Possible Allelopathic Effects of Cyanotoxins, with Reference to Microcystin-LR, in Aquatic Ecosystems

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ABSTRACT: During recent years a shift from macrophyte-dominated to more phytoplankton-dominated lakes has been correlated to the process of eutrophication. The existence of numerous substances exhibiting allelopathic effects on the growth of algae has been shown in different aquatic macrophytes (e.g., *Myriophyllum spicatum*) and is thought to be an important mechanism in stabilizing the macrophyte-dominated clear-water state of a lake. A few recent studies have shown that algae themselves can produce special substances inhibiting growth or photosynthetic processes in other algae. A well-known cyanobacterial secondary metabolite, microcystin-LR, was tested for its allelopathic power on aquatic macrophytes such as *Ceratophyllum demersum* and *Myriophyllum spicatum*, resulting in growth inhibition, reduction in photosynthetic oxygen production, and changes in pigment pattern. This shows that microcystin-LR has a possible role as an allelopathic infochemical. Environ Toxicol 17: 407–413, 2002; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/tox.10071

Keywords: allelopathy; cyanobacterial toxins; photosynthesis; aquatic macrophytes

INTRODUCTION

The phenomenon of chemical interactions among plants, fungi, bacteria, and animals has attracted researchers from all over the world. This special type of communication mainly occurs by organisms releasing or secreting their own chemical substances into a surrounding medium to create an interrelationship among different compartments of the ecosystem.

The term *allelopathy* originally referred to the reciprocal suffering of two organisms, but it is now commonly used as a term for interactions betweens plants plant biochemicals. According to Rice (1984), "Allelopathy is the direct or indirect (harmful or beneficial) effects of a plant (including microbes) on another plant through the release of compounds that escape into the environment".

Almost all plants and many tissues such as leaves, stem, roots, flowers, fruits, and seeds have substances for chemical communication. Einhelling and Leather (1988) used sorghum and the seedlings of the soybean *Glycine max* in an bioassay to demonstrate the inhibitory effects of the Jerusalem artichoke *Helianthus tuberosus*. Most inhibitory compounds in plants are phenolics such as syringic, caffeic, and protocatechuic acids. But allelopathy is not limited to terrestrial ecosystems; it can also exist in aquatic ecosystems. Submerged and floating macrophytes as well as algae play a central role in many aquatic ecosystems. Because they are the main primary producers in oligotrophic habitats, the entire food web depends on their vegetation. It is well known that many of these plant species are able to produce substances for making interrelationships. For example, rice

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(*Oryza sativa*) planted twice a year in a monoculture system reduced the second crop yield by about 25% (Chou, 1990). The phytotoxic compounds detected were identified as *p*-hydroxybenzoic, syringic, vanillic, ferulic, and butyric acids. The most active compounds from rye residues were found to be hydroxamic acids, which occur as glycosides. These compounds are formed from the glucosyl conjugate of 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one upon injury or death of the rye plant (Barnes et al., 1987). Allelopathic activity among phytoplankton in eutrophied systems was detected after examining the sequence of the dominant algal species in an algal bloom. Among these algae the blue-green algae (cyanobacteria) play an important role. Almost all the natural compounds that cause allelopathy in fact are a subset of the array of secondary products synthesized by plants, algae, or microorganisms (Rice, 1984). One of the serious impacts of eutrophication of aquatic ecosystems is the disappearance of submerged macrophytes and the shift to a phytoplankton-dominated state (Körner, 2001). The inhibition of algal growth by tissues of several lake macrophytes has been shown by several groups of researchers (Anthoni et al., 1980; Wium-Andersen et al., 1982; Gross et al., 1996; Nakai et al., 1999). The reverse phenomenon is explored here.

