Bull Environ Contam Toxicol (2011) 86:232–237
DOI 10.1007/s00128-010-0182-y

Observation of Sward Destruction Caused by Irrigation with Toxic *Microcystis* Morphospecies Containing Water in Southern Hungary

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Received: 20 May 2010 / Accepted: 13 December 2010 / Published online: 25 December 2010

Abstract In the summer of 2006 bloom-like phenomenon occurred in a garden pond in Szeged, Southern Hungary. After regular watering of a sward with pond water containing the algal mass, destruction of garden grass occurred. *Microcystis aeruginosa, Microcystis viridis, Microcystis ichthyoblabe,* and *Microcystis wesenbergii* were identified by light microscopy in the water sample; microcystin-FR, -LR, -RR and -YR were determined by matrix-assisted laser desorption/ionization—time-of-flight analysis. There was an 80% decrease in the green mass (87% in chlorophyll-content) of the grass in a 1 m^2 area of the garden irrigated with pond water.

Keywords *Microcystis* · Irrigation · Grass destruction · Microcystins

Microcystis is one of the most common genera of freshwater cyanobacteria and is often the dominating phytoplankton of eutrophic lakes all over the world (Reynolds et al. 1981; Visser et al. 2005). The microcystins, named after their first known producing genera, are far the best known cyanotoxins that can be isolated from surface water blooms and can cause several economic and health problems.

The microcystins inhibit eukaryotic-like protein phosphatase type 1 and 2A both in animal and in plant cells (MacKintosh et al. 1990; Runnegar et al. 1995). Experiments with plants have shown that most plant species—both mono- and dicotyledons—are sensitive to cyanotoxins. Laboratory experiments using mustard seedlings or aquatic plants (e.g. duckweed) as test plants, proved that the toxic cyanobacterial metabolites have effects on nuclease activity, protein pattern, morphology and photosynthetic pigment content of higher plants (Weiss et al. 2000; M-Hamvas et al. 2003). Results from reed cell suspension and reed tissue culture experiments also highlight the morphological and cytological effects of cyanotoxins (Máthé et al. 2007).

It has been shown that microcystins can accumulate in the tissues of cultivated plants if microcystin-producing cyanobacteria have proliferated in the irrigating water. The growth inhibitory effect and the presence of microcystins in plant tissues after irrigation with toxin-containing water was confirmed in the case of lettuce (*Lactuca sativa*, Codd et al. 1999), bean (*Phaseolus vulgaris*, McElhiney et al. 2001); rape (*Brassica napus*) and rice (*Oryza sativa*) (Chen et al. 2004); broccoli (*Brassica oleracea* var. *italica*) and mustard (*Sinapis alba*) (Järvenpää et al. 2007). Grass species have also recently been shown to be affected by microcystins (Pereira et al. 2009). It is important to draw attention to the fact that most works about the effects of microcystins on plants are laboratory experiments; much less are based on field observations.

The establishment of small artificial ponds as honorific elements of gardens and parks is in fashion nowadays. Phytoplankton often proliferate in these ponds. Nutrients

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released from the soil of set plants, direct sunshine, and rapid warming of the pond water contribute to the proliferation of cyanobacteria (Chorus and Bartam 1999).

In June of 2006, an owner of a garden pond in Szeged noticed that the water color had turned green, and a green mass had appeared on the surface of the water. He wanted to get rid of the algae by pouring the water on the grass in the garden, near the pond. The grass, irrigated regularly with the pond water started to sallow, and between June 19th (the first notice of changes in water quality) and July 9th, it perished. The other part of the garden was irrigated with tap water at a similar intensity, and the destruction of the grass did not occur on that area. We assumed that a toxin-producing cyanobacterial bloom caused the coloring of the water and the destruction of the grass. Our aims were to identify the bloom-causing species; to study the effect of the crude extract of the isolate on plant growth; and to identify the main cyanotoxins of the algal mass.

Materials and Methods

The garden pond (1.5 m depth, 15 m^2 area) is an honorific element of the garden of a family home. The owner watered a 30 m^2 part of the garden with $\sim$300 L water per day using an ALKO drain 6001 submersible pump; the other part of the garden was irrigated at the same rate with tap water. For sampling the garden pond, scum from the water surface and pond water was collected in 5 L plastic cans.

