves towards cyanobacterial toxins

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ABSTRACT

Filterfeeders, such as bivalves, are highly affected during toxic cyanobacterial blooms, as they are non-selective and may use the cyanobacteria as main nutrition source. The freshwater mussel *Dreissena polymorpha*, living in lakes and rivers coexisting with cyanobacteria, was exposed to 100 μg L^{-1} microcystin-LR (MC-LR) for up to three days. MC-LR concentration in mussel tissue and surrounding media was quantified by HPLC-PDA during uptake and depuration phase, revealing an immediate, continuous uptake, and release of non-metabolized toxin, and occurrence of reincorporation. The involvement of multi-xenobiotic-resistance protein (P-glycoprotein, P-gp) on the excretion of MC-LR was evidenced by efflux and accumulation version of the Rhodamine Assay as well as on P-gp gene expression. P-gp expression was enhanced after 1 h exposure but no changes were detected after longer (72 h) exposure. P-gp enzyme activity showed a significant increase with exposure time, supporting the hypothesis that P-gp is involved in the excretion of MC-LR.

Induction of biotransformation enzyme such as pi-class glutathione S-transferase (piGST) and antioxidant enzyme catalase (CAT) was immediately inhibited and returned to control values only after more than 72 h expose time. Heat shock protein 70 (hsp70) and protein phosphatase 2A (PP2A) gene expression was not changed due to the treatment with cyanobacterial toxin MC-LR.

1. Introduction

The increase of nutrients such as phosphorous and nitrates into surface waters caused to eutrophication with enhanced phytoplankton growth. Within the phytoplankton community cyanobacteria may dominate in summer months, forming intense blooms. Cyanobacteria have the potential to produce several toxic secondary metabolites, which remain mainly within the cell but are released in high concentrations during cell lysis (Saker and Griffiths, 2000). The negative impacts of those toxins and possible elimination strategies are of particular concern for scientist all over the world (Funari and Testai, 2008).

The most common toxic secondary metabolites of freshwater cyanobacteria are the microcystins (MC), monocyclic hepta-peptides. Alterations in constituent amino acids or side groups result in about 70 structural variants of MC with varying toxicity (Carmichael and Li, 2006). The main natural biodegradation routes of the cyanobacterial toxin MCs in freshwaters are sediment sorption, photolysis and microbial degradation (Harada and Tsuji, 1998). For humans and vertebrates hepatotoxicity has been reported and is of health concern worldwide where toxic surface water is used for human activities

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(Zurawell et al., 2005). Uptake of MC-LR, one of the most common toxin variants, occurs in liver cells via the bile acid transport system (Runnegar et al., 1991), but was also shown to occur by passive diffusion or a specific receptor in embryonic stages of fish eggs, prior to liver development (Wiegand et al., 1999).

MC-LR inhibits protein phosphatases 1 and 2A (PP1 and PP2A) and therewith disrupts the balance with protein kinases, producing hyperphosphorylation of proteins, such as cytosolic and cytoskeletal proteins (Toivola et al., 1994). For PP2A several trimeric holoenzyme forms exist, consisting of a catalytic and a regulatory subunit, associated with variable further regulatory subunits (Wera and Hemmings, 1995). It is yet uncertain if all PP2A holoenzyme forms are targets for MC-LR.

For the cyanobacterial toxin MC biotransformation via glutathione S-transferase (GST) by conjugation to reduced glutathione (GSH) has been reported for aquatic organism, such as fish, crustacean, molluscs and macrophytes (Pflugmacher et al., 1998; Wiegand and Pflugmacher, 2005). The biotransformation pathway implies after conjugation to GSH, the degradation to a gamma-glutamylcysteine and a cysteine conjugate (Pflugmacher et al., 2001). Binding to GSH enhances the water solubility of MC, aiding excretion and providing therewith a decline in parent compound content. Also diminished inhibition on PPase has been reported for the MC-glutathione conjugates compared to the parent compound (Metcalf et al., 2002). Cyanobacterial toxins are also known to cause production of reactive oxygen species (ROS) which may oxidize unsaturated lipids in cell membranes, proteins and DNA, contributing to their toxicity, and hence activate or increase antioxidant defence mechanisms (Zegura et al., 2004). For *Danio rerio* embryos exposed to the toxin variants MC-RR and MC-LF oxidative stress increased, which was indicated by enhanced catalase (CAT) activity following enhanced amounts of H_2O_2 (Cazenave et al., 2006).