Cyanobacteria produce a wide range of secondary metabolites, many of which have adverse effects on mammals, birds, and fish. These cyanobacteria increasingly are being recognized as a potent stress factor and health hazard in aquatic ecosystems (Tencalla and Dietrich, 1997; Rohrlack et al., 1999; Wiegand et al., 1999). Microcystins, one of the main groups of cyanobacterial secondary metabolites, are largely retained within the producer cells during cyanobacterial bloom development (Park et al., 1998). However, these toxins are released into the surrounding medium not only by senescence and lysis of these blooms but also by an active release of toxin, as shown by some researchers (Rapala et al., 1997; Park et al., 1998; Böttcher et al., 2001). An uptake of these substances into other plants has already been shown in the aquatic macrophytes *Ceratophyllum demersum*, *Elodea canadensis*, *Vesicularia dubyana,* and *Phragmites australis.* The amount of uptake ranged from 1.0–120.0 pg/g of fresh weight (FW) after 7 days of exposure to 2.5 mg/L of ^{14}C-MC-LR (Pflugmacher et al., 1999, 2001). The highest uptake was found in the common reed plant *Phragmites australis*.

Every summer, monitoring has been done in many of the lakes of Berlin and Brandenburg, Germany, of the mass development of cyanobacteria at varying concentrations and in varying forms of secondary metabolites, or cyanotoxins (Fastner et al., 1995). In several of these lakes none of the higher aquatic plants such as pondweed (*Potamogeton* sp.), rigid hornwort (*Ceratophyllum demersum*), spatterdock (*Nuphar luteum*), or even the very common duckweed (*Lemna* sp.) could be found. The absence of these plants raises the question: can cyanobacterial toxins act as allelopathic chemicals in the aquatic ecosystem?

MATERIALS AND METHODS

Plant Material

Ceratophyllum demersum and *Myriophyllum spicatum* were taken from cultures that had been grown continuously for 5 years at 20°C under a day/night cycle of 14 h/10 h, with a light intensity of 150 μE m^{-2} s^{-1} in modified Provasoli's medium (Pflugmacher and Steinberg, 1997). *Cladophora* sp., *Elodea canadensis,* and *Phragmites australis* were collected from ponds near the institute and cultivated for 2 month in Provasoli′s medium prior to the experiments.

Measurement of Photosynthetic Oxygen Production

The measurement of macrophyte photosynthetic oxygen production was performed using a Phosy-Mess 4000 (INNOConcept, Berlin), a system for determining the vitality of plants by the amount of oxygen released during photosynthesis. The oxygen measurement is conducted directly on the plant surface by a Clark probe WTW EO 196-1.5 oxygen electrode, measurement light of 630–650 nm at a light intensity of 36 μE m^{-2} s^{-1} with a dark/light/dark cycle of 10 min/12 min/10 min under a constant temperature of 20°C. The rate of photosynthesis was calculated in micromoles of oxygen per hour per gram of fresh weight of plant material. To attain the measurements, 12 independent replicates were done for each plant type used.

Pigment Analysis

Analysis of chlorophyll *a* and *b* content was carried out according to Inskeep and Bloom (1985), using 0.5 mg of plant material and 2.5 mL of *N,N*-dimethylformamide (*N,N*-DMF). Samples were kept in the dark at 4°C for 3 days, until the plant material was completely decolored. The extracts were centrifuged at 5000 × g, and the absorption (O.D.) of the supernatant at 647 and 664.5 nm was measured against *N,N*-DMF. The following formulae were used for calculation of the chlorophyll content:

$$\text{chlorophyll } a = [12.7 \times \text{O.D.}_{664.5}] - [2.79 \times \text{O.D.}_{647}]$$

$$\text{chlorophyll } b = [20.7 \times \text{O.D.}_{647}] - [4.62 \times \text{O.D.}_{664.5}]$$

For all pigment analyses 12 independent replicates were performed.

