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Full Length Research Paper

Development of a rapid and sensitive battery of bioassays for risk assessment of cyanobacterial microcystin-LR in drinking water of rural water treatment plants, South Africa

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Due to the lack of toxin standards and resource limitations for wide scale use of analytical methods for e.g. high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) in cyanobacterial toxin monitoring, it has become necessary to assess and develop additional methods that are rapid, yet realistic and cheap for the detection of cyanobacterial toxins in drinking water of rural conventional water treatment plants in South Africa. A well-known cyanobacterial secondary metabolite (microcystin-LR) which is the dominant cyanotoxin variant in South African surface waters was tested for its adverse effect on the macrophyte plant *Spirodela punctata* (duckweed) and the insects *Periplaneta americana* (American cockroach), *Tenebrio molitar* (yellow mealworm), *Gryllus bimaculatus* (common cricket) as well as the crustacean zooplankton (*Artemia salina*). In this study *Gryllus bimaculatus* and *Artemia salina* LD_{50} body weight values were calculated as 0.45 µg/animal and 0.1 µg/animal, respectively, within the first 48 h after exposure to microcystin-LR. These values obtained indicate that sensitivities of *G. bimaculatus* and *A. salina* to low concentrations of microcystin-LR were comparable to the mouse bioassay. No mortalities of *P. americana* and *T. molitar* were observed after 48 h of exposure to different concentrations of synthetic microcystin-LR (0.5, 1.0, 5.0 µg/L). Moreover, on sub-organism or cellular level, DNA fragmentation occured in *P. americana*, *T. molitar*, *A. salina* and *G. bimaculatus* within the first 48 h after exposure to synthetic microcystin-LR. Although the ease of culturing of the American cockroach and yellow mealworm make them ideal organisms to be included in a battery of bioassays, their 48 h LD_{50} non-response on organism level to low concentrations of microcystin exclude these species from a rapid sensitive battery of bioassays. From the data generated in this study the 5 day *Spirodela punctata* bioassay was the most sensitive bioassay by showing reduction of root growth and fronds weight as well as changes in the chlorophyll *a* and *b* ratio content within the first 12 h after exposure to a low concentration (0.1 µg/L) of synthetic microcystin-LR.

Key words: Battery of bioassays, microcystin-LR, apoptosis, chlorosis, *Spirodela punctata*.

INTRODUCTION

Safe drinking water is one of our most basic needs in life. Although water is essential for all life forms, it can also be a cause of disease and death if it contains harmful substances or organisms. These factors have the potential to act as a brake on the quality of life of impoverished rural people in South Africa and the rest of Africa (Oberholster and Ashton, 2008). Bloom-forming cyanobacteria in eutrophic surface waters of South Africa have caused increasing concern over the last decade as a result of

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their toxic potential (Van Ginkel and Conradie, 2001; Oberholster and Botha, 2007). The most alarming characteristic of cyanobacteria is the ability of many species to produce a range of extremely potent low-molecular-weight cyanobacteria toxins of which *Microcystis* and *Anabaena* are the predominant cyanobacterial species in South African surface waters (Van Ginkel et al., 2000; Oberholster and Botha, 2007).

The ingestion of cyanobacterial microcystins released upon cell lyses, can cause sickness or death of animals and pose serious problems for human drinking water supplies (Oberholster and Ashton, 2008). In China, long-term studies of microcystin concentration within the range of 0.09-0.46 µg/L in well, tap, river and pond drinking water supplies was positively associated with higher incidence of colorectal and liver cancer (Zhou et al., 2002; Ueno et al., 1996). Furthermore Zegura et al. (2002) reported in their study that microcystin-LR induced oxidative DNA damage in Hep G2 human cells at low concentrations (0.01 µg/ml). However, this might be one of the mechanisms by which chronic exposure to low concentrations of microcystins contribute to increase the risk for liver and colorectal cancer development. Moreover, Chong et al. (2000) reported that purified microcystin-LR induced adverse toxic effects at 18.75 µg/ml on 8 different permanent cell lines which include human hepatocellular carcinoma and colon adenocarcinoma cells. From earlier studies it is known that the cyclic heptapeptide, microcystin-LR (MC-LR) has a potent and specific inhibitory effect on animal and plant protein phosphatases type 1 and 2A, respectively (MacKintosh et al., 1990). Incidents of fatal cyanobacterial poisoning in South Africa are not uncommon and these occur annually. However, to date only life stock deaths and some domestic animal poisonings have occurred (Oberholster et al., 2004, 2009; Oberholster and Botha, 2005).

