Journal of Hazardous Materials 190 (2011) 188–196

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Microcystin-LR induced oxidative stress and ultrastructural alterations in mesophyll cells of submerged macrophyte *Vallisneria natans* (Lour.) Hara

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ARTICLE INFO

Article history:
Received 6 December 2010
Received in revised form 7 March 2011
Accepted 8 March 2011
Available online 15 March 2011

Keywords:
Vallisneria natans (Lour.) Hara
Microcystin-LR
Reactive oxygen species
Oxidative stress
Ultrastructure

ABSTRACT

Microcystins produced by cyanobacteria in the aquatic environment are a potential risk to aquatic plants. In the present study, the uptake of microcystin-LR (MC-LR) and related physiological and biochemical effects on *Vallisneria natans* (Lour.) Hara were investigated at concentrations of 0.1–25.0 $\mu g\,L^{-1}$. Results showed that $O_2^{\bullet -}$ intensity was significantly induced at 1.0 $\mu g\,L^{-1}$ and reached a maximum level at 5.0 $\mu g\,L^{-1}$. Superoxide dismutase (SOD) and peroxidase (POD) were induced with increasing MC-LR concentrations as an antioxidant response. Catalase (CAT) was significantly induced while GSH/GSSG (reduced/oxidized glutathione) ratio was significantly reduced at 0.1 $\mu g\,L^{-1}$. The induction of glutathione S-transferase (GST) and inhibition of GSH revealed that GSH was involved in the detoxification of MC-LR in plants. Oxidative damage was evidenced by the significant increase of malondialdehyde content at 1.0 $\mu g\,L^{-1}$. A pigment pattern change and a series of significant ultrastructural alterations were also observed due to MC-LR exposure. The lowest non-effect concentration of MC-LR for *V. natans* at the sub-cellular and molecular level is around 0.5 $\mu g\,L^{-1}$. These results imply that even at relatively low MC-LR concentrations the aquatic plants may still suffer a negative ecological impact.

1. Introduction

Cyanobacterial blooms occur worldwide under certain environmental conditions in eutrophic lakes and reservoirs [1,2]. Many lakes in China, like Lake Taihu, Chaohu and Lake Dianchi have had frequent occurrence of cyanobacterial blooms in recent years due to intensive agriculture and dense population around the lake areas [3]. The blooms are of great ecological concern because many genera of bloom-forming cyanobaceria are known to produce cyanotoxins such as microcystins (MCs). Nearly 80 structural variants of MCs have been identified and the most common and toxic one is microcystin-LR (MC-LR) [4,5]. Concentrations of dissolved MCs in most eutrophic lakes range from 0.1 to 10 $\mu g\,L^{-1}$ [6]. However, the concentration can reach up to 1800 $\mu g\,L^{-1}$ or higher, if most toxins are released into water after lysis of cyanobacterial cells during the collapse of highly toxic blooms [6]. Currently the World Health Organization has recommended a drinking water guideline of 1 $\mu g\,L^{-1}$ for MC-LR [7].

MCs have shown adverse effects on mammals, birds, aquatic organisms and even the whole aquatic ecosystem [8]. There is also evidence that frequent occurrence of cyanobacterial blooms is accompanied by coincident decrease of the abundance and diversity of aquatic plant communities [9,10]. Aquatic plants are an important part of the aquatic ecosystem and are potentially exposed to high levels of MCs during blooms and subsequent cyanobacterial cells lysis [11]. However, the toxic effects of MCs on aquatic plants have not been widely investigated. Further research is needed to clarify the toxic effects and toxicity mechanism of MCs on aquatic plants at relatively low concentrations.

It has been reported that the leaves and roots of aquatic plants, such as *Ceratophyllum demersum*, *Elodea canadensis*, *Vesicularia dubyana* and *Vallisneria natans* can absorb MCs that cause inhibitory effects of germination, growth, development and photosynthesis [12–16]. However, how these absorbed toxins affect the aquatic plants at the subcellular and molecular level was not examined. MCs have been shown to be a potent inhibitor of PP1 and PP2A from higher plants (*Brassica napus* seeds) and for animals [17], but in many cases, their toxicity on aquatic plants seems to be more linked to the induction of oxidative stress manifested by elevated reactive oxygen species (ROS) production and malondialdehyde (MDA) content [13,18–20]. For example, elevated lipid peroxidation (LPO) was observed in cyanobacteria *Synechococcus elongates* in response to MCs exposure [18]. Biochemical studies demonstrated that the activities of detoxication enzymes, such as peroxidase (POD) in *Lemna minor* [21] and superoxide dismutase (SOD), glutathione S-transferase (GST, microsomal and soluble forms), glutathione peroxidase (GPX) and ascorbate peroxidase (APX) in *C. demersum* [13,14] increased following exposure to MCs. Reduced glutathione (GSH), an important cellular thiol against

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doi:10.1016/j.jhazmat.2011.03.023

Table 1
Added and measured concentrations of MC-LR in the solutions.

Added MC-LR ($\mu g\,L^{-1}$)	0.0	0.1	0.5	1.0	5.0	10.0	25.0
MC-LR concentration in solutions ($\mu g\,L^{-1}$) ($n = 5$[a])	0.00	0.09 ± 0.032	0.42 ± 0.072	0.91 ± 0.12	4.43 ± 0.61	8.44 ± 1.07	22.9 ± 1.23

Data are denoted as mean ± SD.
[a] MC-LR concentration in solutions was monitored respectively after 2, 6, 10, 12 and 14 d exposure.

toxic effects of endogenous or exogenous substances, were suggested to detoxify the absorbed MCs in the process of conjugation with toxins [14]. Besides the oxidative damage, exposure to MCs could led to reduction in chlorophyll content, alteration in pigment pattern and consequent inhibition of photosynthesis in plant [12,20].

V. natans (Lour.) Hara is a perennial submerged clonal macrophyte which is widely distributed in different freshwater habitats in China [22] and some waters in South and Southeast Asia Area (e.g. India, Malaysia, Vietnam, Nepal). It was selected due to its favorable experimental properties such as highly adaptive capability and easy to collect, culture and maintain in the laboratory. Frequent occurrence of cyanobacterial blooms may play an important role in the decline of submerged plants such as *V. natans* in some waters. However, the toxicological effects of MCs on *V. natans* at environmentally relevant concentrations remain unclear. Therefore, the aim of the present study was to investigate the uptake of MC-LR by and the related physiological and biochemical effects of MC-LR on *V. natans* at environmentally relevant concentrations.

2. Materials and methods

2.1. Chemicals and reagents

MC-LR (purity ≥ 96%) was purchased from Alexis Biochemicals (Läufelfingen, Switzerland). 1,2-dihydroxybenzene-3,5-disulfonic acid (Tiron), phenylmethanesulfonyl fluoride (PMSF), uranyl acetate and lead citrate were from Sigma Chemical (St. Louis, MO, USA). Methanol, acetone, acetonitrile and trifluoroacetic acid (TFA) with HPLC grade were from Tedia (Fairfield, OH, USA). Other reagents were analytical grade and obtained from chemical companies in China.