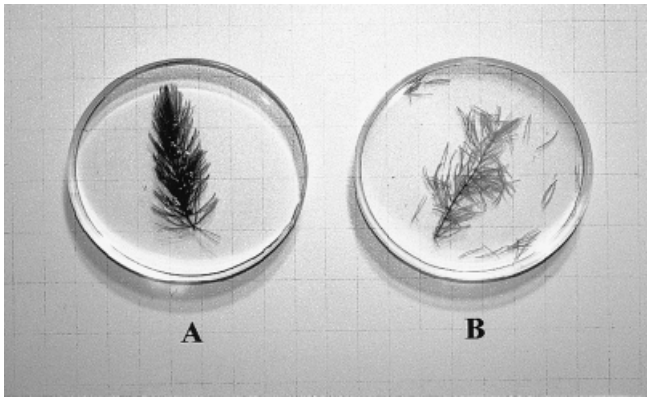

Fig. 1. *Ceratophyllum demersum* after a 24-h exposure to microcystin-LR at a concentration of 5.0 mg/L: (a) control plant, (b) exposed plant. During exposure loss of pigmentation in and of leaves from *C. demersum* was observed, ultimately leading to the plant's death.

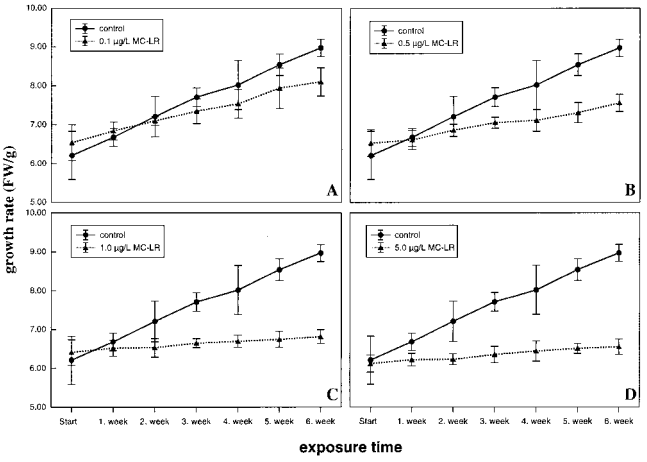

Fig. 2. Growth rates (FW/g) of *Ceratophyllum demersum* with several concentrations of microcystin-LR: (a) 0.1 μg/L, (b) 0.5 μg/L, (c) 1.0 μg/L, and (d) 5.0 μg/L. Significant growth inhibition was found at all concentrations tested but significance differences in starting times of total exposure also was found. Significance was tested using the ANOVA and Newman–Keuls tests ($p < 0.05$).

Determination of Cyanobacterial Toxins

Microcystin concentrations were determined after freeze-drying the sample and dissolving it in a 70:30 mixture of methanol and water by high-performance liquid chromatography (HPLC) using the method described in Pflugmacher et al. (1998). Briefly, the analysis of microcystin-LR (MC-LR) was performed using a Waters HPLC system, a 5-μm ODS LiChrospher 100, 4.6 i.d. $\times$ 250 mm (Merck, Darmstadt, Germany). Detection was at 238 nm, column temperature was maintained at 40°C, and the injection volume was 80 μL. Microcystin-LR was purchased from Alexis Biochemicals (Grünberg, Germany). A gravimetric standard of MC-LR for calibration was a gift from G. A. Codd (University of Dundee, Scotland).

RESULTS

After exposing *Ceratophyllum demersum*, a common lake macrophyte, to a nonenvironmentally relevant concentration of MC-LR (5 mg/L) for 24 h, the plant died. Pigment bleaching from dark green to pale yellow was observed, as well as the loss of its leaves (Fig. 1). To do growth experiments at environmentally relevant MC-LR concentrations, a range of 0.1–5.0 μg/L was selected. The lowest of these concentrations to show a significant reduction in growth ($p < 0.05$) only after 6 weeks' exposure was 1.0 μg/L. At the higher MC-LR concentration (5 μg/L) a significant reduction in growth appeared much faster, after 3 weeks (Fig. 2). After measurement of the photosynthetic oxygen production in *C. demersum* using the same range of MC-LR concentrations as above, significant inhibition (by a factor of 1.3) of the photosynthetic oxygen production was observed at an MC-LR concentration of 0.5 μg/L. The highest concentration used (5.0 μg/L) inhibited photosynthetic oxygen production by a factor of 18, which is comparable with the inhibition factor of 26 achieved after exposure of *C. demersum* to the herbicide atrazine at a concentration of 0.5 μg/L (Fig. 3). Monitoring the chlorophyll pattern (chlorophyll *a* [Chl *a*] and chlorophyll [Chl *b*]) of *C. demersum*,