The deemed health risk to humans in rural areas of South Africa is via chronic exposure to low levels of cyanotoxins in drinking water supplies since it is estimated that only 21% of households in rural areas have access to pipe indoor water (DWAF, 2004). Moreover, water treating plants in rural areas also do not produce water of acceptable quality for domestic consumption (Momba et al., 2004). Long-term chronic exposure to low levels of cyanotoxins by residence drinking water from these water treatment plants may also occur as rural conventional treatment processes are ineffective in removing cyanobacterial toxins from drinking water (Hoffman, 1976). Additionally, in studies conducted on conventional water treatment plants it was pointed out that cell-bound microcystins decreased while extracellular toxin concentrations remained constant after flocculation and filtration processes (Himberg et al., 1989; Hitzfeld et al., 2000). Another study conducted by Pietsch et al. (2002) showed that flocculation and filtration resulted in an increase of extracellular toxin after experiments with *Microcystis aeruginosa* and *Planktothrix rubescens*, suggesting that turbulences in pipes and the pressure gradients in the filters may be the reasons for elevated extra cellular toxin concentrations.

Therefore, South Africa is faced with the need to monitor extra cellular cyanobacterial toxin in drinking water of rural water treatment plants. Presently, there are several types of bioassays developed for detection and analysis of cyanotoxins, of which the mouse bioassay is the most extensively used (Carmichael, 1992). However, there are growing objections in different countries to the use of mammalian bioassays (e.g. mouse bioassays) due to moral reasons. Futhermore, these bioassays requires expensive husbandry which is not always feasible in developing countries (Bell and Codd, 1996). Because of a lack of toxin analytical standards and due to resource limitations it has become necessary to assess and to develop additional methods for the detection and estimation of cyanobacterial toxins in drinking water of rural water treatment plants in South Africa and in other developing countries. The aim of the present study was (1) to use locally available organisms of different trophic levels to test for their sensitivity to low concentrations of synthetic MC-LR and a crude extract of *M. aeruginosa*, (2) to evaluate these organisms for their cost-effectiveness for broader use, and (3) to evaluate sub-cellular responses of the different test organisms after exposure to low levels of synthetic MC-LR and crude extract of *Microcystis aeruginosa*. For this purpose a battery of bioassays using different end points were conducted on *Periplaneta americana* (American cockroach), *Gryllus bimaculatus* (common cricket), *Tenebrio molitar* (yellow mealworm), *Artemia salina* (brine shrimp) and *Spirodela punctata* (duckweed).

MATERIALS AND METHODS

Cyanobacterial culturing

For the assessment of toxicity a cyanobacterial crude extract was prepared from an axenic *Microsystis aeruginosa* Kürtzing strain (UP051) isolated from the semi-rural lake Roodeplaat, South Africa. The strain was cultured in liquid MA (Ichimura, 1979) medium at 25 ± 2ºC under continuous illumination of 25 µmol photons m^{-2} s^{-1}. At 21 days growth, 50 ml of the culture was transferred to a serum vial and lyophilized.

A methanol extract was prepared following the method of Hiripi et al. (1998). In brief *Microcystis* cells were homogenized in 10 mg/ml methanol, centrifuged at 5,000 rpm/10 min and the supernatant evaporated. The residue was dissolved in 1.3% NaCl containing 0.03% Brij-35. An aliquot (1 ml) of the supernatant was used for injection of the different insect test specimens.

