Uptake and Growth Effects of Cyanotoxins on Aquatic Plants *Ludwigia Adscendens* and *Amaranthus Hybridus* in Raw Surface Waters

Glynn K. Pindihama and Mugera W. Gitari

***Abstract*—Not much has been done to investigate the uptake and synergic effect of multiple classes of cyanotoxins on aquatic plants. Two aquatic plants Ludwigia adscendens and Amaranthus hybridus (biomass ranging 3.27 to 9.4 gL-1) were exposed to contaminated water for 14 days to test the synergic effects of cyanotoxins in solution on the plants. The plants were exposed to concentrations of 5.385-7.641 µg mL-1 for MC-LR; 8.905 µg mL-1 for MC-RR). L.adscendens accumulated microcystins up to a concentration of 0.04595 ± 0.0004 ng mg-1 wet weight and A.hybridus accumulated microcystins up to a concentration of 0.0166 ± 0.0016 ng mg-1 wet weight of plant material over the 14 days. Except in beaker experiments where the plants were exposed to artificial water dosed with crude extracts, the treatments and their respective controls did not show any significant difference in toxin removals (one-way ANOVA at p=0.05), showing that the plants were unable to significantly remove the toxins from solution. When exposed to raw dam water, both plants were negatively affected as demonstrated by the loss in fresh wet weight. It was thus concluded that the cyanotoxins had adverse effects on aquatic plants at the concentrations used.**

***Index Terms*—Cyanotoxins, aquatic plants, Ludwigia adscendens, Amaranthus hybridus, microcystins.**

I. Introduction

Cyanobacteria are prokaryotic organisms of which more than 2000 species are known to date. A worrisome sign of their living ecological success is the recent frequency and visibility of harmful cyanobacterial blooms, or CyanoHABs, in many aquatic environments world over [1], [2] since most species of CyanoHABs are known to produce extremely potent low-molecular weight cyanobacteria toxins [3]. The growth in human population and climate change have contributed to an increase in the water temperatures and the load of nutrients reaching many surface waters and thus intensifying the proliferation of the HABs even in temperate climates [4]. The intensity and incidences of CyanoHABs has been on the increase in the last decade, and their ecotoxicological potential has also received growing attention [5], since animal deaths or animal-poisoning episodes attributed to CyanoHABs have been documented in many studies [6], [7].

Eutrophic conditions are known to exist in one in five of the 75 major impoundments in South Africa and in 18 of the 25 major river catchments [8]. As is the global experience, eutrophication constitutes a major threat to the supply of water free from cyanobacteria and/or cyanobacterial metabolites and toxins in South Africa. Many studies have been focusing on the harmful effects of the most studied variant of cyanotoxins, microcystins on aquatic plants because of the likelihood of these organisms being exposed to these toxins in the surrounding water [9]. Aquatic plant biomass reductions have been noted after uptake of cyanotoxins due to their ability to bioaccumulate in tissues [9]. The effects of cyanotoxins on aquatic plants has been studied to a lesser extent [10], but being part of the aquatic ecosystem they could be exposed to high concentrations of extracellular cyanobacterial toxins [10], [11]. Ref. [10] also reported the decrease in abundance of submerged plants and reduced diversity of aquatic plants communities in aquatic systems with cyanobacterial blooms.

Cyanotoxins particularly microcystins are usually cell-bound (75%) but can also be dissolved in the surrounding aqueous media at varying concentrations depending on the cyanobacterial biomass and decay [11]. It has been demonstrated that aquatic plants whether submerged and emergent absorb microcystins from very low concentrations in the surrounding water and accumulate them in their tissue. When exposed to microcystins, aquatic plants have been found to exhibit growth inhibition, slowed photosynthetic oxygen production and bleaching of chlorophyll pigments [12].

Several studies have investigated the uptake of the most studied congener of the hepatotoxin Microcystin, Microcystin-LR (MC-LR) [13]-[15]. Since no single cyanotoxin exists in a water body at any given scenario, little information has been gathered with respect to the ability of aquatic plants to survive under such conditions and their effectiveness in taking up a range of cyanobacterial metabolites under conditions that would be experienced in the natural environment. This is particularly relevant since multiple classes of these metabolites are now being simultaneously detected in water bodies [16].

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cyanobacterial biomass and decay [11]. It is therefore likely that aquatic plants are exposed to levels of dissolved extracellular cyanotoxins at or above applied in this study in hypertrophic dams particularly cyanobacterial bloom incidents, scum accumulation and decay.

Biological processes have for centuries been applied for the remediation and treatment of polluted and/or drinking water. They are generally low technology processes that require little or no maintenance and possess the advantage of being 'natural' treatment [16]. This study thus evaluates the cyanotoxin bioaccumulation potential of two naturally occurring aquatic macrophytes namely; *Ludwigia adscendens* and *Amaranthus hybridus*, their ability to withstand the levels of toxins in the dam and combined effect of a range of cyanobacterial metabolites on the plants and the ability to bioaccumulate the toxins under those conditions. This will be of significance as it will also assess the potential for application of aquatic plants in-situ bioremediation of cyanotoxin-polluted raw surface water.

II. Experimental Procedures

A. Sampling and Extraction of Cyanotoxins from Cyanobacterial Cells

Raw lake water samples were collected from Hartbeesport Dam in the North West Province of South Africa. Hartbeespoort Dam is known world-over for its heavy presence of heavy *Microcystis aeruginosa* cyanobacterial blooms and related toxins [8]. Cyanobacterial bloom material mainly consisting of *M. aeruginosa* was collected in the months January to March 2015 in 5 L containers and kept frozen at −20◦C till required. Samples were lyophilised to dryness in a VIRTIS benchtop freeze-drier (2KBTES) at -55◦C.

The method used to extract the cyanotoxins (microcystins and cylindrospermopsin) from the bloom material was according to [17]. In brief Lyophilized samples were extracted three times with 10 mL of BuOH: MeOH:H2O (1:4:15), sonicated for 3 min (30% amplitude, 60 W, 20 kHz,), then centrifuged at 4000 rpm for 20 min at room temperature. The dry-weight of the extracted material ranged from 0.8 to 1.1 g. Clean-up and concentration of the samples was achieved by extracting the samples through hydrophilic–lipophilic balance (HLB) (Oasis® , 60 mg). A small portion of the extracted sample was taken for quantification of microcystins and cylindrospermopsin, using HPLC-PDA and the remaining portion was introduced to the beakers for the removal experiments.

