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CYLINDROSPERMOPSIN INHIBITS GROWTH AND MODULATES PROTEASE ACTIVITY IN THE AQUATIC PLANTS *LEMNA MINOR* L. AND *WOLFFIA ARRHIZA* (L.) HORKEL

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The toxic effects of cylindrospermopsin (cyanobacterial toxin) on animals have been examined extensively, but little research has focused on their effects on plants. In this study cylindrospermopsin (CYN) caused alterations of growth, soluble protein content and protease enzyme activity were studied on two aquatic plants *Lemna minor* and *Wolffia arrhiza* in short-term (5 days) experiments. For the treatments we used CYN containing crude extracts of *Aphanizomenon ovalisporum* (BGSD-423) and purified CYN as well. The maximal inhibitory effects on fresh weight of *L. minor* and *W. arrhiza* caused by crude extract were 60% and 54%, respectively, while the maximum inhibitory effects were 30% and 43% in the case of purified CYN at 20 μg ml^{-1} CYN content of culture medium. In CYN-treated plants the concentration of soluble protein showed mild increases, especially in *W. arrhiza*. Protease isoenzyme activity gels showed significant alterations of enzyme activities under the influence of CYN. Several isoenzymes were far more active and new ones appeared in CYN-treated plants. Treatments with cyanobacterial crude extract caused stronger effects than the purified cyanobacterial toxins used in equivalent CYN concentrations.

Keywords: Aphanizomenon ovalisporum – cylindrospermopsin – protease – *Wolffia arrhiza* – *Lemna minor*

INTRODUCTION

The toxin cylindrospermopsin (CYN) is produced by a variety of cyanobacterial species: *Cylindrospermopsis raciborskii*, *Anabaena bergii, Aphanizomenon ovalisporum, Umezakia natans* and *Raphidiopsis curvata* [35], which became prevalent from tropical regions towards temperate climatic zones in natural lakes or in newly built reservoirs [19]. In an aquatic ecosystem lysis of cyanobacterial blooms leads to the release of a mixture of cyanotoxins and other cyanobacterial components into water, acting on aquatic organisms. Stability of these blooms, the extent of CYN release from cells depends on various environmental factors such as light intensity and water temperature, etc. [20, 22]. Photodegradation of CYN seems to be highly dependent

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Abbreviations: cylindrospermopsin (CYN); fifty percent inhibitory concentration (IC_{50}); phenylmethanesulfonyl fluoride, protease inhibitor (PMSF)

on UV-A light and it is very low under natural conditions [38]. CYN was not degraded by co-occuring natural bacterial communities after 40 days, either [37]. The stability of the cyanotoxin could be one of the reasons of high extracellular CYN concentration found in diverse waterbodies.

Cylindrospermopsin is a tricyclic guanidine derivative containing a hydroxymethyluracil group [17]. CYN is a highly water-soluble molecule with a relatively low molecular mass of 415 Da [27, 28], so its uptake into cells seems to occur by diffusion [6, 35]. Cattle mortalities and human poisonings in Queensland have been attributed to the occurrence of *Cylindrospermopsis raciborskii* blooms producing CYN [35]. In mammals CYN caused liver, kidney, thymus and heart damage, including hepatic cellular vacuolisation, membrane proliferation and cytotoxicity and in addition, renal epithelial necrosis [7, 8, 30]. CYN is a significant and irreversible inhibitor of eucaryotic protein biosynthesis, although the molecular mechanism is yet to be established [8, 30]. CYN inhibited pyrimidine nucleotide synthesis in mouse liver cell-free extracts [24], induced DNA adducts formation, DNA strand breaks and disruption of the kinetochore spindle leading to chromosome loss (aneuploidy), exhibiting the mutagenic as well as carcinogenic activity [23]. In natural waterbodies CYN is able to alter the diversity of zooplankton (e.g. Crustaceae, mussels) communities [35] as well.

In contrast to animals, there are only a limited number of studies on the effects of CYN on plants. Vasas et al. [32] proved that this cyanotoxin inhibits the growth of white mustard *(Sinapis alba)* seedlings, with an 50% inhibitory concentration (IC_{50}) of 18.2 μg ml^{-1}. Germination of tobacco pollen was inhibited by CYN between 5 and 1000 μg ml^{-1} [14]. Aquatic plants have a potentially increased risk of negative effects of cyanotoxins due to their simple anatomical structure and lack of well-developed mechanisms to resist toxicity compared with aquatic vertebrates. Plants have a limited ability to decrease toxin exposure, as dissolved toxins are capable of diffusing into almost all macrophyte habitats [11]. Kinnear et al. [11] demonstrated that whole-cell extracts of *C. raciborskii* containing 0.4 μg ml^{-1} CYN induced a general growth stimulation and decrease of chlorophyll content of *Hydrilla verticillata*, an aquatic plant. The work of Beyer et al. [4] showed that 0.5–40 μg ml^{-1} CYN disturbes reed *(Phragmites australis)* growth and anatomy through the alteration of organization of microtubule system. CYN-treated common reed roots showed several phenomena of general stress responses of plants. In freshwater ecosystems the cosmopolitan species *Lemna minor* and *Wolffia arrhiza* (*Lemnaceae*, Monocotyledons) have an important role in the food chain since they are primary producers and regulators of oxygen level of water. They are sensitive bioindicator species to environmental pollution [9] and these species are of use for testing specific biosynthesis inhibitors [2] and hormones [21]. Microcystins, cyanobacterial toxins with protein phosphatase inhibitory effect caused growth inhibition on *Lemna minor* and *Wolffia arrhiza* after 5 days exposure at 10–20 μg ml^{-1} concentration regime [16]. The effects of CYN have not been studied in these aquatic plants.

Parallel with protein synthesis, proteolysis is essential for many aspects of plant physiology and development. It is responsible for cellular housekeeping and stress response by removing abnormal/misfolded proteins for supplying amino acids needed

to synthesize new proteins, for assisting in the maturation of zymogens and peptide hormones by limited cleavages, for controlling metabolism, homeosis and development by reducing the abundance of key enzymes and regulatory proteins, and for the programmed cell death of specific plant organs or cells. Protein degradation in plants is a complex process involving a multitude of proteolytic pathways organised by proteases or sets of protease enzymes [33]. Unlike other enzymes, classification of proteases is based on the essential amino acids/metals within their active sites, so they can be cysteine-, serine-, aspartic acid-, or metalloproteases. Gelatine containing proteinase activity gels, used in our experiments are suitable to detect all of the four groups of proteases.

There are two approaches in experiences focusing on the effects of cyanotoxins on plants. The first applies purified cyanotoxins for laboratory experiments, while the second investigates the "real-life" situation of a cyanobacterial lysis event with crude extracts [4, 11]. The comparison of the results of different approaches has been missing in the literature of CYN and plant relation. This paper presents a study concerning the influence of CYN upon the growth, protein content and protease activity of two aquatic plant species in short-term (5 d) experiments. Our results allow comparing the effects of CYN in cell free extracts of *Aphanizomenon ovalisporum* (BGSD-423) culture to the effects of purified CYN on plants.