2.2. Plant material and exposure experiments

The sterilization and germination of *V. natans* seeds were carried out as described in Ref. [16]. After germination, *V. natans* seedlings were then cultured with modified 10% Hoagland nutrient solution (containing 6.0 mg L^{-1} K^+, 32.0 mg L^{-1} Ca^{2+}, 12.0 mg L^{-1} Mg^{2+}, 46.0 mg L^{-1} Cl^- and 63.0 mg L^{-1} SO_4^{2-}, pH 7.0–8.0), under a light/dark cycle of 12/12 h, a light intensity of 2000 lx and a temperature of 22 ± 1 °C.

When the seedlings grew up to 10–15 cm in length, plants with healthy and uniform growth were randomly selected to be treated with 0, 0.1, 0.5, 1.0, 5.0, 10.0 and 25.0 $\mu g\,L^{-1}$ MC-LR for 14 d. Plant seedlings were planted in aquariums (16 cm × 16 cm × 25 cm) with quartz sand (5 cm of thickness) at the bottom. Four replicates were performed in each treatment.

The nutrient solutions in the aquariums were renewed and amended every 48 h with MC-LR stock solution to maintain a constant concentration of MC-LR. MC-LR concentrations in nutrient solutions were monitored periodically and the results indicated that there were no remarkable differences between the measured and the added MC-LR concentrations in solutions during the experimental period (Table 1).

2.3. Microcystin analysis

To determine MC-LR concentrations in solutions, 1 L water sample was vacuum filtered using glass microfiber filters (47 mm, 1.2 μm, Whatman, England), concentrated by solid phase extraction using an ODS-C18 cartridge (Agilent AccuBOND, USA) and washed with 20% methanol. MC-LR in cartridge was eluted with 90% methanol containing 0.1% TFA, evaporated to dryness and reconstituted with methanol. Extracts were analyzed according to Aranda-Rodriguez et al. [23] using an HPLC (Agilent 1100 Series, USA) on a Zorbax Eclipse SB-C18 column (250 mm × 4.6 mm, 5 μm). MC-LR concentration in test solutions was monitored periodically and the results indicated that there were no remarkable differences between the measured and added initial MC-LR concentrations in test solutions during the experimental period.

To determine the uptake of MC-LR by *V. natans*, the pretreatment of leaf samples was performed according to Yin et al. [16] with minor modifications. Leaf tissues were grounded to fine powder under liquid nitrogen, and then extracted with 5% acetic acid followed by 90% methanol. The extracts were pooled, centrifuged at 15,000 rpm for 20 min and diluted with Milli-Q water before applied to Oasis HLB cartridges (Waters, Ireland). MC-LR in cartridge was eluted with 90% methanol and evaporated to dryness. The dried exacts were reconstituted with Milli-Q water and analyzed by ELISA (Microcystin plate kit, Institute of Hydrobiology, Chinese Academy of Sciences). All extraction and centrifugation were conducted at 4–8 °C.

2.4. $O_2^{\bullet -}$ determination

$O_2^{\bullet -}$ levels were determined by electron paramagnetic resonance (EPR) according to the method described by Lynch and Thompson [24] with some modifications. About 0.3 g fresh leaf tissues were grounded into a fine powder in a mortar under liquid nitrogen and homogenized in 50 mM CHES (N-(2-cyclohexylamino) ethanesulfonic acid) buffer (containing 10 mM Tiron and 0.5% (v/v) Tween-20, pH 8.6). All operations were performed in a sealing box that was purged continuously with nitrogen gas. Homogenates were centrifuged at 12,000 rpm for 20 min at 4 °C and the supernatant was used for EPR analysis. The EPR spectra were recorded with Bruker EMX 10/12 X-band spectrometer at a center field, 3470 G; scan range, 50 G; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; receiver gain, 2×10^4; scans, 1 times; microwave power, 20 mW. The EPR signals were used to calculate the height of the second peak and interpreted as intensity of $O_2^{\bullet -}$.

2.5. Enzyme extraction and activity assays

About 0.2 g frozen leaf tissues were crushed to a fine powder in a mortar under liquid nitrogen. The powder was resuspended in 2 mL 50 mM PBS, containing 1 mM EDTA, 1 mM PMSF, 5 mM ascorbic acid, 0.3% Triton X-100 and 4% polyvinylpyrrolidone, pH 7.8. The extracts were centrifuged at 12,000 rpm for 20 min. The supernatant was then divided into aliquots and stored at −80 °C for further analysis. All operations were performed at 0–4 °C.

SOD (EC 1.15.1.1) activity was determined according to the photochemical method as described by Giannopolitis and Ries [25].

Catalase (CAT, EC 1.11.1.6) activity was determined according to Bestwick et al. [26] with some modifications. Three millilitre reaction mixture contained 50 mM PBS (pH 7.0), 20 mM H_2O_2, and 50 μL of enzyme extracts. One CAT unit was defined as the amount of enzyme necessary to decompose half of the concentration of H_2O_2 in 100 s at 25 °C. POD (EC 1.11.1.7) activity was assayed according to Kochharm et al. [27]. GST (EC 2.5.1.18) activity was determined according to Habig et al. [28] using 1-chloro-2,4-dinitrobenzene as a substrate. In all cases total protein (Pr) content was measured according to the Bradford method [29] with bovine serum albumin as a standard.

2.6. Glutathione determination

The levels of GSH and oxidized glutathione (GSSG) in plant leaves were determined according to Hissin and Hilf [30] with some modifications. Frozen leaf tissues were extracted with 100 mM sodium phosphate–EDTA buffer (pH 8.0) and 6.5% trichloroacetic acid. The homogenates were centrifuged at 12,000 rpm for 20 min at 4 °C, and the supernatant was stored at −80 °C for further analysis.

GSH level in supernatant was measured fluorometrically after incubation with o-phthaldialdehyde in phosphate–EDTA buffer. The fluorescence intensity was recorded at 420 nm after excitation at 350 nm on a fluorescence spectrophotometer (Hitachi, Japan). To measure GSSG levels, an aliquot of 0.5 mL of the supernatant was incubated at room temperature with 200 μL of 0.04 M N-ethylmaleimide for 20 min.

2.7. MDA determination

The MDA content was measured using a commercialized chemical assay kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

2.8. Chlorophyll determination

Following extraction of leaves with 80% acetone, the chlorophyll (Chl *a* and Chl *b*) contents were determined according to Porra et al. [31].

2.9. Microscopic analysis

Rectangular segments (1 mm × 2 mm) were cut from the young leaf tips and the location to cut was chosen to include veins. Segments were immediately fixed in 0.2 M PBS (pH 7.2) with 4% (v/v) glutaraldehyde. The leaf samples were then rinsed in 0.1 M PBS, post-fixed in 1% OsO_4 in PBS, and rinsed with buffer again. Subsequently, they were dehydrated in graded acetone series and embedded in epon 812. Polymerization was conducted respectively at 30 °C for 24 h, 40 °C for 24 h and 60 °C for 48 h. Ultrathin sections were sliced with LKB-8800 Ultramicrotome, stained with uranyl acetate and lead citrate and examined under a H-600 transmission electron microscope (Hitachi, Japan).