Sessile bivalves may be the most obvious group of organisms threatened by the presence of toxic cyanobacteria (Lindholm et al., 1989) having no chance to escape by movement. On the other hand they might use these toxic cyanobacteria as main feeding sources during cyanobacterial blooms. Insensitivity to MCs has been reported; expulsion via pseudo-faeces has been described as a primary defence mechanism, as *Dreissena polymorpha* produces large quantities of mucous so called 'pseudo-diarrhoea' periodically expelled through the pedal gape by shell valve adduction when exposed to highly toxic strains (Vanderploeg et al., 2001; Juhel et al., 2006). Besides that, acceptance exists that also other mechanisms provide insensitivity, as e.g. for *D. polymorpha* opposed results were found suggesting no prevention on feeding (Pires et al., 2004, 2007). Also biotransformation via GST has been evidenced in different organs of the marine mussel *Mytilus galloprovincialis* (Vasconcelos et al., 2007). Nevertheless negative effects have been described when feeding on toxic phytoplankton such as altered larvae development and survival (Pires et al., 2003) or changes in feeding behavior and energy balance in *D. polymorpha.* Furthermore, especially marine mussels, such as *Mytilus edulis* and *M. galloprovincialis* accumulating high amounts of toxins, either via the uptake of dissolved or intracellular toxins, are possible vectors for toxin transfer to consumers, including humans (Vasconcelos, 1995; Sipia et al., 2001).

Other studies on the co-occurrence of *D. polymorpha* and cyanobacteria focus on long-term ecosystem changes, as in some cases a shift in phytoplankton composition to cyanobacteria dominance was observed for lakes invaded by *D. polymorpha*. Bykova et al. (2006) suggest that high abundances of *D. polymorpha* provoke alterations in the processing of nitrogen and phosphorus, which could contribute to the re-emergence of *Microcystis* blooms. Contrasting, a long-term study on the interaction of phytoplankton composition and invasion by *D. polymorpha* found no correlation between the parameter 'mussel filtration rate' and 'cyanobacterial biomass', but 90% of the variance of cyanobacterial dominance was explained by temperature and dissolved SiO_3 (Fernald et al., 2007).

Knowing about accumulation and biotransformation of MCs the mechanism of excretion of the toxin in mussels was investigated. Multidrug resistance (MDR) proteins have been identified in human tumor cells providing an effective mechanism to resist distinct drugs or chemicals by immediate discharge and therefore prevention of cell abidance of the chemicals (e.g. Bolhuis et al., 1997). A similar mechanism called multi-xenobiotic-resistance (MXR), based on the activity of MXR proteins such as the P-glycoprotein (P-gp), located in the membrane, pumping non-metabolized and metabolized toxins directly out of the cell, has been found in different organism (e.g. Bard, 2000). This mechanism might be a further defence mechanism against bioaccumulation of MCs in mussels. It has been shown to be responsible for mussels' insensitivity to other toxins, such as the marine dinoflagellate diarrheic shellfish poison (Svensson et al., 2003).

It was of special interest if mussels' insensitivity towards exposure to the typical cyanobacterial toxin MC-LR is also mediated by increased MXR mechanism. Thus, toxin uptake and depuration, as well as accumulation, were evidenced in a short-term experiment. P-gp synthesis and determination of protein activity were expected to give insight on the dynamics and degree of the immediate cellular response. mRNA changes of pi-class GST (piGST) and CAT shall provide information if due to biotransformation and antioxidant mechanism gene induction is required. Hsp70 and PP2A expression should give indication on protein damage and disruption in the signalling pathway due to toxin exposure.

2. Material and methods

2.1. *Rearing of* D. polymorpha

For laboratory and field studies *D. polymorpha* mussels within a size of 22–25 mm were collected during summer time two weeks prior each experiment from Lake Liepnitzsee, located in a remote area north of Berlin.

During a two-week acclimatization period mussels were kept in 10 L glass tank each with approximate hundred individuals at 18 ± 0.5 °C. Mussels were fed daily with a monospecies culture of *Monoraphidium minutum* cultivated in Z4-medium (Zehnder and Gorham, 1960). The

artificial freshwater (AFW, Milli-Q-grade, 100 mg L^{-1} Instant Ocean sea salt, 200 mg L^{-1} $CaCl_2$, 103 mg L^{-1} $NaHCO_3$) was changed every second day. Light and dark phases were set to 14:10 h.