The bloom-forming organisms were identified from the collected algal mass by light microscopy (Leica light microscope). We identified the morphospecies on the basis of morphological characteristics, as described by Komárek and Anagnostidis (1998).

The collected water samples were filtered by plankton net, the cell mass was lyophilized (Christ Alpha 1–2 LD plus lyophilizer) and stored at −20°C until further processing. The lyophilized cells were disrupted by sonication (Transsonic T470/H sonicator), centrifuged (Fisher Model 59A microcentrifuge, 8,000 rpm, 5 min) and the supernatant (the crude extract) was used for the cyanotoxin tests and for the capillary electrophoretic measurements (see below).

The main mass of the lyophilized cells was disrupted by freezing and thawing several times; the cyanotoxin composition and the toxin concentration of the crude extract was determined by micellar electrokinetic chromatography (MEKC) (Prince CEC-770 instrument; polyimide coated fused silica capillary [Supelco, 60 cm × 50 μm id., effective length: 52 cm]; hydrodynamic injection 100 m bar × s^{-1}; applied voltage: 25 kV; 25 mM sodium-tetraborate—100 mM SDS buffer, pH: 9.3; detection by diode-array detector at 239 nm). Dax 3D 8.1 software was used for the evaluation of the electropherograms. The peptide-like compounds were extracted with 70% methanol from the crude extract; the methanolic extract was evaporated to dryness and then resuspended in 10% acetonitrile. The cyanotoxins in the acetonitrile extract were separated by semi-preparative HPLC (Shimadzu LC-10AD VP HPLC instrument; SUPELCO SupelcosilTM LC-18 25 cm × 10 mm, 5 μm column; diode-array UV–VIS detector). The determination of the molecular structure of the purified toxins was carried out by matrix-assisted laser desorption/ionization—time-of-flight mass spectrometry (MALDI-TOF MS) and post-source-decay fragmentation; the mass spectra were qualitatively analyzed as described in Welker et al. 2007.

DNA extraction was achieved by the phenol–chloroform method. PCR was used to analyze the presence or absence of the *mcyB* and *mcyE* specific gene region, which are important parts of the gene cluster responsible for microcystin biosynthesis (Tillett et al. 2000). PCR amplification was accomplished as described in Ouahid et al. (2005). The molecular mass marker was GeneRuler^{TM} 1 kb DNA Ladder (Fermentas), and the photo preparation and the analysis of results were achieved using a Cleaver GelDoc system.

For the quantitative determination of grass destruction, the above-ground phytomass was collected from 1 m^2 of the pond-water irrigated and 1 m^2 of the tap-water irrigated area of the garden on the third week of irrigation, in triplicate. The green masses of the collected vegetation (mainly consisting of perennial rye-grass; *Lolium perenne* L.) were weighed (Orma model bc analytical scale); phytomass samples were dried (65°C, 24 h) and the dry masses were identified. A 2-g mass of grass was disrupted in 40 mL 80% acetone by using a pestle and mortar with sand. The homogenate was centrifuged (Beckman Avanti J-25 centrifuge, 10,000 rpm, 10 min) and the chlorophyll-a content of the samples was determined by measuring the absorbance in 80% acetone at 663 nm (Shimadzu UV-1603 spectrophotometer) (Bendall et al. 1988).

To study the toxic effects of the collected cyanobacteria on rye-grass (*Lolium perenne* L.), a laboratory method for culturing rye-grass was developed. G-Renovation grass-seed mix was used for the experiments. The grass seedlings were grown in Petri dishes on 3 layers of filter paper, and watered with a tenfold dilution of Allen medium. The best results were observed on 3 layers of filter paper, watered with 5 ml 10× diluted Allen medium. One set of the seeds was treated with crude extracts containing 1, 2, 3, 4, 8 mg lyophilized pellet per mL; another set of the seeds was treated with purified microcystin-LR (5, 10, 20, 40, 80 μg × mL^{-1}). Seedlings grown in tenfold diluted Allen medium were used as controls. The seeds were not

previously treated; 30 seeds of similar size were placed in each dish. The percentage of germination was checked every day during the 8-day experiment; the length of the roots and the shoots were measured on days 4, 6 and 8. Every experiment was carried out in triplicate.