In total, eight segments were cut from each group and the photographed cell in each cell layer was randomly chosen, but the location inside the cell was selected to include chloroplasts.

2.10. Data analysis

Data were reported as mean ± standard deviation (SD) and analyzed using one-way ANOVA. Significant differences between means were determined by *LSD-t* test. Modeling fitting was performed in Origin 8.0.

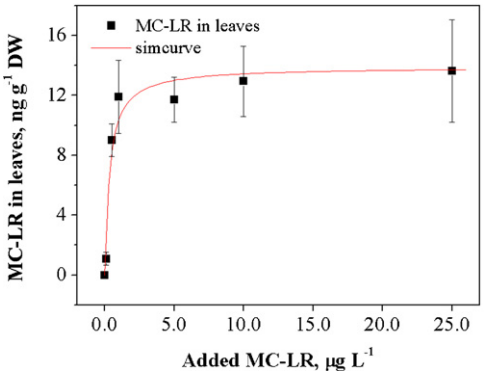

Fig. 1. MC-LR accumulation in leaves of *V. natans* seedlings exposed to MC-LR at different concentrations for 14 d. Data are denoted as mean ± SD ($n = 4$). MC-LR was not detected in the control group.

3. Results and discussion

3.1. Uptake of MC-LR by V. natans

Fig. 1 shows that the MC-LR accumulated in leaves increased and reached a plateau at MC-LR > 1.0 μg L^{-1}. To avoid the possibility of false positive results in determination of MC-LR by ELISA method, the leaf tissues extracts were further qualitatively analyzed by LC–ESI-MS. Both ELISA and ESI-LC–MS analyses (Fig. S1) showed the presence of MC-LR in *V. natans* leaves. The maximum value was 13.63 ± 3.42 ng g^{-1} DW at 25.0 μg L^{-1} MC-LR, which was 12.5 times higher than that at 0.1 μg L^{-1} MC-LR (1.09 ± 0.042 ng g^{-1} DW). However, when exposed to 10 mg L^{-1} MC-RR, it was reported that the toxin absorbed in *V. natans* leaves peaked at 7 d and the maximum amounts reached up to 0.3 ± 0.03 μg g^{-1} FW [16], which is about 110 times the maximum concentration of MC-LR accumulation in the current study (a coefficient of 5 was used to convert DW to FW). This great discrepancy may arise from the difference between the two types of toxins, but mainly from the difference in treatment dosage in the two studies: the treatment dosage of MC-RR in Yin et al. [16] was 400 times higher than that of MC-LR in the current study. The plateau observed in the present study may arise from the inhibition of transfer of toxins from roots to leaves. The inhibition was induced by roots damage under the exposure of MC-LR at high concentrations (>1.0 μg L^{-1}).

A Langmuir equation was applied to fit the MC-LR accumulation in leaves of *V. natans* seedlings exposed to MC-LR at different concentrations for 14 d:

$$q = \frac{q_m kC}{(1 + kC)}$$

where q is the uptake of MC-LR in leaves, ng g^{-1} DW; q_m is the possible maximum concentration of MC-LR absorbed by *V. natans* under environmentally relevant concentrations, ng g^{-1}; k is a constant, L μg^{-1}; and C is the exposed MC-LR concentration, μg L^{-1}.

The parameter estimates are as follows: 13.9 ng g^{-1} DW and 2.91 L μg^{-1} for q_m and k, respectively. Fig. 1 shows that Langmuir equation fits well for the uptake of MC-LR by *V. natans* (the residual sum of squares is 4.19). The ability of *V. natans* to take up MCs under environmentally relevant concentrations indicates that MCs may enter the food chain because *V. natans* is an important food source for many kinds of aquatic animals.

3.2. Effects of MC-LR on $O_2^{\bullet -}$ production

Endogenous ROS such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxy radical ($^{\bullet}OH$) are formed in many normal cellular reactions [32]. They also can be generated as by-

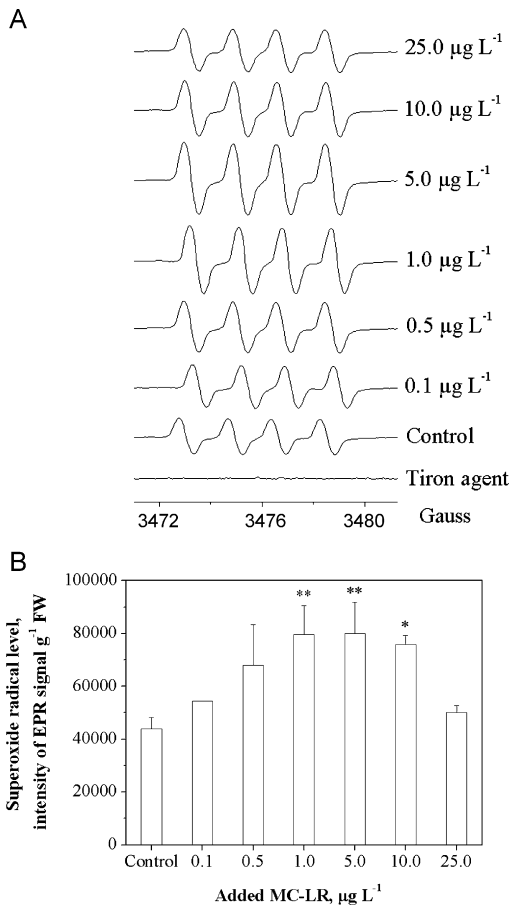

Fig. 2. Effect of MC-LR on $O_2^{\bullet-}$ production in the leaves of *V. natans* seedlings. (A) Tiron-$O_2^{\bullet-}$-EPR spectra detected in leaves of *V. natans* seedlings exposed to MC-LR at different concentrations for 14 d. (B) Signal intensity of Tiron-$O_2^{\bullet-}$ adducts in leaves of *V. natans* seedlings exposed to MC-LR at different concentrations for 14 d. Data are denoted as mean ± standard deviation ($n = 3$). Significant differences from the control are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

products of biotransformation reactions of xenobiotics or toxins. Few studies have been carried out to directly detect these short-lived ROS induced by MCs in aquatic plant tissues. So far, the spin trapping technique, followed by analysis of the resulting adducts by EPR spectroscopy, has been proved to be the most reliable and direct technique for detecting short-lived radicals *in vivo* [33]. In the present study, via spin trapping by EPR spectroscopy, the Tiron-$O_2^{\bullet-}$ adducts were detected in leaf tissues of *V. natnas* seedlings (Fig. 2A), indicating that MC-LR could stimulate the production of $O_2^{\bullet-}$. Because the whole trapping operation was performed in an oxygen-free system, the influence of oxygen is negligible.

As shown in Fig. 2B, after 14 d exposure, the signal intensity of $O_2^{\bullet-}$ increased with the increasing of MC-LR concentrations and reached a maximum at 5.0 μg L^{-1} MC-LR. When MC-LR concentration was above 5.0 μg L^{-1}, the signal intensity declined. Significant increases in the signal intensity of $O_2^{\bullet-}$ were observed at MC-LR concentrations from 1.0 to 10 μg L^{-1} ($p < 0.05$, $p < 0.01$). At the highest MC-LR concentration (25.0 μg L^{-1}), a sharp reduction in $O_2^{\bullet-}$ level was shown, which might be a result of antioxidant system responses or subcellular damages of membranes and cellular organs in mesophyll cells under MC-LR exposure (see Section 3.7).

