Title: Toxicity and toxin composition of *Microcystis aeruginosa* from Wangsong reservoir

Running title: Wangsong *Microcystis aeruginosa* toxicity

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Abstract

Objective

The increasing world population, resulting in increased anthropogenic water pollution, is negatively impacting the limited available water resources. In South Korea, this similarly affects the water quality of reservoirs. As water is a basic necessity for life, water quality monitoring is essential but typically does not include toxicity testing. However, as toxic bloom event frequencies are increasing, this previously neglected aspect becomes pertinent. Therefore, in the present study, the toxin composition and toxicity of a *Microcystis aeruginosa* strain isolated from a persistent bloom in lake Wangsong, South Korea, was investigated.

Methods

A combination of bioassays and chemical analysis was used for this purpose. The bioassay species included terrestrial and aquatic plants, an alga, a rotifer, a tubificid annelid, and crustaceans, representing various trophic levels.

Results

The strain was found to produce microcystin-LR, -RR, and YR, as well as β-*N*-methylamino-L-alanine. The bioassays indicated that the primary producers were less sensitive to the crude extract.

Conclusion

The presence of absence of a visible cyanobacterial bloom is also not an indication of the toxins that may be present in the afflicted waters, and thus does not predict exposure risk. Similarly, the presence and absence of toxins and mixtures thereof does not indicate the ecological effect. Therefore, it would be advantages to include toxicity testing into routine water testing regimes to better understand the impact of harmful algal blooms.

Keywords:

Cyanobacteria, microcystin congeners, bioassays, toxicity

1. Introduction

Eutrophication, accepted as the main reason for the outbreak of potentially toxic cyanobacterial blooms[1], is also one of the principal driving factors for bloom formation in South Korea[2] where, in general, the four major rivers Han, Geum, Nakdong, and Yeongsan, are most heavily affected[3-5]. As they also function as potable water sources and are used for recreational purposes, the water quality is a major focus in these rivers and the lakes they collect into[6]. Typically, lake water quality and the trophic state thereof are evaluated using a variety of parameters including pH, total organic carbon, chlorophyll-a, total phosphorus, and turbidity[7], but not toxin content or toxicity. In terms of toxin content, microcystin concentrations of 0.057 µg L^{-1} up to 2612 µg L^{-1} have been detected in these different river systems[5,8], however, to date toxicity testing seems to have been neglected. Aside from microcystins (MCs), anatoxin-a has been detected in the Daecheong reservoir[9], yet toxin characterization data for the Wangsong lake, a major urban reservoir, is lacking.

The Wangsong reservoir, a shallow eutrophic reservoir located in Uiwang City, was built to secure a stable water resource for the area and is classified as a water supply, as a recreational feature, and is used for industrial purposes, as well as agricultural and landscape irrigation[10,11]. The dam was also constructed as a flood control mechanism and for hydroelectric power generation. Due to ongoing expansion and housing projects, pollution of the Wangsong reservoir has steadily increased, accompanied by cyanobacterial bloom formation[7]. Hence, great attention has been paid to water quantity and quality problems of the reservoir.

Cyanotoxins constitute a threat to the health of humans in contact with contaminated waters since they have toxic effects in living organisms[12]. *Microcystis aeruginosa* is the most common bloom-forming cyanobacterial species in freshwaters and has the ability to produce secondary metabolites such as the potent hepatotoxins, especially MCs[13]. To date, the dominant cyanobacterial genera which occur in the four main river systems in South Korea include

78 *Microcystis*, *Anabaena,* and *Oscillatoria*[3,5,8,14], with microcystin-LR, -RR, and -YR as the most
79 frequently detected MC isomers[3].
80 Most of the available studies describe the toxic effects of single MCs in aquatic organisms such
81 as fish species, cladocerans, and mussels[15-20]. Only a few studies include exposure of
82 phytoplankton and macrophytes to crude extracts of *M. aeruginosa,* also evaluating the
83 oxidative stress responses, which resemble a closer approach to actual environmental
84 scenarios[21-24]. Information regarding how water quality affects primary producers will
85 furthermore shed light on how higher trophic levels will be affected.
86 The aim of the present study was to elucidate the toxin composition of the *M. aeruginosa* strain
87 isolated from the Wangsong reservoir, South Korea. Besides the toxin composition, the
88 potential toxicity was evaluated using different bioassay systems, thereby assessing the
89 potential health risk at various trophic levels.

