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Uptake and Metabolism of the Cyanobacterial Hepatotoxin Microcystin-RR by *Spirodela intermedia* from Brazil

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Summary

Spirodela intermedia was exposed to low (10 µg L^{-1}) and high concentration (100 µg L^{-1}) of microcystin-RR (Arginin-Arginin) over a period of two weeks. Depuration in the water phase, as well as uptake in the plant was determined using HPLC with photodiode array (PDA) detection. After the toxin uptake, metabolism of microcystin-RR occurred resulting in the formation of a glutathione conjugate. This conjugate was further processed yielding in a cysteine conjugate, a well known breakdown product in the biotransformation pathway of microcystins. The results indicate the uptake, accumulation and metabolism of microcystin-RR in a surface floating aquatic plant and raise the possibilities, to use these plants within water purification or toxin removal. Further implication on aquatic ecosystem and the transfer of toxin in the food web might occur and needs more investigation.

Introduction

Due to eutrophication processes and the global warming the massive growth of cyanobacteria (blue-green algae) can occur which can result in extensive blooms and scums in freshwater bodies. Several freshwater bloom-forming cyanobacterial genera including *Microcystis*, *Anabaena*, *Oscillatoria*, *Nostoc*, and *Nodularia* produce microcystin-LR (Leucin-Arginin) (CARMICHAEL and FALCONER, 1993; CODD 1995). *Microcystis* is one of the most common freshwater bloom-forming cyanobacterial genera throughout the world (CARMICHAEL, 1994). Many of these cyanobacterial species are well known to produce a wide range of secondary metabolites, so called cyanotoxins. Under these toxins, cell toxins like cylindrospermopsin, neurotoxins like anatoxins and hepatotoxins like the microcystins can occur (CARMICHAEL and FALCONER, 1993). The last mentioned are the most important and relevant toxins and here the microcystins, cyclic heptapeptides exhibiting the unusual hydrophobic β-amino acid Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4E,6E-dienoic acid), are the most commonly known.

The natural role of these cyanobacterial toxins is still unknown, but evidence exist that in lakes exhibiting massive blooms every year the abundance of submerged macrophytes has decreased (ABE et al., 1996) and the biodiversity of the overall aquatic plant community getting smaller (HARPER, 1992; CASANOVA et al., 1999).

Effects on aquatic plants in freshwater systems were seen as growth reductions, which might be also not only related toxin effects, but also to bad light conditions, oxygen depletion or a shift in pH value during blooming of the cyanobacteria. Laboratory studies showed that purified toxins inhibit the growth of several macrophytes such as *Elodea*, *Ceratophyllum* and *Lemna* species and *Phragmites australis* (KIRPENKO, 1986; YAMASAKI, 1993; WEISS et al., 2000; PIETSCH et al., 2001; PFLUGMACHER, 2004) in environmentally by relevant toxin concentrations.

Lemna species are one of the most widespread aquatic plants in freshwater bodies. They have doubling times between 1-4 days and because of their high sensitivity to xenobiotics, they are frequently used in biotest (LANDOLT and KANDELER, 1986; KAYA, 1996; LEWIS 1994)

The objective of this study was to assess the possible effects of the hydrophilic microcystin-RR in *Spirodela intermedia* in concerns to growth, toxin uptake and metabolism of the toxin within the plant. Furthermore a possible use of these plants for removal of toxin from water bodies will be discussed.

Materials and methods

Plant material

The macrophytes *Spirodela* sp. were chosen because of the possibility that these plants may live in microcystin-containing waterbodies. The aquatic macrophyte *Spirodela intermedia* was provided from the Water Quality Laboratory of the Universidade Federal de Ouro Preto (UFOP) by Professor Mauro Schettino of the Department of Biological Sciences. It originated in the Pantanal region (central west Brazil), and was transferred to UFOP for the Brazilian Company of Agricultural Research (Embrapa) Vali Joana Pott and was cultivated for over two years at UFOP. The culture was carried out in nutrient solution of Hoaglan with 1/5th of the original strength at pH 7 (HOAGLAND et al., 1950) under constant aeration, a temperature of 22 ± 2 °C and a photoperiod 12 h cold fluorescent light and 12 h darkness. Light intensity was the order of 40 µmoles of photons $m^{-2}s^{-1}$.