$O_2^{\bullet-}$ will be generated if electrons are misdirected and donated to oxygen. The mitochondrial electron transport and the electron transport chain of the photosynthetic apparatus within chloroplasts are well known as the two sources of superoxide anion [32]. Therefore, injuries to these cellular organs may result in the disturbance of various cellular events and the reduction of $O_2^{\bullet-}$ levels. In addition to its direct deleterious effects on organisms, $O_2^{\bullet-}$ accumulation in *V. natans* seedlings can react with H_2O_2 in a Haber–Weiss reaction under catalysis of metal ions (such as iron) to form hydroxyl radicals. If the cellular antioxidant defense is deficient, the highly reactive hydroxyl radicals (and their derivatives) are able to rapidly attack macromolecules and result in LPO, proteins denaturation, and DNA mutation [32,34].

3.3. Effects of MC-LR on antioxidant enzymes

It is well known that organisms have developed an antioxidant system involving antioxidant enzymes or free-radical traps (e.g. GSH) as protection from oxidative stress. As shown in Table 2, SOD activities were elevated with increasing concentrations of MC-LR concentration and significant differences occurred at the MC-LR concentrations of 10.0 (105.3% of the control) and 25.0 μg L^{-1} (206.9% of the control). In addition, a good correlation was found between the SOD activities and the MC-LR concentrations ($r = 0.944$, $p < 0.05$). CAT activities increased significantly by 135.3% at the lowest MC-LR concentration (0.1 μg L^{-1}) and then decreased with the increasing concentrations of MC-LR. For POD activities, a similar trend to that of the SOD activities was observed and significant differences in POD activities were found at all levels of MC-LR concentrations between 0.5 and 25.0 μg L^{-1} except for the 5.0 μg L^{-1}.

Induction of antioxidant enzymes like SOD, CAT, POD and GST indicated the ongoing detoxification process. Oxidative stress in plants caused by MCs exposure has been documented previously for different species of spinach (*Spinacia oleracea* L.) variants, *Lepidium sativum*, *C. demersum* and *Medicago sativa* [13,19,35,36]. Elevation of SOD indicates an increase of $O_2^{\bullet-}$ production in plants, which dismutated to form H_2O_2 [37,38]. The induction of SOD resulted in an accumulation of H_2O_2 which in turn activated the CAT and POD. CAT was sensitive to low dose of MC-LR, but insensitive at higher MC-LR levels because of its poor efficiency in removing H_2O_2 at low levels and an inhibition effect by a reaction product of $O_2^{\bullet-}$, H_2O_2 and CAT [39]. GST plays a key role in the biotransformation of MCs to a glutathione conjugate and detoxification of the metabolites of lipid preoxidation [13,35,36]. Pflugmacher et al. [40] found that soluble GST in *C. demersum* was activated when MC-LR was above 0.5 μg L^{-1} after 7 d exposure. In the current study, significant promotion of GST activities at higher MC-LR levels indicated that the conjugation reaction occurred and therefore GSH would be necessary to act as a co-substrate.

3.4. Effects of MC-LR on glutathione content

As shown in Table 2, after 14 d exposure, GSH content in treated groups was slightly induced at 0.1 μg L^{-1} and then reduced with increasing MC-LR concentrations. Significant differences were observed for GSH content when MC-LR concentrations increased from 5.0 to 25.0 μg L^{-1} ($p < 0.05$, $p < 0.01$). GSSG content showed no significant differences in all treated groups as compared to the control. The GSH/GSSG ratios in all treated groups were significantly inhibited as compared to the control and reached a minimum value at the MC-LR concentration of 5 μg L^{-1}.

Glutathione is one of the most important components of antioxidant system in plants and the GSH/GSSG ratio is stable in the normal functioning of plant metabolism [13]. The initial increase of GSH content was probably because of synthesis of new GSH triggered by exposure to low concentrations of MC-LR. The subsequent decrease of GSH contents was considered to be related to forming glutathione conjugate via the GST system or nonenzymatically to detoxify MC-LR [11,14,41], or inhibition of the GSH synthesis in plants [36]. Furthermore, there is a transformation trend from GSH to GSSG under oxidative stress [38], remaining relative stable GSSG

Table 2
Biochemical responses of *V. natants* exposed to MC-LR.

Biochemical measurements	Added MC-LR concentrations ($\mu g\,L^{-1}$)						
	0.0	0.1	0.5	1.0	5.0	10.0	25.0
SOD (U mg^{-1} Pr)	1.89 ± 0.52	2.91 ± 0.96	3.17 ± 1.20	3.04 ± 0.62	3.49 ± 1.06	3.88 ± 0.70*	5.80 ± 0.43**
CAT (U mg^{-1} Pr)	17.2 ± 1.8	40.1 ± 0.4*	31.7 ± 8.1	27.4 ± 6.4	23.6 ± 3.7	17.2 ± 4.9	23.2 ± 8.4
POD (U mg^{-1} Pr)	318 ± 48	411 ± 15	431 ± 64*	418 ± 20*	409 ± 59	421 ± 21*	530 ± 51**
GST (U mg^{-1} Pr)	178 ± 35	161 ± 22	178 ± 26	136 ± 5	219 ± 32*	260 ± 18**	241 ± 10**
GSH ($\mu g\,mg^{-1}$ Pr)	5.21 ± 0.52	5.85 ± 0.99	4.59 ± 0.67	4.53 ± 0.38	3.90 ± 0.85*	3.68 ± 0.30**	3.37 ± 0.50**
GSSG ($\mu g\,mg^{-1}$ Pr)	4.65 ± 0.30	5.39 ± 0.20	4.92 ± 0.83	4.73 ± 0.33	4.65 ± 1.10	4.00 ± 0.30	3.84 ± 0.35
GSH/GSSG	1.05 ± 0.05	1.01 ± 0.06**	0.91 ± 0.03**	0.87 ± 0.08**	0.84 ± 0.02**	0.88 ± 0.02**	0.89 ± 0.05**
MDA (nmol TBARS mg^{-1} Pr)	2.79 ± 0.58	2.79 ± 0.46	3.07 ± 0.41	3.75 ± 0.34**	3.40 ± 0.41	3.39 ± 0.22	1.88 ± 0.13*
Chl *a* ($mg\,g^{-1}$ FW)	1.05 ± 0.02	0.99 ± 0.02	1.04 ± 0.01	1.00 ± 0.06	1.02 ± 0.04	0.95 ± 0.04*	0.90 ± 0.01**
Chl *b* ($mg\,g^{-1}$ FW)	0.38 ± 0.05	0.35 ± 0.03	0.42 ± 0.03	0.47 ± 0.09	0.44 ± 0.05	0.41 ± 0.01	0.49 ± 0.08*

Data are denoted as mean ± SD ($n = 4$). Significant differences from the control are indicated as *($p < 0.05$) and **($p < 0.01$).

contents. It is worth noting that the cell membrane damage and consequent GSH efflux, as previously proposed by Ding et al. [42], would also contribute to the depletion of intracellular GSH under the exposure of high concentrations of MC-LR. Besides the loss of detoxification capability, the alteration in intracellular GSH can also alter the redox status of the cell [43] and therefore may favor ROS generation and disturb the related signaling pathways. The depletion of GSH contents resulted in the reduction of the GSH/GSSG ratio and limited the ability of *V. natans* seedlings to detoxify MC-LR.