MATERIALS AND METHODS

Aphanizomenon ovalisporum *(Forti) culture, preparation of crude cyanobacterial extract and purification of cylindrospermopsin*

Cylindrospermopsin (CYN) producing *Aphanizomenon ovalisporum* (Forti) strain ILC-164 isolated from Lake Kinneret, Israel in 1994 [3] (in our collection BGSD-423) was grown as described [1, 31, 32] in our laboratory.

For preparation of crude cyanobacterial extract: 14 days old culture of *A. ovalisporum* was centrifuged (6000×*g,* 10 min, Beckman Avanti J-25 centrifuge) at 4 °C, then cell sediments were freeze-dried and stored at –20 °C until further experiments. The freeze-dried cyanobacterial cell mass was resuspended in sterile distilled water; the cells were disrupted by freezing and thawing several times (4×). The suspension was centrifuged (6000×*g,* 10 min, 4 °C, Beckman Avanti J-25 centrifuge) and the pellet- and cell-free supernatant was used as crude extract for the treatments. The CYN content of the supernatant was determined with the help of capillary electrophoresis (Fig. 1) as described in our laboratory earlier [31, 32].

For CYN purification cyanobacterial filaments were harvested by centrifugation and kept at –20 °C until use. The thawed cell pellet was extracted by 90% methanol; the methanolic extract was evaporated to dryness and then resuspended in 50% ethanol. The extract was loaded onto Toyopearl HW-40 size exclusion column and further purified by semi-preparative HPLC (Supercosil TM SPLC-18 column, Supelco, Bellefonte, USA) [31, 32].

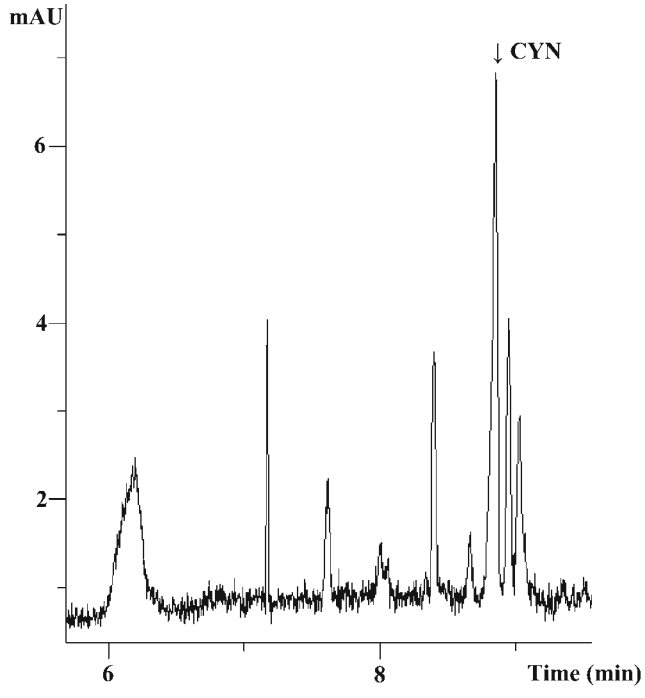


Fig. 1. Electropherogram of crude cell free extract of *Aphanizomenon ovalisporum* culture

Plant material

Experiments were performed on sterile clones of *Lemna minor* L. and *Wolffia arrhiza* (L.) Horkel *(Lemnaceae)*. Axenic stock monocultures were maintained in our laboratory on Allen medium [1]. These test organisms were grown in 25 ml sterile Erlenmayer flasks on 21 °C and 14/10 h photoperiod, 60 μE m^{-2} s^{-1}.

Lemna minor *L. and* Wolffia arrhiza *(L.) Horkel tests*

Plant cultures were aseptically transferred to Allen medium [1] into 3-ml sterile well container (Titer-Tech® test plate). Nine (3 × 3) fronds of *L. minor* were placed in test plate containing CYN extract or purified CYN with equal CYN concentrations (0.01, 0.1, 1, 10 and 20 μg ml^{-1} corresponding to 0.024–96.4 μM, respectively) in Allen medium [1]. In the case of *W. arrhiza* the experiments were carried out in the same way as for *L. minor*, with 20 fronds as an initial number. Three independent experiments were performed and each bioassay was prepared with four replicates for every CYN concentration. The plant growth was estimated by counting frond number and measuring fresh weight of the initial population and the plant cultures after 5 days of

exposure. The significance of differences between growth of controls and CYN treatments was analyzed by one-way ANOVA (Sigma Plot 11.0 software, USA).

Results were presented with the aid of graphical and statistical facilities of Sigma Plot 11.0 software (USA).

Enzyme activity measurement/Gelatin-zymography

For the determination of protease activity, control and cyanotoxin treated plants were collected, pulverized in liquid nitrogen and homogenized with 100 mM Tris-HCl buffer pH 8.0 (Sigma-Aldrich) containing 150 mM NaCl (Reanal, Budapest, Hungary) and 1 µl ml^{-1} 2-mercaptoethanol (Sigma-Aldrich). The homogenates were sonicated and shaked to disintegrate remaining organelles as described by Schlereth et al. [26]. After centrifugation (13,000×*g,* 2 × 5 min, Biofuge) the supernatants were used as crude protein extracts. Protein content was determined by the method of Bradford [5], using bovine serum albumin as standard.

Protease activity analysis of crude protein extracts of aquatic plants (10–20 µl of 10 µg protein) was carried out using gelatine-containing SDS slab gels according to Schlereth et al. [26]. Protein samples were dissolved in SDS-probe buffer without heating and loaded on 10% SDS-polyacrylamide gels [12] containing 0.04% gelatine. The gels were run at 4 °C in dark. To renature proteolytic enzymes, SDS was removed from gels by two 10 min washes in 20% (v/v) 2-propanol in reactivating buffer [15, 26]. The gels were incubated for 5 hours at 37 °C in reactivating buffer (0.1 M sodium phosphate buffer (Reanal, Budapest, Hungary), pH 5.0 or 0.1 M Tris-HCl buffer pH 8.0, both with 5 mM β-mercaptoethanol) in the dark. For characterisation of proteases the gels were incubated in absence and presence of β-mercaptoethanol and phenylmethanesulfonyl fluoride (PMSF), respectively (data not shown).

Local gelatine degradations were visible after Coomassie Blue staining (Coomassie Brilliant Blue R250) and revealed the sites of active proteinases [26].

Relative molecular mass and band intensity were evaluated by UVI-TEC® and CpAtlas® softwares. All the measured parameters were presented graphical with Sigma Plot 11.0 software. Experiments were repeated three times and representative gels are presented in the Results section.

RESULTS

Growth inhibition and alteration in protein content

Both extract (containing CYN) and purified CYN induced growth alterations of *Lemna minor* and *Wolffia arrhiza* (Figs 2, 3). Exposure of *L. minor* to crude extract and purified toxin at 1–20 µg ml^{-1} CYN concentrations caused significant decreases in growth as shown by frond numbers (Figs 2a–c, 3a). In case of purified cyanotoxin significant decreases were experienced at 0.01 and 0.1 µg ml^{-1} concentrations as well.