B. Plant Exposure Experiments

The two aquatic plant species were collected from the Luvuvhu River catchment in Limpopo Province, South Africa. The plants were left in river water for 48 hours to acclimatise to the laboratory conditions. The plants were then washed first with tap water, and then rinsed with milli-pore water followed by weighing before introducing them into 1 L beakers for the experiments to determine their ability to remove cyanotoxins over time. Three types of different treatments were used for the experiments.

1) Treatment 1

The first treatment consisted of individual plants from the two species exposed to artificial water (milli Q water, CaCl2 [±0.2 g L^{-1}], NaHCO3 [±0.103 g L^{-1}] and sea-salts [±0.1 g L^{-1}]) [15], dossed with 3 mL of the cyanobacterial crude extracts in 1 L glass beakers.

2) Treatment 2

The second batch of treatments consisted of individual plants from the two species which were exposed to raw dam water collected from Hartbeespoort Dam. The raw dam water used had a pH of 6.52 and Electrical Conductivity of 388µS and chlorophyll *a* concentration of ±16 mg/m^{-3}. The levels of important anions and cations in the dam water are shown in Table I.

3) Treatment 3

The third treatment consisted of the two plant species exposed to 20 mL of raw dam water in 980 mL of milli-pore water. (Since cyanotoxins are also known to have toxic effects on plants at high concentrations diluting the raw dam water was expected to minimise the negative effects of the cyanotoxins on the plants and improve their uptake).

Since whole plants or fresh shoots were used, the plant biomass differed in each beaker, but ranged from 3.27 to 9.4 grams in a litre of sample water. All experiments were conducted in the laboratory under natural lighting conditions and at average temperatures of 25 (±1 ℃). All treatments were performed in triplicate and controls were run concurrently with the treatments, hence with no plant material. Sampling was conducted on the 1st, 2nd, 4th, 7th and 14th days. Samples consisted of 10 mL extracted from each beaker on the specified intervals which were kept frozen at -20°C prior to preparation for HPLC analysis. After the 14 day experiment plant biomass was recorded and so as the volume of the remaining water in the beakers recorded.

C. Preparation of Samples for Cyanotoxins Analysis with HPLC-PDA

During the experiments, sampling was conducted as stated earlier. For clean-up and concentration of the samples, 10 mL of the sample were filtered through 0,45µm syringe filters (Minisart NYLON with GF) then followed by solid phase extraction (SPE). SPE was achieved by running the samples through HLB (Oasis® , 3cc 60 mg). The cartridges were conditioned by passing through 3 mL of 100% methanol followed by 6 mL of 100% milli-pore water. The eluent was then dried at 50°C under a stream of nitrogen gas and the reconstituted material in 50% methanol, was then used for HPLC-PDA analysis.

D. HPLC-PDA Analysis of Cyanobacterial Toxins

The determination of the MCs (MC-RR; MC-YR & MC-LR) and Cylindrospermopsin (CLY) content in the samples was achieved using a Thermo Scientific - Finnigan Surveyor Plus HPLC System (Thermo Scientific, USA) comprising a thermo surveyor auto sampler, a surveyor MS pump and a surveyor PDA system. Separation was achieved through a reverse phased XBridge C18 Column (130Å , 3.5 µm, 4.6 mm X 75mm). The mobile phases consisted of solvent A: Milli-Q water with 0.1% TFA and solvent B:

acetonitrile with 0.1% TFA (60: 40 isocratic elution) for MCs and solvent A: Milli-Q water with 0.1% TFA and solvent B: methanol with 0.1% TFA (60: 40 isocratic elution) for CLY. The sample injection volume was 25 µL throughout and a flow rate of 1 mL min^{-1} was used. Toxins were characterized by comparison of retention time and PDA spectra with those of the standards used. The typical chromatogram of the pure standards used is shown in Fig. 1.

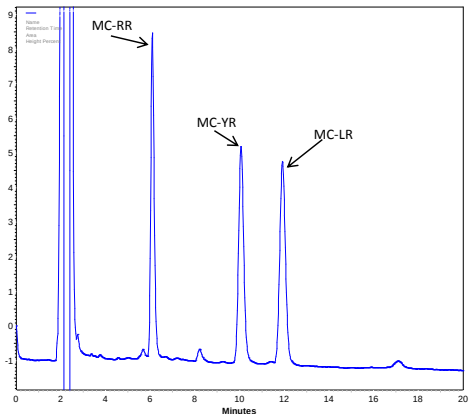

Fig. 1. HPLC–PDA chromatogram of the pure MCs standards.

E. Extraction of Cyanotoxins from Plants

An accurately weighed portion of the plants from all treatments, were removed from the beakers/tank and washed twice with deionized ultrapure water and were blotted dry between the washes with clean tissue paper. Each sample was homogenized in 30 mL of a 3:1 methanol: water mixture using an electric homogenizer. The homogenate was extracted for 30 min [14] and then sonicated for 3 min (30 % amplitude, 60 W, 20 kHz,), then centrifuged at 4000 rpm for 20 min at room temperature and the supernatant removed and transferred into glass amber 100 mL sampling bottles. This extraction was repeated three times [17] and the extracts were combined in the amber bottles.

The combined extracts, were evaporated to dryness at 50°C using an electric water bath, under a stream of nitrogen gas and then frozen until analysis. Before analysis, the toxin was re-suspended in 1mL of ultrapure water. Fig. 2 shows the schematic diagram for the procedure used to extract MCs from plants.

F. Enzyme-Linked Immunosorbent Assay (ELISA) for Toxins Accumulated in L.adscendens and A.hybridus

Toxins accumulated in the plants were analysed by enzyme-linked immunosorbent assay (ELISA) using a procedure based on the Standard Operating Procedure (SOP) that comes with the Envirologix QuantiPlateTM Kit for Microcystins. The ELISA kit used was an Envirologix QuantiPlateTM kit for Microcystins (Kit lot number: 090475; Expiry date: 30/05/2016). The Microplate Reader was a Bio-TekK Instrument with KC4 software Version 3.4; Revision number 21. Both the reader and software were supplied by Bio-TekK Instruments, Inc, U.S.A.

The ELISA method was used for this purpose since ELISAs typically have the ability to detect toxins at low levels. For quantitative analysis purpose, the calibration curve was produced with the standard calibrators, according to manufacturer's instructions (linear range of 0.16-2.5 µg L-1). Samples with concentrations above the detection limit were further diluted by ultrapure water till the concentrations were within the linear range of the standard calibrators.