3.5. Effects of MC-LR on lipid peroxidation

Evidences showed that elevated LPO, indicative of oxidative damage, is an important aspect of the toxicity of MCs to aquatic plants [12,13,18,20]. In the current study, the LPO level in *V. natans* leaf tissues was determined by the production of MDA, which is commonly used as an indicator of oxidative stress in biological systems [44]. Results showed that MDA level was significantly increased at the MC-LR concentration of 1.0 $\mu g\,L^{-1}$ ($p < 0.01$) compared to the control (Table 2), indicating the plant suffered from oxidative damage. However, MDA level showed a significant decrease at 25.0 $\mu g\,L^{-1}$ of MC-LR, which might relate to the subcellular damage of membrane structure in mesophyll cells at this concentration. Normally, MDA levels can be well correlated with ROS levels [38,45]. A similar result was also observed in the current study ($r = 0.817$, $p < 0.05$) (Fig. 3), indicating that there exists a close connection between MDA production and cellular redox status.

In the current study, we found an apparent dualistic response of MDA level in plants toward the MC-LR treatment, which was not reported in previous studies. In addition, among the other investigated parameters, changes of GST activity and GSH content could also be recognized in a dualistic pattern. In fact, a known dualistic effect of MC on animal cells has been well investigated, with both apoptosis and increased cellular proliferation being reported, which is believed to be the result of inhibition of PP1 and PP2A [46]. In this study, we infer that ROS or the cellular redox may play a critical role in the dualistic responses of these oxidative stress biomarkers. The low-intensity or the high-intensity responses apparently depend on the MC-LR exposure concentrations, indicating a limit tolerance of *V. natans* to relatively high MC-LR concentrations.

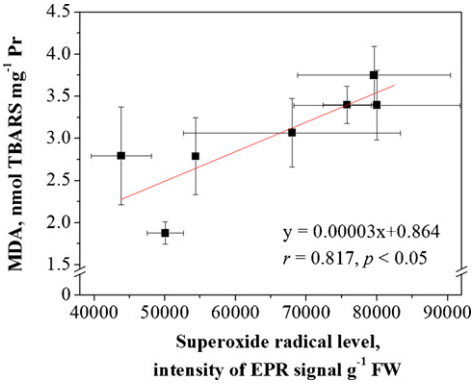

Fig. 3. Correlation between $O_2^{\bullet -}$ levels and MDA levels in leaves of *V. natans*.

3.6. Effects of MC-LR on chlorophyll content

Although there was no death of *V. natans* seedlings in all treated groups after 14 d exposure to MC-LR, retarded growth, declined root length and root hairs, as well as a phenomenon of pigment bleaching were observed. A decrease of Chl *a* content (significant at MC-LR concentrations of 10.0 and 25.0 $\mu g\,L^{-1}$) accompanied by an increase of Chl *b* content (significant at the MC-LR concentration of 25.0 $\mu g\,L^{-1}$) was observed (Table 2), indicating that MC-LR caused a pigment pattern change. A similar effect on chlorophyll was observed by exposing *C. dermesum* to MC-LR of 0–5.0 $\mu g\,L^{-1}$ for 24 h, and a clear dose-dependent switch from Chl *a* to Chl *b* was also indicated [12]. The decrease of Chl *a* and related damages in the chloroplasts (see Section 3.7) under the environmentally relevant concentrations suggest that MCs, as secondary metabolites of cyanobacteria, do act as allelochemicals in the aquatic plant–algae interactions.

3.7. Effects of MC-LR on ultrastructure of mesophyll cells

So far, little attention was paid to ultrastructural alterations of aquatic plants exposed to MC-LR under the environmentally relevant concentrations. Fig. 4A and B shows mesophyll cells of young leaves from the control group. Organelles in the cytoplasm were abundant and chloroplasts, mitochondria, and golgi apparatus were significant with normal and intact morphology. Most chloroplasts are located near the cell wall with a few osmiophilic granules within them. Thylakoid lamellae arranged in order and could be observed easily. Note that the ingrown cell wall extending into the cytoplasm formed lots of wall ingrowths. After exposing to 0.1 $\mu g\,L^{-1}$ MC-LR for 14 d, only the number of osmiphilic granules increased slightly and a slight plasmolysis could be observed in some cells (Fig. 4C). However, when treated with 1.0 $\mu g\,L^{-1}$ MC-LR (Fig. 4D–F), a series of morphological changes, such as plasmolysis, swollen chloroplasts with partly disappeared outer membrane, distended or indistinct thylakoid lamellae and condensed chromatin, could be observed. It was also found that mitochondria swelling and cristae vague appeared in the cells. The degree of cell and organelle structural damages increased as MC-LR concentrations increased. At 10.0 or 25.0 $\mu g\,L^{-1}$, the number of mitochondria kept decreasing,

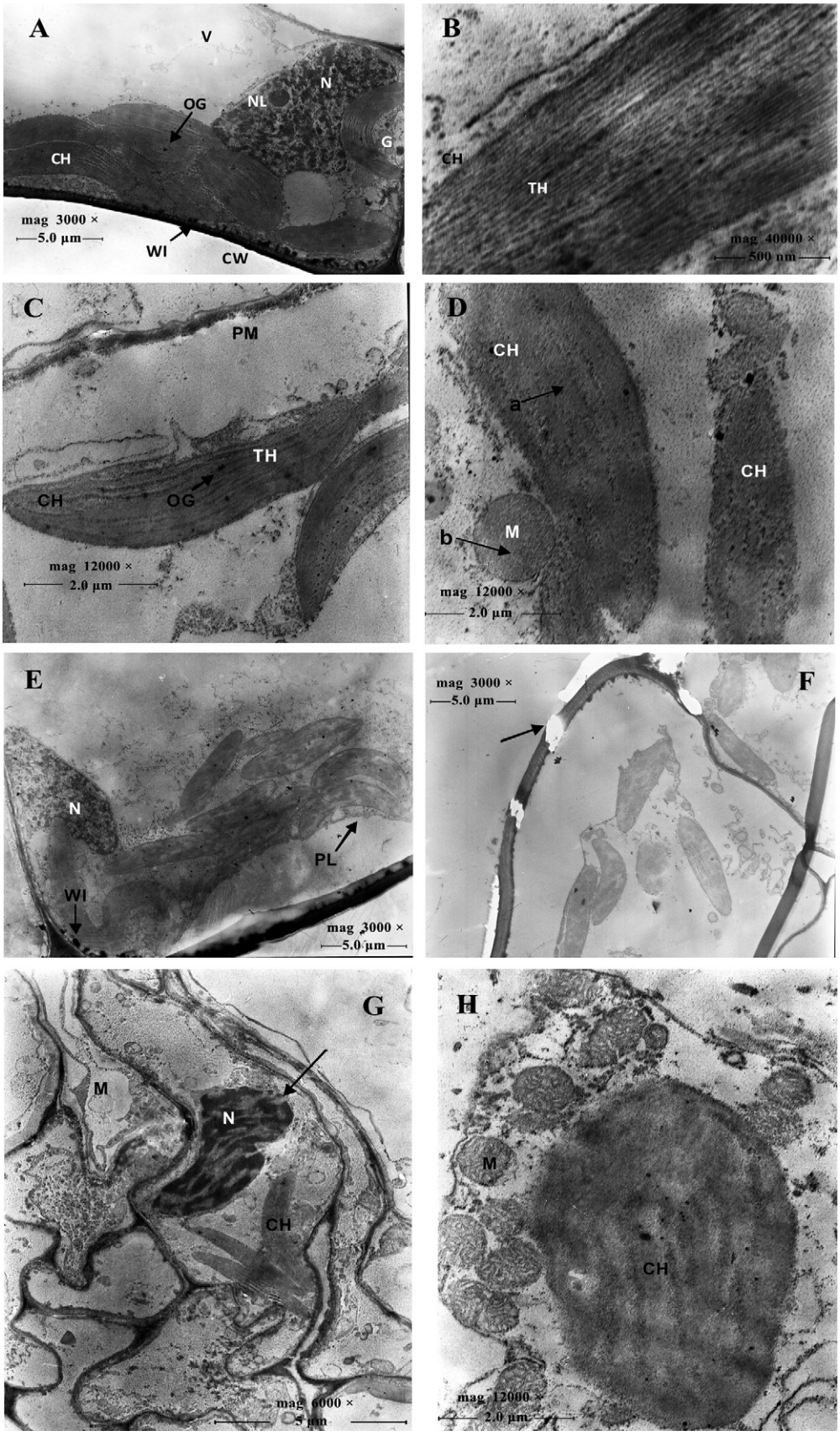
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Fig. 4.

