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Cyanobacterial Toxin Elimination *via* Bioaccumulation of MC-LR in Aquatic Macrophytes: An Application of the "Green Liver Concept"

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Cyanobacterial blooms and their corresponding toxins are a major concern to human health when surface waters of eutrophicated lakes are the only source for drinking water supply. The aim of the study was to test effective methods for cyanotoxin elimination by using the bioaccumulation potential of aquatic macrophytes in order to reduce microcystin LR (MC-LR) concentrations from raw lake surface water before entering the drinking water plant for further processing. Laboratory assays with aquatic macrophytes were performed in order to assess the most favorable species and optimal biomass for cyanotoxin elimination, where *Lemna* sp., *Myriophyllum* sp., and *Hydrilla* sp. were shown to be the most efficient macrophytes. In a second phase a pilot scale pond system (e.g. replica of the outdoor pond system) was constructed to assess the toxin elimination efficiency of 5.0 g L^{-1} biomass of combined macrophytes. The applied macrophytic biomass reduced an initial MC-LR concentration of 12.1 and 9.2 μg L^{-1} to values below the WHO guidelines for drinking water of 1.0 μg L^{-1} (MC-LR) in only three days. Applying these results in a specially constructed outdoor pond system resulted in >84% of toxin elimination at an initial concentration of 1.1 μg L^{-1} MC-LR within the raw lake water.

Introduction

Limnic ecosystems have experienced an increase of nutrient levels (i.e., eutrophication) due to anthropogenic activities within the past decades (*1*). Eutrophication, together with other environmental conditions such as increasing temperature, has become a driving force for massive proliferations of primary producers, like prokaryotic cyanobacteria in limnic ecosystems (*2*−*4*). Massive blooms of cyanobacteria have adverse effects on freshwater bodies used for recreation and drinking water supplies (*5*−*7*), as many cyanobacteria have the ability to synthesize toxic secondary metabolites (i.e., cyanotoxins) (*2*). The most studied, widespread, and prevalent cyanotoxins are the microcystins (MCs), and over 70 isoforms have been detected (*8, 9*). Since MCs are known to be tumor promoters (liver and colon) (*10*−*12*), they pose a risk to human health when surface waters are used as a drinking water source and/or for recreational purposes (*7*). The World Health Organization (WHO) published a provisional guideline value for MC-LR in drinking water of 1.0 μg L^{-1} (*13*). The increase in the human population is generating a higher demand for drinking water. Furthermore, many surface waters are being utilized as a source for drinking water production, especially in developing and underdeveloped countries, but not exclusively. Therefore drinking water suppliers have to adopt strategies to avoid massive blooms within their raw water source and/or implement treatment options to eliminate both cyanobacterial cells and their secondary metabolites (e.g., cyanotoxins, taste and odor compounds) (*14*).

During the past few years, several lakes in the P.R. China have experienced increasingly massive cyanobacterial blooms. One of the lakes affected was Lake Chaohu and with a surface area of *ca.* 760 km^2 is the fifth largest freshwater lake in the P.R. China, located in the Anhui Province, *ca.* 400 km west of Shanghai. With a mean depth of 3.10 m (shallow property) and a high input of nutrients from anthropogenic activities like agriculture and sewage discharge, between others, Lake Chaohu has been increasingly eutrophicated, with concentrations of total phosphate of 0.72 mg L^{-1} and total nitrogen of 3.99 mg L^{-1} (summer 2006), but higher values up to 5.16 mg L^{-1} have been detected (*15*). Next to the use as a fishery resource and for recreational purposes, Lake Chaohu surface waters are an important drinking water source for the local population of Hefei City (*15*). Consequently there is a need for local water works to implement effective technologies for cyanotoxin elimination to guarantee adequate drinking water quality.