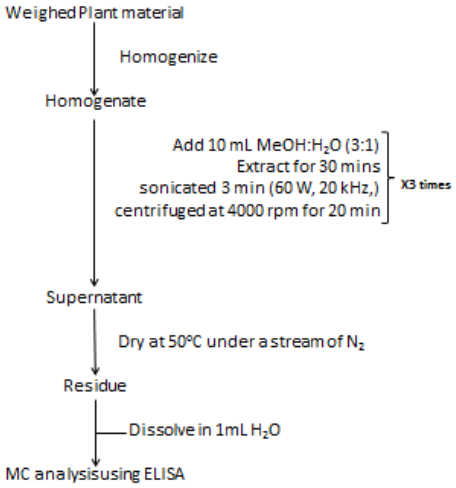

Fig. 2. Procedure for the extraction of microcystins in plants.

G. Data Analysis

The differences in the mean plant biomass for each treatment were subjected to a one-way analysis of variance (ANOVA) followed by a Tukey-Kramer post-hoc Multiple Comparisons Test. Results were considered significant at a P level <0.05. Since whole plants or shoots were used, regression analysis using SPSS version 22 was used to correlate plant biomass and the removal efficiency of cyanotoxins in the samples.

III. Results

A. Characterization of the Raw Dam Water

The raw dam water used had a pH ranging from 6.44 to 6.77 and Electrical Conductivity ranging from 272 µS to 388µS and chlorophyll a concentration of ±16 mg/m-3. The levels of important anions and cations in the dam water are shown in Table I. Except for pH which was within the range preferred by the largest variety of freshwater aquatic organisms of 6.5 to 8.0 [18], the other parameters were way outside the range/limits expected for the maintenance of aquatic life [19-20].

B. Levels of Cyanotoxins in the Samples Crude Extracts

The crude extracts varied in composition, but of the MC variants of interest, they contained MC-RR and MC-LR (Fig. 3). In some of the extracts, the HPLC-PDA data showed that the crude extracts were dominated by unidentified microcystins (not the three variants used in the current study: MC-RR; MC-YR & MC-LR). Fig. 4 shows the typical chromatograms of the extracted material and peaks for compounds which were identified to be microcystins. Of the five extracted samples of the bloom material only one contained traces of the alkaloid toxin cylindrospermopsin.

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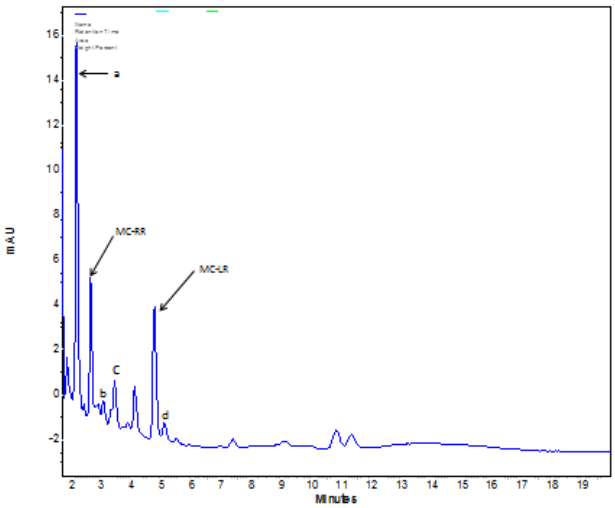

Fig. 3. HPLC-PDA chromatogram (at 238 nm) of the extracted bloom material containing MC-RR and MC-LR and 'a'; 'b' and 'c' showing unidentified MCs: Thermo Scientific HPLC. 25 µl of standard was injected onto a C18 column (XBridge C18 Column, 130Å , 3.5 µm, 4.6 mm X 75mm) a flow rate of 1 ml/min. The mobile phase for the HPLC process was Milli-Q water with 0.1% TFA and acetonitrile with 0.1% TFA (60: 40 isocratic elution).

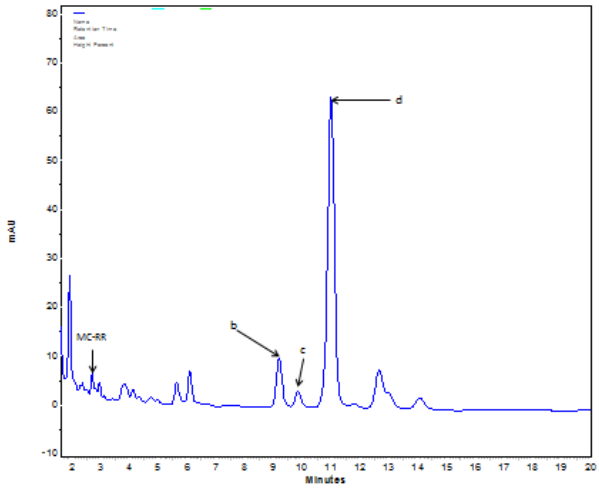

Fig. 4. HPLC-PDA chromatogram (at 238 nm) of the extracted bloom material dominated by unidentified MCs (b, c & d): Thermo Scientific HPLC. 25 µl of standard was injected onto a C18 column (XBridge C18 Column, 130Å , 3.5 µm, 4.6 mm X 75mm) a flow rate of 1 ml/min. The mobile phase for the HPLC process was Milli-Q water with 0.1% TFA and acetonitrile with 0.1% TFA (60: 40 isocratic elution)

C. Raw Hartbeespoort Dam water samples

The raw dam water used for the experiments was dominated by MC-LR with traces of MC-RR. The water also contained other variants of MCs as was demonstrated by the UV Maximum absorption spectra of the peaks observed. Fig. 5 shows the typical chromatograms of the raw dam water used for the experiments and the peaks for compounds which were identified to be microcystins.

D. Toxins Removal in Solution

1) Treatment 1

The artificial water was dosed with crude extracts from the cyanobacterial bloom material. The water had initial concentrations of 6.4512 µg mL^{-1} for MC-RR and 5.481 µg mL^{-1} for MC-LR.