2. Results and discussion

2.1. Culture toxin composition

The seasonal variation of *Microcystis* species in South Korean reservoirs has previously been monitored[11,25].

In the aqueous cell-free crude extract of the *M. aeruginosa* strain, three different microcystin congeners in total, namely MC-LR, MC-RR, and MC-YR, were detected (Fig. 1). The highest concentrations were detected for MC-LR (176.35 µg g^{-1}) followed by MC-RR (50.27 µg g^{-1}) and the lowest concentration for MC-YR (9.25 µg g^{-1}). β-*N*-methylamino-L-alanine (BMAA) was detected and quantified amounting to an average concentration of 0.906 ± 0.016 µg g^{-1}. In the extract, neither anatoxin-a nor cylindrospermopsin was detected by the employed quantitative analysis methods.

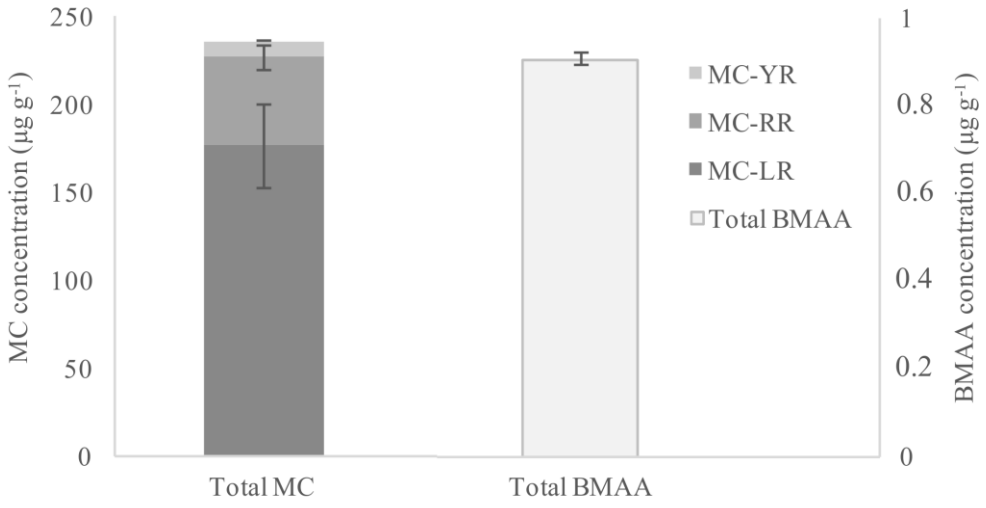


Figure 1: Cyanobacterial toxin composition of the *M. aeruginosa* KW strain isolated from Wangsong reservoir (South Korea). Data represent mean toxin concentration ± standard deviation (n = 4)

2.2. Toxicity analysis using commercial and non-commercial assays

The toxicity of the crude extract in various dilutions was tested using various commercially available TOXKITs in combination with non-commercially available bioassays such as the toxicity towards *T. tubifex* and the oxidative stress status in aquatic macrophytes.

Using the commercial TOXKIT bioassays (Table 1), the aqueous crude extract resulted in a relatively high toxicity response using the THAMNOTOX-F^{TM} kit with an LC50 amounting to 0.1 µg L^{-1} followed by the DAPHTOX pulex kit with an EC50 of 1.1 µg L^{-1} and therefore 10-fold less sensitive compared to the THAMNOTOX-F^{TM} kit. The 24-h LC_{50} for the strain obtained using the THAMNOTOX-F^{TM} kit corresponded to previously reported toxicities for *M. aeruginosa* isolated from Hungary, Germany and Brazil[26]. The toxicity of the extract was much 8.7 times higher than the previously reported toxicity of an *M. aeruginosa* extract with *Daphnia pulex* (48-h LC_{50} 9.6 µg ml^{-1})[27]. The ALGALTOX (EC50 of 3.7 ±1.2 µg ml^{-1}) and PHYTOTOX kits (average IC50 of 3.9 µg ml^{-1}) demonstrated the lowest responses with the crude extract exposure, demonstrating lower sensitivities for primary producers. Previously, an IC_{50} of 3 mg ml^{-1} was reported for *M. aeruginosa* using the Blue-green *Sinapis alba* test[28], approximately a 1000-fold higher concentration. Using the TUBIFEX toxicity test the sensitivity towards the crude extract was similar to that obtained with the DAPHTOX pulex kit, interestingly as both as primary consumers.