Several treatment options for cyanobacterial cells and dissolved toxins have been developed and adopted by waterworks in different countries (e.g., slow sand filtration, coagulation, activated carbon, membrane filtration, chlorination, ozonation) (*16*−*20*). Management and treatment of cyanobacteria and their toxins generally raises costs within the drinking water production process, being acceptable for developed countries, but might be a problem in other countries with limited economic and technological resources. Consequently, alternative, effective, and cheap treatment options have to be developed. For that purpose the application of the *Green Liver Concept* was suggested. Briefly, this concept is based on the fact that both animals and plants exhibit a similar metabolization of toxic compounds (e.g., biotransformation). Whereas in animals this process is connected generally to the excretion of byproduct of metabolization *via* urine and/or feces, plants have the ability to isolate these metabolites (*21*). These metabolites are exported out of the cells into vacuoles or into the extracellular space and then deposited into cell wall components such as lignins resulting in a compartimentation for nontoxic long time storage (*22*). Since uptake and accumulation of MC-LR in aquatic macrophytes was shown by Pflugmacher et al. (*23*) and Mitrovic et al. (*24*), and biotransformation of MC-LR *via* glutathione S-transferase (GST) was confirmed by Pflugmacher et al. (*25*), the aim of the present investigation was to develop an effective water treatment system for cyanotoxin elimination *via* bioaccumulation in aquatic macrophytes, as a preliminary purification step before entering the waterworks for further processing. In order to develop a toxin elimination system the experiments were carried out at three different levels. The first level consisted of a pilot scale experiment (one liter volumes) with the aim of choosing the appropiate macrophyte species configuration for further steps. During the second level a laboratory pilot pond system (mesocosm experiment, 50 L volume) was constructed with the purpose of testing the combined toxin elimination performance of the previously chosen aquatic

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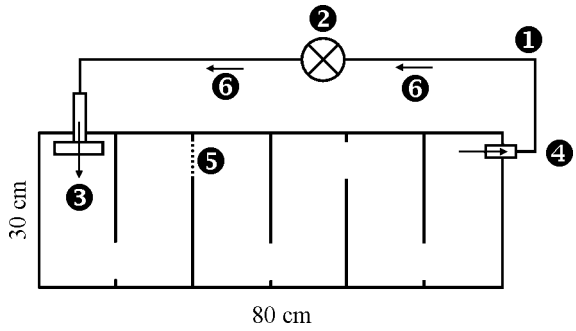

FIGURE 1. Technical drawing of the pilot pond system upper view (Experiment 2; dimensions: height 25 cm, width 30 cm, length 80 cm, total capacity 60 L, during experiment filled up to 50 L) made of plexiglass. In and outflow were connected by an Eheim 104821993 recirculation pump (series: 33051; type 2048; 10 L min^{-1}) connected by Teflon tubes (1 cm diameter). Symbol legend: 1, Teflon tube; 2, recirculation pump; 3, inflow; 4, outflow; 5, *Lemna* mesh; 6, flow direction.

macrophytes in a small scale system constructed with similar proportions as the pond system used for the field studies. The third level consisted of a field experiment conducted in a pond system of *ca.* 1,200,000 L volume to validate the toxin elimination under environmental conditions.

Experimental Section

Microcystin (MC-LR) Elimination Experiments. *A.. Laboratory Scale Evaluation Experiment - Static, Separate Species - Experiment 1.* Aquatic macrophytes from the species *Lemna minor*, *Myriophyllum elatinoides*, *Hydrilla verticillata*, and *Ceratopteris thalictroides* were haltered in 60 L aquaria filled with artificial freshwater (AFW) (milli Q water, $CaCl_2$ [0.2 g L^{-1}], $NaHCO_3$ [0.103 g L^{-1}] and sea-salts [0.1 g L^{-1}]), with a photoperiod of 14:10 h (light:dark), irradiance of 100 [μE m^2 s^{-1}], and at a temperature of 20 $\pm$ 1 °C (26). After acclimatization of the above-mentioned macrophyte species (*L. minor, M. elatinoides, H. verticillata*, and *C. thalictroides*) plants were transferred into one liter glass beakers in order to determine the time dependence of toxin elimination via bioaccumulation in aquatic plants. A specific single species biomass of the selected aquatic plants was exposed to an initial nominal microcystin-LR concentration of 2.5 μg L^{-1} (MC-LR), for a period of 14 days (14:10 h (light:dark), irradiance: 100 [μE m^2 s^{-1}], T°: 20 $\pm$ 1 °C). For *L. minor* a biomass of 1.0 g per liter (e.g., 0.1 g/100 mL) was used.