To determine the toxicity of the cyanobacterial mass, a modified version of the Blue Green Sinapis Test (BGST, *Sinapis alba*) was used (Kós et al. 1995; Vasas et al. 2002). The concentrations of the cell free cyanobacterial extracts applied for the treatments were as follows: 0.01; 0.1 and 10 mg lyophilized pellet × mL^{-1}.

For the statistical analysis of the data One-Way Anova and Tukey-test were used.

Results and Discussion

During light microscopy we established that the water bloom in the garden pond was caused by several *Microcystis* morphospecies. *Microcystis aeruginosa, Microcystis viridis, Microcystis ichthyoblabe,* and *Microcystis wesenbergii* were identified in the water sample. These are common morphospecies in the temperate region and often cause water blooms in Hungary. Since these species are known to be potentially toxic, we analyzed the cyanobacterial sample to determine the toxic components.

The toxicity of the samples isolated from the garden pond was studied by the well known mustard seedling test (Kós et al. 1995; Vasas et al. 2002). This test is a fast and easy applicable model system to study the effect of toxic compounds on plants. Compounds isolated from the bloom were toxic; the root and shoot lengths of mustard were shorter in the case of mustard seedlings treated with the crude extract than in control plantlets, at all of the applied concentrations (0.01; 0.1; 1 and 10 mg lyophilized pellet × mL^{-1}; data not shown). The statistical analysis (One-Way Anova and Tukey-test) showed that the crude extract caused significant decrease in the growth of mustard seedlings at all of the applied crude extract concentrations (0.01–10 mg × mL^{-1}; $N = 3$, $F = 319.283$, $p = <0.001$). In the case of shoots significant differences among the control and the treated plantlets appeared only at 1 and 10 mg × mL^{-1} concentrations ($N = 3$, $F = 302.218$, $p = <0.001$).

Using standards purified in our laboratory and applying the MEKC method, we characterized the cyanotoxin pattern and determined the total cyanotoxin content of the *Microcystis* bloom (Fig. 1, the arrows show the microcystin peaks). Crude extract containing 10 mg lyophilized pellet × mL^{-1} was used for the capillary electrophoretic measurements. On the basis of the standards the cell mass collected from the garden pond contained 16.8 μg MCY-LR equivalent cyanotoxin per mg dry mass.

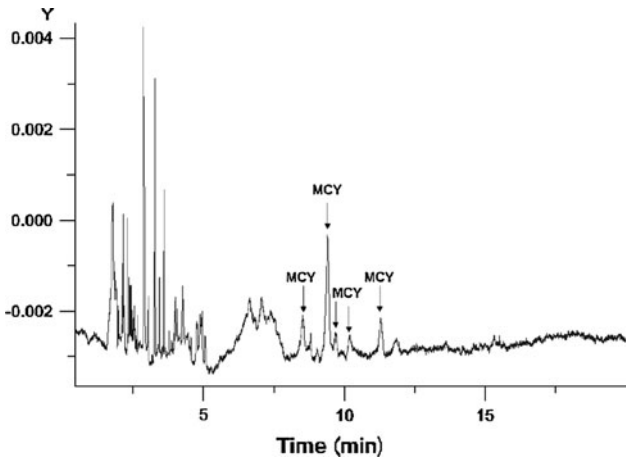

Fig. 1 The result of the applied MEKC method: the cyanotoxin pattern of the collected *Microcystis* cell mass. The *arrows* show the microcystin peaks on the electropherogram

Four of the 9 separated variants were present in sufficient amount for molecular structure determination. Table 1 shows the results of the PSD fragmentation.

As an alternative determination of the toxigenic potential of the *Microcystis* bloom, *mcyB* and *mcyE* genes from the *mcy* gene cluster were used. The chosen gene fragments were amplified in the *Microcystis* samples collected from the garden pond (Fig. 2). These results proved that the *Microcystis* population proliferated in the garden pond had microcystin-producing ability at the genetic level.