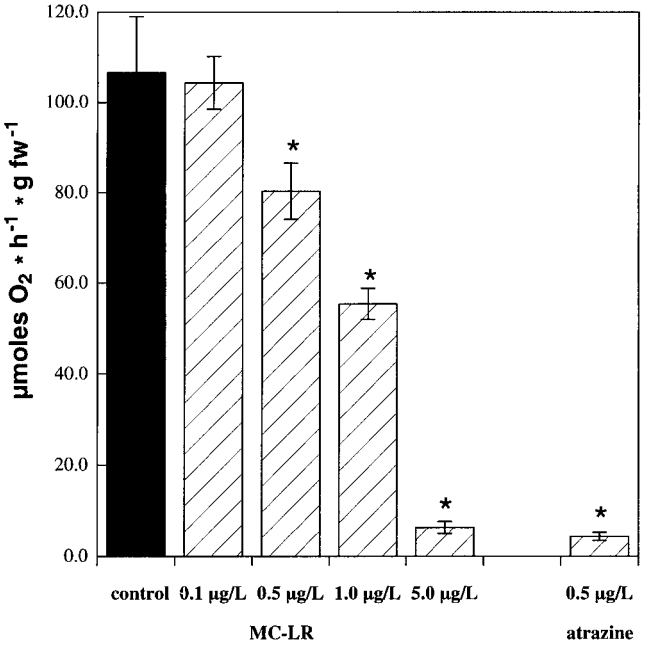

Fig. 3. Dose-dependent inhibition of photosynthetic oxygen production in *Ceratophyllum demersum* using varying concentrations of microcystin-LR, with the herbicide atrazine at a concentration of 5.0 μg/L used as a comparison. Asterisks indicate significance according to the ANOVA and Newman–Keuls tests ($p < 0.05$).

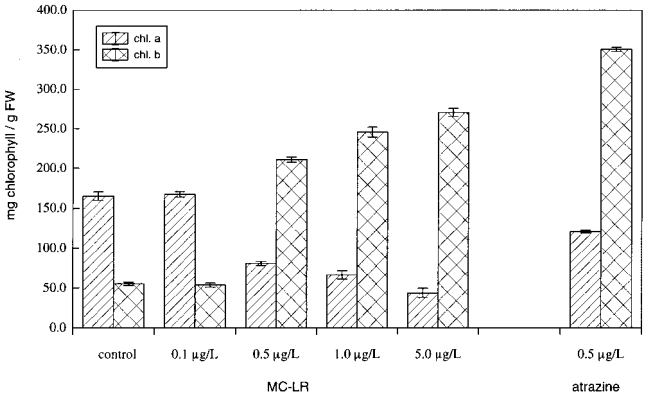

Fig. 4. Dose-dependent switch in the chlorophyll pattern of *Ceratophyllum demersum* after a 24-h exposure to various concentrations of microcystin-LR and to the herbicide atrazine, used as a control.