2.2. Exposure scenario and sampling

In all experiments exposure concentration was 100 μg L^{-1} MC-LR (Alexis Biochemical Products, Grünberg Germany) in AFW, and each replicate consisted of one mussel (size: 22–25 mm) exposed in 150 mL medium (toxin in AFW or AFW for control) in a static manner. Mussels were not fed during the experiments.

a) Sampling of medium: during uptake, concentration of MC-LR was determined in the exposure medium after 1, 4, 8, 12, 24, 48 and 72 h ($n = 10$). After 1, 24 and 72 h mussels were transferred to toxin-free AFW for further 24 h. Depurated MC-LR was determined after 1, 4, 8, 12 and 24 h in the AFW. To quantify MC-LR concentration of the medium 1000 μL of each replicate were sampled for direct HPLC analysis (Section 2.3).
b) Tissue samples: to determine MC-LR tissue content, whole mussel tissue was sampled after 1, 24 and 72 h MC-LR exposure. Furthermore tissue concentration was measured after 12 and 24 h of depuration in toxin-free medium for the 1 h exposure duration ($n = 5$ for each sampling, Section 2.3).
c) Physiological response on molecular level: to study changes in gene expression, gills were sampled after 1, 24 and 72 h ($n = 6$) of exposure duration and shock-frozen in liquid nitrogen. Alterations in mRNA levels of P-gp, piGST, CAT, hsp70 and PP2A were determined by semi-quantitative RT-PCR. Gill tissue was selected as gills are the first organ in contact with the surrounding medium and therefore expected to react immediately to toxin exposure (Sections 2.4 and 2.5).
d) Excretion activity: to study *in vivo* changes in P-gp enzyme activity by the efflux version of the Rhodamine Assay mussels were exposed for 1 h to MC-LR and Verapamil (positive control). The accumulation version of the assay was performed after 1 and 24 h MC-LR exposure (Section 2.6).

2.3. Toxin extraction and quantification

Tissue content of MC-LR in mussels was extracted as follows: after determining fresh weight, samples were homogenized with an Ultra Turrax in 5 mL 70% methanol, followed by agitation for 30 min. Homogenates were centrifuged at 4000 g (Multifuge 1 S-R) for 10 min at 4 °C, and the resulting pellet was suspended in 2 mL 70% methanol and extracted again. Supernatants of both extraction steps were combined and completely evaporated. Remains were re-dissolved in 1000 μL methanol in three steps. Samples were transferred into HPLC vials. MC-LR concentration was quantified by HPLC and related to mussel fresh weight. Samples from exposure and depuration medium were directly applied to HPLC analysis.

Analysis of MC-LR in water samples and tissue extracts was performed according to Pflugmacher et al. (1998). A Waters HPLC system with photodiode array detection and a reverse phase column (RP 18 5 μM LIChroSpher 100) was used. Injection volume of the samples was 10 μL. Bioconcentration factor (BCF) was calculated by relating the tissue content to medium concentration.

2.4. RNA isolation

RNA isolation from the gill tissue was done according to the manufacturers instructions using the phenolic reagent TRIZOL (Invitrogen, Karlsruhe, Germany). Addition of chloroform followed by centrifugation (20,000 g) separates aqueous from organic phase. After transfer of the aqueous phase, the RNA was precipitated by isopropyl alcohol. The pellet obtained by centrifugation (20,000 g) was dissolved in 50 μL RNase-free diethyl pyrocarbonate-treated water (DEPC water). RNA purification using RNeasy Mini Kit (Qiagen, Hilden, Germany) was performed according to the supplier's protocol with an additional step of on-column DNA digestion using RNase-Free DNase Set (Qiagen, Hilden, Germany).

2.5. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA isolated was adjusted to 0.1 μg μL^{-1} using RNase-free DEPC water. 1 μg total RNA was used for cDNA synthesis by incubating with 0.8 μL (0.5 μg μL^{-1}) Oligo $(dT)_{12-18}$ primers (Invitrogen, Karlsruhe, Germany) at 70 °C for 5 min. RT was performed using 3 μL reverse transcriptase buffer (5 times conc. M-MLV, Promega, Mannheim, Germany), 0.8 μL deoxynucleotide triphosphates (dNTPs, 10 mM) and 0.4 μL M-MLV reverse transcriptase (Promega, Mannheim, Germany) and incubated at 42 °C for 1 h. The process was terminated by heating at 94 °C for 4 min and the resulting cDNA samples were stored at −20 °C.

cDNA of the elongation factor 1-α (EF1-α), P-gp, CAT, piGST, hsp70 and PP2A specific for *D. polymorpha* was amplified by semi-quantitative RT-PCR using a Thermal Mastercycler (Eppendorf, Hamburg, Germany). Primers were designed (Clone Manager, Professional Suite for Windows) according to sequences published in the National Centre for Biotechnology Information (NCBI). Accession numbers of the original sequences, their references and the sequences of the designed primers are listed in Table 1. All primers were optimized regarding best annealing temperatures and durations, as well as optimal cycle numbers.