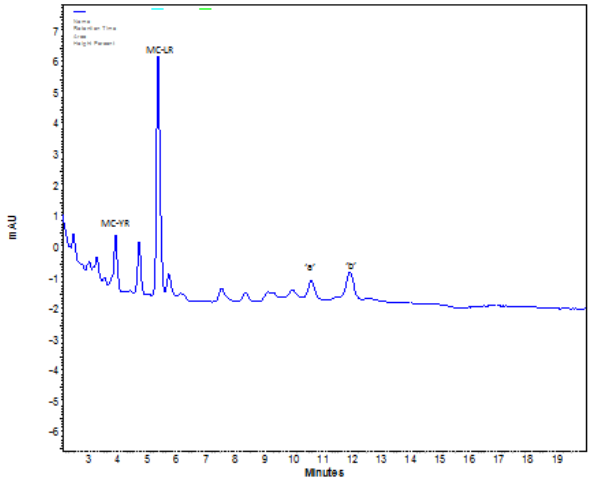

Fig. 5. HPLC-PDA chromatogram (at 238 nm) of the raw dam water used, showing MC-YR; MC-LR and unidentified MCs (a & b): Thermo Scientific HPLC. 25 µl of standard was injected onto a C18 column (XBridge C18 Column, 130Å , 3.5 µm, 4.6 mm X 75mm) a

TABLE I: PHYSICO-CHEMICAL PARAMETERS OF THE RAW DAM WATER USED

Parameter	Concentration
Lithium (ppm)	0.861±0.08
Sodium (ppm)	57.801±1.13
Ammonium (ppm)	29.498±2.45
Potassium (ppm)	22.685±3.76
Calcium (ppm)	9.339±2.39
Magnesium (ppm)	36.606±4.55
Fluoride (ppm)	_
chloride (ppm)	91.870±2.18
phosphate (ppm)	4.545±1.07
sulphate (ppm)	103.092±4.86
bromide (ppm)	_
pH	6.520±0.76
EC (µS)	388.000±9.39
Chlorophyll *a* (mg/m^3)	15.200±4.41

E. Removal of Microcystin-RR (MC-RR)

After 24 hours of exposing the plants to the polluted water, the levels of the toxin MC-RR went down by 94% and 88% in the beakers with *L.adscendens* and *A.hybridus* respectively. This can be compared to only 33% by the sole action of microbial activity in the controls (Fig. 6). A one way Analysis of Variance (ANOVA at p-0.05) followed by a Tukey-Kramer post-hoc Multiple Comparisons Test, showed that there was a significant difference in the levels of toxin removal after the first 24 hours of exposures between the treatments and their respective controls, but thereafter no significant differences were recorded thereafter over the remaining part of the 14 day period (Fig.7). Significance was tested using a one way ANOVA test (p - 0.05). This indicated that the rate at which the plants were taking up the toxin was significant as compared to the sole microbial activity in the control beakers (with no plant material).

The toxin was below the detection limit in the beakers with *L.adscendens* and in the control beakers after two days of the experiment, whilst traces of the toxin (0.429 µg mL^{-1}) were

detected after the same duration of exposure in the beakers with *A.hybridus* (Fig. 7). This difference in the levels of MC-RR in the beakers after two days of exposure did not represent a significant difference using a one way ANOVA (p-0.05).

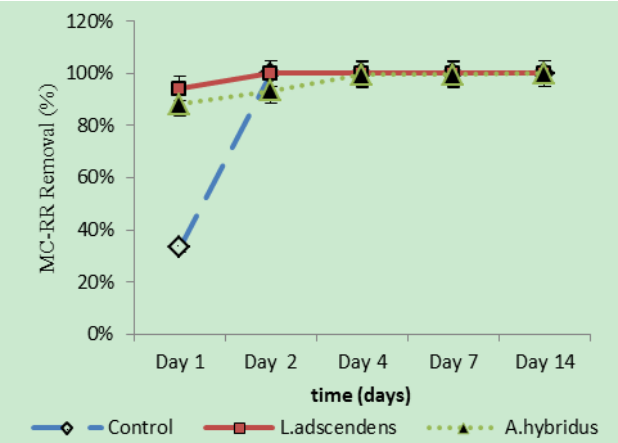

Fig. 6. MC-RR Removal efficiency of the two plants and the control beakers after exposure to raw Hartbeespoort Dam water for a period of 14 days.

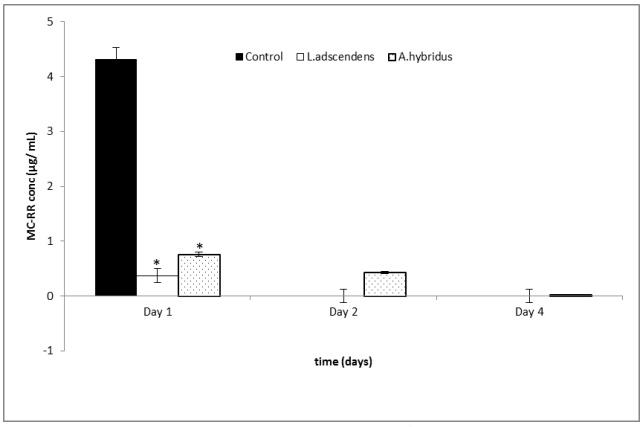

Fig. 7. Individual plant beaker experiments for cyanotoxins removal experiments with the two macrophytes (Experiment 1). Each bar represents remaining MC-RR in solution.

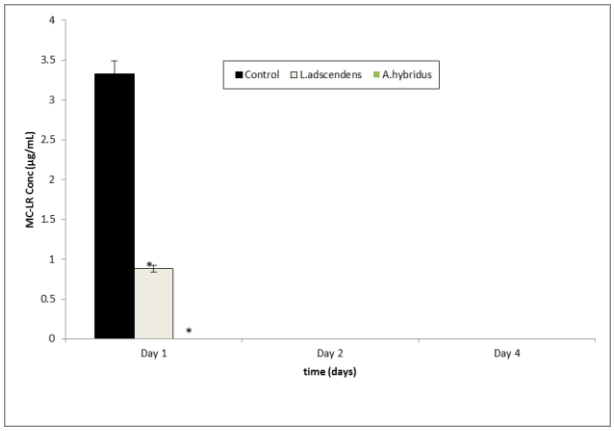

Fig. 8. Individual plant beaker experiments for cyanotoxins removal experiments with the two macrophytes (Experiment 1). Each bar represents remaining MC-LR in solution.

F. Removal of Microcystin-LR (MC-LR)

On exposure to the two plants, a nominal concentration of 5.481 µg mL^{-1} for MC-LR was reduced to below detection limit and 0.532 µg mL^{-1} for beakers with *A.hybridus* and *L.adscendens* respectively after 24 hours (Fig. 8). In the control beakers, the nominal concentration of 5.481 µg mL^{-1} for MC-LR was only reduced to 3.3245 µg mL^{-1} after 24 hours of exposure. This difference in the levels of MC-LR in solution in the beakers of different treatment represented a significant difference when tested using a one way ANOVA (p-0.05) followed by a Tukey-Kramer post-hoc Multiple Comparisons Test. This indicated that the toxin was being taken up by the plants at a faster rate than the sole degradation by microbial activity in solution. A significant difference was also noted between the percentage removals of the toxin between the two plants using a one way ANOVA (p-0.05). Fig. 8 shows that, the species *A.hybridus* was taking up the toxin at a faster rate as compared to *L.adscendens*. After two days of exposure, the toxin levels had dropped to below the detectable limits in all the treatments.