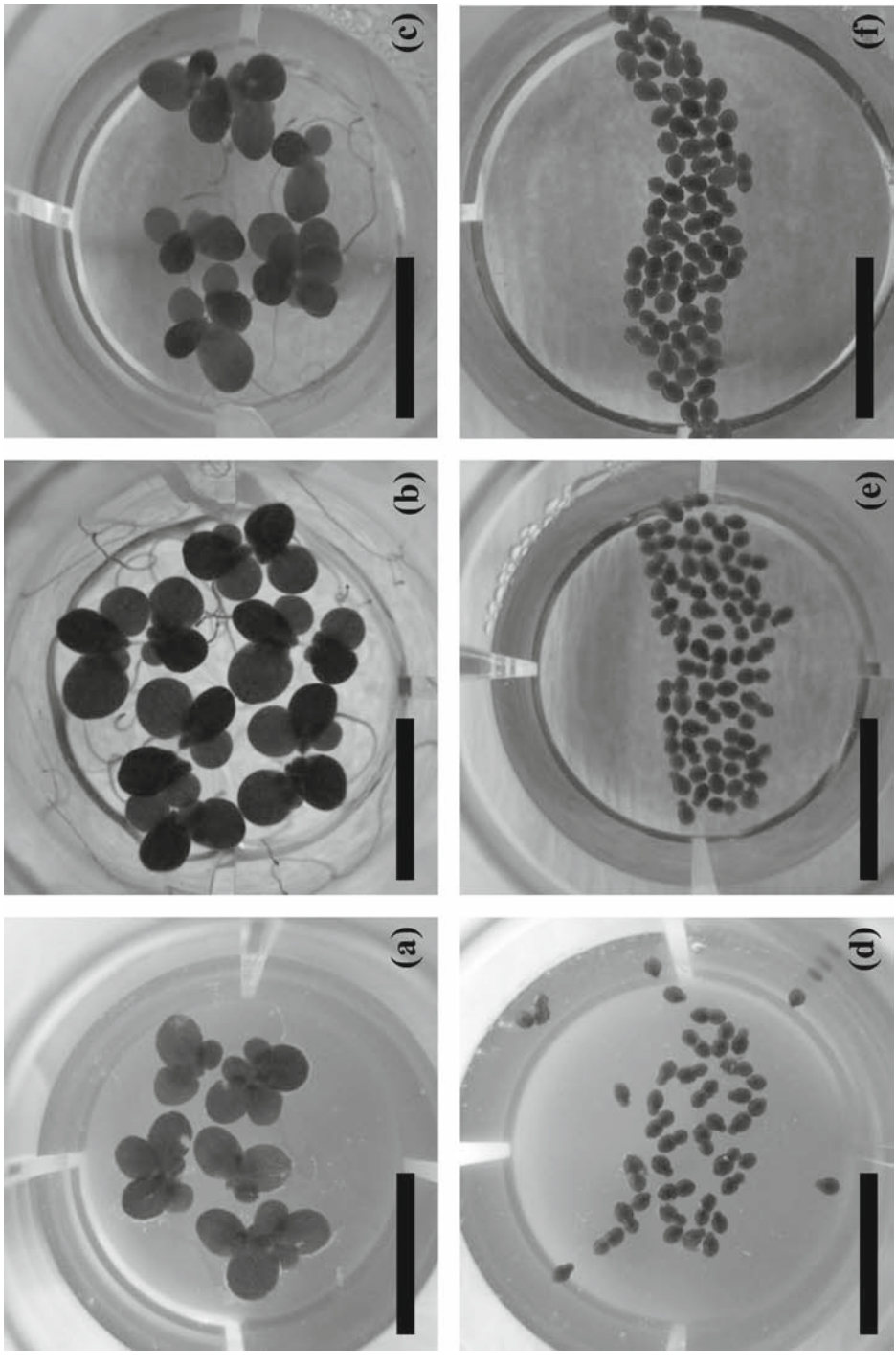


Fig. 2. The effects of cylindrospermopsin on the growth of *Lemna minor* (**a–c**) and *Wolffia arrhiza* (**d–f**) on the fifth day of treatment. **b, c**: control, **a, d**: plants treated with extract containing 20 μg ml^{-1} CYN and **c, f**: plants treated with purified CYN (20 μg ml^{-1}). Bars: 1 cm

The fresh weight of *L. minor* appeared to be a more variable parameter, than the number of fronds. The crude extracts caused significant differences between fresh weights of control and of CYN treated plants at 10 and 20 µg ml^{-1} CYN concentrations. Purified CYN induced significant decrease in fresh weights at 0.1; 10; 20 µg ml^{-1} concentrations (Fig. 3b). Figure 2d–f and Fig. 3d, e illustrate the CYN caused