PCR reaction for all gene expressions consisted of 3 μL of cDNA sample 10-fold diluted with DEPC water, 14.1 μL DEPC-treated sterile de-ionized water, 2 μL PCR-buffer, 0.2 μL forward and reverse primers, 0.4 μL dNTPs (10 mM) and 0.1 μL (5 U μL^{-1}) HotStar Taq DNA polymerase (Qiagen, Hilden, Germany). RT-PCR conditions were optimized and set specifically for each sequence (Table 1). The PCR settings for all sequences were 94 °C for 15 min, followed by specific durations at 94 °C, specific annealing times and temperatures, as well as specific durations at 72 °C. Final extension

Table 1
Accession numbers for NCBI of the original sequences of the examined genes, their sequences of the designed primers and NCBI references. RT-PCR conditions were optimized and set specifically for each sequence.

	P-gp	CAT	piGST	Hsp70	PP2A	EF-1α
Description	P-glyco-protein	Catalase	pi-class Glutathione S-transferase	Heat Shock Protein 70	Protein phosphatase 2A	Elongation Factor 1-alpha
Accession number in NCBI	AJ506742	EF681763	DQ459993	EF526096	AF508223	AJ250733
Primer sequence forward	5′-GGGTGAATCTC AAGGACATC-3′	5′-TCAGCCTGCG ACCAGAGACG-3′	5′-AGCCCAAGACCC AGTTCATC-3′	5′-CCTGGGGAGC AGCGTCCGTG-3′	5′-AGACCGAGGTT ACTACTCAG-3′	5′-TCATTGGGC ACGTCGACTCC-3′
Primer sequence reverse	5′-CGTCCAGCAGG AGGATCTTG-3′	5′-TCATGACTTGG ATCTTAAGG-3′	5′-GCCTTGAATTCG TCGGAGTG-3′	5′-TCCGAGCGGT CGCCGGACAA-3′	5′-CAGAGATGTCC TGTCCAAAG-3′	5′-ACGCACATGGGCTT AAATGG-3′
Reference in NCBI	Tutundjian et al. (2002)	Peuthert (2007a)	Doyen et al. (2006)	Peuthert (2007b)	Lamers et al. (2002)	Tutundjian (1999)
PCR settings						
Time at 94 °C	45 s	30 s	30 s	45s	30s	45s
Annealing	45 s at 54 °C	30 s at 45 °C	30 s at 54 °C	45s at 52 °C	30s at 49 °C	45s at 54 °C
Time at 72 °C	1 min	30 s	30 s	1 min	1 min	1 min
Cycle numbers	20	32	27	26	30	20

was performed at 72 °C for 10 min equal for all sequences, whereas cycle numbers were also set specifically.

Following separation of PCR products on ethidium bromide-stained agarose gel (1%), the intensity of bands was quantified densitometrically using image analyzer (Gel Doc 2000, Bio-Rad, Munich, Germany). EF1-α was used as a control gene for DNA level normalization, because levels of EF1-α in *D. polymorpha* were not changed by the treatment. Relative mRNA expression levels of exposed animals were compared to the respective control.

2.6. Rhodamine Assay for determination of excretion activity

The efflux and accumulation version of the Rhodamine Assay according to Smital et al. (2000) was used to study changes in P-gp enzyme activity due to MC-LR exposure. MC-LR was assumed to have an effect on P-gp activity, as it was observed that P-gp mRNA levels were induced after 1 h exposure. The assay bases on the detection of the P-gp substrate Rhodamine B, a fluorescent dye, either accumulated in gill tissue or depurated into dye free medium and gives therewith evidence of altered P-gp enzyme activity.

In each step of both assays (exposure, loading, pre-efflux and efflux phase, respectively), it was verified if animals opened their shells, indicating active filtering, after 5–10 min. If this requisite was not obtained, those mussels were rejected and the experiment was re-performed until a replicate number of $n = 10$ for each treatment and the control was reached, respectively. Both versions of the Rhodamine Assays were performed with light protected exposure vessels located at a magnet stirrer at 350 rpm stirring speed.

A) For the efflux version of the assay, mussels were primarily exposed for 1 h to 100 μg L^{-1} MC-LR or toxin-free medium (control). Mussels were then transferred singly to 50 mL of 5 μM Rhodamine B (Sigma, Germany) solution for 1 h, followed by three short washing steps with 150 mL AFW and a "pre-efflux" phase of 5 min in 150 mL AFW. After anew transfer to AFW, depuration of Rhodamine was monitored for 1 h, by sampling of 1000 μL water of each replicate at every 5 min, and measuring excitation at 535 nm and emission at 590 nm.

B) For the accumulation version of the assay mussels were exposed either to 100 μg L^{-1} MC-LR or 20 μM Verapamil (LKT Laboratories, Germany) solution (positive control, as it inhibits P-gp activity) or toxin-free AFW (control) for 1 and 24 h, followed by loading with Rhodamine for 1 h in 5 μM Rhodamine B solution (50 mL per single mussel). The loading was followed by three short washing steps with 150 mL AFW and sampling of gills of ten mussels per exposure. Gill samples were weighed, homogenized and centrifuged for 10 min at 10,000 g (miniSpin) and Rhodamine content of the supernatant was measured by excitation at 535 nm and emission at 590 nm.