Table 2 shows the green mass, dry mass and the chlorophyll a data after 3 weeks irrigation of the different areas of the garden. The pond water-irrigated phytomass was dried, its colour was tawny, briefly the sward looked drained, none the less it was irrigated. The green mass was more than fivefold greater in 1 m^2 of the tap water-irrigated area, than in 1 m^2 of the pond water-irrigated area, and the dry mass of the vegetation was also higher in the tap water-irrigated part of the garden. A 7.5 fold difference in chlorophyll a was observed between the two areas.

In the rye-grass bioassay with crude extract from the cyanobacteria, some inhibition of growth was evident in all of the applied concentrations (1, 2, 3, 4 and 8 mg lyophilized pellet per mL; Fig. 3). A stronger growth inhibitory effect was recognizable from 4 mg × ml^{-1} concentration,

Table 1 The results of the PSD fragmentation: the name and the molar mass of the identified microcystin variants

The identified microcystin variant	m/z
MCY-FR	1029.5
MCY-LR	995.6
MCY-RR	1038.6
MCY-YR	1045.5

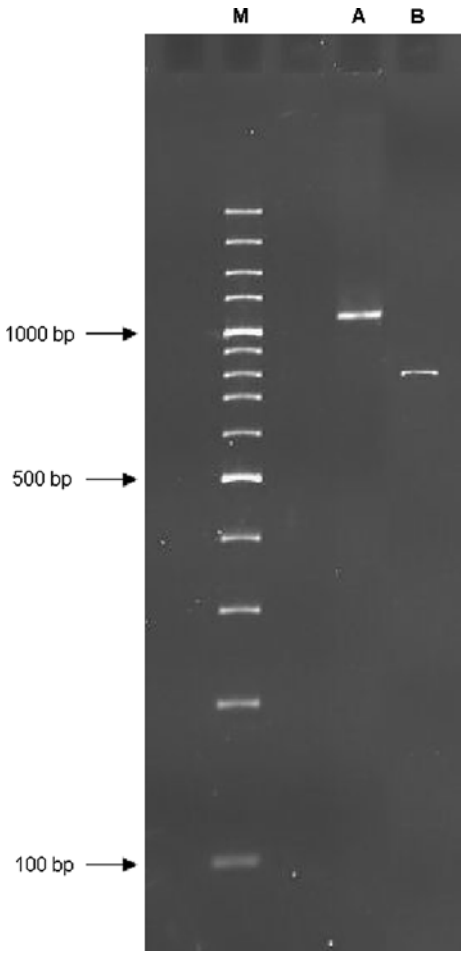

Fig. 2 PCR amplification of the selected gene from the *mcy* gene cluster (*mcyB* and *mcyE*) in the *Microcystis* cell mass collected from the garden pond. M shows the molecular marker (GeneRuler^{TM} 1 kb DNA Ladder; Fermentas); A shows the amplified *mcyB*; and B shows the amplified *mcyE* gene fragments (955 and 755 bp, respectively) in the sample

with the inhibition being most pronounced in the 6–8th days. Roots were more sensitive than shoots (Fig. 3), after 8 days, roots were inhibited even at the lower exposure concentrations of crude extract (1 or 2 mg × mL^{-1}). At the highest crude extract concentration (8 mg × mL^{-1}) roots were inhibited more than three times that of shoots. Statistical analysis of the data showed significant differences among the control and the treated plantlets at all of

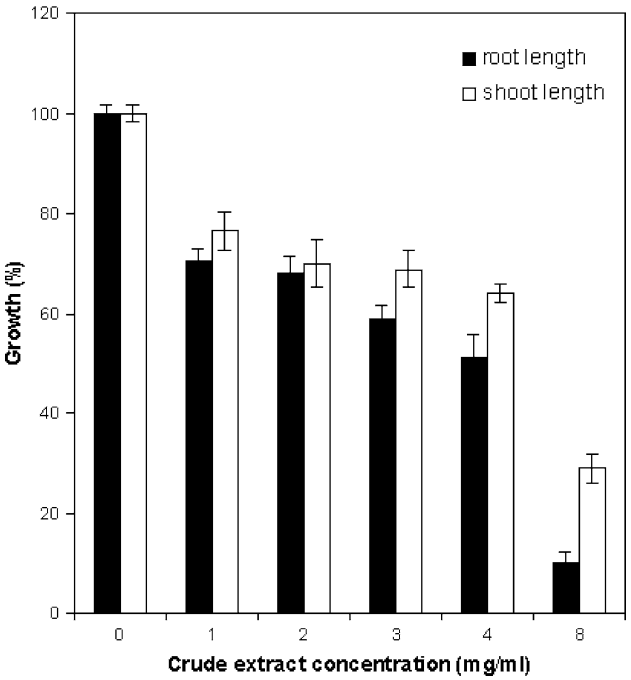

Fig. 3 The roots and shoots lengths on the 8th day of the crude extract treatment. *Black columns* roots; *white columns* shoots