In the case of the rooted species like *M. elatinoides*, *H. verticillata*, and *C. thalictroides* a biomass of 3.0 g per liter was used. All treatments were performed in the absence of soil/sediment within the test system. Since only whole plants were used in this survey, the exposure volume varied according to the fresh weight of the aquatic macrophyte in order to maintain the adequate biomass-exposure volume relation. Controls were carried out simultaneously to the exposures under the same conditions, thus without plant material. All experiments were carried out under nonsterile conditions. After exposure plants were removed, and the remaining volume of water was determined before passing through a solid phase extraction column as described further on. Sampling was carried out after 1, 2, 4, 7, and 14 days of exposure.

B. Laboratory Pilot Scale System Experiment - Recirculation, Mixed Species - Experiment 2. The combined MC-LR elimination efficiency of the selected macrophytes species was assessed in a recirculating system designed to be scaleable for use in the field. The pilot pond system made of plexiglass with the following dimensions: height 25 cm, width 30 cm, length 80 cm with a total capacity of 60 L (Figure 1). In- and outflow were connected by an Eheim 104821993 recirculation pump (series: 33051; type 2048; 10 L min^{-1}) connected by Teflon tubes (1 cm diameter), and water flow was maintained at 2 L min^{-1}.The following aquatic macrophytes *L. minor*, *M. elatinoides*, and *H. verticillata* were acclimatized to the previously described testing conditions. A combined plant biomass (total biomass 5 g L^{-1}) consisting of *L. minor* (25 g), *H. verticillata* (125 g), and *M. elatinoides* (100 g) was exposed in a volume of 50 L AFW enriched with MC-LR. No sediment or soils were added to the test system. In a first experimental run the above-mentioned plant biomass (combination of plant species) was exposed to a concentration of 9.2 μg L^{-1} MC-LR (dissolved in AFW). In a second run the same MC-LR concentration was applied, but no plant biomass was added (i.e., control). In a third survey a combined plant biomass (*L. minor* - 25 g; *H. verticillata* - 125 g; *M. elatinoides* - 100 g) was exposed to 12.1 μg L^{-1} MC-LR equivalents of *Microcystis aeruginosa* (strain M12 was a gift from Prof. V. M. Vasconcelos, CIIMAR, Porto, Portugal) crude extract (CE). The control (e.g., no plants) and the exposure runs (e.g., with macrophytes) were carried out consecutively, under the same conditions as described above for the acclimatization of the aquatic macrophytes. Exposure duration was set to five days. Sampling was carried out every 24 h where three aliquots of one liter water samples were taken and filtered through solid phase extraction column as described in a specific section later in the article.

C. Field Experiment (at the Hefei Water Works, Lake Chao, Anhui, P.R. China) - Experiment 3. For the field experiments, the plant material was collected within the catchment area of Lake Chaohu, where whole plants including roots (*Lemna* sp., *Hydrilla* sp., *Myriophyllum* sp., and *Phragmites* sp.; all collected from the Chaohu catchment area) were transferred into the corresponding section of the field pond (Figure 2) at the location of the water works Hefei (P.R. China). For the field experiment a pond system (dimensions: length 60 m, width 20 m, max. water depth 1 m) was constructed in cooperation with the Anhui Environmental Protection Bureau (AEPB) on the area of the water works in Hefei (Anhui, P.R. China). The pond system (Figure 2) was supplied by raw lake water without any pretreatment (e.g. surface water from Lake Chaohu as it would enter the waterworks). Water inflow was controlled, ranging between 1.2 and 2.8 L s^{-1}, and the residence time of lake water ranged between 3 and 7 days. The selected aquatic plants were transferred into their corresponding compartment (Figure 2). *Lemna* sp. was introduced into the first compartment for colonization. Into the second compartment of the pond system, a density of 10 (plants m^{-2}) of both *Hydrilla* sp. and *Myriophyllum* sp. was planted. The third compartment was planted with *Phragmites* sp. (10 plants m^{-2}). Excessive *Lemna* sp. biomass as well as dead and dry plant material was removed from the pond system periodically. At the beginning of the vegetation period also withered *Phragmites* sp. parts were cut down and removed. The aim of this experiment was to scale up the laboratory pilot scale experiment in order to confirm the toxin elimination performance of the aquatic macrophytes under environmental conditions and enlarged size. Sampling was carried out during the vegetation period together with the development of a cyanobacterial mass population in the lake system. Samples consisted of one liter of lake water filtered through glass fiber filters to separate cellular from dissolved toxin fraction and then concentrated by solid phase extraction as described in the corresponding section.