Mass cultivation of *M. protocystis*

For toxin production a monospecies strain of *Microcystis protocystis* (HBRF01), known to produce high levels of microcystin-RR, was provided by the laboratory of Hydrobiology of the Water and Waste Water Treatment Company of Minas Gerais (COPASA). This strain was isolated in 2003 from natural bloom of cyanobacteria in the reservoir of Furnas (21.27' 29.3" S/45° 59'47" O), located to the south Minas Gerais. The mass cultivation of *M. protocystis* was performed in ASM-1 medium (according to GORHAM et al.,1964) in 9 L glass bottles under sterile conditions, constant aeration, and a temperature of 22 ± 1 °C continuous lighting with cold fluorescent light (40 µmoles of photons $m^{-2}s^{-1}$). Cell counts using a Sedgwick-Rafter chamber were taken weekly to observe growth of the culture.

Isolation and purification of microcystin-RR

To lyse the cells and to set free the toxin, the cells were subjected three times to a freeze thaw cycle. After this treatment, the cells were freeze dried within 48 h and dry weight was calculated in mg L^{-1} of culture. The dry material was extracted according to FASTNER et al. (1998) using 75 % methanol. Further purification of MC-RR was performed using the method of KRISHNAMURTHY et al. (1986) were the sample is centrifuged for 10 min at 2.200 x g, the supernatant is collected and evaporated completely with gently heating at 40 °C. After resolving the concentrate in 1 L methanol, this extract is

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purified using an octadecylsilane (ODS-C18) cartridge previously activated with 6 mL of 100 % methanol followed by 6 mL of MilliQ water. For extraction the columns were washed with two volumes of 6 mL MilliQ water and 6 mL of 20 % methanol. Then elution of the columns were performed using 75 % methanol containing 0.1 % of trifluoroacetic acid. The eluent was dried completely by gently heating and suspended in 6 mL of MilliQ water.

Toxin analysis

The samples were analyzed using a HPLC-PDA equipped with a Merck LiChrospher 100 RP-18 (5 mm) according to PFLUGMACHER et al. (1998). For determination of MC-RR a mobile phase gradient of MilliQ + 1% TFA (eluent A) and acetonitrile + 1% (eluent B) at 1 mL min^{-1} was used and detection was done by photo-diode array at a wavelength of 200-300 nm. Total run time of one analysis was 25 min, at a column temperature of 40 °C and an injection volume on column of 80 µL. The terms of linear gradient were determined as 65 % and 35 % eluent as initial conditions. After the analysis the rate was readjusted to the original terms. The total time spent in a race was twenty-five minutes using a temperature of 40 °C for the column. For the quantification of MC-RR a commercial available reference substance (MC-RR) from Axxora (Grünberg, Germany) with 98 % of purity was used.

LC-MSMS analysis

Identification of MC-RR and metabolites in LC-MSMS analyses is based on the retention times in a reverse phase HPLC and the observed m/z values. For this analysis a combination of an Agilent 1200 and a quadrupole mass detector Applied Biosystems 3200 was used. For the HPLC a STAR RP-18 endcapped column 30 mm x 4 mm I.D. 3 µm particles were used with a linear gradient of acetonitrile and water both containing 0.5 % formic acid.
For the MSMS part the ionization parameters were: capillary voltage 3.8 kV, cone voltage 80 V, source temperature 150 °C, desolvation temperature 300-350 °C, cone gas flow 50 L h^{-1}, desolvation gas 650 L h^{-1}.