3. Results

3.1. Uptake and depuration

In the MC-LR uptake experiment the toxin was immediately and continuously cleared from the exposure medium. After 1 h MC-LR concentration decreased to 70.6 ± 5.7 μg L^{-1}, after 24 h to 59.1 ± 2.9 μg L^{-1} and after 72 h to 34.8 ± 13.4 μg L^{-1} (Fig. 1, upper part). Release of the non-metabolized parent compound into toxin-free AFW was detected following 1 and 24 h uptake, but no parent compound was detectable after exposure for 72 h. In the 1 and 24 h exposure it could be observed that the toxin was reincorporated after 8 h (Fig. 1, lower part), and newly released after 12 h.

3.2. Bioconcentration

Tissue concentration was quantified in exposed mussels and mussels that had undergone a depuration phase (Fig. 2). After exposure to 100 μg L^{-1} MC-LR all the replicates of the 1 h exposure contained MC-LR with a mean value of 21 μg gWW^{-1}; after 24 h and 72 h out of five mussels only $n = 4$ and $n = 2$ contained MC-LR, thus 66 and 85% less, compared to the 1 h treatment. The BCF decreased with exposure durations starting from 295 after 1 h to 127 and 111 after 24 and 72 h, respectively. After 12 and 24 h

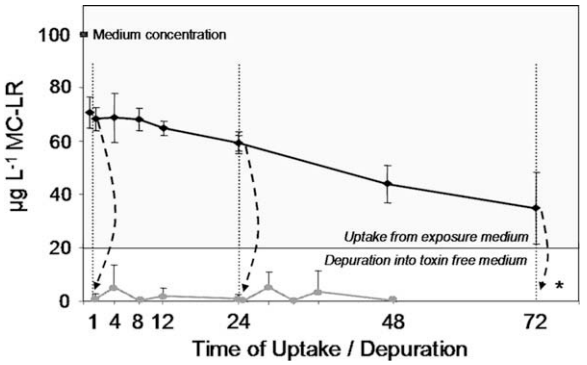

Fig. 1. Exposure of *D. polymorpha* to 100 µg L^{-1} MC-LR for up to 72 h and transfer (dotted arrows) into toxin-free medium for further 24 h after 1, 24 and 72 h former exposure. Decreasing MC-LR toxin concentration in exposure medium (upper part, black line) and depurated toxin after transfer into toxin-free medium (lower part, grey line) was determined by HPLC [µg L^{-1} MC-LR]. The asterisk indicates that after 72 h no depurated parent compound could be detected.

depuration in toxin-free medium of formerly 1 h exposed mussels, out of five only $n = 3$ and $n = 1$ still contained MC-LR amounts of 60 and 50%, respectively, compared to the mean content detected in mussels before depuration.

The amounts of MC-LR as parent compound decreased with duration of exposure and depuration.

3.3. Molecular response in gills

mRNA levels of P-gp were twofold higher after 1 h exposure, thus not statistically significant (Fig. 3A). After 24 and 72 h mRNA levels were only slightly higher compared to non-exposed mussels. This leads to the assumption that after this short increase of protein synthesis levels are

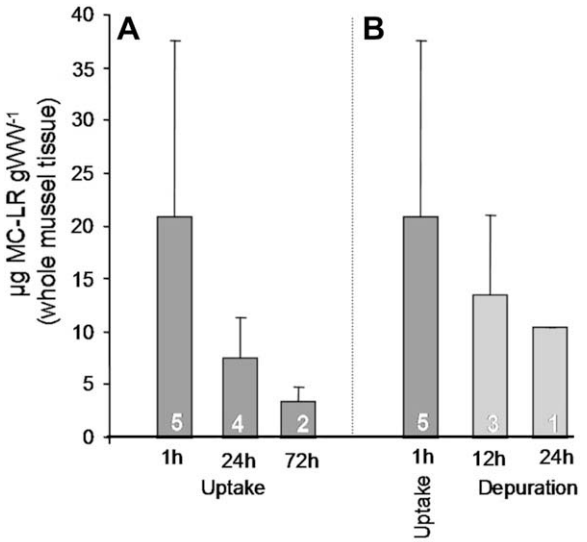

Fig. 2. Tissue content [µg MC-LR gWW^{-1} (whole mussel tissue)] of MC-LR (A) after exposure of *D. polymorpha* to 100 µg L^{-1} MC-LR for 1, 24 and 72 h, and (B) for the 1 h MC-LR exposure after transfer into toxin-free medium for further 12 and 24 h. Tissue toxin concentration was determined by HPLC. White numbers in bars indicate number of individuals (out of five) containing toxin in their tissue.