using the same plant material as that used for the photosynthetic oxygen production measurement, a clear switch in the ratio of Chl *a* to Chl *b* could be seen. In the control and in plants exposed to 0.1 µg/L there was always more Chl *a* than Chl *b*. After exposure to an MC-LR concentration of 0.5 µg/L and above, a sharp increase of Chl *b* was found, with the level of Chl *a* slowly decreasing in parallel (Fig. 4). The same switching of Chl *a* to Chl *b* could be found after exposure to atrazine at a concentration of 0.5 µg/L. A time-kinetic inhibition of photosynthetic oxygen production during exposure to an MC-LR concentration of 5.0 µg/L showed a rapid decrease in oxygen production during the first 10 min. After 45 min practically no oxygen production could be measured. After 1 day a slight increase in the oxygen level appeared (Fig. 5), and after measuring for 6 days a recovery of the photosynthetic oxygen production in *C. demersum* back to the original level was detected. Using the standard HPLC method, practically no MC-LR could be detected in the exposure medium after 1 day. On monitoring other aquatic plants, the macroalgae *Cladophora* sp., the submergent macrophyte *M. spicatum,* and the emergent macrophyte *Phragmites australis*, a significant inhibitory effect ($p < 0.05$) on the photosynthesis of these plants was detected after exposure to an MC-LR concentration of 0.5 µg/L. For *Elodea canadensis* and *C. demersum* with the same concentration MC-LR, this inhibitory effect was even more pronounced—90% inhibition (Fig. 6).

DISCUSSION

Microcystin-LR was used at environmentally relevant concentrations in this study and was similar to the lower values found by Fastner et al. (1995) in their study on the microcystin content of some German lakes. Higher concentrations of microcystins generally occur in late summer.

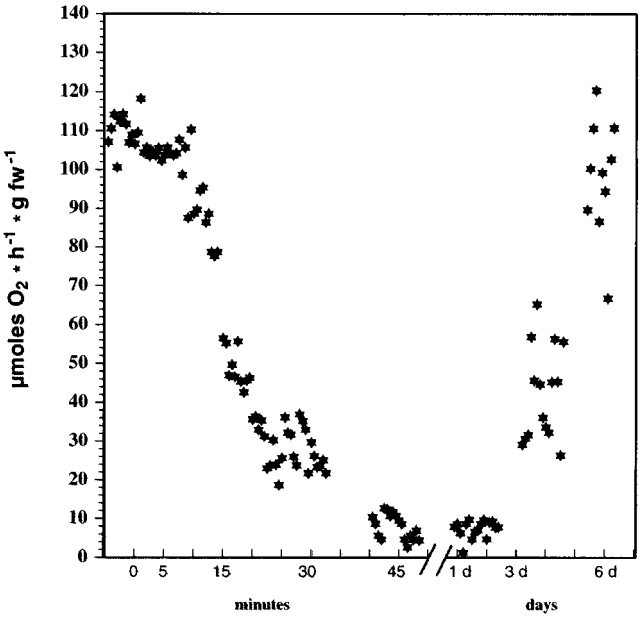

Fig. 5. Time-kinetic response of photosynthetic oxygen production of *Ceratophyllum demersum* after exposure to 5.0 µg total microcystin-LR (0.5 µg/L) for 6 days, showing an inhibition of photosynthetic oxygen production after 10 min going nearly to zero after the first day and showing a recovery of photosynthetic oxygen production during the next 5 days of the experiment.

The dominance of cyanobacteria has been discussed in relation to several environmental factors and is, in principle, discussed as a factor in the dieback of reed in Japanese lakes (Yamasaki, 1993). The presence of cyanobacteria might influence reed morphology and stability by decreasing sclerenchyma tissues because of nitrogen limitation (Kühl and Kohl, 1993). Other allelopathic effects associated with the increase in cyanobacterial blooms include a reduction of

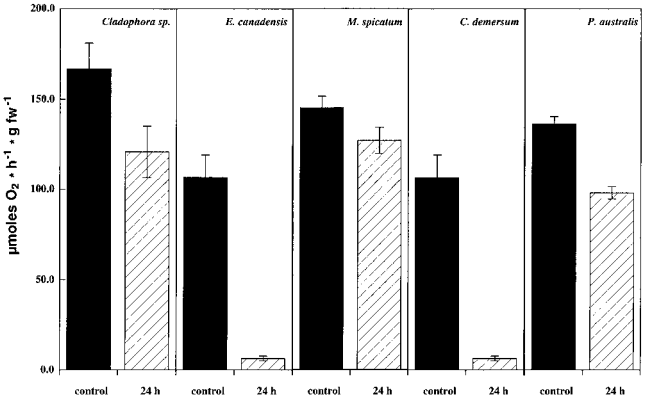

Fig. 6. Monitoring of photosynthetic oxygen production in several aquatic macrophytes showing an inhibition after exposure to a microcystin-LR concentration of 0.5 µg/L, with the most visible inhibition in *Elodea canadensis* and *Ceratophyllum demersum,* as compared with controls without MC-LR.