Water Sample Preparation for MC-LR Analysis. During Experiments 1 and 2 (pilot scale and pilot pond) sampling was carried out in defined intervals as described in the above section Experiments 1 and 2, where samples consisted of one liter of AWF with the remaining toxin content of each treatment. Water samples were concentrated using a solid phase extraction column (Sep-Pak plus tC18, Waters, pore

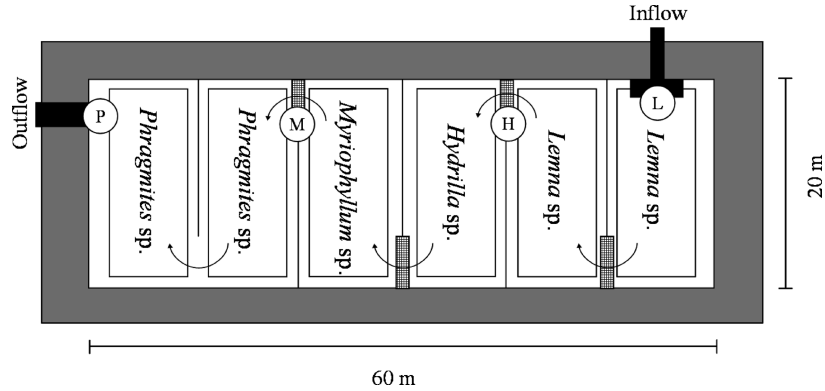

FIGURE 2. Technical drawing of the field pond system (Experiment 3; dimensions: length 60 m, width 20 m, water depth max. 1 m) on the area of the water works in Hefei (Anhui, P.R. China). Inflow range: 1.2−2.8 L s^{-1}; water residence time: 3−7 days. (grid pattern) *Lemna* net (mesh); ↶ flow direction; ○ sampling points [L = Lemna (inflow); H = Hydrilla; M = Myriophyllum, and P = Phragmites (outflow)].

size 125 Å, particle size 45 μm, hold up volume 1.60 mL/filled cartridge). Sep-Pak cartridges were eluted with 10 mL of pure methanol (100% MeOH) into glass beakers and then evaporated at 35 °C. Samples were redissolved in one mL of 100% MeOH for quantification. Field pond samples were first filtered through GF-C (Whatman) in order to separate particulate matter from liquid fraction. The filtered fraction of the water samples (1 L) was concentrated using a solid phase extraction column (Sep-Pak plus tC18, Waters) and eluted with 10 mL of pure methanol (100% MeOH), which was evaporated at 35 °C and redissolved in one mL of 100% MeOH for MC-LR analysis *via* HPLC-PDA. GF-C filters were cut into small pieces and extracted in 15 mL of 100% methanol (MeOH) in falcon tubes and placed in a turnover shaker for 24 h. Then samples were centrifuged at 4500 rpm, and the supernatant was transferred into glass beakers for evaporation at 35 °C and redissolved in one mL of MeOH for quantification.

High-Performance Liquid Chromatography (HPLC) Analysis of MC-LR. The determination of the MC-LR content in the samples was performed as described in ref *27*. Briefly, a Waters high-performance liquid chromatography (HPLC) system (Waters Alliance, Eschborn, Germany) with a photodiode array detector (Waters 2996) and a separation module (Waters 2695) was used for MC-LR analysis. The separation of the samples was carried out on a reverse phase column RP18, 5 μm, 25 cm (LiChroCART 250-4; LiChrospher 100). The temperature of the column was set at 40 °C, and the injection volume was 80 μL. The mobile phase consisted of Milli-Q water and acetonitrile (Rathburn, Walkerburn, U.K.), both enriched with 0.1% (v/v) trifluoroacetic acid (TFA; Merck, Germany) at a flow rate of 1 mL min^{-1}. The acetonitrile (0.1% TFA) phase was increased linearly from 30 to 45% within a period of 10 min. Microcystin LR for all laboratory experiments and analysis was purchased from Axxora (Germany). Vials of one mg of MC-LR were redissolved in one mL of MeOH and used as reference stock. From the MC-LR reference stock solution dilutions for quantification and exposures were prepared. The limit of detection (LOD) was set to 0.01 μg L^{-1}, corresponding to the lowest concentration of a dilution series (10, 1.0, 0.1, 0.01 μg mL^{-1} MC-LR) producing a visible peak including a typical PDA spectrum. The limit of quantification (LOQ) for the HPLC-PDA method was set to 0.1 μg mL^{-1} MC-LR in concentrated samples.