Total microcystin concentrations of the lyophilized cells were quantified using the enzyme-linked immunosorbent assay kit (ELISA) (EnviroLogix, Portland, Me, USA). The MC-LR concentration was determined by employing a standard curve of MC-LR and the results were obtained by reading it on a multiskan ascent plate reader (Thermo Labsystems, USA) at 450 nm within 30 min after the addition of the stop solution (Uneno et al., 1996).

Plant cultivation and microcystin-LR application

All experiments have been carried out with axenic plants of the duckweed *Spirodela punctata*. Plants were collected in spring from

waterbodies around the city of Pretoria, South Africa. The plants were cultivated for several months in modified Provasoli's medium (Pflugmacher and Steinberg, 1997). The *S. punctata* plants were sterilized by employing the method of Bowker et al. (1980). Plants were then precultivated for 2 weeks in 100 ml Erlenmeyer flask under continuous illumination of approximately 60 µmol photons.$m^{-2}.s^{-1}$ at 22-24ºC before exposure to different concentrations of synthetic MC-LR and the crude *M. aeruginosa* extract. Ten plants of *S. punctata* with 2 fronds each were then transferred from the preliminary culture into sterile (5 cm-diameter) polystyrene petri dishes with constant stirring. These plants were then exposed to modified Provasoli's medium (2 ml) supplemented with several standardized concentrations of synthetic MC-LR (0, 0.1, 0.5 and 1.0 µg/L) obtained from Sigma (USA), as well as the cyanobacterial crude extract from strain UP051. Three sets of petridishes in triplicate were used. The first set was used for chlorophyll *a* and *b* ratio, the second set for root reduction and the third set for frond growth. Incubation took place for 5 days under the same culture conditions as was used for the preliminary cultures in the growth chambers. The modified Provasoli's medium with or without (negative control) synthetic MC-LR was replaced every 2 days to maintain concentrations of nutrients, synthetic MC-LR and crude *M. aeruginosa* extract. The entire experiment was repeated 3 times.

Growth end points

The number of fronds of *S. punctata* plants was counted every day and a growth rate was calculated on the basis of the increase in fronds over a period of 5 days according to an exponential growth model. Growth rate was calculated as:

$$t^{-1} \cdot \log (F_t / F_0) \cdot (\log 2)^{-1} \quad (1)$$

Where t is the duration of the test in days, F_0 is the initial number of fronds, and F_t is the number of fronds at the end of the test. Root elongation (mm) was also measured on a daily basis.

Pigment analysis

Analysis of chlorophyll (chl *a* and chl *b*) content was carried out every 12 h by shock frozen only the fronds of *S. punctata* plants in liquid nitrogen after exposure to different concentrations of microsystin. Thereafter chlorophyll content was extracted with 80% acetone at 4ºC and determined spectrophotometrically (647 and 664 nm wavelengths) according to the method of Porra et al. (1989).

Insect bioassays

For insect toxicological tests, fully grown, 6 times moulted *P. americana* (mean weight 1.7 g) and *G. bimaculatus* (mean weight 1.9 g) were used. The insects were taken from a stock culture reared in our laboratory at 30ºC with a photoperiod of 12 h (light and dark cycle). For the experiments 1.5 - 2 g weight adults were used after 4 h starvation. Synthetic MC-LR was dissolved in deionized water containing 0.03% Brij-35 after which 1 ml was injected with a stainless steel needle into the body cavity of the different test insects. The needle was inserted parallel to the body for at least ¼ to avoid damaging the gut and to ensure that the toxin concentration did not escape from the needle hole. The injection apparatus was a micro-applicator with a 1 ml syringe. Thirty *P. americana* and *G. bimaculatus* were injected in triplicate to test for the adverse effects of a single dose. In each case several concentrations of synthetic MC-LR (0.5, 1.0, 5.0 µg/L) were used and compared with the negative controls (injected deionized water containing 0.03% Brij-35) as well as to insects injected with the crude extract from the *Microcystis* lyophilized cells (Strain UP051). The injected insects were placed in plastic screw-topped jars (10 injected insects per container) containing the same diet as the stock culture and kept at 30ºC at a light:dark cycle of 12 h. The LD_{50} of the different test insects were calculated after the first 48 h of exposure.