Exposure of *S. intermedia* to MC-LR

a) growth experiment

For the evaluation of the growth under constant exposure to MC-RR one gram of fresh weight of *S. intermedia* was placed in a 150 mL beaker containing 100 ml of nutrient solution of Hoagland (HOAGLAND et al., 1950) and cultivation conditions described above. The experiment was conducted in quintuplicate. Plant material was collected on day 7, 14, 21.

b) Uptake experiment

For the evaluation of the toxin uptake and metabolism under constant exposure to MC-RR one gram of fresh weight of *S. intermedia* was placed in a 150 mL beaker containing 100 ml of nutrient solution of Hoagland and cultivation conditions described above. MC-RR was given to the medium in a methanol solution in two separated concentration: 10 and 100 µg L^{-1}. Control experiments were conducted using 150 mL of medium without toxin. The experiment was conducted in quintuplicate. Plant material was collected on day 0, 1, 2, 3, 7, 14 and 21.

Plant extraction method

After end of exposure time, the water and plants were separated. The water was filtered with a microfilter fibreglass (GF-1, 47 mm, from Macherey-Nagel) to get rid of material in suspension. The filtered sample (100 ml) was applied to a solid phase extraction column (SPE plus tC18, Waters) according to the manufactures guidelines. The plants were rinsed three times with 10 mL Milli Q water to remove adsorbed MC-RR and ground in 4 mL of 100 % methanol to extract microcystin. The resulting slurry was shaken for 1 h and then ultrasonicated for 1 min. After that the samples were again shaken for 24 h at 20 °C temperature. The extract was then centrifuged at 2200 g s^{-1} for 10 min at 4 °C. The supernantant was then evaporated with gentle heating at 40 °C and resuspended in 1 mL of 100 % methanol. After filtration on a Mirex 0.45 µm filter the extracts were stored at 4 °C until analysis.

Results

Uptake kinetics of MC-RR by *S. intermedia*

Experiment 1: 10 µg L^{-1} MC-RR

In the controls without plants, the MC-RR concentration was reduced from initially 9 µg L^{-1} to 6 µg L^{-1} in 21 days of exposure (Fig. 1). This decrease might be due to bacterial degradation or binding of toxins to the surface of the exposure vessel.
In the experiments with plants (Fig. 1), the analyses of MC-RR revealed that toxin concentration in the medium was drastically reduced during the first two days until no toxin was detectable anymore. In parallel MC-RR detected in *S. intermedia* was increasing during the first three days up to a concentration of 4 µg L^{-1}. This maximum concentration was then reduced slightly during the 21 days exposure time, indicating a possible metabolism of the toxin within the plant.

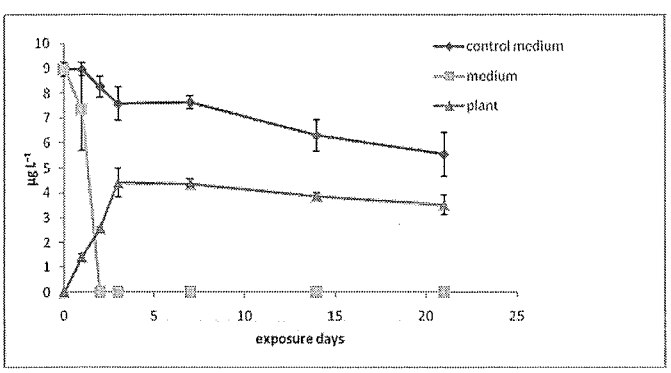

Fig. 1: Analysis of 10 µg L^{-1} MC-RR in medium and plants as well as in a control without plants to see possible bacterial degradation. Bars correspond to +/- standard error from seven independent experiments.

Experiment 2: 100 µg L^{-1} MC-RR

In the controls without plants, the MC-RR concentration was reduced from initially 90 µg L^{-1} to 5 µg L^{-1} in 21 days of exposure (Fig. 2). This decrease might also be due to bacterial degradation or binding of toxins to the surface of the exposure vessel.
In the experiments with plants (Fig. 2), the analyses of MC-RR revealed that toxin concentration in the medium was drastically reduced during the first three days and on day 14 no toxin was detectable anymore. In parallel MC-RR detected in *S. intermedia* was increasing during the first three days up to a concentration of 65 µg L^{-1}. This maximum concentration was then reduced slightly during the 21 days exposure time, indicating a possible metabolism of the toxin within the plant like in the first experiment.