Data Analysis. Statistically significant differences between treatments and their corresponding controls were verified *via* a one-way analysis of variance (ANOVA) followed by a Tukey's posthoc test, at $p < 0.05$ (Statistica, StatSoft, Inc. 2000).

Results and Discussion

A. Laboratory Scale Evaluation Experiment - Static, Separate Species - Experiment 1. Within this experiment the MC-LR elimination capacity of different single aquatic macrophytes was assessed in a static system. Figure 3 shows that all four tested macrophytes species were able to reduce the MC-LR concentration in the exposure media at a higher rate than the sole microbial reduction observed in the controls. All tested macrophytes, rooted at a biomass of 3 g L^{-1} and free floating at 1 g L^{-1}, were able to reduce an initial MC-LR concentration of 2.5 μg L^{-1} below the WHO guideline value for drinking water (1.0 μg L^{-1} MC-LR) in only one day of exposure, whereas the sole microbial degradation (i.e., control without plants) needed 7 days to reach similar elimination level. The mean net to MC-LR elimination of the macrophytes (i.e., the difference of the remaining MC-LR concentrations of the control (no plant addition) and the remaining MC-LR concentrations of the corresponding treatment with plant addition) indicates the net to elimination capacity (NEC) of the macrophytic biomass for each time point. The REC corresponds to the average of NEC values of the whole experiment. *Lemna* sp. showed the highest values for REC (1.116), followed by *Hydrilla* sp. (1.064) and *Myriophyllum* sp. (0.952), whereas the lowest REC was recorded for *Ceratopteris* sp. (0.862). The aquatic macrophyte *Ceratopteris* sp. was eliminated from further experiments due to the lowest mean toxin elimination capacity. The best MC-LR elimination capacity showed *Lemna* sp. (biomass: 0.1 g/100 mL^{-1} wet weight), since after one day of exposure (initial MC-LR concentration 2.5 μg L^{-1}) only 0.2 μg L^{-1} MC-LR was detected within the remaining exposure medium. Scale effects should be taken into account, because testing volume and applied biomass for this particular assay with *Lemna* sp. was 1 order of magnitude lower than for the other macrophyte species within the static assay. Figure 3 shows that at low MC-LR concentrations (below 1.0 μg L^{-1} MC-LR) the toxin elimination efficiency seems to be reduced in all tested species (i.e., low MC-LR concentration - low elimination rate). MC-LR uptake in aquatic plants has been investigated by Pflugmacher et al. (*25, 28*), where aquatic macrophytes such as *Ceratophyllum demersum*, *Elodea canadensis*, *Vesicularia dubyana*, and *Phragmites australis* were able to take up MC-LR in a range 1.0−120 pg g^{-1} fresh weight (FW). MC-LR uptake in *Lemna gibba* was confirmed by Saqrane et al. (*29*), where an exposure of 12 days to 300 μg L^{-1} (MC-LR equivalents) resulted in an uptake of 2.24 μg g^{-1} dry weight (DW) MC-LR. Physiological effects (e.g., growth, photosynthetic oxygen production, and pigment analysis) on environmentally relevant MC-LR concentrations (0.1 − 5.0 μg L^{-1}) on the aquatic macrophytes *C. demersum*, *E. canadensis*, *M. spicatum*, and *P. australis* were reported by Pflugmacher (*30*). The results showed that all tested plants suffered from negative effects on growth, photosynthesis, and pigment pattern, thus *M. spicatum* and *P. australis* presented lower sensitivity against MC-LR than the other test species (*30*).