To assess apoptosis and 48 h LD_{50} of *T. molitar* larvae, late instar mealworm larvae (mean weight 1.06 g/specimen) were obtained from a local supplier. Different concentrations of synthetic MC-LR (0.5, 1.0, 5.0 µg/L) were administered (10 test specimens for each concentration in triplicate and deionized water containing 0.03% Brij-35 for the negative control specimens), as well as the extract from the lyophilized cells (UP051). The different concentrations of synthetic MC-LR and the cyanobacterial extract from strain UP051 were injected through the exoskeleton of the test specimens, parallel to the long axis of the abdomen with a fixed injection needle imbedded beneath the dorsal surface of the second abdominal segment. Groups of 10 mealworms were placed in individual 100 ml plastic containers. Each container was filled with approximately 40 g of rolled oats, which provided nutrition and a substrate for the duration of the experiment. The mealworms were kept at 21ºC under a 12 h light : 12 h dark photoperiod.

Brine shrimp (*Artemia salina*) bioassay

For the assessment of 48 h LD_{50} and the appearance of genomic DNA laddering patterns (PCD) in zooplankton the brine shrimps, *A. salina* were used. 50 adult brine shrimps were exposed to each concentration of synthetic MC-LR in triplicate. These were cultured in a saline solution containing (g/L): NaCl, 23; $MgCl_2.6H_2O$, 11; Na_2SO_4, 4; $CaCl_2.6H_2O$, 2 and KCl, 0.7; pH 8 to 9 according to the protocol of Lewan et al. (1992). Adult brine shrimps were exposed to 3 different concentrations of synthetic MC-LR (0.5; 1.0 and 5.0 µg/L) in 5 cm-diameter petri dishes. The negative controls were also performed in triplicates (150 adult brine shrimps in culture saline solution) as described above. After 48 h the number of affected adult brine shrimps was determined and LD_{50} was calculated.

Detection of DNA fragmentation and/or breakdown in *P. americana*, *T. molitar*, *G. bimaculatus* and *A. salina*.

Briefly, the key biochemical event of apoptosis is endonucleolysis that results in cleavage of nuclear DNA into oligonucleosome-sized fragments when exposed to low levels of toxic substances (Arends et al., 1990). For determination of DNA fragmentation and/or breakdown, genomic DNA were isolated from all test specimens accept *S. punctata* plants using the DNAzol®-Genomic DNA Isolation reagent following the manufacturers' procedures (Molecular Research Center, Inc., USA). The genomic DNA was extracted from the different specimens at 48 h after exposure to different concentrations of synthetic microcystin-LR and the cyanobacterial crude extract (UP051). DNA fragmentation patterns were analyzed on 1% agarose (Techcomp Ltd.) dissolved in 1 x TAE buffer (Tris-acetate-EDTA buffer, pH 7.5) containing 3 µl Goldview™ Nucleic Acid Stain (Sylvean Biotech, South Africa). The generated fragments were separated at 85 Mv for 1 h, and visualized under UV-light and photographed.

Data analyses

The results were recorded on standard excel spreadsheets for data processing. Statistical differences were analyzed using the Pearson correlation coefficient and *t* test Sigma Stat (Jandel Scientific) program. Values of $p \leq 0.05$ were regarded as significant.