P. australis shoot length and dry weight and reduced access to nutrients and oxygen (Sukopp and Markstein, 1989; Yamasaki, 1993).

The results presented here have shown a clear dose-dependent inhibition of growth of the submerged macrophyte *C. demersum* at concentrations of MC-LR ranging from 0.1 to 5.0 μg/L. To our knowledge this is only the second article (after Yamasaki, 1993) to show growth inhibition of a macrophyte in the presence of cyanobacterial secondary metabolites, in this case MC-LR. In Yamasaki's research he described the presence of a cyanobacterial bloom but did not measure its microcystin content.

A clear inhibitory effect of MC-LR on photosynthetic oxygen production of the aquatic macrophytes *C. demersum, E. canadensis,* and *M. spicatum* at environmentally relevant concentrations was shown here. This inhibition of the photosynthetic process, of growth inhibition, and of cell lysis was also detected by Singh et al. (2001) after exposing the cyanobacteria *Nostoc muscorum* and *Anabaena* strain BT 1 to MC-LR for 6 days. The MC-LR concentrations used in that study were in the range of 25–50 mg/L and therefore beyond environmental relevance. Using a fifth of the lowest concentration used by Singh et al., the aquatic macrophyte *C. demersum* was seen to die (Fig. 1). In purified microcystins (MC-LR, MC-RR) a concentration of 0.5 μg/L had no inhibitory effect on the photosynthesis of the green algae *Scenedesmus armatus,* as shown by Pietsch et al. (2001). In this study, within 10–15 min of exposure to the cyanobacterial toxin MC-LR the inhibitory effect on the photosynthetic oxygen production was significant, showing a possible time difference between submerged macrophytes and microalgae such as *Nostoc* or *Anabaena* in uptake mechanisms. Differences in reaction to purified MC-LR also were found in the aquatic plants used in this study. Whereas the macroalgae (*Cladophora* sp.) and the emergent macrophyte (*P. australis*) had only a 10% reduction in the production of photosynthetic oxygen, the three submerged macrophytes had a 50%–90% reduction of this activity during a 24-h exposure. After 3 days of the time-course experiment a 50% recovery in photosynthetic oxygen production was detected, and after 6 days a near complete recovery to the original level was found. In the current experiment, when calculating the amount of MC-LR that would be taken up by *C. demersum* after a 7-day exposure, which a previous study (Pflugmacher et al., 1999) found to be 1.98 μg/g of FW, it was estimated that a proportion of the MC-LR applied (5 μg total toxin in 10 L of medium) might be taken up by the plant, but the complete removal had to be a result of other mechanisms. A hypothesis for the possible reactions from MC-LR uptake in plant cells is given in Figure 7. After uptake of MC-LR into a plant, a certain amount (under 10%) will be conjugated nonenzymatically to glutathione (GSH). A second quantity will be conjugated enzymatically to GSH via the glutathione S-transferases system (Pflugmacher et al., 1998, 2001). A part