1) Treatment 2

After exposure to the initial nominal concentrations of 9.081 µg/mL of MC-LR, the concentrations of the concentration of the toxin decreased gradually over the 14 day period to concentrations of 0.4153 µg mL^{-1}; 0.4137 µg mL^{-1} and 0.232 µg mL^{-1} in beakers containing *L.adscendens*; *A.hybridus* and control beakers respectively. The levels of the toxin in the surrounding medium did not show any significant difference over the whole 14 day period in the three treatments (one ANOVA at p-0.05).

2) Treatment 3

On exposure to initial concentrations of about 2.0132 µg mL^{-1} of MC-LR, the concentrations went down gradually in all the three treatments (*L.adscendens*; *A.hybridus* & the control) till the 4th day and thereafter no MC-LR was detected. The findings were in line with other studies for example [21] who studied the persistence of microcystin-LR in different natural surface waters and found that the toxin persisted for 3 days to 3 weeks; however, more than 95 % loss occurred within 3 to 4 days. The decrease in the levels of the toxin from the first day did not show any significant differences over the four days when tested using a one way ANOVA (p-0.05)

G. Effect of Plant Biomass on Toxin Removal

Since different plant biomasses were used in the experiments, the relationship between plant biomass and the rate of microcystins removal was tested using a standard correlation and regression analysis. The null hypothesis tested was: There is no relationship between plant biomass and the rate of microcystins removal after 24 hours of exposure.

Plant biomass was used in a standard regression analysis to predict MC removal (%). The scatter plot for the two variables is presented in Fig. 9. As can be seen in Fig. 9, the correlation was very weak and there was no statistically significant relationship between the two variables. The prediction model was not statistically significant, p=0.998 ($p > .05$), and accounted for approximately 0 % of the variance of MC removal (%) ($R^2 = .001$, Adjusted $R^2 = .000$).

Since the 'p' value was found to be 0.998, which was greater than our alpha level (p-0.05) we accepted the null hypothesis and concluded that, there was no sufficient evidence to support the assertion that plant biomass had a relationship with MC removal (%) in solution.

The scatter plot and the regression analysis indicated that

there was no correlation between plant biomass and the removal efficiency of MC in the artificial water dosed with crude cyanobacterial extracts (Pearson Correlation value of -.001 which is less than .3 indicated a very weak correlation).

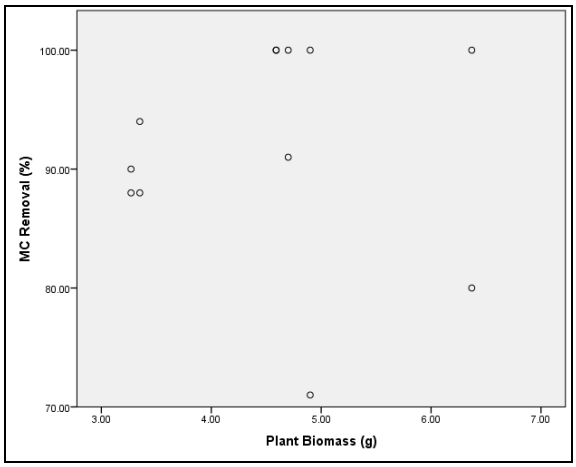

Fig. 9. Scatter plot showing the relationship between plant biomasses used in experiments with crude extracts and MC percentage removal.

H. Effects of Cyanotoxins on L.adscendens and A.hybridus

Several studies have demonstrated the possible negative effects of cyanotoxins on plants (both aquatic and terrestrial) [14], [22], [23]. Microcystins have been observed to increase glutathione S-transferase activities in aquatic plants [24] and inhibit plant growth [14].

In this study the use of different treatments in experiment 1 (laboratory individual plant beaker experiments) made it possible to detect the possible effects of cyanotoxins on the growth of the plants. When exposed to the different treatments for 14 days in experiment 1, only plants exposed to artificial water contaminated with crude extracts (treatment 1) showed an overall gain in fresh wet weight after the experiments (Fig. 10). An Analysis of Variance (ANOVA) (at p -0.05) showed that there was a significant difference in the fresh wet weight of the plants in treatment 1 before and after the experiment. When exposed to raw dam water (treatment 2) and diluted dam water (treatment 3), both plants seemed to be negatively affected as demonstrated by the loss in fresh wet weight over the 14 day period. Even though the overall weight loss in the fresh wet weight of the plants was apparent (Fig. 10), the differences in the fresh weights in treatment 2 and treatment 3 for both plants before and after the experiments were not statistically significant (ANOVA, at p -0.05).

Looking at the overall weight loss or gain of the two plants when exposed to the three treatments (artificial water contaminated with crude extracts; raw Dam water and diluted dam water), results showed that the plants only gained weight in treatment 1 and there were overall fresh wet weight losses in the other two treatments (Fig. 11). Results showed that when exposed to artificial water with crude cyanobacterial extracts (treatment 1), *A.hybridus* species gained weight significantly as compared to *L.adscendens* (ANOVA at p -0.05).

Inasmuch as both plants had an overall fresh weight loss when exposed to raw dam water and diluted dam water, there were no significant differences in the overall fresh weight losses of the two plants (*A.hybridus* and *L.adscendens*) over the 14 days (ANOVA, p-0.05). It was expected that *L.adscendens* would suffer more deleterious effects when exposed to raw dam water compared to *A.hybridus* since it was found to bioaccumulate microcystins much faster (in the next section). Results in Fig. 11 however showed that *L.adscendens* had an overall less fresh weight loss when exposed to raw dam water (treatment 2) as compared to *A.hybridus*, even though there was no statistically significant difference in the overall fresh weight loss (ANOVA, p-0.05).