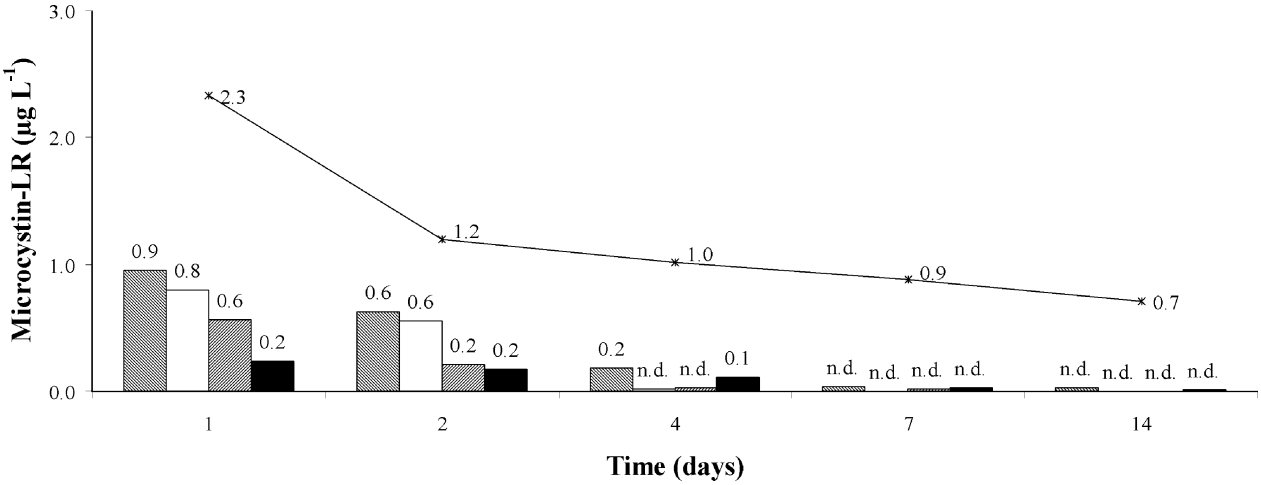

FIGURE 3. Pilot scale microcystin-LR elimination experiment with selected macrophytes (Experiment 1). Values represent remaining toxin concentration (MC-LR) within the surrounding medium, (n = 1). n.d. = not detectable. -*- control: initial 2.5 μg L^{-1} (MC-LR) without plant biomass (volume: 1000 mL); (grid pattern) *Ceratopteris* sp.: initial 2.5 μg L^{-1} (MC-LR) + 3 g L^{-1} plant biomass (volume: 1000 mL); □ *Myriophyllum* sp.: initial 2.5 μg L^{-1} (MC-LR) + 3 g L^{-1} plant biomass (volume: 1000 mL); (grid pattern) *Hydrilla* sp.: initial 2.5 μg L^{-1} (MC-LR) + 3 g L^{-1} plant biomass (volume: 1000 mL); ■ *Lemna* sp.: initial 2.5 μg L^{-1} (MC-LR) + 1 g L^{-1} plant biomass (volume: 100 mL).

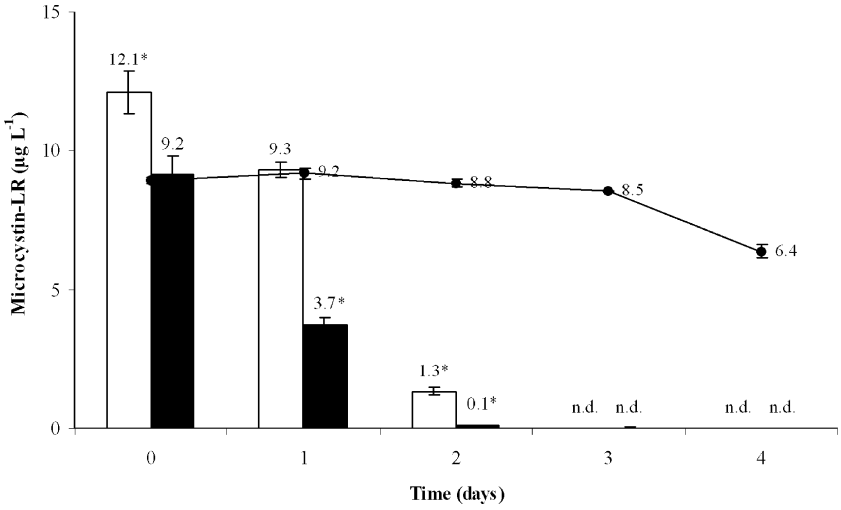

FIGURE 4. Pilot pond microcystin-LR elimination experiment with selected macrophytes (Experiment 2). Values represent remaining toxin concentration within the surrounding medium. Significant differences were calculated *via* oneway ANOVA followed by Tukey's posthoc test (n = 3; α = 0.05). n.d. = not detectable. −•− control: microcystin-LR without plant biomass; ■ 9.2 μg L^{-1} microcystin-LR + 5 g L^{-1} plant biomass; □ M12 crude extract with added MCLR [eq. 12.1 μg L^{-1} (MC-LR)] + 5 g L^{-1} plant biomass; volume 50 L.