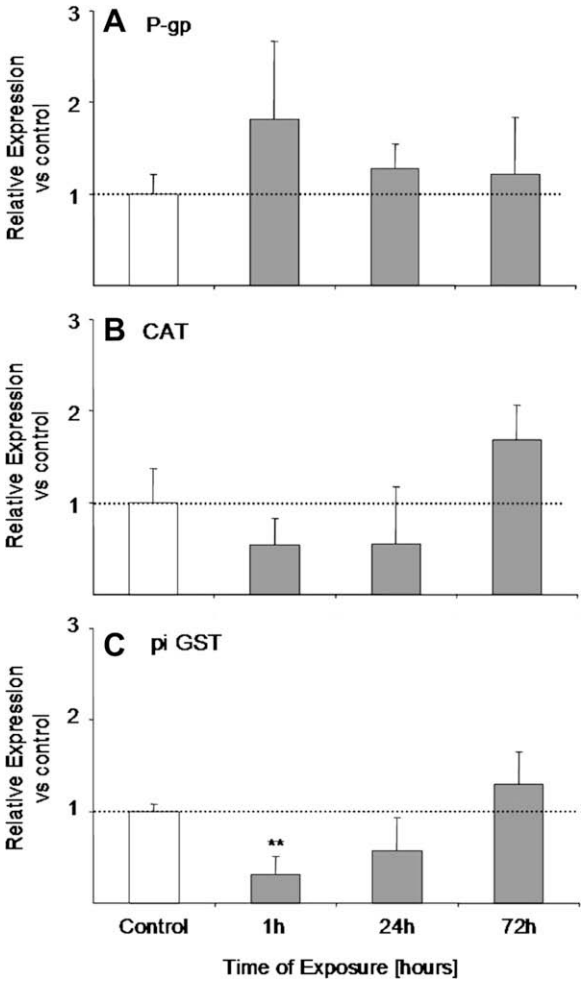

Fig. 3. Relative mRNA expression of (A) P-gp, (B) CAT and (C) piGST in gills of *D. polymorpha* exposed for 1, 24 and 72 h to 100 µg L^{-1} MC-LR (grey bars). Results are expressed relative to the controls (white bars), normalized to elongation factor 1-α (EF1-α). Asterisks indicate significant differences to control of the respective time point by Duncan's test ($^{**}p = 0.01$).

sufficient for excretion activity, making a further and more intense gene induction unnecessary.

CAT mRNA transcription was inhibited after 1 and 24 h exposure (Fig. 3B). After 72 h 80% higher mRNA amounts compared to non-exposed mussels were detected. Alterations of CAT mRNA levels were not statistically significant. piGST mRNA levels were significantly lower after 1 h exposure ($p < 0.01$). After 24 h levels were still lower than in non-exposed mussel, but returned to control values after 72 h (Fig. 3C). As both enzymes piGST and CAT obtain the same induction mechanism it can be assumed that disturbance of the signalling pathway occurred.

mRNA levels of hsp70 and PP2A did not differ significantly due to the toxin exposure compared to the respective controls.

3.4. P-gp activity

The efflux version of the Rhodamine Assay was used to study the immediate increase in P-gp enzyme activity

after MC-LR exposure for 1 h. Following a 1 h Rhodamine uptake phase, depuration of the dye showed an immediate release by all mussels (Fig. 4). Formerly MC-LR exposed mussels released the dye Rhodamine faster than control mussels.

The accumulation version of the assay evidenced that mussels pre-exposed for 1 and 24 h to MC-LR contained after further loading with Rhodamine 40 and 60% less of the dye in their gills compared to control animals. This was statistically significant for the 24 h treatment ($p < 0.05$) and is a confirmation for enhanced P-gp activity (Fig. 5). In the 1 and 24 h inhibition treatment with Verapamil it could be observed that Rhodamine content was 80 and 40%, respectively, higher than in the gills of control animals. This was statistically significant for the 1 h treatment ($p < 0.001$) and confirms inhibited P-gp activity.

4. Discussion

Although cyanobacterial blooms are a natural phenomenon, anthropogenic eutrophication contributes to increased bloom occurrence (Carmichael et al., 1988; Zurawell et al., 2005). As acute toxicity of cyanobacterial toxins to many vertebrates and invertebrates has been evident, cyanobacterial blooms are included concerning the security and management of public and environmental health.

D. polymorpha occurs in high abundances in freshwater ecosystems despite possible toxic cyanobacteria. This is a phenomenon and suggests that mussels must have a strategy to overcome toxicity.