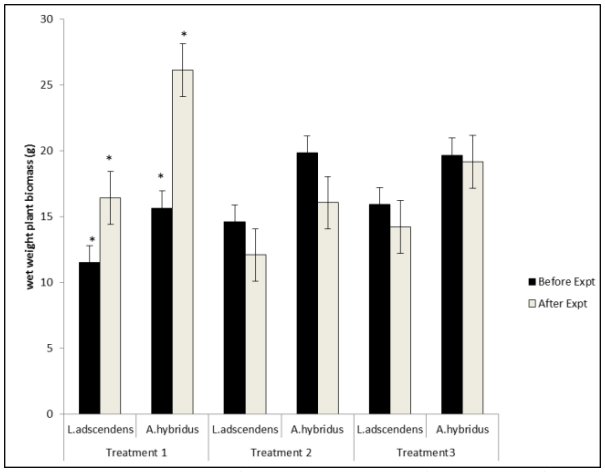

Fig. 10. Average wet weight of the two plants in g after 14 days of exposure to the various treatments (Treatment 1=artificial water contaminated with crude extracts; Treatment= raw Dam water; Treatment 3 = diluted dam water). Bar indicates mean wet weight, and error bars indicate standard error of three replicates. *Significantly different ($p < 0.05$) between wet weight before the experiment and after in each treatment (ANOVA)

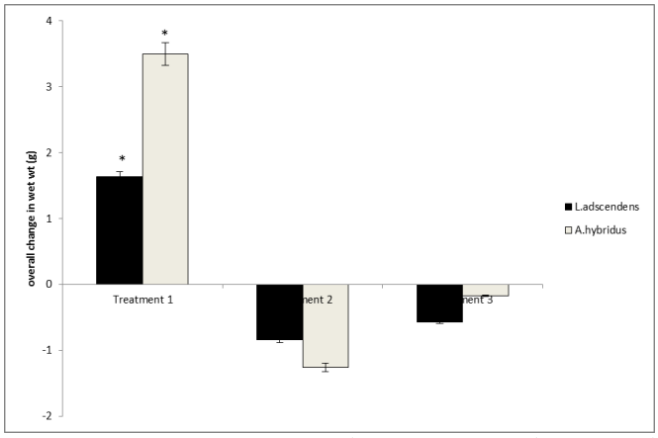

Fig. 11. Average change in wet weight of the two plants in g after 14 days of exposure to the various treatments (Treatment 1=artificial water contaminated with crude extracts; Treatment 2 = raw Dam water; Treatment 3 = diluted dam water). Bar indicates mean wet weight, and error bars indicate standard error of three replicates. *Significantly different ($P < 0.05$) between the overall wet weight change of the two plants before and after the experiment (ANOVA).

In previous studies, significant decreases in the growth of aquatic plants were observed for MC-LR concentration of 10 to 20 µg mL^{-1} [14] and MC-RR was observed to inhibit the growth and decreased chlorophyll content of *L.minor* at a concentration of 3 µg mL^{-1} [25]. In the current study all the microcystin concentrations used were below the 10 µg mL^{-1}, so it is not surprising that besides stunted plant growth no other significant observable side effects to the plants were observed even though the concentration appeared to be very high than naturally available under normal environmental

conditions.

Besides the stunted growth reported in beakers with raw dam water and the bleaching of leaves in some of the plants, no other significant side effects to the plants were recorded. This is in consistent with the findings by [23] who did not observe any negative effects on the growth and chlorophyll of the plants due to the mature age of the plants they used in a similar study with cyanotoxins.

I. Accumulation of Microcystins within L.adscendens and A.hybridus

The bioaccumulation of Microcystins in the two plants *L.adscendens* and *A.hybridus* when exposed to raw Hartbeespoort dam water in beaker experiments was measured (Table II). *L.adscendens* was found to accumulate Microcystins at a much faster rate compared to *A.hybridus*. An unpaired Student 't' test was done to test for the significance of the differences in the mean bioaccumulation potentials of *L.adscendens* and *A.hybridus* and it was observed to differ significantly. The two-tailed P value was found to be less than 0.0001, considered extremely significant (at p-0.05). The difference in the bioaccumulation rate could be attributed to the fact that *L.adscendens* has horizontal extensions which usually spread over the surface of the water and long fibrous roots, giving it a much more surface area exposed to the water hence a greater ability to take up toxins in solution.

TABLE II: BIOACCUMULATION OF MICROCYSTINS IN *L.ADSCENDENS* AND *A.HYBRIDUS*

Test species	Accumulated toxin (ng mg^{-1} wet wt plant material)
L.adscendens	0.04595 ± 0.0004
A.hybridus	0.0166 ± 0.0016

L. adscendens accumulated microcystins up to a concentration of 0.04595 ± 0.0004 ng mg^{-1} wet weight of plant material over the 14 days of the experiment, equivalent to an accumulation rate of 0.003282 ng mg^{-1} day^{-1}. *A. hybridus* accumulated microcystins up to a concentration of 0.0166 ± 0.0016 ng mg^{-1} wet weight of plant material over the 14 days of the experiment, equivalent to an accumulation rate of 0.001186 ng mg^{-1} day^{-1}.

In several other studies, the bioaccumulation potential of microcystins in terrestrial and aquatic plants has been demonstrated [14], [15], [24]. Ref. [14] investigated the effects and possible uptake of the most common cyanotoxins microcystin-LR on the duckweed, *L. minor* and filamentous macroalgae, *C. fracta* in controlled laboratory tests and found that *L. minor* accumulated microcystin-LR up to a concentration of 0.28870.009 ng mg^{-1} wet wt. plant material, equivalent to an accumulation rate of 0.058 ng mg^{-1} day^{-1} and *C. fracta* accumulated a microcystin-LR concentration of 0.04270.015 ng mg^{-1} wet wt plant material over the 5 days of the experiment, equivalent to an accumulation rate of 0.008 ng mg^{-1} day^{-1} wet wt. plant material when exposed to 10 µg mL^{-1} MC-LR over the 5 days of the experiment. This was very much comparable to the concentrations of 0.04595 ± 0.0004 ng mg-1 wet weight of plant material, microcystin uptake for *L.adscendens* and 0.0166 ± 0.0016 ng mg^{-1} wet weight of plant material for *A. hybridus* over the 14 days of the experiment in the current study. Even though, the duration of exposure differed, the results are very much comparable considering the fact that microcystins usually degrade and disappear in solution after about four days.

IV. DISCUSSION

A. Use of Environmental Relevant Levels of Cyanotoxins

The raw dam water used in this study was collected from Hartbeespoort Dam. The dam has for long been classified as hypertrophic [26], hence high levels of toxic cyanobacterial blooms. The dam is also known to be a commonplace for cyanobacteria of the species *Microcystis* and *Anabaena*, which are known to produce the most common cyanotoxins microcystins. Based on that, the high levels of microcystins found in the raw water (± 5 µg mL^{-1} for both MC-LR and MC-RR) are common in the dam.

Although high levels in the parts per million (µg mL^{-1}) region are rare, they do occur. Concentrations of microcystins as high as 5 µg mL^{-1} [27] and 47.5 µg mL^{-1} [14] have been reported. Concentrations of up to 25 µg mL^{-1} have also been reported in waters contaminated with cyanobacterial blooms [28], hence it is not surprising that this study employed concentrations in the parts per million (µg mL^{-1}) region since these concentrations are relevant under local conditions tested.