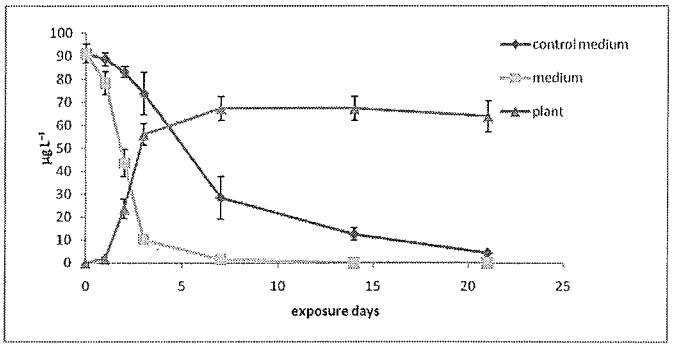

Fig. 2: Analysis of 100 µg L^{-1} MC-RR in medium and plants as well as in a control without plants to see possible bacterial degradation. Bars correspond to +/- standard error from seven independent experiments.

Metabolism of MC-RR by *S. intermedia*

In plant extracts exposed for 21 d to MC-RR a metabolism study was carried out using LC-MSMS detection for possible metabolites. As seen in Fig. 4 (exposure) the parent compound MC-RR was clearly detected in extracts of *S. intermedia* showing a *m/z* of 1038.2 and a related *m/z* at 519.8. Besides the parent compound a glutathione conjugate of MC-RR with a *m/z* of 1345.3 and a breakdown products of this conjugate with a *m/z* of 1158.6 was detected. According to mass calculations this breakdown product is a cysteine conjugate. In plant extracts of the control these metabolites is not showing (Fig. 3).

Discussion

Based on exposure of whole plants to purified MC-RR in medium, uptake of MC-RR in *S. intermedia* could be clearly demonstrated using HPLC-PDA analysis. The both concentration of MC-RR used in this study were in the environmental range, easily found in different water bodies around the world (LAMBERT et al., 1994; HARADA et al., 1996; LAMBERT et al., 1996; PARK et al., 1998; COSTA et al., 2006).

The uptake of cyanobacterial toxins such as MC-LR was shown by PFLUGMACHER et al. (1998, 2001). Here microcystin uptake by the common freshwater macrophyte *C. demersum* was detectable within the first 12 h of exposure resulting in an uptake of about 1.11 % of the applied microcystin concentration. Also microcystin uptake was found in *Elodea canadensis* and *Vesicularia dubyana* both being submerged makrophytes. The uptake of MC-LR by reed plants and the metabolism of MC-LR within the plants were also shown previously (PFLUGMACHER et al., 2001).

The family of *Lemnaceae* are floating makrophytes on the water surface mainly. WEISS et al. (2000) and MITROVIK et al. (2004) showed the influence of MC-RR rsp. MC-LR on *Lemna minor* in concentrations between 0.1 and 5.0 mg L^{-1} on growth and frond size during an exposure of 6 days. The influence of extracts of toxic cyanobacteria on growth and morphology of aquatic plants *Spirodela oligorrhiza* was compared with purified and commercial available MC-LR (ROMANOWSKA-DUDA and TARCZYNSKA, 2002). This was done by exposure of the plants for 96 hours to high concentrations of microcystins. The reduction in the number of fronds was observed after 24 hours of exposure in the presence of 0.2 and 0.1 mg L^{-1} of MC-LR.

Furthermore MITROVIK et al. (2005) found an accumulation of MC-LR in a concentration of 0.288 ng mg^{-1} plant material after an exposure of 5 days. In growth experiments using *Lemna japonica* and a culture of *Microcystis aeruginosa* JANG et al. (2006) were able to show allelophatic effects resulting in a growth inhibition of the *Lemna* species.

In experiments using *Spirodela oligorrhiza* the highest concentration of MC-LR was detected after 96 h of exposure with 0.344 mg MC-LR L^{-1} also showing a growth reduction on 50 % and a reduced frond size.