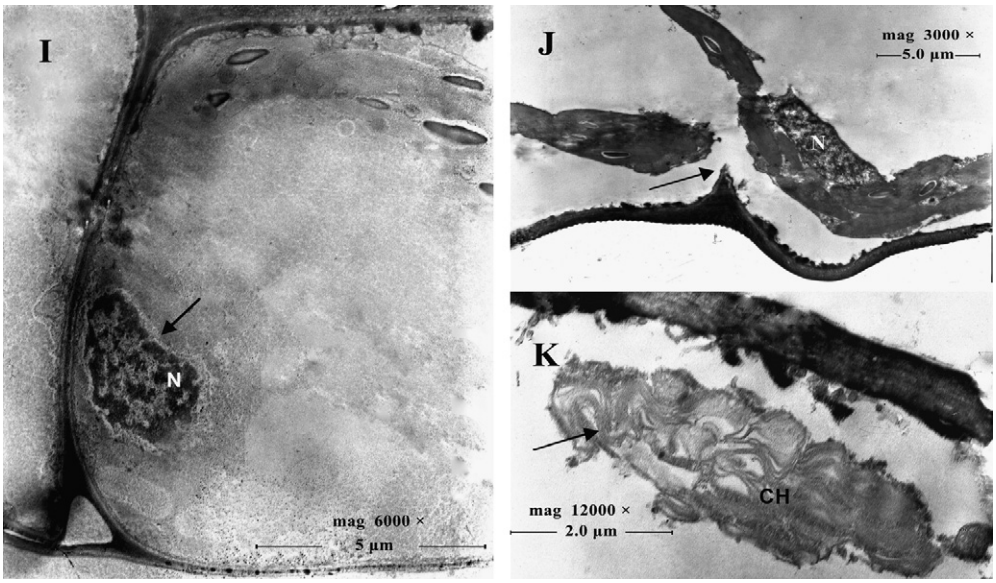

Fig. 4. Effects of MC-LR on ultrastructure in mesophyll cells of *V. natans* after exposure to MC-LR for 14 d. (A) Part of mesophyll cell of *V. natans* in control group. Note intact organelles and membranes and lots of wall ingrowths. (B) Chloroplast in control group with thylakoid lamellae arranged in order. (C) Part of mesophyll cell of *V. natans* exposed to 0.1 $\mu g\,L^{-1}$ MC-LR. Note the increased number of osmiphilic granules. (D) Chloroplast and mitochondria in a mesophyll cell of *V. natans* exposed to 1.0 $\mu g\,L^{-1}$ MC-LR. Note swollen chloroplast with distended and indistinct thylakoid lamellae (arrow a) and swollen mitochondria with distended vague cristae (arrow b). (E) Plasmolysis (arrow) and (F) part of broken cell wall (arrow) and damaged organelles in a mesophyll cell of *V. natans* exposed to 1.0 $\mu g\,L^{-1}$ MC-LR. (G) Note the karyopyknosis and chromatin condensation (arrow) and (H) distorted cell wall, and seriously damaged chloroplast and mitochondria in mesophyll cells of *V. natans* exposed to 10.0 $\mu g\,L^{-1}$ MC-LR. (I) Note the strongly chromatin condensation (arrow) and (J) distorted or even broken cell wall (arrow) and (K) totally damaged chloroplast (arrow) in mesophyll cells of *V. natans* exposed to 25.0 $\mu g\,L^{-1}$ MC-LR. Abbreviations: CH, chloroplast; CW, cell wall; G, golgi apparatus; M, mitochondria; N, nucleus; NL, nucleolus; OG, osmiophilic granules; P, plasmamembrane; PL, plasmolysis; Th, thylakoids.

most structure of chloroplasts and mitochondria were disrupted, outer membrane disappeared, severe plasmolysis, karyopyknosis and chromatin condensation appeared, and the cell wall became distorted or even broken in some cells at 25.0 $\mu g\,L^{-1}$ (Fig. 4G–K). MC-LR induced damage to morphology of mesophyll cells was further supported by the scanning electron microscope observations on leaves surface of *V. natans* exposed to MC-LR for 14 d (Fig. S2). Considering that MC-LR is a potent inhibitor of PP1 and PP2A, these subcellular damages might relate to the disturbance of MC-LR on protein phosphorylation and the consequent induction of oxidative stress. It is worth noting that the severe damage to cellular organs like chloroplasts and mitochondria induced by high level MC-LR exposure may explain the reduction of $O_2^{\bullet -}$ as mentioned in Section 3.2.