B. Uptake of Cyanotoxins by L. adscendens and A. hybridus

The current study revealed that the two plants *Ludwigia adscendens* and *Amaranthus hybridus* when exposed to artificial water contaminated with crude cyanobacterial extracts from cyanobacterial bloom material with initial concentrations of 6.4512 µg mL^{-1} for MC-RR and 5.481 µg mL-1 for MC-LR were able to eliminate the toxins within 24 hours of exposure. However when exposed to raw Hartbeespoort dam water the two plants could not eliminate Microcystins (MC-LR and MC-RR) at the concentrations tested. These concentrations were much higher compared to those used by [15] who managed to reduce MC-LR from concentrations of 12.1 and 9.2 µg L^{-1} to less than 1.0 µg L^{-1} (MC-LR) in three days.

The study aimed at exposing the two plant species to three variants of microcystins namely; MC-RR; MC-YR and MC-LR and the tricyclic alkaloid cylindrospermopsin (CYN) (mainly produced by the species *Cylindrospermopsis raciborskii* in freshwater). However MC-LR was found to be the most prominent and consistent toxin in most if not all the samples and MC-RR was also found to be consistent in the raw dam water. This finding was in consistent with the available literature which confirmed that, microcystin-LR is the more common microcystin found in bloom samples [29] and thus have a wider application.

The current study used two plant species not previously researched for the bioaccumulation of microcystins namely: *Ludwigia adscendens* and *Amaranthus hybridus* but using a comparable biomass of 5.0 g L^{-1} as used by [15]. Since whole plants were used in this study and constant water volumes were used for the beaker experiments, it was expected that the reduction of microcystins would be biomass dependent.

Previous studies have demonstrated that MCs can be taken up by plants and, when present in sufficient concentrations [23] and accumulation of MC-LR in *L.minor* species was shown to be linearly related to toxin concentration in the water [14]. The highest concentration tested by ref. [14] was 10 µg mL^{-1}, thus concentrations in the region of 5 µg mL^{-1} for microcystins used in this study provided an abundant of the toxin to demonstrate a good correlation between plant biomass and toxin uptake, but however no correlation between the two variables could be established. The reason for this could be the presence of many cyanobacterial metabolites and other organics, which made it impossible for any uptake to be detected in the surrounding medium. Previous studies demonstrated that when exposed to a medium containing 10 µg mL^{-1} MC-LR, *L. minor* accumulated MC-LR to a concentration of around 0.11 ng mg^{-1} and 0.04 ng mg^{-1} in *C. fracta* over a five day period [14]. Ref. [24] also found that *C. demersum* could take up to 1.98 g/g of frsh weight (FW) after a 7-day exposure to MC-LR at more or less similar concentrations.

V. Conclusion

The following were the significant findings of the study:

An analysis of the physicochemical properties of the raw dam water used showed that, except for pH the other parameters were way outside the range/limits expected for the maintenance of aquatic life. The raw water contained excessive amounts of nutrients to support the growth of plants in the experiments.

The raw dam water samples contained MC-LR, MC-RR and other unidentified congeners of MCs but did not contain the alkaloid toxin cylindrospermopsin.

The extracted bloom material introduced to artificial water contained MC-LR, MC-RR and other unidentified congeners of MCs and traces of the alkaloid toxin cylindrospermopsin.

On exposure to artificial water contaminated with crude cyanobacterial cell extracts, there was a significant difference in the levels of toxin removal (for both MC-RR and MC-LR) after the first 24 hours of exposures between the treatments and their respective controls, but thereafter no significant differences were recorded thereafter over the remaining part of the 14 day period.

In all experiments were raw dam water or diluted raw dam water was used the plants were unable to significantly remove the toxins from solution under those conditions.

A standard correlation and regression analysis indicated that there was no correlation between plant biomass and the removal efficiency of MC in the artificial water dosed with crude cyanobacterial extracts (Pearson Correlation value of -.001 which was less than .3 indicated a very weak correlation).

L.adscendens was found to accumulate Microcystins at a much faster rate compared to *A.hybridus* (unpaired Student 't' test, p-0.05). *L.adscendens* accumulated microcystins up to a concentration of 0.04595 ± 0.0004 ng mg^{-1} wet weight of plant material over the 14 days of the experiment, equivalent to an accumulation rate of 0.003282 ng mg^{-1} day-1. *A.hybridus* accumulated microcystins up to a concentration of 0.0166 ± 0.0016 ng mg^{-1} wet weight of plant material over the 14 days of the experiment, equivalent to an accumulation rate of 0.001186 ng mg^{-1} day^{-1}.

Plants exposed to artificial water contaminated with crude extracts (treatment 1) showed an overall gain in fresh wet weight after the experiments. An Analysis of Variance (ANOVA) (at p -0.05) showed that there was a significant difference in the fresh wet weight of the plants when exposed to artificial water contaminated with cyanobacterial extracts before and after the 14 day experiment period.

When exposed to raw dam water (treatment 2) and diluted dam water (treatment 3), both plants seemed to be negatively affected as demonstrated by the loss in fresh wet weight over the 14 day period. The differences in the fresh weights before and after the experiments in treatment 2 and treatment 3 for both plants before and after the experiments were not statistically significant (ANOVA, at p -0.05).

Based on the findings of the study, it was evident that the cyanotoxins had adverse effects on aquatic plants at the concentrations used. Thus the possible use of aquatic plants for treatment and remediation of freshwater regularly contaminated with harmful cyanobacterial blooms is not a realistic option.

References

[1] M. R. Neffling, E. Lance, and J. Meriluoto, "Detection of free and covalently bound microcystins in animal tissues by liquid chromatography–tandem mass spectrometry," *Environmental Pollution* vol. 158, p. 948, 2010.

[2] H. W. Paerl, "Mitigating harmful cyanobacterial blooms in a human- and climatically impacted world," *Life (Basel).*, vol. 4, p. 988, 2014.

[3] P. J. Oberholster, T. E. Cloete, C. E. Ginkel, A. M. Botha, and P. J. Ashton, "The use of remote sensing and molecular markers as early-warning indicators of the development of cyanobacterial hyperscum crust and microcystin-producing genotypes in the hypertrophic Lake Hartebeespoort, South Africa," *Report edn. Pretoria: CSIR Natural Resources and the Environment*, 2009.