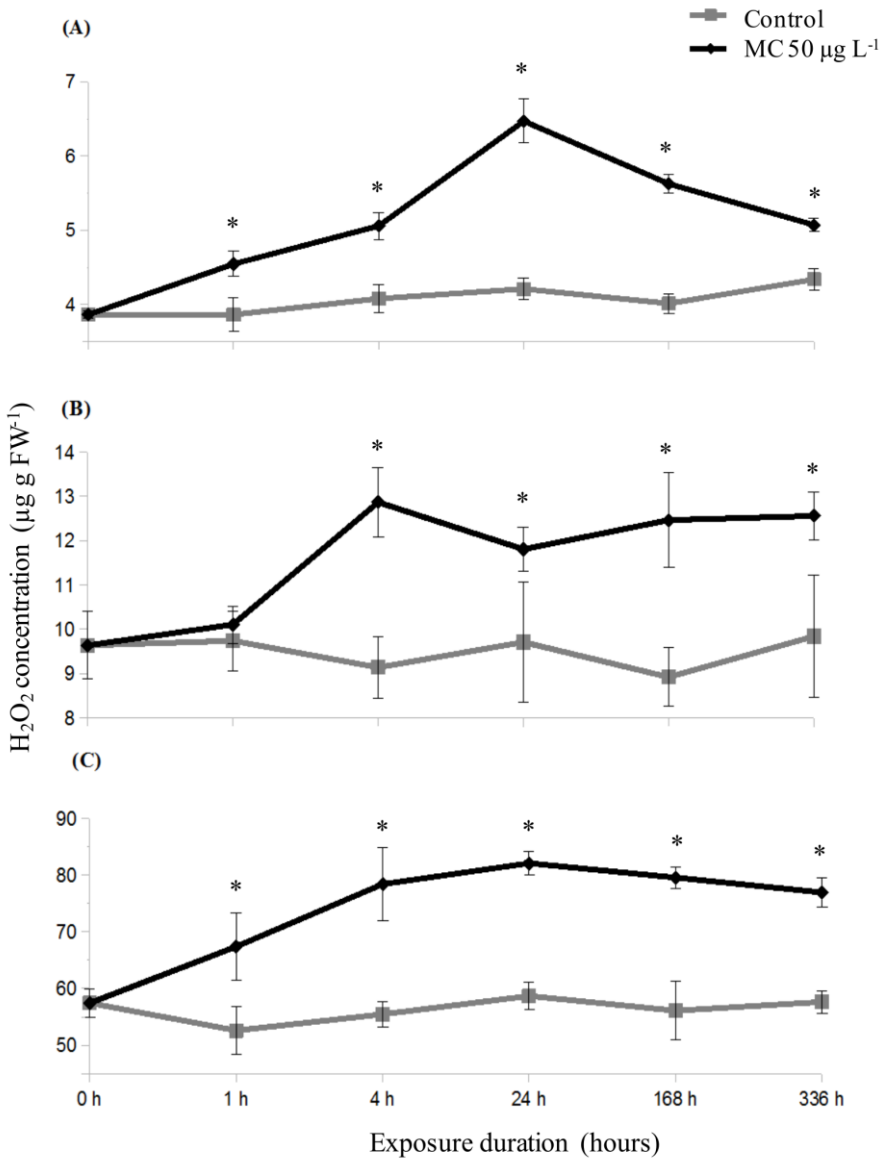
126 **Table 1:** Determination of LC_{50}, EC_{50} and IC_{50} using various bioassays, commercially available ones as
127 well as others.