In the present study the cyanotoxin MC-LR was immediately incorporated by *D. polymorpha*, but also instantly released, when mussels were transferred to toxin-free medium. Immediate uptake and rapid release of MC-LR was also observed by Pires et al. (2004) for *D. polymorpha* and by Amorim and Vasconcelos (1999) for *M. galloprovincialis*. It was possible to show in our study that exposure to MC-LR was related to an immediate increase in P-gp activity. Both the accumulation and efflux experiment using the P-gp model substrate Rhodamine B evidenced the increase in enzyme activity of P-gp. Already a short MC-LR exposure of 1 h provoked enhanced P-gp activity, which was observable

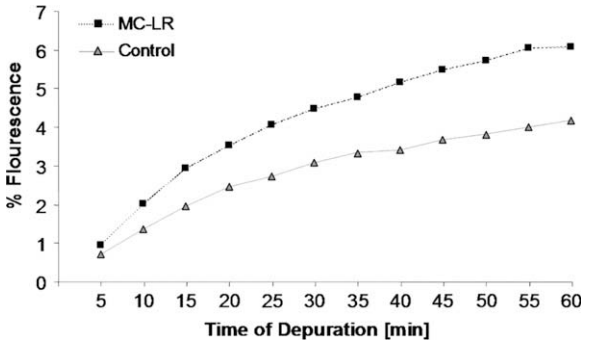

Fig. 4. Efflux version of the Rhodamine Assay: after former exposure for 1 h of *D. polymorpha* to 100 μg L^{-1} MC-LR mussels depurated the experimental dye more effectively than non-exposed mussels [% Fluorescence]. More depurated dye indicates augmented activity of the transmembrane pump P-gp ($n = 10$).

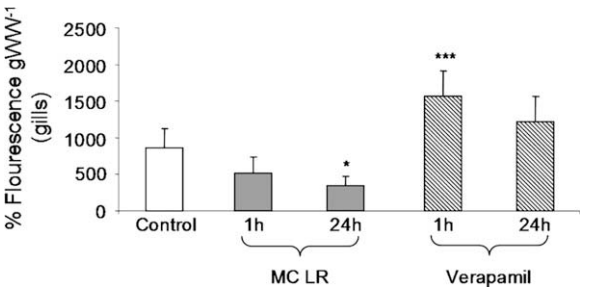

Fig. 5. Accumulation version of the Rhodamine Assay: after former exposure for 1 and 24 h of *D. polymorpha* to 100 μg L^{-1} MC-LR and 20 mM Verapamil, accumulated dye is determined in gill tissue [% Fluorescence g^{-1} (gills)].

in increased discharge of the dye compared to control mussels. Further exposure yielded to a time dependent increase in P-gp activity, as less of the dye was accumulated in the gill tissue. The inverse effect on P-gp activity was demonstrated with Verapamil, a well-known P-gp inhibitor. This leads to the conclusion that the MXR mechanism is clearly triggered by MC-LR exposure. Also mRNA levels of P-gp were slightly induced instantaneous in MC-LR exposed mussels, but induction did not persist. The high variance in mRNA level for P-gp may be explained by shell closure, a primary defence mechanism of mussels (Rajagopal et al., 2005) and hence different contact times due to decreased filtering activity. In general, exposure to 100 μg L^{-1} MC-LR did not cause the closure effect. Furthermore, presumably levels of this protein were already constitutively high, making a further gene induction unnecessary and possibly new synthesis at the beginning ($\sim$1 h) is sufficient for longer time at low rates of conversion.

Increased P-gp activity also explained insensitivity to okadaic acid in marine mussel blood cells (Svensson et al., 2003). Furthermore P-gp has been shown to be involved in cell defence against accumulation of various xenobiotics, such as moderately hydrophobic drugs by providing fast excretion in *D. polymorpha* (Eufemia and Epel, 2000; Pain et al., 2007). A study conducted with human tumoral cells revealed the mammalian analogue multidrug resistance (MDR) mechanism to be partly responsible for MC insensitivity (de Souza Votto et al., 2007). Besides the assumption of defence by immediate export the authors emphasize the higher antioxidant competence of MDR phenotype cells, evidenced by lower ROS concentrations. If the same mechanism applies to *D. polymorpha* the combination of higher export rates and decreased ROS formation might contribute to the insensitivity of *D. polymorpha*, towards cyanobacterial toxins. MXR is also involved in excretion of various xenobiotics in *D. polymorpha* (Minier et al., 2006).

Despite mussels responded to toxin exposure with increased MXR mechanism, accumulation in mussel tissue was observed as well in our study. Amounts of detected parent compound in mussel tissue, as well as amounts of released parent compound into toxin-free medium, decreased with exposure duration till below detection limit after 72 h, indicating that biotransformation processes helped to detoxify the MC-LR.