[4] A. Zamyadi, S. L. MacLeod, Y. Fan, N. McQuaid, S. Dorner, S. Sauvé, and M. Prévost, "Toxic cyanobacterial breakthrough and accumulation in a drinking water plant: A monitoring and treatment challenge," *Water Res.*, vol. 46, p. 1511, 2012.

[5] F. E. Khalloufi, K. Oufdou, M. Lahrouni, I. E. Ghazali, S. Saqrane, V. Vasconcelos, and B. Oudra, "Allelopatic effects of cyanobacteria extracts containing microcystins on Medicago sativa-Rhizobia symbiosis," *Ecotoxicology and Environmental Safety*, vol. 74, p. 431, 2011.

[6] T. Qiu, P. Xie, Z. X. Ke, L. Li, and L.G. Guo, "In situ studies on physiological and biochemical responses of four fishes with different trophic levels to toxic cyanobacterial blooms in a large Chinese lake," *Toxicon.*, vol. 50, p. 365, 2007.

[7] L. Li, P. Xie, H. Lei, and X. Zhang, "Renal accumulation and effects of intraperitoneal injection of extracted microcystins in omnivorous crucian carp," (*Carassius auratus*). *Toxicon.*, vol. 70, p. 62, 2013.

[8] M. W. Matthews and S. Bernard, "Eutrophication and cyanobacteria in South Africa's standing water bodies: A view from space," *S Afr J Sci.*, vol. 111, p. 1, 2015.

[9] M. C. Bittencourt-Oliveira, T. C. Hereman, M. K. Cordeiro-Araújo, I. Macedo-Silva, C. T. Dias, F. F. C. Sasaki, and A. N. Moura, " Phytotoxicity associated to microcystins: a review," *Braz. J. Biol.* vol. 74, p. 753, 2013.

[10] S. Saqrane and B. Oudra, "Cyanobacterial toxins: A short review on phytotoxic effect in an aquatic environment," *African Journal of Environmental Science and Technology.*, vol. 5, p. 1146, 2011.

[11] D. Gutiérrez-Praena, Á. Jos, S. Pichardo, I. M. Moreno, and A. M. Cameán, "Presence and bioaccumulation of microcystins and cylindrospermopsin in food and the effectiveness of some cooking techniques at decreasing their concentrations: A review," *Food and Chemical Toxicology.*, vol. 53, p. 139, 2014.

[12] D. Drobac, N. Tokodi, J. Simeunović, V. Baltić, D. Stanić, and Z. Svirčev, "Human exposure to cyanotoxins and their effects on health," *Arh Hig Rada Toksikol.*, vol. 64, p. 305, 2013.

[13] S. Pflugmacher, C. Wiegand, K. A. Beattie, G. A. Codd, and C. E. W. Steinberg, "Uptake of the cyanobacterial hepatotoxins microcystin-LR by aquatic macrophytes," *J. Appl. Bot*., vol. 72, p. 228, 1998.
[14] S. M. Mitrovic, O. Allis, A. Furey, and K. J. James, "Bioaccumulation and harmful effects of microcystin-LR in the aquatic plants *Lemna minor* and *Wolffia arrhiza* and the filamentous alga *Chladophora fracta," Ecotox. Environ. Saf*., vol. 61, p. 345, 2005.
[15] J. Nimptsch, C. Wiegand, and S. Pflugmacher, "Cyanobacterial toxin elimination via bioaccumulation of MC-LR in aquatic macrophytes: An application of the green liver concept," *Environ. Science & Technol.*, vol. 42, p. 8552, 2008.
[16] L. Ho, E. Sawade, and G. Newcombe, "Biological treatment options for cyanobacteria metabolite removal — A Review," *Water Research*, vol. 46. p. 1536, 2012.
[17] L. Xie and H. Park, "Determination of microcystins in fish tissues using HPLC with a rapid and efficient solid phase extraction," *Aquaculture*, vol. 271, p. 530, 2007.
[18] S. Behar, "Definition of water quality parameters. Testing the waters: Chemical and physical vital signs of a river," *Montpelier, VT: River Watch Network*, ISBN-0-782-3492-3, 1997.
[19] Department of Water Affairs and Forestry (DWAF), "South African Water quality guidelines. Volume 7: Aquatic ecosystems," Department of Water Affairs and Forestry, Republic of South Africa, 1996.
[20] U. S. Enderlein, R. E. Enderlein, and W. P. Williams, "Water quality requirements. In: Water pollution control," 2007.
[21] G. J. Jones, S. I. Blackburn, and N. S. Parker, "A toxic bloom of Nodularia spumigena Mertens in Orielton Lagoon, Tasmania," *Australian Journal of Marine and Freshwater Research*., vol. 45. p. 787, 1994.
[22] S. Pflugmacher, "Possible allelopathic effects of cyanotoxins, with reference to microcystin-LR, in aquatic ecosystems," *Environ. Toxicol*., vol. 17, p. 407, 2002.
[23] S. Järvenpää, C. Lundberg-Niinistö, L. Spoof, O. Sjövall, E, Tyystjärvi, and J. Meriluoto, "Effects of microcystins on broccoliand mustard, and analysis of accumulated toxin by liquid chromatography–mass spectrometry," *Toxicon.*, vol. 49, p. 865, 2007.
[24] S. Pflugmacher, G. A. Codd, and C. E. W. Steinberg, "Effects of the cyanobacterial toxin microcystin-LR on detoxication enzymes in aquatic plants," *Environ. Toxicol.*, vol. 14, p. 111, 1999.
[25] J. Weiss, H. P. Liebert, and W. Braune, "Influence of microcystins-RR on growth and photosynthetic capacity of the duckweed *Lemna minor L*," *J. Appl. Bot.*, vol. 74. p. 100, 2000.
[26] C. E. Ginkel, "National assessment report. National eutrophication monitoring programme 2004," *Internal Report No.N/0000/00/DEQ/0305 for the Department of Water Affairs and Forestry (DWAF)*, Pretoria, South Africa, 2005.
[27] G. J. Jones and P. T. Orr, "Release and degredation of microcystin following algicide treatment of a Microcystis aeruginosa bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay," *Water Res.*, vol. 28, p. 871, 1994.
[28] S. Pavagadhia and R. Balasubramanian, "Toxicological evaluation of microcystins in aquatic fish species: Current knowledge and future directions," *Aquatic Toxicology.*, vol. 142–143, p. 1, 2013.
[29] V. M. Vasconcelos, K. Sivonen, W. R. Evans, W. W. Carmichael, and M. Namikoshi, "Microcystin (heptapeptide hepatotoxins) diversity in cyanobacterial blooms collected in Portuguese fresh waters," *Water Res*., vol. 30. p. 2377, 1996.

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