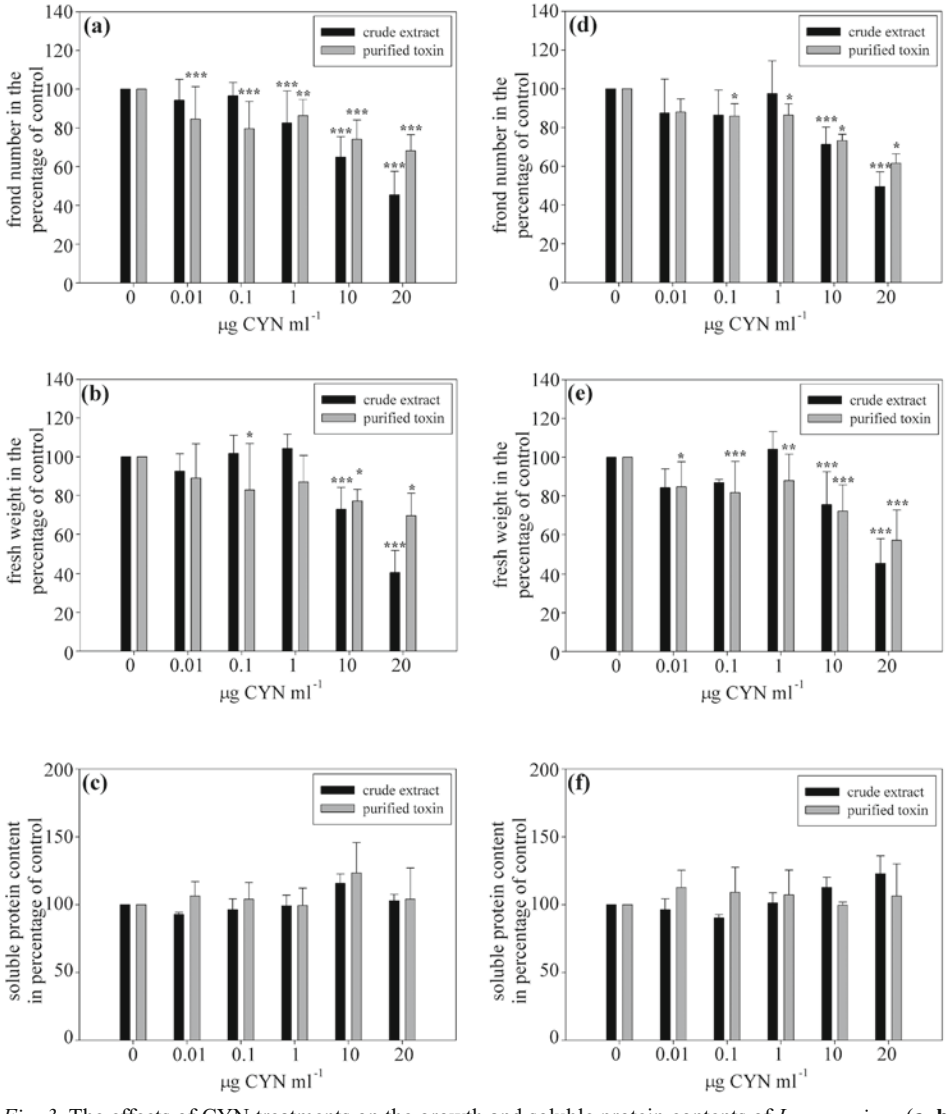


Fig. 3. The effects of CYN treatments on the growth and soluble protein contents of *Lemna minor* (**a, b, c**) and *Wolffia arrhiza* (**d, e, f**) after 5 days of exposure. Bars indicate standard errors, at n>8310. Control of each experiment was considered as 100%. Significant differences between controls and treatments are indicated by $*P<0.05$; $**P<0.01$ and $***P<0.001$

inhibition of *W. arrhiza* growth. Both frond number and fresh weight were significantly reduced only at concentrations of 10 and 20 μg ml^{-1} when crude extracts were used for treatment, but purified CYN caused decreases in fresh weight at 0.01–20 μg ml^{-1} (Fig. 3e) and in frond number at 0.1–20 μg ml^{-1} (Fig. 3d). The maximal inhibitory effects of CYN were measured on fresh weight of plants. At 20 μg ml^{-1} CYN the inhibition was 60% and 54% for crude extract treated *L. minor* and *W. arrhiza,* respectively. These values were 30% and 43%, for purified CYN treated plants (Fig. 3b, e).

No significant differences in soluble protein contents were observed between the two aquatic plant species (Fig. 3c, f). In CYN treated plants the concentration of soluble protein did not decrease even mild increases were characteristic in both investigated plants (Fig. 3c, f).

The effect of CYN on the activity of protease isoenzymes

The effects of crude extracts of *A. ovalisporum* and purified CYN on the activity of protease isoenzymes of *L. minor* and *W. arrhiza* can be detected on gelatine zymograms (Figs 4 and 5). Nine bands in *L. minor* (Fig. 4a and Table 1: signed L_I–L_{IX}) and eight to ten bands (W_I–W_{VIII} and W_+, W_{++}) in *W. arrhiza* protein extracts (Fig. 5a, b and Table 2) were detected. The proteases of both plant species could be classified as three sets of proteases based on molecular mass of protease isoenzymes (see Tables 1 and 2).

Table 1
Protease isoenzyme alterations of *Lemna minor* exposed to the crude extracts of *Aphanizomenon ovalisporum* and purified CYN

Set	Isoenzyme (kDa)		pH 5			pH 8		
			extract treated	control	purified toxin treated	extract treated	control	purified toxin treated
1	L_I	101±2	↑	++	NC	↑	+	NC
	L_{II}	89±3	↑	+	↑ [0.01–1]	↑	++	↑ [0.01–1]
	L_{III}	83±1	↑	++	↑ [0.01–1]	↑	++	↑ [0.01–1]
2	L_{IV}	71±2	↑	+	↑ [0.01–1]	↑	+	↑ [0.01–1]
	L_V	60±2	↑	+	↓	NC	++	NC
	L_{VI}	50±2	↑	+	↓	NC	++	NC
3	L_{VII}	41±2	NC	++	NC	NC	+	NC
	L_{VIII}	35±2	↑	++	↓	NC	+	NC
	L_{IX}	32±1	↑	–	–	–	–	–

+ detectable; ++ high enzyme activity; – not detectable; ↑ CYN caused increase in enzyme activity in 0.01–20 μg ml^{-1} concentration regime; ↓ CYN caused decrease in enzyme activity in 0.01–20 μg ml^{-1} concentration regime; [0.01–1] it is characteristic in 0.01–1 μg ml^{-1} concentration regime; NC not changed as compared to control.

In case of *L. minor* a set of high molecular mass (>75) proteases (set_1) were common on gels incubated at acidic and basic pH as well. In control *L. minor* three proteases were separated ($L_I - L_{III}$) in this set (Fig. 4a). The treatment with CYN caused increase in their activity, the inductive effect of purified CYN was transient, the highest activity was measured for plants treated with crude extract containing 20 μg ml^{-1} CYN (Fig. 4b, c, e, f and Table 1). Proteases with molecular mass between 50 and 70 kDa (set_2) had high activity and it seemed that they have alkaline pH optimum. Isoenzyme L_{IV} was sensitive to CYN, its activity changed similarly to enzymes of set_1. The activities of the two other bands (L_V, L_{VI}) were elevated only by crude extracts. Proteases of set_3 all were acidic proteases with molecular masses of <45 kDa. On gels incubated at pH 5.0, proteases signed L_{VII} and L_{VIII} were very active in control and in CYN-treated plants. These were sensitive to CYN, especially enzyme L_{VIII}. Its activity was increased by the crude extract, but did not change or slightly decreased by purified CYN, consequently their activity was not depended of CYN. The treatment with crude extract induced appearance of a new isoenzyme at pH 5.0 (Fig. 4a, b; Table 1, signed L_{IX}) as well. In case of *L. minor*, crude extract (containing CYN) induced increase of total protease activity at pH 5.0 and at pH 8.0 incubated gels as well, while purified CYN caused only transient elevation of total protease activity at pH 8.0 (Fig. 4d, g).

In case of control *W. arrhiza*, set_1 of high molecular mass (>75) proteases included only one or two enzyme(s) depending on pH and treatment (Fig. 5a and Table 2). At

Table 2
Protease isoenzyme alterations of *Wolffia arrhiza* exposed to the crude extracts of *Aphanizomenon ovalisporum* and purified CYN

Set	Isoenzyme Mw (kDa)		pH 5			pH 8		
			extract treated	control	purified toxin treated	extract treated	control	purified toxin treated
1	W_I	94±4	↑	+	NC	↑	++	NC
	W_{II}	84±3	↑	–	–	↑	–	–
	W_{III}	78±2	↑	–	↑	↑	++	NC
2	W_+	72±3	–	–	–	NC	+	NC
	W_{IV}	63±3	↑	++	NC	↑	+	↓ [10–20]
	W_V	56±4	↑	–	–	↑	++	↓ [10–20]
	W_{VI}	53±2	↑ [0–1]	+	↓	–	–	–
	W_{VII}	49±1	+	–	–	↑	++	↓
3	W_{++}	44±1	–	–	–	↑	–	–
	W_{VIII}	41±1	NC	+	NC	–	–	–

+ detectable; ++ high enzyme activity; – not detectable; ↑ CYN caused increase in enzyme activity in 0.01–20 μg ml^{-1} concentration regime; ↓ CYN caused decrease in enzyme activity in 0.01–20 μg ml^{-1} concentration regime; [0.01–1] it is characteristic in 0.01–1 μg ml^{-1} concentration regime; NC not changed as compared to control.