BCF in our study was 295 after 1 h exposure and subsequently decreased over time. To compare our results to experiments conducted with *M. edulis*, fed on toxic strains (Vasconcelos, 1995; Amorim and Vasconcelos, 1999), bioconcentration was calculated from toxin content in the cyanobacterial cells and cell density used in their studies in relation to the detected mussel tissue content. Results reveal that BCFs were in the same range (~300) after 4 and 10 days in the feeding experiment as it was in our study by exposure to the dissolved toxin. Considering the decreasing BCF with exposure time we could demonstrate that activity of the P-gp increases due to exposure to MC-LR, hence we assume that the incorporated MC-LR and all of the metabolites, such as the glutathione-, gamma-glutamylcystein or cystein-conjugates, were excreted more rapidly with exposure time. Moreover it is to concern that the parent compound MC-LR had undergone biotransformation processes and metabolites were not considered in the calculation, thus led to an underestimation, if MC-LR and its metabolites are regarded in sum. Protein bound MC-LR has not been extracted and may account for decreasing recovery of MC-LR during the study.

Decreased expression of piGST and CAT due to toxin exposure were observed as further physiological changes already after 1 h. The decrease might be due to a disturbance of the signalling pathway, as both enzymes obtain the same induction mechanism, as MC-LR is known to inhibit protein phosphatase (PP), which in turn is involved in cell signalling processes (Rivas et al., 2000). The dinoflagellate toxin okadaic acid also specifically inhibits PP1 and PP2A causing major effects on signal transduction pathways and hence essential cellular functions (Svensson et al., 2003). Studies on PPase inhibition are complex, as several trimeric holoenzyme forms of PP2A exist, consisting of a catalytic and a regulatory subunit, associated with variable further regulatory subunits (Wera and Hemmings, 1995). As only one isoform of PP2A was analyzed in the present study, revealing no mRNA level changes at the applied MC-LR concentration, no further conclusions can be drawn on the inhibitory effect and possible proximate transcription increase. To get an integrated view on the cause-and-effect chain of MC-LR on *D. polymorpha* PP2A, the two physiological endpoints, enzymatic alterations and changes in gene transcription of varius isoforms have to be regarded in further studies.

The slight elevations above control levels of piGST and CAT expression after 72 h MC-LR exposure indicate an enhanced demand for new synthesis of both enzymes, suggesting involvement of piGST in the detoxification of the tested toxin and oxidative stress for the cells. For with the cyanobacterial toxin nodularin exposed marine mussels no involvement of GST in the detoxification but emerging oxidative stress was evidenced by activity changes of the respective enzyme (Kankaanpaa et al., 2007). In turn, for MC exposed *D. polymorpha* the clear involvement of GST in the detoxification was evidenced by time- and concentration-dependent increase of this enzyme (Peuthert and Wiegand, 2004). The enhanced consumption of GSH together with increased GR activity supported furthermore occurring biotransformation processes.

Even though enhanced ROS formation due to MC-LR exposure and hence possible protein damage was assumed, this was not confirmed in the present study, as expression of hsp70 did not change.

Compared to zooplankton, as e.g. Daphnids, mussels are much more efficient in minimizing the assimilation of MC (Ibelings et al., 2005). The interaction of various defence mechanisms against MC toxicity in the freshwater mussel *D. polymorpha*, as immediate depuration due to high constitutive levels of P-gp and possible biotransformation via GST, explains mussels' survival success, even when exposed to high concentrations as in this study. The insensitivity to toxins and xenobiotics of freshwater mussels can give implications for lake restoration, whereas Pires et al. (2007) point out to prefer the use of native semi-insensitive species as e.g. *Anodonta anatina*. For this approach it is to consider that high dominance of *D. polymorpha* can in turn also enhance the growth and hence dominance of toxic cyanobacteria due to selective feeding and a shift in the nutritional supply has been assumed by Bykova et al. (2006). Fernald et al. (2007) followed in a long-term study the association of the introduction of *D. polymorpha* with increased dominance of cyanobacteria in the Hudson River Estuary during 1993–2005. They revealed no correlation of absolute or relative amounts of total cyanobacteria or *Microcystis* sp. content to mussels' filtration rate. Instead, 90% of variances of cyanobacterial abundances were related to temperature and availability of silicium (Fernald et al., 2007).

In conclusion this study contributes to the findings on mussels' insensitivity to cyanobacterial toxins highlighting the involvement of the MXR mechanism in toxin depuration as an immediate response to exposure. Thus, the inhibitory effect on piGST and CAT expression indicates the need of an instant counteraction against MC toxicity in the mussels, supporting the assumption of insensitivity but only with augmented energy cost for depuration, biotransformation and gene induction.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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