3.8. Proposed biochemical response pathway of *V. natans* to MC-LR

As Fig. 5 shows, forming glutathione conjugates via the GST system or nonenzymatically is the first step for plants to detoxify MC-LR after uptake in mesophyll cells of *V. natans*. Then the plant transfers the conjugate into vacuoles for storage and further processing [12,47]. In mesophyll cells, MC-LR can disturb the normal protein phosphorylation balance to cause a series of toxic

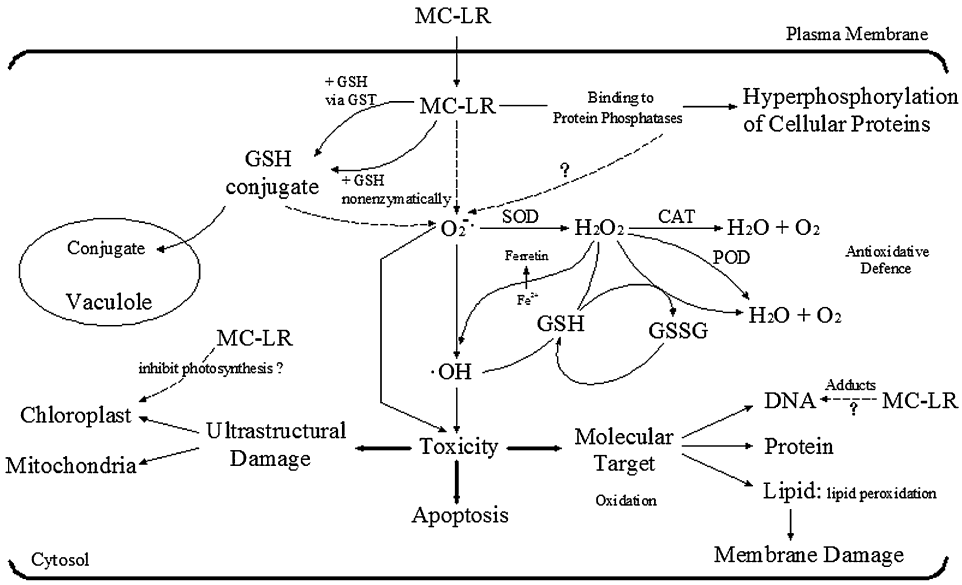

Fig. 5. Proposed biochemical response pathway after uptake of MC-LR in mesophyll cells of *V. natans*.

effects after binding to protein phosphatases and may therefore favor ROS production in an unspecific way. Enhanced formation of ROS can in turn trigger the responses of the antioxidant system, involving antioxidant enzymes or glutathione for protection from oxidative stress. A series of negative effects occur under the disturbance of protein phosphorylation and oxidative stress upon MC-LR exposure. These negative effects include increased MDA content, decreased Chl *a* content and induction of subcellular damages. Further research should pay more attention to the witnessed decrease of the $O_2^{\bullet -}$ production and MDA level at high concentration of MC-LR ($25\ \mu g\ L^{-1}$). This phenomenon indicates that ROS formation and related oxidative damage are results from complex cascades of cellular events in response to MCs. Other more toxic effects should be investigated to clarify the toxicity mechanism of MCs on aquatic plants at environmentally relevant concentrations. For instance, exposure to MCs may also be capable of causing apoptosis, a well accepted MC-induced process in plants and animal cells, which is believed to be closely related to excessive formation of ROS and the subsequent induction of mitochondrial permeability transition [46,48,49].

4. Conclusion

The uptake and accumulation of MC-LR in leaves of *V. natans* seedlings showed that MC-LR may enter the food chain. Low concentrations of MC-LR can lead to ROS production and oxidative stress in *V. natans*, which is evidenced by changes in the activities of SOD, CAT, POD, GST, glutathione content and MDA content. Among these parameters, the CAT activity and GSH/GSSG ratio are sensitive to low concentration of MC-LR ($0.1\ \mu g\ L^{-1}$). In addition, exposure to MC-LR can result in decrease of Chl *a* concentration and induce ultrastructural damage of mesophyll cells of *V. natans*. The severity of cell and organelle structural damages increased as MC-LR concentrations increased. Considering the oxidative effects and other adverse effects assessed in this study, the critical toxic threshold of MC-LR to *V. natans* at the subcellular and molecular level is between 0.5 and $1.0\ \mu g\ L^{-1}$. These results indicate that even when the cyanobacterial blooms have not occurred in a large scale, low concentrations of MCs may still exert a negative ecological impact on aquatic plants.

Acknowledgements

This work was supported by the National Basic Research Program of China (2008CB418102) and the Key Special Program on the S&T for the Pollution Control and Treatment of Water Bodies (2008ZX07316-004).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.03.023.