The absorption cyanobacterial toxins using *Lemna gibba* was confirmed by SAQEANE et al. (2007), where an exposure for 12 days to 300 mg L^{-1} MC-LR equivalents resulted in an absorption of 2.24 mg g^{-1} dry weight.

The metabolism of MC-RR in *S. intermedia* seems to follow the same scheme like shown for MC-LR in *Phragmites australis*, yielding in the formation of a glutathione conjugate by the activity of a

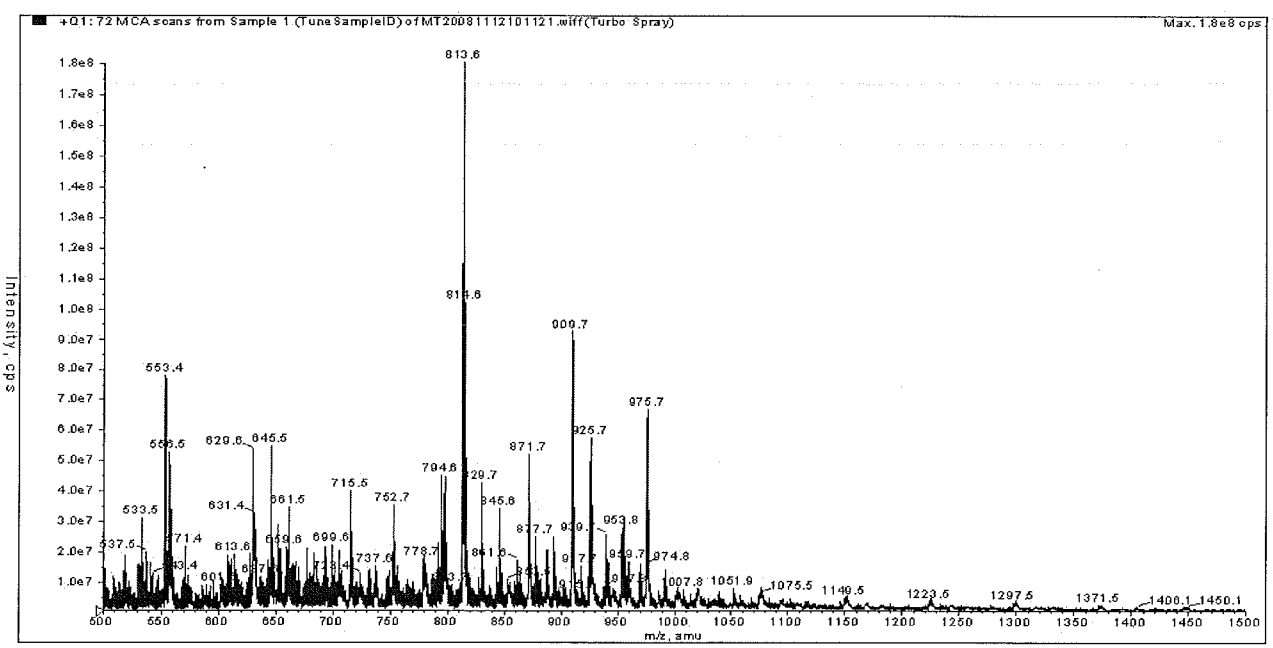

Fig. 3: LC-MSMS analysis of the metabolisation after 21 days in the control of *S. intermedia.*