Bioassay	Test organisms	Trophic level	Test outcome (LC_{50}, EC_{50}, IC_{50}*)	Toxicity as total MC concentration (µg MC ml^{-1})
THAMNOTOX-F™	*Thamnocephalus platyurus*	Primary consumer	24-h LC_{50}	0.1 ± 0.2
ROTOTOX-F	*Brachionus calyciflorus*	Primary consumer	24-h EC_{50}	6.5 ± 1.2
DAPHTOX pulex	*Daphnia pulex*	Primary consumer	24-h EC_{50}	1.1 ± 0.5
TUBIFEX TOX	*Tubifex tubifex*	Detritivore	24-h EC_{50}	1.5 ± 0.7
ALGALTOX	*Pseudokirchneriella subcapitata*	Primary producer	72-h EC_{50}	3.7 ±1.2
PHYTOTOX	*Sorghum saccharatum*	Primary producers	72-h IC_{50}	3.4 ± 0.5
	Sinapis alba		72-h IC_{50}	4.4 ± 0.9
	Lepidium sativum		72-h IC_{50}	3.9 ± 1.2

128 * LC_{50} = lethal concentration, EC_{50} = effect concentration, IC_{50} = inhibitory concentration
129
130 Morphological changes monitored in three different aquatic macrophytes exposed to the bloom
131 extract showed severe changes only in *P. perfoliatus* for which all plants became chlorotic
132 within the exposure time of 14 days (Table 2). *C. demersum*, as well as L. sessilifora, did not
133 show any visible effects; however, in *L. sessiliflora* the leaves seemed to crinkle more than
134 compared to the control (Table 2).

Table 2: Altered morphology of macrophytes exposed to cyanobacterial cell-free crude extract containing MCs at a concentration of 50 µg L^{-1} for 14 days

	Ceratophyllum demersum	*Limnophila sessiliflora*	*Potamogeton perfoliatus*
Control			
MCs exposure (50 µg L^{-1})			



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139 **Figure 2:** Oxidative stress response monitored as changes in cellular H_2O_2 level in three
140 submerged macrophytes: *C. demersum* (**A**), *L. sessiliflora* (**B**) and *P. perfoliatus* (**C**) during
141 14-day exposure to a cyanobacterial cell-free crude extract containing 50 µg L^{-1} total MCs.
142 Data represent average H_2O_2 content ± standard deviation (n = 3); * denotes statistical
143 significance compared to the control ($p > 0.05$)

144

145 Significantly enhanced H_2O_2 levels compared to the control ($p < 0.05$; Fig. 2) were evident for
146 *C. demersum* and *P. perfoliatus* from the onset of exposure, however, the H_2O_2 content only
147 increased for *L. sessiliflora* after 1 hour of exposure ($p > 0.05$; Fig. 2). For *C. demersum* and *P.*
148 *perfoliatus*, the H_2O_2 content increased until 24 hours of exposure, indicating that the level of

reactive oxygen species started to exceed the anti-oxidative capacity of the plants, where after the H_2O_2 decreased, hinting at recovery. However, after 14 days, the normal H_2O_2 level, as seen in the control, was not regained.

The aquatic macrophytes indeed showed adverse effects due to exposure the crude extract containing a concertation of 50 µg ml^{-1} total MC. However, compared to the PHYTOTOX kits, for which an average IC50 of 3.9 µg ml^{-1} was achieved, the aquatic macrophytes seemed less sensitive as plant death was only observed in exposures with *P. perfoliatus* albeit the12.8-fold higher concentration.

The results show the importance of testing toxicity at various trophic levels as the different organism displayed different sensitives. In the present study, primary producers were found to be less sensitive to a crude extract containing MC, compared to primary consumers and detritivores such as for example the *T. platyurus, T. tubifex,* and *D. pulex*. In general, the strain was found to be in some cases equally toxic (as seen with *T. platyurus*) and in others more toxic (as seen with *D. pulex*) compared to blooms reported elsewhere. The study illustrates that toxicity testing is an essential test parameter that should be considered together with routine water quality evaluations.

3. Material & Methods

3.1. Cyanobacterial Strain and Crude Extract

Samples were collected from the Wangsong reservoir, South Korea, during a bloom event between July and October in 2007. The bloom consisted mainly of *M. aeruginosa* with a minor proportion of other cyanobacteria such as *Anabaena* and *Oscillatoria*. The strain, *M. aeruginosa* KW, was isolated from the bloom material and cultivated in 1 L Erlenmeyer flasks containing 500 mL of BG 11 medium[29] under 30-40 mmol photon m^{-2} s^{-1} with a photoperiod of 14:10 h photoperiod at 22 ± 1°C. Culture purity was evaluated microscopically using brightfield. The crude extracts were prepared as described by Romero-Oliva et al.[30].