References

[1] G.A. Codd, Cyanobacterial toxins: occurrence, properties and biological significance, Water Sci. Technol. 32 (1995) 149–156.
[2] K. Sivonen, Cyanobacterial toxins and toxin production, Phycologia 35 (1996) 12–24.
[3] Q.L. Wu, P. Xie, L.Y. Yang, G. Gao, Z.W. Liu, G. Pan, B.Z. Zhu, Ecological consequences of cyanobacetrial blooms in lakes and their countermeasures, Adv. Earth Sci. (in Chinese) 23 (2008) 1115–1123.
[4] D.R. Dietrich, S.J. Hoeger, Guidance values for microcystin in water and cyanobacterial supplement products (blue-green algae supplements): a reasonable or misguided approach? Toxicol. Appl. Pharmacol. 203 (2005) 273–289.
[5] S. Pérez, D.S. Aga, Recent advances in the sample preparation, liquid chromatography tandem mass spectrometric analysis and environmental fate of microcystins in water, TrAC Trends Anal. Chem. 24 (2005) 658–670.
[6] K. Lahti, J. Rapala, M. Färdig, M. Niemelä, K. Sivonen, Persistence of cyanobacterial hepatotoxin microcystin-LR in particulate material and dissolved in lake water, Water Res. 31 (1997) 1005–1012.
[7] WHO, Guidlines for drinking-water quality, in: Health Criteria and Other Supporting Information, Second ed., World Health Organization, Geneva, 1998 (addendum to vol. 2).
[8] W.W. Carmichael, Cyanobacteria secondary metabolites—the cyanotoxins, J. Appl. Bacteriol. 72 (1992) 445–459.
[9] M.T. Casanova, M.D. Burch, M.A. Brock, P.M. Bond, Does toxic *Microcystis aeruginosa* affect aquatic plant establishment? Environ. Toxicol. 14 (1999) 97–109.
[10] S. Körner, Development of submerged macrophytes in the shallow Lake Müggelsee (Berlin, Germany) before and after its switch to the phytoplankton-dominated state, Arch. Hydrobiol. 152 (2001) 395–409.
[11] C. Wiegand, S. Pflugmacher, Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review, Toxicol. Appl. Pharmacol. 203 (2005) 201–218.
[12] S. Pflugmacher, Possible allelopathic effects of cyanotoxins, with reference to microcystin-LR, in aquatic ecosystems, Environ. Toxicol. 17 (2002) 407–413.
[13] S. Pflugmacher, Promotion of oxidative stress in the aquatic macrophyte *Ceratophyllum demersum* during biotransformation of the cyanobacterial toxin microcystin-LR, Aquat. Toxicol. 70 (2004) 169–178.
[14] S. Pflugmacher, C. Wiegand, A. Oberemm, K.A. Beattie, E. Krause, G.A. Codd, C.E.W. Steinberg, Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication, Biochem. Biophys. Acta 1425 (1998) 527–533.
[15] S. Pflugmacher, C. Wiegand, K.A. Beattie, G.A. Codd, C.E.W. Steinberg, Uptake of the cyanobacterial hepatotoxin microcystin-LR by aquatic macrophytes, J. Appl. Bot. 72 (1999) 228–232.
[16] L.Y. Yin, J.Q. Huang, D.H. Li, Y.D. Liu, Microcystin-RR uptake and its effects on the growth of submerged macrophyte *Vallisneria natans* (Lour.) Hara, Environ. Toxicol. 20 (2005) 308–313.
[17] C. Mackintosh, K. Beattie, S. Klumpp, P. Cohen, G.A. Codd, Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants, FEBS Lett. 264 (1990) 187–192.
[18] Z.Q. Hu, Y.D. Liu, D.H. Li, A. Dauta, Growth and antioxydant system of the cyanobacterium *Synechococcus elongatus* in response to microcystin-RR, Hydrobiologia 534 (2005) 23–29.
[19] S. Pflugmacher, K. Jung, L. Lundvall, S. Neumann, A. Peuthert, Effects of cyanobacterial toxins and cyanobacterial cell-free crude extract on germination of alfalfa (*Medicago sativa*) and induction of oxidative stress, Environ. Toxicol. Chem. 25 (2006) 2381–2387.
[20] J. Leflaive, L. Hage-Ten, Algal and cyanobacterial secondary metabolites in freshwaters: a comparison of allelopathic compounds and toxins, Freshwater Biol. 52 (2007) 199–214.
[21] S.M. Mitrovic, O. Allis, A. Furey, K.J. James, Bioaccumulation and harmful effects of microcystin-LR in the aquatic plants *Lemna minor* and *Wolffia arrhiza* and the filamentous alga *Chladophora fracta*, Ecotoxicol. Environ. Saf. 61 (2005) 345–352.
[22] C. Wang, S.H. Zhang, P.F. Wang, J. Hou, W. Li, W.J. Zhang, Metabolic adaptations to ammonia-induced oxidative stress in leaves of the submerged macrophyte *Vallisneria natans* (Lour.) Hara, Aquat. Toxicol. 87 (2008) 88–98.
[23] R. Aranda-Rodriguez, A. Tillmanns, F.M. Benoit, F.R. Pick, J. Harvie, L. Solenaia, Pressurized liquid extraction of toxins from cyanobacterial cells, Environ. Toxicol. 20 (2005) 390–396.
[24] D.V. Lynch, J.E. Thompson, Lipoxygenase-mediated production of superoxide anion in senescing plant tissue, FEBS Lett. 173 (1984) 251–254.
[25] C.N. Giannopolitis, A.K. Ries, Superoxide dismutase. I. Occurrence in higher plants, Plant Physiol. 59 (1977) 309–314.
[26] C.S. Bestwick, A.L. Adam, N. Puri, J.W. Mansfield, Characterisation of and changes to pro- and anti-oxidant enzyme activities during the hypersensitive reaction in lettuce (*Lactuca satia* L.), Plant Sci. 161 (2001) 497–506.
[27] S. Kochharm, V.K. Kochhar, S.D. Khanduja, Changes in the pattern of isoperoxidases during maturation of grape berries cv. Gulabi as affected by ethephon (2-chloroethyl phosphoric acid), Am. J. Enol. Vitic. 30 (1979) 275–277.
[28] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione S-transferases: the first enzymatic step in mercapturic acid formation, J. Biol. Chem. 249 (1974) 7130–7139.
[29] M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[30] P.J. Hissin, R. Hilf, A fluorometric method for determination of oxidized and reduced glutathione in tissues, Anal. Biochem. 74 (1976) 214–226.
[31] R.J. Porra, W.A. Thompson, P.E. Kriedemann, Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy, Biochim. Biophys. Acta 975 (1989) 384–394.
[32] C. Bowler, M.V. Montagu, D. Inze, Superoxide dismutase and stress tolerance, Annu. Rev. Plant Physiol. Plant Mol. Biol. 43 (1992) 83–116.
[33] K. Takeshita, K. Fujii, K. Anzai, *In vivo* monitoring of hydroxyl radical generation caused by X-ray irradiation of rats using the spin trapping/EPR technique, Free Radic. Biol. Med. 36 (2004) 1134–1143.
[34] E. Cadenas, Biochemistry of oxygen toxicity, Annu. Rev. Biochem. 58 (1989) 79–110.

[35] S. Pflugmacher, M. Aulhorn, B. Grimm, Influence of a cyanobacterial crude extract containing microcystin-LR on the physiology and antioxidative defence systems of different spinach variants, New Phytol. 175 (2007) 482–489.
[36] J. Stüven, S. Pflugmacher, Antioxidative stress response of *Lepidium sativum* due to exposure to cyanobacterial secondary metabolites, Toxicon 50 (2007) 85–93.
[37] R. Mittler, Oxidative stress, antioxidants and stress tolerance, Trends Plant Sci. 7 (2002) 405–410.
[38] Y Yin, X.R. Wang, Y.Y. Sun, H.Y. Guo, D.Q. Yin, Bioaccumulation and oxidative stress in submerged macrophyte *Ceratophyllum demersum* L. upon exposure to pyrene, Environ. Toxicol. 23 (2007) 328–336.
[39] K. Asada, The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 601–639.
[40] S. Pflugmacher, G.A. Codd, C.E.W. Steinberg, Effects of the cyanobacterial toxin microcystin-LR on detoxication enzymes in aquatic plants, Environ. Toxicol. 14 (1999) 111–115.
[41] S. Takenaka, Covalent glutathione conjugation to cyanobacterial hepatotoxin microcystin-LR by F344 rat cytosolic and microsomal glutathione S-transferases, Environ. Toxicol. Pharmacol. 9 (2001) 135–139.
[42] W.X. Ding, H.M. Shen, C.N. Ong, Microcystic cyanobacteria extract induces cytoskeletal disruption and intracellular glutathione alteration in hepatocytes, Environ. Health Perspect. 108 (2000) 605–609.
[43] L.L. Amado, J.M. Monserrat, Oxidative stress generation by microcystins in aquatic animals: why and how, Environ. Int. 36 (2010) 226–235.
[44] J.M.C. Gutteridge, B. Halliwell, The measurement and mechanism of lipid peroxidation in biological systems, Trends Biochem. Sci. 35 (1990) 129–135.
[45] C.R. Wang, X.R. Wang, Y. Tian, H.X. Yu, X.Y. Gu, W.C. Du, H. Zhou, Oxidative stress, defense response, and early biomarkers for lead-contaminated soil in *Vicia faba* seedlings, Environ. Toxicol. Chem. 27 (2008) 970–977.
[46] M.M. Gehringer, Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response, FEBS Lett. 557 (2004) 1–8.
[47] J.O.D. Coleman, M.M.A. Blakekalff, T.G.E. Davis, Detoxification of xenobiotics by plants: chemical modifications and vacuolar compartmentation, Trends Plant Sci. 2 (1997) 144–151.
[48] W.M. Huang, W. Xing, D.H. Li, Y.D. Liu, Microcystin-RR induced apoptosis in tobacco BY-2 suspension cells is mediated by reactive oxygen species and mitochondrial permeability transition pore status, Toxicol. In Vitro 22 (2008) 328–337.
[49] A. Campos, V. Vasconcelos, Molecular mechanisms of microcystin toxicity in animal cells, Int. J. Mol. Sci. 11 (2010) 268–287.