the applied crude extract concentrations (1–8 mg × mL^{-1}), both in the case of roots and shoots (N = 3, F = 116.765, p = <0.001 in the case of roots; N = 3, F = 147.083, p = <0.001 in the case of shoots). There was noticeable necrosis in the roots starting at 2 mg × mL^{-1} crude extract treatment.

MCY-LR from *Microcystis aeruginosa* was used for the purified toxin treatments. Growth inhibition was observed only at relatively high toxin levels; 20 μg × mL^{-1} was the lowest concentration which caused a 5% decrease in root or shoot length (Fig. 4). The inhibitory effect was much stronger at 40 and 80 μg × mL^{-1} toxin concentration (Fig. 4). Statistical analysis of the data of purified MCY-LR treatments showed that significant differences appear only at 20 μg × mL^{-1}, and higher cyanotoxin concentration in the case of roots (N = 3, F = 116.765, p = <0.001). The shoots differed significantly from control when the plantlets were treated with 40 and 80 μg × mL^{-1} MCY-LR concentrations (N = 3, F = 117.162, p = <0.001).

Table 2 Green mass, dry mass (in 1 m^2 area) and chlorophyll a content (per g dry mass) of the vegetation from the different areas of the garden on the 3rd week of irrigation

	Green mass on 1 m^2 area	Dry mass on 1 m^2 area	Chlorophyll-a content per g dry mass
Tap-water irrigated part of the garden ("control")	146.4 g	37.29 g	1.5822 mg × g dry mass^{-1}
Pond-water irrigated part of the garden ("treated")	27.52 g	19.44 g	0.2109 mg × g dry mass^{-1}

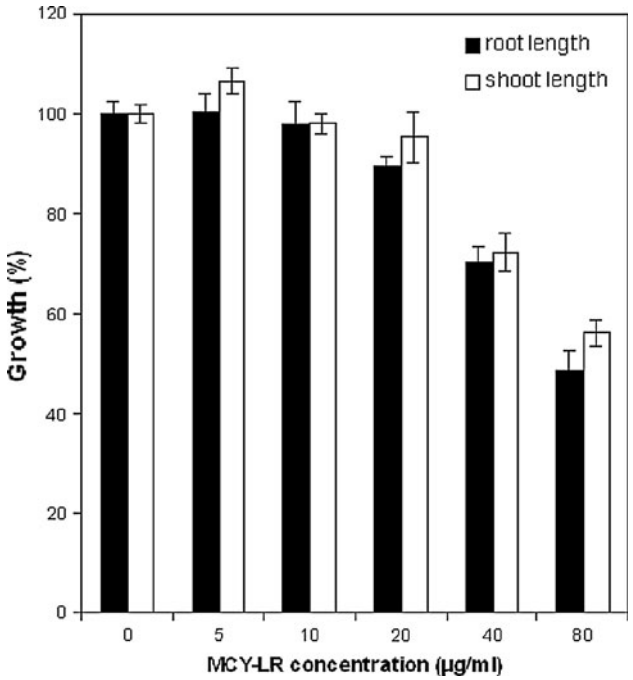

Fig. 4 The roots and shoots lengths on the 8th day of the purified microcystin-LR treatment. *Black columns* roots; *white columns* shoots

As in the case of the crude extract, the roots were more sensitive then the shoots (Fig. 4). Máthé et al. (2007) showed that reed roots in direct contact with cyanotoxin-containing culture medium were more sensitive to the effect of cyanotoxin than shoots. Similar data were obtained by M-Hamvas et al. (2003) for mustard seedlings and by Chen et al. (2004) for rice. Smith et al. (1994) showed that in Arabidopsis, root development is primarily affected by the type 1 and type 2A protein phosphatase inhibitors okadaic acid and calyculin-A, and longitudinal cell growth was inhibited in the elongation zone of roots. These large complex molecules are rapidly taken up by the plants and have similar biochemical effects to those of MCY-LR. This could be the reason for such organ-dependent growth inhibition.