3.2. Analytics of the cyanobacterial toxins

Microcystin congener (MC-LR, -RR, and -YR) determination and quantification were performed as detailed in Romero-Oliva et al.[30]. Calibrations were linear ($R^2 = 0.999$) between 5 and 500 µg L^{-1}. Limit of detection (LOD) was set at 1 ng mL^{-1} (signal to noise S/N > 3) and limit of quantification at 5 ng mL^{-1} (S/N > 5) for all MCs congeners.

Anatoxin-a chromatographic detection and quantification was performed as detailed in Ha et al.[31]. Calibrations were linear ($R^2 = 0.999$) between 5 and 250 µg L^{-1}. LOD and LOQ were 1 (S/N > 3) and 5 µg L^{-1} (S/N > 5), respectively.

BMAA was detected and quantified after derivatization using a Phenomenex EZ:Faast kit as detailed by Esterhuizen-Londt et al.[32]. Calibrations were linear between 0.1 and 1000 µg L^{-1}, with the limit of detection set at 100 fg on column (S/N > 3) and the limit of quantification set at 1 pg on column (S/N > 5).

Chromatographic detection and quantification of CYN were performed as detailed by Esterhuizen-Londt et al.[33]. Calibrations for this method were linear ($R^2 = 0.998$) between 0.01 and 100 µg L^{-1}.

3.3.Toxicity Assays

All TOXKITS were purchased from Microbiotests, Belgium. Producer protocols were strictly followed, including verification of culture media, pH, and the quality of the controls. The dilutions of the crude extract, were prepared in the appropriate exposure media in final concentrations of 100, 20, 4, 0.8, 0.16 and 0.03 mg dw biomass mL^{-1}, i.e. 99.00, 19.80, 3.96, 0.79, 0.16, and 0.03 µg total MC L^{-1}.

THAMNOTOXKIT F™, using the fairy shrimp *Thamnocephalus platyurus* instar II-III larvae was used for the first investigation. The test was carried out in six replicates of 30 animals each incubated with the various crude extract dilutions at 25°C in the dark for 24 h. Dead larvae were counted, and the % mortality was calculated as well as the 24 h LC_{50} using standard methods[34].

For the ROTOXKIT F, juveniles of the rotifer *Brachionus calyciflorus* were utilized for the acute 24 h toxicity test, with 30 animals per test concentration in six replicates. The plates were incubated at 25°C in darkness. After 24 h, the dead animals were counted, and the % mortality, as well as the LC50, was calculated[35].

For the DAPHTOXKIT pulex, *Daphnia pulex* neonates were hatched from ephippia four days before the start of the tests. The test was with 50 neonates per test concentration in replicates of six. Hatching was initiated in Petri dishes with 15 mL standard freshwater at 20°C under continuous illumination with 8000 lux, at 25°C in darkness. After 24 h, deceased animals were counted, and the % mortality, as well as the LC50, was calculated.

For all of the kits mentioned above, the tests were only valid with mortalities in controls being less than 10%. Positive controls were performed using potassium dichromate ($K_2Cr_2O_7$) (1000 ppm stock solution) diluted to a series of 1.8, 1.0, 0.56, 0.32, and 0.18 mg L^{-1}.

TUBIFEX Toxicity TEST utilizes the oligochaete *Tubifex tubifex* for toxicity testing[36]. The test was performed in small glass beakers with 50 animals per test concentration in replicates of ten. Mortality of the oligochaete was evaluated microscopically after the exposure time of 24 h.

The ALGALTOXKIT used *Selenastrum capricornutum* (renamed as *Pseudokirchneriella subcapitata*) in a 72 h algal growth test. Optical density, as a measure of growth, was measured using a spectrophotometer at 670 nm strictly according to the protocol.

The PHYTOTESTKIT employed seeds of three different terrestrial plants *Sorghum saccharatum* (monocotyledon), *Lepidium sativum* and *Sinapis alba* (dicotyledons) to test for toxic effects, i.e. effects on germination and early development. The tests were performed in three replicates in a climate chamber for three days at 25°C in the dark. For the germination, the germinated seeds were counted and values compared to those of controls as a measure of toxicity.