(a) I. II. III. IV. V. VI.VII. VIII.IX.
(b)
crude extract
pH 5
(c)
purified cyanotoxin
I.
II.
III.
IV.
V.
VI.
VII.
VIII.
IX.
66
45
34.7
0 0.01 0.1 1 10 20
(d)
total band intensity
(number of square pixels)
in the percentage of control
200
150
100
50
0
crude extract
purified toxin
0 0.01 0.1 1 10 20
μg CYN ml-1

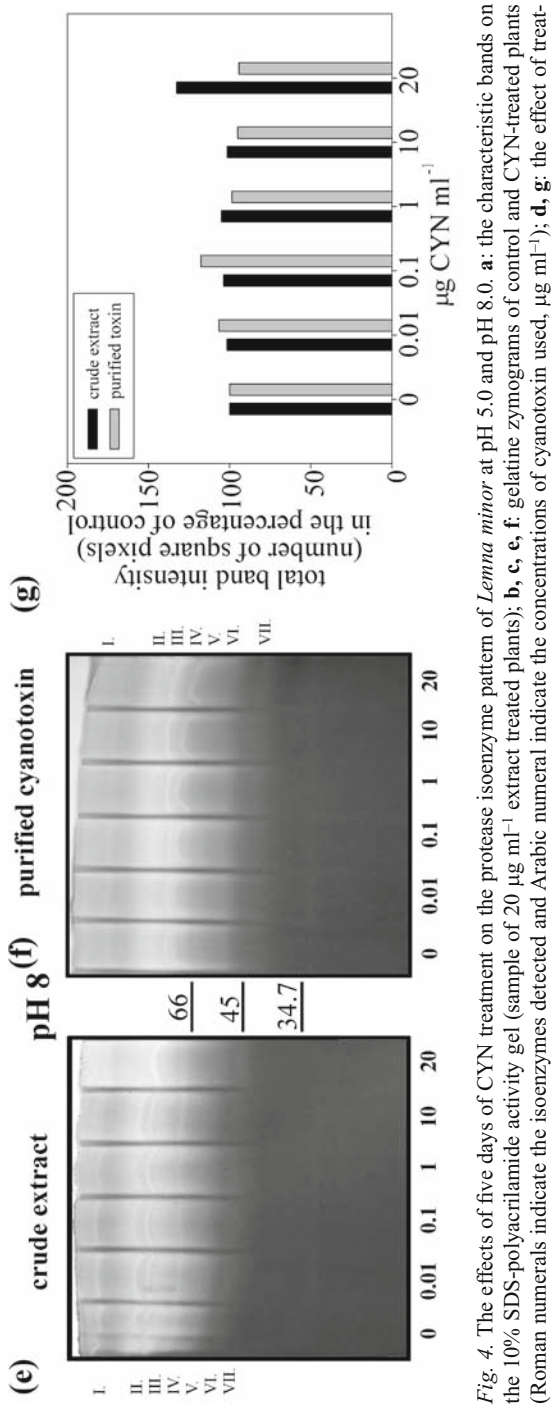
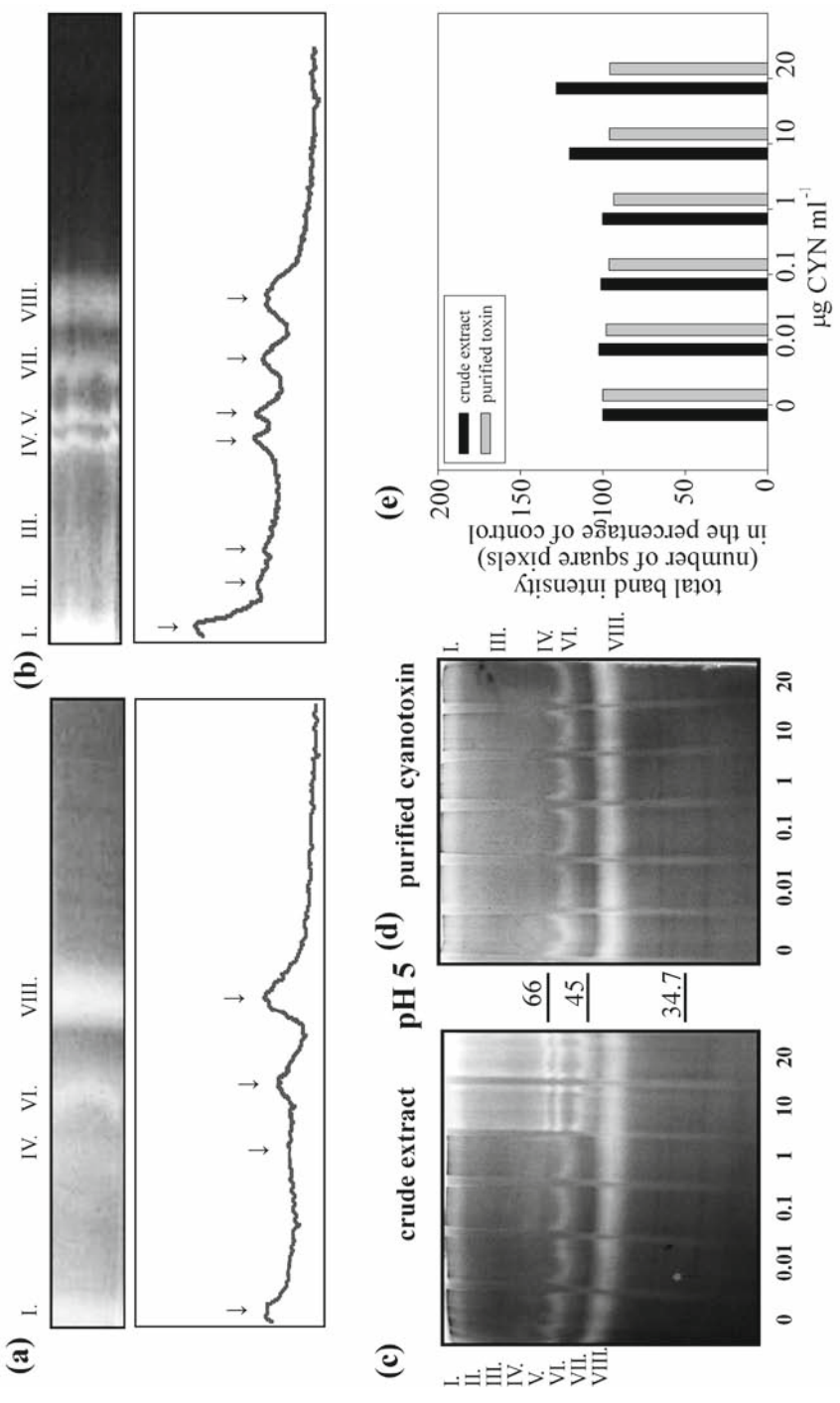