Weiss et al. (*31*) showed that microcystin-RR (MC-RR) concentrations in the range of 100−1000 μg L^{-1} MC-RR had no significant effect on the photosynthesis of *Lemna minor* L., whereas higher concentrations (3000−5000 μg L^{-1} MC-RR) were able to inhibit photosynthesis. LeBlanc et al. (*32*) tested environmentally relevant concentrations ranging between 0.1−10 μg L^{-1} MC-LR on *Lemna gibba* L. and observed no significant negative effects on growth, frond number, biomass, and chlorophyll. Previous studies on uptake kinetics of MC-LR in aquatic plants have shown a linear pattern of uptake, thus it seems that uptake is related to the corresponding plant species (*30*); generally uptake of harmful substances displays saturation at higher concentrations. The results of the present study together with the results of previous investigations from other researchers suggest that *Lemna* sp, *Myriophyllum* sp., and *Phragmites* sp. are less sensitive to MC-LR exposure than other aquatic macrophytes (*23, 30, 32*). Within the present investigation the selection of *Lemna* sp., *Myriophyllum* sp., *Hydrilla* sp., and *Phragmites* sp. for further experiments is suggested, with the restriction of keeping MC-LR concentrations below critical levels, in order to maintain plant health, promoting plant growth, and microcystin metabolization.

B. Laboratory Pilot Scale System Experiment - Recirculation, Mixed Species - Experiment 2. During this experiment the MC-LR elimination performance of three selected aquatic macrophytes species was tested in combination. Higher toxin concentrations within an increased volume were applied, since the results of this experiment will lead to the selection of macrophyte type and macrophytic biomass to be used within the field experiment in China. Figure 4 shows that a concentration of 9.2 μg L^{-1} MC-LR in a volume of 50 L was reduced by a combined biomass of 5 g L^{-1} wet weight *L. minor*, *H. verticillata*, and *M. elatinoides*. This macrophyte combination was able to reduce MC-LR concentrations of the exposure media below WHO drinking water guidelines value (1 μg L^{-1}) for MC-LR in only 2 days, whereas in the control samples after 4 days 6.4 μg L^{-1} MC-LR was detected. The cyanobacterial crude extract with an equivalent of 12.1 μg L^{-1} MC-LR (HPLC-PDA) was used to approach more natural realistic conditions. Figure 4 shows that the same amount and composition of macrophytes were able to reduce

the initial concentration of 12.1 μg L^{-1} MC-LR to values below 1 μg L^{-1} MC-LR within three days. No MC-LR was detectable after three days of the experiment. As discussed in the previous section uptake of MC-LR in aquatic plants seems to display a linear pattern, thus our results show that when pure MC-LR is applied to the test system a linear fitting correlation coefficient with $r^2 = 0.99$ was achieved, whereas when MC-LR enriched cyanobacterial crude extract was applied the linear fitting correlation coefficient was reduced to $r^2 = 0.93$. The results of this experiment demonstrated the suitability of the suggested pond system for MC-LR elimination *via* bioaccumulation in aquatic macrophytes. Since *Lemna* sp. seems to be the least sensitive of the tested species (*29*−*31*) and has a biomass duplication rate ranging from 1−4 days (*31*) it appears to be the adequate macrophyte type for the inflow area because of the following reasons: 1. it reduces light penetration, decreasing the available light for cyanobacterial growth (covers the surface); 2. it reduces the amount of nutrients available for cyanobacterial growth; 3. it tolerates higher microcystin levels than other macrophytes; and 4. it reduces initial toxin concentration to tolerable levels of other macrophytes. In view of the fact that *Lemna* sp. bioaccumulates MC-LR mainly from water surface since vertical mixing within the pond system is limited both *Myriophyllum* sp. and *Hydrilla* sp. were planted within the middle area of the pond system, given that these macrophytes are rooted, grow from the bottom to the water surface, and therefore are more in contact with water of the deeper layers and sediments (*33*). *Phragmites* sp., the macrophyte with the highest uptake potential (*30*), was planted within the last portion of the pond system in order to eliminate the remaining MC-LR, even at low concentrations.