MORPHOLOGICAL CHANGES of MACROPHYTES were determined using three different aquatic macrophytes, namely *Ceratophyllum demersum*, *Limnophila sessilifora,* and *Potamogeton perfoliatus*. Macrophytes were exposed to the crude extract at a biomass density of 10 mg fw L^{-1} amounting to 22.5 µg MC-LR L^{-1}, 24.7 µg –RR L^{-1} and 2.8 µg -YR L^{-1} (50 µg L^{-1} in total). Morphological changes between the controls and the exposed plants were visibly assessed after 14 days.

OXIDATIVE STRESS RESPONSES of MACROPHYTE were measured in *C. demersum* in a 24 h static renewal exposure experiment. Plant material (3 g wet weight) was exposed in 100 mL medium containing the crude extract (50 ± 0.8 µg L^{-1} total MCs, as before) in replicates of five in parallel with an unexposed control. The level of cell internal H_2O_2 as a marker for oxidative stress was colorimetrically determined according to the method of Jana and Choudhuri[37].

3.4.Data analyses

The TOXKIT assay effect levels were calculated using the Microtox statistical analysis software program, which calculates effect concentrations (EC1, EC10, EC20, and EC50) and associated 95% confidence intervals for 15 and 30-min exposure periods. Statistical significant differences and Pearson Correlation coefficients were calculated using Statistica software. Concentration-response curves were evaluated using Probit analysis[34], and the 50%-effective concentrations (LC_{50}, EC_{50}, or IC_{50}) for the respective assay. The differences and statistical significance were evaluated using ANOVA, followed by Duncan's post-hoc test. Statistical significance was considered at $p < 0.05$.

4. Conclusion

The presence of absence of a visible cyanobacterial bloom is also not an indication of the toxins that may be present in the afflicted waters and thus does not predict exposure risk. Similarly, the presence and absence of toxins and mixtures thereof do not indicate the ecological effect.

Therefore, it would be advantages to include toxicity testing into routine water testing regimes to better understand the impact of harmful algal blooms.

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Figure titles:

Figure 1: Cyanobacterial toxin composition of the *M. aeruginosa* KW strain isolated from Wangsong reservoir (South Korea). Data represent mean toxin concentration ± standard deviation (n = 4)

Figure 2: Oxidative stress response monitored as changes in cellular H_2O_2 level in three submerged macrophytes: *C. demersum* (**A**), *L. sessiliflora* (**B**) and *P. perfoliatus* (**C**) during 14-day exposure to cyanobacterial cell–free crude extract containing 50 μg L^{-1} total MCs. Data represent average H_2O_2 content ± standard deviation (n = 3); * denotes statistical significance compared to the control ($p > 0.05$)

377 **Table 1:** LC_{50}, EC_{50} and IC_{50} of extract using various bioassays

Bioassay	Test organisms	Trophic level	Test outcome (LC_{50}, EC_{50}, IC_{50}*)	Toxicity as total MC concentration (µg MC ml^{-1})
THAMNOTOX-F™	*Thamnocephalus platyurus*	Primary consumer	24-h LC_{50}	0.1 ± 0.2
ROTOTOX-F	*Brachionus calyciflorus*	Primary consumer	24-h EC_{50}	6.5 ± 1.2
DAPHTOX pulex	*Daphnia pulex*	Primary consumer	24-h EC_{50}	1.1 ± 0.5
TUBIFEX TOX	*Tubifex tubifex*	Detritivore	24-h EC_{50}	1.5 ± 0.7
ALGALTOX	*Pseudokirchneriella subcapitata*	Primary producer	72-h EC_{50}	3.7 ±1.2
PHYTOTOX	*Sorghum saccharatum*	Primary producers	72-h IC_{50}	3.4 ± 0.5
	Sinapis alba		72-h IC_{50}	4.4 ± 0.9
	Lepidium sativum		72-h IC_{50}	3.9 ± 1.2

378 * LC_{50} = lethal concentration, EC_{50} = effect concentration, IC_{50} = inhibitory concentration

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380 **Table 2:** Altered morphology of macrophytes exposed to cyanobacterial cell-free crude extract
381 containing MCs at a concentration of 50 µg L^{-1} for 14 days

	Ceratophyllum demersum	*Limnophila sessiliflora*	*Potamogeton perfoliatus*
Control			
MCs exposure (50 µg L^{-1})			

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