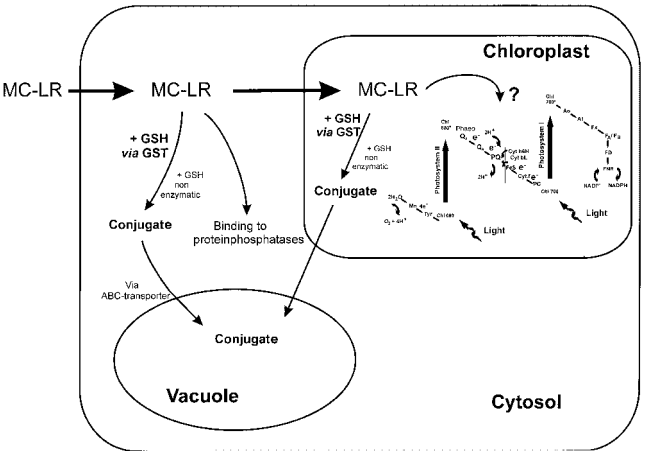

Fig. 7. A schematic hypothesis showing pathways in plant cells after uptake of microcystin-LR, with possible reactions of MC-LR from nonenzymatic binding to glutathione protein binding to protein phosphatases and enzyme-linked conjugation reactions plus transport of the conjugates via an ABC transporter into the vacuole of the plant cell; as well as a postulated uptake of MC-LR into chloroplasts along similar pathways showing a possible explanation for the inhibitory effects of MC-LR on the photosynthetic oxygen production of the cell.

of the remaining MC-LR will bind to protein phosphatases (MacKintosh et al., 1990) and possibly to other cellular proteins. Finally, any remaining MC-LR could be taken up by the chloroplasts. In the chloroplasts three main pathways were postulated, nonenzymatic binding to GSH, enzymatic conjugation to GSH via the GST system, and reactions with proteins or structures of the photosynthetic apparatus of the chloroplast, resulting in the detected inhibitory effects. To remove the GSH conjugates, the plants transfer these conjugates into the vacuole via multidrug-resistant associated proteins (MRPs), which are part of the ABC transporter family, for preliminary storage and further processing of the GSH conjugate (Martinoia et al., 1993; Coleman et al., 1997; Walbot et al., 2000). Recovery of photosynthetic oxygen production in *C. demersum* might be explained in two ways: (1) ongoing detoxication processes in the plant cytosol as well as in the chloroplasts via the GST pathway, ending in several conjugates such as GSH, γ-glutamylcysteine, and cysteine conjugates, as described in more detail by Pflugmacher et al. (1998, 2001), reducing the MC-LR concentration in the plant cell; (2) binding of MC-LR perhaps D1 proteins of the photosynthetic apparatus, which could exhibit a very rapid protein turnover, allowing photosynthetic oxygen production to recover quickly only if the concentration is not too high.

Looking at the pigment pattern of *C. demersum* after exposure to varying concentrations of MC-LR raised an additional reason for possible damage to the photosystems: a change in the pigment pattern. The switch from Chl *a* to

using more Chl *b* is always a sign of stress in plants and could therefore be correlated to MC-LR exposure.

Keeping in mind both the definition of Rice (1984) and the mostly negative effects of cyanotoxins on other aquatic macrophytes led to the conclusion that these cyanotoxins, as secondary metabolites of cyanobacteria, do have allelopathic power in plant–algae interactions. Algae–algae interactions have been shown with compounds produced by cyanobacteria such as the volatile geosmins or geranylactone (Ikawa et al., 2001). An allelochemical, fischerellin, produced by the cyanobacterium *Fischerella muscicola*, was found to exhibit inhibitory effects on the photosynthesis of other cyanobacteria (Papke et al., 1997). The site of action was located in photosystem II (Gross et al., 1991). Other compounds isolated from *Fischerella* (12-epi-hapalindole E isonitrile) and *Calothrix*, (calothrixin A), showed very severe modes of action by inhibition of protein synthesis and DNA replication (Doan et al., 2000). However, an increasing number of bioactive compounds in cyanobacteria have been found to inhibit direct oxygenic processes, and they have been discussed both as regulators for natural populations and as potential natural herbicides or biocontrol agents (Smith and Doan, 1999). Another reason that cyanobacterial toxins could be used as allelopathic substances is that an evolutionary enzyme system such as the glutathione S-transferases, effective for the detoxification of man-made compounds, uses microcystins as natural substrates, initiating the detoxication/elimination process of these infochemical compounds in plants. Additional study is needed to clarify whether cyanobacterial toxins, as cyanobacterial secondary metabolites, are normal infochemicals in aquatic ecosystems, regulating plant–algae or algae–algae interactions.

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