C. Field Experiment (at the Hefei Water Works, Lake Chao, Anhui, P.R. China) - Experiment 3. The previously selected aquatic macrophytes (*Lemna* sp., *Hydrilla* sp., *Myriophyllum* sp., and *Phragmites* sp.) were planted into their corresponding section of the pond system as shown in Figure 2. Sampling within the field experiment was limited to the vegetation period which ranges in Hefei (Anhui, P.R. China) generally from April to October. During sampling on 06/29/2006, at the pond inflow (before *Lemna* sp. section), a concentration of 0.6 μg L^{-1} MC-LR was measured, whereas within the *Hydrilla* sp. section only 0.1 μg L^{-1} MC-LR, in the *Myriophyllum* sp. section <0.1 μg L^{-1} MC-LR, and at the end of the *Phragmites* sp. section <0.1 μg L^{-1} MC-LR were detected in the water. This corresponds to a MC-LR elimination capacity of the field pond system of >84%. A lower MC-LR elimination capacity is expected when aquatic plant biomass is reduced (e.g. winter, early spring) since *Lemna* sp. may not cover the whole surface of their corresponding pond section and during cut down of *Phragmites* sp. at the beginning of the vegetation period. Further on, it has been described by Dziga et al. (*34*) that submerged aquatic macrophytes produce allelopathic substances like polyphenols (e.g., pyrogallol, hydroquinone) which at a sufficiently high concentration can inhibit or reduce the growth of cyanobacteria. Consequently, it is possible that the biomass of aquatic macrophytes was not sufficient to produce enough allelopathic substances to reduce sufficiently the growth of the toxin producing cyanobacteria in addition to the incomplete surface coverage of the *Lemna* section together with the cut down of *Phragmites* sp., since the second sampling was conducted out during the beginning of the vegetation period. In the future different plant combinations need to be tested in order to replace *H. verticillata*, since recent investigations of avian vacuolar myelinopathy (AVM) have suggested a close correlation between AVM and the presence of a cyanobacterium (Stigonematales) grown as epiphyte on aquatic macrophyte *Hydrilla verticillata* (*35*), although the cause of the disease has not been elucidated yet (*36*). Therefore it is suggested that in the next step *H. verticillata* will be substituted as a precaution by another suitable aquatic macrophyte species.

Elimination of MC-LR by the use of aquatic macrophytes in an experimental pond system could be proven under environmental conditions. An MC-LR elimination rate of >84 was reached depending on plant density and toxin concentration of the water. Since only the parent compound MC-LR was monitored, further attention to the presence of microcystin metabolites and other microcystin isoforms in the water after pond treatment should be monitored in further studies. Previous studies on the bioaccumulation of MC-LR in aquatic plants and algae by Mitrovic et al. have suggested a linear uptake pattern of MC-LR, thus at higher concentrations where growth reduction was observed, bioaccumulation of MC-LR was reduced (*24*). For that reason an important aspect is the accurate maintenance of the macrophytic pond system. On one hand the inflow and residence time of the water should be controlled together with the active plant biomass in order to avoid excessively high concentrations of MC-LR since it is known that aquatic plants suffer deleterious effects, such as growth reduction and lower photosynthetic rates, when exposed to extremely high concentrations of cyanotoxins (*30*), reducing toxin bioaccumulation. Consequently the water inflow and residence time should be in balance with the input of cyanotoxins into the pond system keeping cyanotoxin concentrations below critical levels supporting plant growth. Special attention should be given to the fate of entrapped microcystins within dead plant material. Since plants bioaccumulate MC-LR, dead plant material and especially excess of *Lemna* sp. (before die-off) should be removed from the pond system because accumulated toxins and metabolites may be released during plant die-off and following degradation, thus further studies on release rates of entrapped microcystins in aquatic plants should be carried out.

Finally the outdoor macrophyte pond proved that the system is functioning, thus further vegetation periods and different plant combinations should be tested and monitored as well as plant density adjusted in order to improve the efficiency of the macrophytic pond system for cyanotoxin elimination.

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