The lyophilized cell mass contained 16.8 µg MCY-LR equivalent per mg dry weight (see above). On the basis of this data, we can say that there is only a slight difference between the inhibitory effect of the crude extract and of the purified toxin: In small concentration the crude extract cause stronger inhibition; 1 and 2 mg × mL^{-1} crude extract—containing 16.8 and 33.6 µg × mL^{-1} MCY-LR equivalent toxin—caused a greater decrease in growth of the shoots and the roots than 20 and 40 µg × mL^{-1} MCY-LR (Figs. 3, 4). At higher concentration the inhibitory effect of the crude extract and the purified MCY-LR is more comparable.

There are a lot of data in the literature about the sensitivity of different plants to microcystins. Máthé et al. (2007) showed that the sensitivity of reed plants was comparable to that of the aquatic plants *Lemna minor* and *Wolffia arrhiza* (Mitrovic et al. 2005), whereas *Ceratophylum demersum* and *Spirodela oligorrhiza* were more sensitive (IC_{50} 0.5 µg × mL^{-1} and 0.1–0.2 µg × mL^{-1}, respectively) to MCY-LR than reed plants (Pflugmacher 2002; Romanowska-Duda and Tarczynska 2002). In contrast, Arabidopsis plants were less sensitive to MCY-LR (IC_{50} values of ≥30 µg × mL^{-1}; Baskin and Wilson 1997). Chen et al. (2004) showed that rice seeds endure higher concentration of microcystins than rape; MCY-treated rice seeds had a higher germination percentage and the inhibitory effect on rice was weaker than on rape. White mustard (*S. alba*), a model system, has an IC_{50} to MCY-LR of up to 3 µg × mL^{-1} (Kós et al. 1995; McElhiney et al. 2001). In the study of Pereira et al. (2009) grass species, *Lolium perenne* or *Festuca rubra* seemed to be not so sensitive on microcystins; the applied MCY concentrations (5.9–56.4 µg × L^{-1}) did not affect seed germination in the case of the two grass species. On the basis of the results of our laboratory experiments we can say that the rye-grass is even less sensitive to microcystins (Figs. 3, 4).

In our laboratory test system the IC_{50} of *Lolium perenne* appeared at an extremely high (80 µg × ml^{-1}) MCY-LR concentration (and at 67.2 MCY-LR equivalent in the case of the crude extract). This means that there could be some mechanisms that protect the rye-grass from the harmful effect of the MCY-LR, or the plantlets do not take up the whole amount of cyanotoxin. It is also known that the different microcystins have different toxic properties (McElhiney et al. 2001), and the cell mass could contain other inhibitory compounds in considerable amount. The answers to these questions require further study.

The laboratory tests showed that the rye-grass is not highly sensitive to microcystins; but the data shows that in 1 L irrigating pond water the amount of contaminating cyanobacterial cells was equivalent to 2.5266 g dry cell mass and 42.4469 mg MCY. It has to be emphasized that this amount of toxin was concentrated in the scum (which was mainly watered on the grass) and presumably this value was not present continuously; it could change in time. Still, this amount of microcystin equivalent cyanotoxin "treatment" during 3 weeks—together with other inhibitory compounds—could lead to the grass destruction; since (as the laboratory tests showed) the crude extract caused inhibition already in smaller concentration; and 40 µg × mL^{-1} toxin concentration also decreased the growth of the plantlets within 8 days.

According to our knowledge, this is the first time that the harmful effect of irrigation with cyanotoxin-containing water on grass species—namely rye-grass—has been reported based upon field observations. The phenomenon

described here attracts attention that not only vegetables but grass species may be included in the hazards associated with irrigation by water containing a bloom of cyanobacteria.

Acknowledgments The work was supported by Hungarian National Research Foundation Grants OTKA F046493 and OTKA K81370, GVOP-3.2.1.-2004-04-0110/3.0., Bolyai Foundation.

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