Fig. 4. The effects of five days of CYN treatment on the protease isoenzyme pattern of *Lemna minor* at pH 5.0 and pH 8.0. **a**: the characteristic bands on the 10% SDS-polyacrilamide activity gel (sample of 20 µg ml^{-1} extract treated plants); **b, c, e, f**: gelatine zymograms of control and CYN-treated plants (Roman numerals indicate the isoenzymes detected and Arabic numeral indicate the concentrations of cyanotoxin used, µg ml^{-1}); **d, g**: the effect of treatment on total protease activity


(a)
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VI.
VIII.
(b)
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IV. V.
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crude extract
pH 5
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II.
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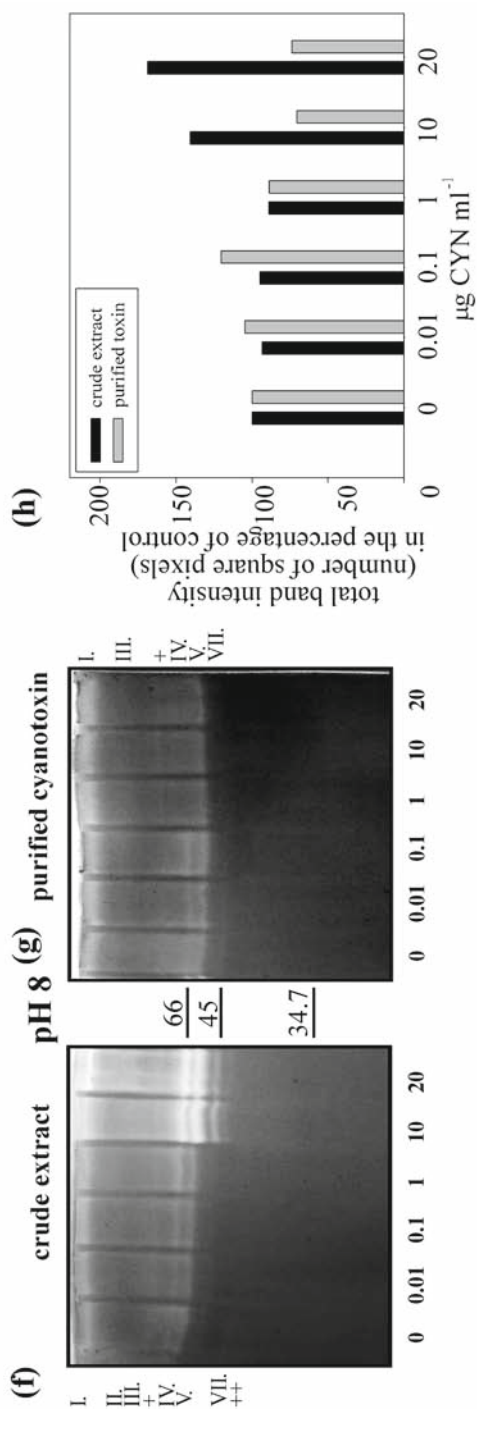

Fig. 5. The effects of five days of CYN treatment on the protease isoenzyme pattern of *Wolffia arrhiza* at pH 5.0 and pH 8.0. **a**: the characteristic bands on the 10% SDS-polyacrilamide activity gel of control and **b**: plants treated with *A. ovalisporum* extracts containing 20 µg ml^{-1} CYN; **c, d, f, g**: gelatine zymographies of control and CYN-treated plants (Roman numerals indicate the isoenzymes detected and Arabic numerals indicate the concentrations of cyanotoxin used, µg ml^{-1}); **e, h**: the effect of CYN treatment on total protease activity

10–20 μg ml^{-1} CYN we detected three isoenzymes in crude extract treated plants and two isoenzymes in purified toxin treated ones (Fig. 5b and Table 2). The zymograms of proteases with molecular masses between 47 and 70 kDa (set_2) are variable depending on pH and treatments. The most conspicuous changes were on the gels incubated at pH 5.0 (Fig. 5c). There was an enzyme band (W_{VI}), which was characteristic in control, purified cyanotoxin and crude extract treated plants, but for the latter one only at 0.01–1 μg ml^{-1} CYN concentration regime (Fig. 5c; Table 2). In the crude extract (containing 10–20 μg ml^{-1} CYN) treated plants it was not detected and two new bands (W_V, W_{VII}) were observed. Band (W_{IV}) increased in crude extracts treated plants as well (Fig. 5c). In contrast to crude extract, purified CYN did not influence the enzyme pattern on gels pH 5.0 (Fig. 5d). There was an enzyme with alkaline pH optimum (signed "W_+" on Table 2), which was not recognised on acidic gel and CYN did not influence its activity (Fig. 5f). It is worth mentioning, that bands W_V and W_{VII} were similarly inducible under both acid and alkaline conditions in crude extract treated plants. Proteases with molecular mass of <45 kDa (set_3) included only two enzymes, one is an acidic protease with high activity (W_{VIII}), was not influenced by CYN. At alkaline pH a new protease isoenzyme activity was induced by 10 and 20 μg ml^{-1} CYN in crude extract (Fig. 5f and Table 2, signed "W_{++}"). The total protease activity (sum of the activity of isoenzymes) of crude extract treated *W. arrhiza* showed an increase at the highest concentrations (10 and 20 μg ml^{-1} CYN), while purified cyanotoxin increased only transiently the activity of enzymes at pH 8.0 (in 0.01–0.1 μg ml^{-1} CYN; Fig. 5e, h). However, this transient increase could be attributed to the increase in the activities of isoenzymes W_{IV} and W_V at 0.01 and 0.1 μg ml^{-1} CYN only. The activities of these isoenzymes decreased at higher cyanotoxin concentrations (10–20 μg ml^{-1} CYN, Fig. 5e, h, Table 2).

DISCUSSION

Macrophytes from the *Lemnaceae* family in freshwater bodies are widespread and fast-growing plants with doubling times of 1–4 days. They can be good indicators of environmental pollution [13, 18] and metabolits of other organisms for example algal species living together [29]. In the present work the toxic effects of purified CYN and a CYN containing cyanobacterial extracts were examined on *Lemna minor* and *Wolffia arrhiza*. Significant reduction in fresh weight and frond-number were observed in both treatments on the fifth day of the treatment. There was not a large difference between the toxic effects of crude extract and purified cyanotoxin. Twenty μg ml^{-1} CYN in crude extract decreased the fresh weight of plants by 60–55%. The inhibitory effect of purified CYN was a bit weaker (30–40%). Vasas et al. [32] described that CYN inhibited the growth of *Sinapis alba* seedlings grown in dark. The IC_{50} for *Sinapis alba* was in good agreement with our results. *Hydrilla verticillata,* a rooted, submersed aquatic plants was exposed to whole-cell extracts of *Cylindrospermopsis raciborskii* containing nominal treatment concentrations of 25–400 μg l^{-1} extracellular CYN over 14 days. Plants were able to uptake CYN in a concentration

dependent manner and accumulated CYN at a maximum of 176 ng g^{-1} freeze-dried concentration [11], which is below the exposure concentration. In spite the lack of bioaccumulation *Hydrilla* showed alterations in growth, chlorophyll components, and root production without chlorosis or necrosis [11, 34]. *Phragmites australis* plantlets originated from tissue cultures were sensitive to CYN applied in 0.5–40 μg ml^{-1} concentration regime [4]. As general stress responses of plants to cyanotoxins, CYN increased root numbers, induced the formation of callus-like tissue and necrosis, correlated with alterations in orientation of microtubules. We confirmed the growth inhibitory effects of CYN on aquatic plants, but without necrosis and plant death at a short exposition time.