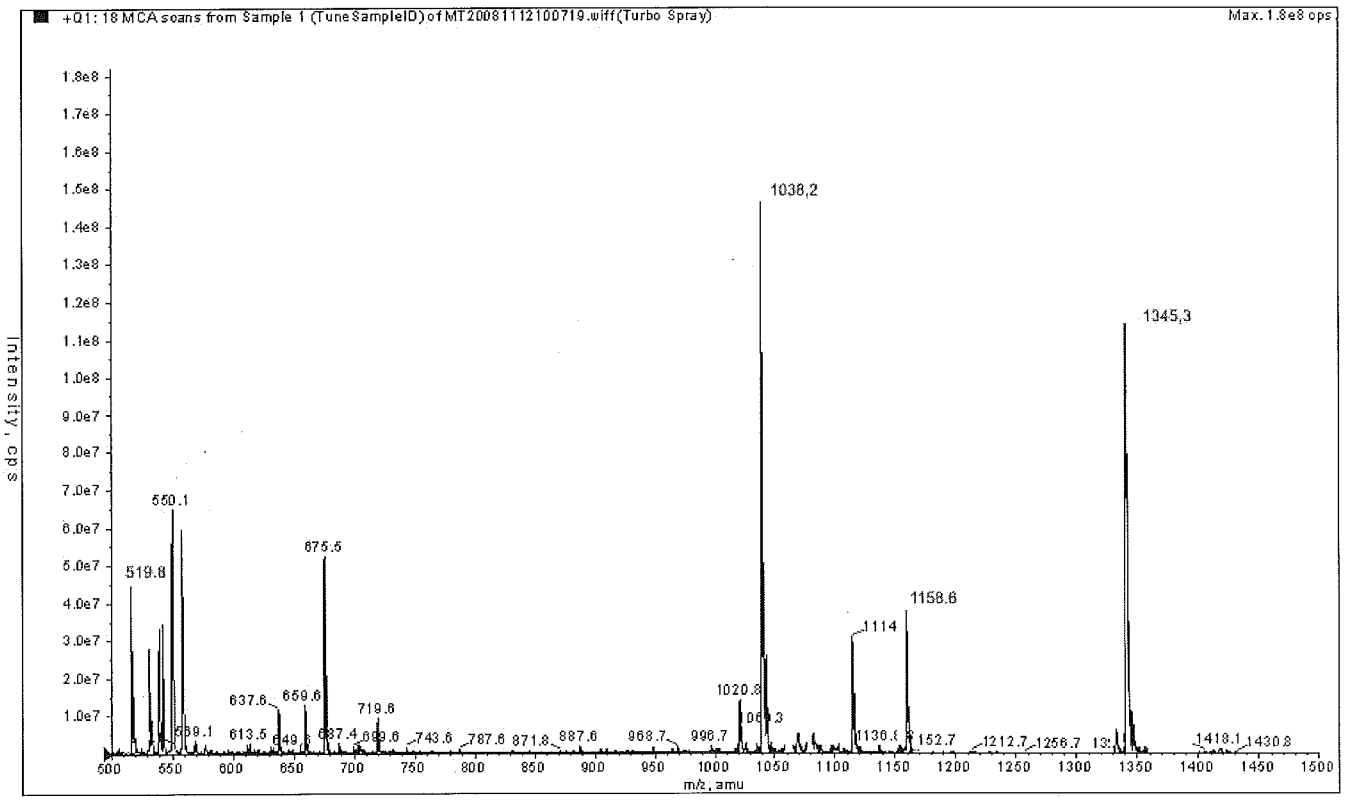

Fig. 4: LC-MSMS analysis of the metabolisation of MC-RR after 21 days in *S. intermedia* showing the presence of MC-RR and two metabolites.

glutathione S-transferase. This conjugate is then further metabolized ending up in a cysteine conjugate (PFLUGMACHER et al., 2001). Also in this study, for the first time using MC-RR, the formation of a MC-RR glutathione conjugate was seen in *S. intermedia* and the further degradation of this metabolite to a cysteine conjugate by the activity of a carboxypeptidase responsible for the cleavage of the glycine moiety (PFLUGMACHER et al., 2001). So this pathway seems to be a general one in aquatic plants and may apply also for different microcystins such as shown by MC-LR and LC-RR.

The uptake of cyanobacterial toxins by plants may play an important role in future for the management of freshwater bodies exhibiting regularly cyanobacterial blooms and also for water purification these ability of aquatic plants to take up and metabolize cyanobacterial toxins might be very important, like shown by NIMPTSCH et al. (2008), using aquatic plants for the removal of cyanobacterial toxins from raw water used for drinking water purposes.

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