Soluble protein content is a good parameter for the characterization of key processes in plant life, like senescence or responses to environmental stress conditions. In these processes the elevation of soluble protein content parallel with the increase of stress induced enzymes (e.g. catalase, superoxide dismutase, peroxidase) activity is characteristic [10, 21]. CYN is a protein-synthesis inhibitor in animals and plants [8, 14, 30]. In our experiments the maximum exposure concentrations was 20 μg ml^{-1} and it did not cause decrease in soluble protein content per unit fresh weight. We can suppose, that the synthesis of certain proteins may be inhibited by CYN, while the synthesis of others does not change or it can even increase (e.g. in case of stress proteins). Beyer et al. [4] showed that CYN induces increases of tubulin content in *Phragmites australis* roots. We recognized that the relative amount of active protease enzymes in the protein pool of cells increased in consequence of 10 and 20 μg ml^{-1} CYN treatments. Crude extract (containing CYN) induced an increase of total protease activity of both *L. minor* and *W. arrhiza* plants on gels incubated at pH 5 and pH 8 (Figs 4 and 5). In case of purified CYN treated plants the increase in total enzyme activity appeared at lower concentration regimes (0.01–1 μg ml^{-1}), but this increase did not exceed the effect of extract containing 10 and 20 μg ml^{-1} CYN (Figs 4 and 5). It is known that crude extracts of toxic cyanobacteria contain several substances [35], which could contribute to the harmful effects of cyanotoxins. This was established by comparing the effects of purified microcystin-LR with the effects of cyanobacterial extract on *Spirodela oligorrhiza* [25]. The CYN treatments induced new isoenzyme activities on gelatine containing gels: in case of *L. minor* at pH 5 (Fig. 4b; Table 1, signed L_{IX}). It seemed that isoenzyme L_{IX} together with isoenzymes L_{VII} and L_{VIII} require, β-mercaptoethanol (5 mM) in the incubation buffer and are inhibited by PMSF (10 mM in incubation buffer, data not shown) and are most active at acidic pH, so they may be cysteine-proteases. In case of crude extract treated *W. arrhiza* a new band appeared at gel pH 8 (Fig. 5f; Table 2, signed W_{++}). Bands with high activity were characteristic on gels of crude extract treated plants (Fig. 5c, f; Table 2, signed W_{II}, W_{III}, W_{VII}). Their activity was decreased by PMSF. Band W_{VII} required β-mercaptoethanol, in opposite to izoenzymes W_{II} and W_{III}, which were active in the absence of it (data not shown). PMSF has inhibitory effect to serine proteinases and several cysteine proteinases, so partially could suppress cell death, for example in pathogen infected tobacco leaves and menadione-induced protoplasts [36]. Cysteine and serine proteases are very important enzymes in processes such as

hypersensitive response, signal transduction, programmed cell death [33, 36]. They exist in different plant organs and as vacuolar proteases they are very important in protein turnover. It is worth mentioning, that vacuolar proteases account for most of the proteolytic activity measured in plant extracts [26, 33]. β-Mercaptoethanol (for reducing conditions) could be more important for cysteine proteases, because they have cysteine (with thiol residue) within their active sites [26]. The increased amount and altered pattern of these enzymes in CYN-treated plants proved that despite the temperate effect on growth, CYN, a cyanobacterial cyanotoxin can disturb such a key process as proteolysis. Proteolysis of cellular proteins is a constant and ongoing process in plant cells. Abnormal proteins arise in cells as a result of stresses. We can suppose that such protein alterations can be induced by cyanotoxins and those proteins are subjects of proteolytic processes. The changes of *L. minor* and *W. arrhiza* protease patterns after CYN exposure support this idea and they may be important in regulating numerous biological processes in cyanotoxin exposed plants.

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REFERENCES

1. Allen, M. M. (1968) Simple conditions for the growth of unicellular blue-green algae on plates. *J. Phycol*. *4,* 1–4.
2. Bajguz, A., Asami, T. (2005) Suppression of *Wolffia arrhiza* growth by brassinazole, an inhibitor of brassinosteroid biosynthesis and its restoration by endogenous 24-epibrassinolide. *Phytochemistry 66,* 1787–1796.
3. Banker, R., Carmeli, S., Hadas, O., Teltsch, B., Porat, R., Sukenik, A. (1997) Identification of cylindrospermopsin in *Aphanizomenon ovalisporum* (Cyanophyceae) isolated from Lake Kinneret, Israel. *J. Phycol*. *33,* 613–616.
4. Beyer, D., Surányi, G., Vasas, G., Roszik, J., Erdődi, F., M.-Hamvas, M., Bácsi, I., Bátori, R., Serfőző, Z., Szigeti, Z. M., Vereb, G., Demeter, Z., Gonda, S., Máthé, C. (2009) Cylindrospermopsin induces alterations of root histology and microtubule organization in common reed *(Phragmites australis)* plantlets cultured in vitro. *Toxicon 54,* 440–449.
5. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem*. *72,* 248–254.
6. Chong, M. W. K., Wong, P. K. S., Shaw, G. R., Seawright, A. A. (2002) Toxicity and uptake mechanism of cylindrospermopsin and lophyrotomin in primary rat hepatocytes. *Toxicon 40,* 205–211.
7. Falconer, I. R., Hardy, S. J., Humpage, A. R., Froscio, S. M., Tozer, G. J., Hawkins, P. R. (1999) Hepatic and renal toxicity of the blue-green alga (cyanobacterium) *Cylindrospermopsis raciborskii* in male Swiss albino mice. *Environ. Toxicol*. *14,* 143–150.
8. Froscio, S. M., Humpage, A. R., Burcham, P. C., Falconer, I. R. (2003) Cylindrospermopsin-induced protein synthesis inhibition and its dissociation from acute toxicity in mouse hepatocytes. *Environ. Toxicol*. *18,* 243–251.

9. Horvat, T., Vidaković-Cifrek, Ž, Oreščanin, V., Tkalec, M., Pevalek-Kozlina, B. (2007) Toxicity assessment of heavy metal mixtures by *Lemna minor* L. *Sci. Total Environ. 384,* 229–238.
10. Huang, B., Zhang, G. (2000) Physiological and biochemical changes during seed filling in relation to leaf senescence in soybean. *Biol. Plantarum 43,* 545–548.
11. Kinnear, S. H. W., Fabbro, L., Duivenvoorden, L. J. (2008) Variable growth responses of water thyme *(Hydrilla verticillata)* to whole-cell extracts of *Cylindrospermopsis raciborskii*. *Arch. Environ. Contam. Toxicol*. *54,* 187–194.
12. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227,* 680–685.
13. Lakatos, G., Mészáros, I., Bohátka, S., Szabó, S., Makádi, M., Csatlós, M., Langer, G. (1993) Application of Lemna species in ecotoxicological studies of heavy metals. *Sci. Total Environ. 44,* 773–778.
14. Metcalf, J. S., Barakate, A., Codd, G. A. (2004) Inhibition of plant protein synthesis by the cyanobacterialhepatotoxin, cylindrospermopsin. *FEMS Microbiol. Letters 235,* 125–129.
15. M.-Hamvas, M., Máthe, Cs., Molnár, E., Vasas, G., Grigorszky, I., Borbély, Gy. (2003) Microcystin-LR alters the growth, anthocyanin content and single-stranded DNase enzyme activities in *Sinapis alba* L. seedlings. *Aquat. Toxicol*. *62,* 1–9.
16. Mitrovic, S. M., Allis, O., Furey, A., James, K. J. (2005) Bioaccumulation and harmful effects of microcystin-LR in the aquatic plants *Lemna minor* and *Wolffia arrhiza* and the filamentous alga *Chladophora fracta*. *Ecotox. Environ. Safe*. *61,* 345–352.
17. Ohtani, I., Moore, R. E., Runnegar, M. T. C. (1992) Cylindrospermopsin: a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii. J. Am. Chem. Soc*. *114,* 7941–7942.
18. Oláh, V., Combi, Zs., Szőllősi, E., Kanalas, P., Mészáros, I. (2009) Anthocyanins: possible antioxidants against Cr(VI) induced oxidative stress in *Spirodela polyrrhiza*. *Cer. Res. Commun. 37,* 533–536.
19. Padisák, J. (1997) *Cylindrospermopsis raciborkii* (Woloszynska) Seenayya et Subba Raju, an expanding, highly adaptive cyanobacterium: worldwide distribution and review of its ecology. *Arch. Hidrobiol./Suppl. 4,* 563–593.
20. Pietsch, C., Wiegand, C., Amé, V. M., Nicklisch, A., Wunderlin, D., Pflugmacher, S. (2001) The effects of a cyanobacterial crude extraction different aquatic organisms: Evidence for cyanobacterial toxin modulating factors. *Environ. Toxicol. 16,* 535–542.
21. Piotrowska, A., Bajguz, A., Godlewska-Żyłkiewicz, B., Czerpak, R., Kamińska, M. (2009) Jasmonic acid as modulator of lead toxicity in aquatic plant *Wolffia arrhiza* (Lemnaceae). *Environ. Exp. Bot. 66,* 507–513.
22. Preußel, K., Wessel, G., Fastner, J., Chorus, I. (2009) Response of cylindrospermopsin production and release in *Aphanizomenon flos-aquae* (Cyanobacteria) to varying light and temperature conditions. *Harmful Algae 8,* 645–650.
23. Rastogi, R. P., Sinha, R. P. (2009) Biotechnological and industrial significance of cyanobacterial secondary metabolites. *Biotechnol. Adv. 27,* 521–539.
24. Reisner, M., Carmeli, S., Werman, M., Sukenik, A. (2004) The cyanobacterial toxin cylindrospermopsin inhibits pyrimidine nucleotide synthesis and alters cholesterol distribution in mice. *Toxicol. Sci. 82,* 620–627.
25. Romanowska-Duda, Z., Tarczynska, M. (2002) The influence of microcystin-LR and hepatotoxic cyanobacterial extract on the water plant *Spirodela oligorrhiza*. *Environ. Toxicol. 17,* 434–440.
26. Schlereth, A., Becker, C., Horstmann, C., Tiedemann, J., Müntz, K. (2000) Comparison of globulin mobilization and cysteine proteinases in embryogenic axes and cotyledons during germination and seedling growth of vetch (*Vicia sativa* L.). *J. Exp. Bot. 51,* 1423–1433.
27. Shaw, G. R., Sukenik, A., Livne, A., Chiswell, R. K., Smith, M. J., Seawright, A. A., Norris, R. L., Eaglesham, G. K., Moore, M. R. (1999) Blooms of the hepatotoxic cyanobacterium, *Aphanizomenon ovalisporum* (Forti) in newly constructed lakes, Queensland, Australia. *Environ. Toxicol. 14,* 167–177.

28. Sivonen, K., Jones, G. (1999) In: Chorus, I., Bartman, J. (eds) *Toxic Cyanobacteria in Water. A Guide to their Public Health Consequences, Monitoring and Management.* E&FN Spon, London and New York, pp. 41–111.
29. Szabó, S., Braun, M., Balázsy, S., Reisinger, O. (1998) Influences of nine algal species isolated from duckweed-covered sewage miniponds on *Lemna gibba* L. *Aquat. Bot. 60,* 189–195.
30. Terao, K., Ohmori, S., Igarashi, K., Ohtani, I., Watanabe, M. F., Harada, K. I., Ito, E., Watanabe, M. (1994) Electron-microscopic studies on experimental poisoning in mice induced by cylindrospermopsin isolated from blue-green-alga *Umezakia natans*. *Toxicon 32,* 833–843.
31. Vasas, G., Gáspár, A., Páger, C., Surányi, G., Máthé, C., M.-Hamvas, M., Borbély, G. (2004) Analysis of cyanobacterial toxins (anatoxin-a, cylindrospermopsin, microcystin-LR) by capillary electrophoresis. *Electrophoresis 25,* 108–115.
32. Vasas, G., Gáspár, A., Surányi, G., Batta, G., Gyémánt, G., M.-Hamvas, M., Máthé, C., Grigorszky, I., Molnár, E., Borbély, G. (2002) Capillary electrophoretic assay and purification of cylindrospermopsin, a cyanobacterial toxin from *Aphanizomenon ovalisporum*, by plant test (blue–green *Sinapis* test). *Anal. Biochem. 302,* 95–103.
33. Vierstra, R. D. (1996) Proteolysis in plants: mechanisms and functions. *Plant Molecular Biology 32,* 275–302.
34. White, S. H., Duinvenvoorden, L. J., Fabbro, L. D. (2005) Absence of free-cylindrospermopsin bioconcentration in water thyme (*Hydrilla verticillata*). *B. Environ. Contam. Tox. 75,* 574–583.
35. Wiegand, C., Pflugmacher, S. (2005) Ecotoxicological effects of selected cyanobacterial secondary metabolites: a short review. *Toxicol. Appl. Pharmacol. 203,* 201–218.
36. Woltering, E. J., van der Bent, A., Hoeberichts, F. A. (2002) Do plant caspases exist? *Plant Physiol. 130,* 1764–1769.
37. Wormer, L., Cires, S., Carrasco, D., Quesada, A. (2008) Cylindrospermopsin is not degraded by co-occurring natural bacterial communities during a 40-day study. *Harmful Algae 7,* 206–213.
38. Wormer, L., Huerta-Fontela, M., Cires, S., Carrasco, D., Quesada, A. (2010) Natural photodegradation of the Cyanobacterial toxins, microcystin and cylindrospermopsin. *Environ. Sci. Technol. 44,* 3002–3007.