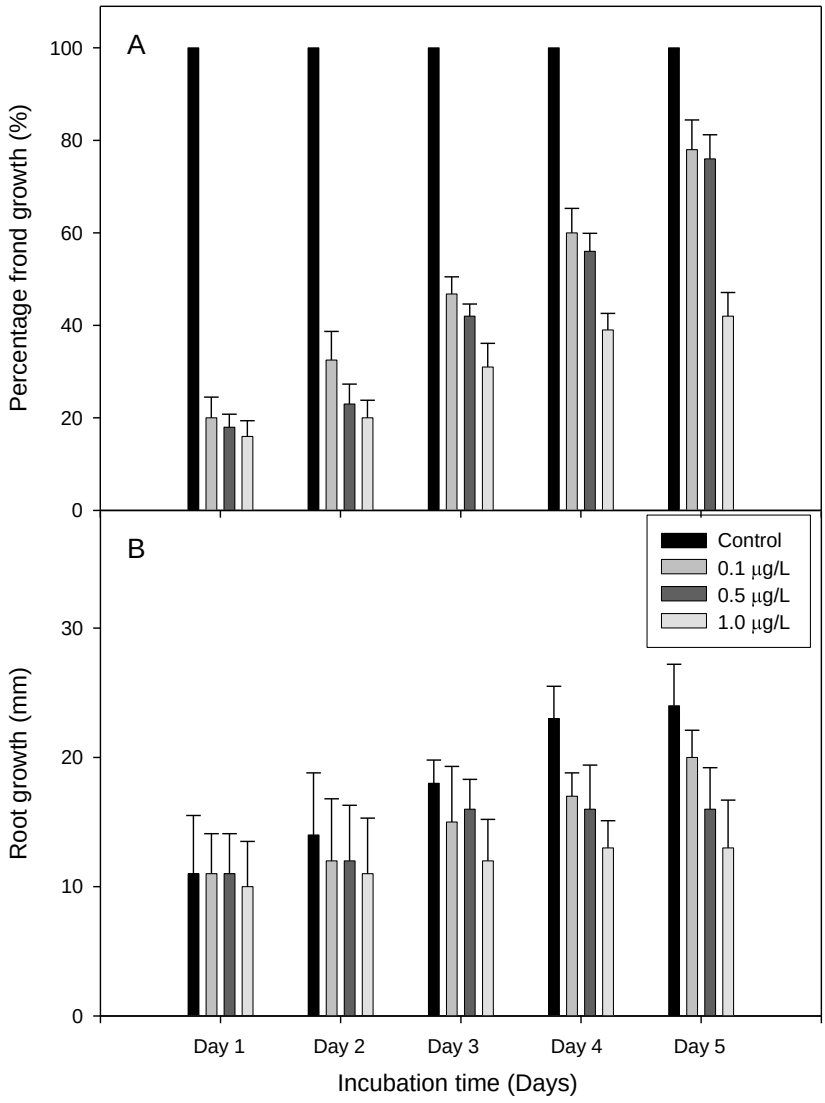

Figure 1. (A). The adverse effect of different concentrations of synthetic microcystin-LR on the frond growth of *S. punctata* plants in comparison with the frond growth of the control plants (100%) over a period of 5 days. (B). The adverse effect of different concentrations of synthetic microcystin-LR on the root growth (mm) of *S. punctata* plants in comparison with the control plants over a period of 5 days.

RESULTS

Effect of pure microcystin-LR and lyophilized crude cyanobacterial extract on the growth of *S. punctata*.

In this study a 78% reduction in frond growth of *S. punctata* plants in comparison to the control plants was observed after 120 h of incubation in 1 µg/L synthetic MC-LR. This concentration (1 µg/L) is also the maximum allowable concentration prescribed by the world health organization (WHO) in human drinking water (Figure 1). Hence, frond reduction was observed within the first 24 h at low concentrations of synthetic MC-LR (0.1, 0.5 µg/L). Reduction in root growth correlated positively ($r = 0.9876$; $p \leq 0.05$) with low concentrations of the synthetic MC-LR (0.1 µg/L) when compared to the control plants over the exposure period of 5 days (Figure 1).

A clear reduction in the total chlorophyll content *µg*/g fresh weight, as well as differences between chl *a* and chl *b* ratio were observed within the first 24 h after exposure to a low synthetic MC-LR concentration (0.1 µg/L) (Figure 2). The most significant difference between chl *a* and chl *b* ratio were observed at exposure to 0.5 µg/L of synthetic MC-LR when the ratio change from 4:1 at the commencement of the experiment to 3:2 after 96 h of exposure. A negative correlation ($r = -0.9269$; $p \leq 0.05$) was observed

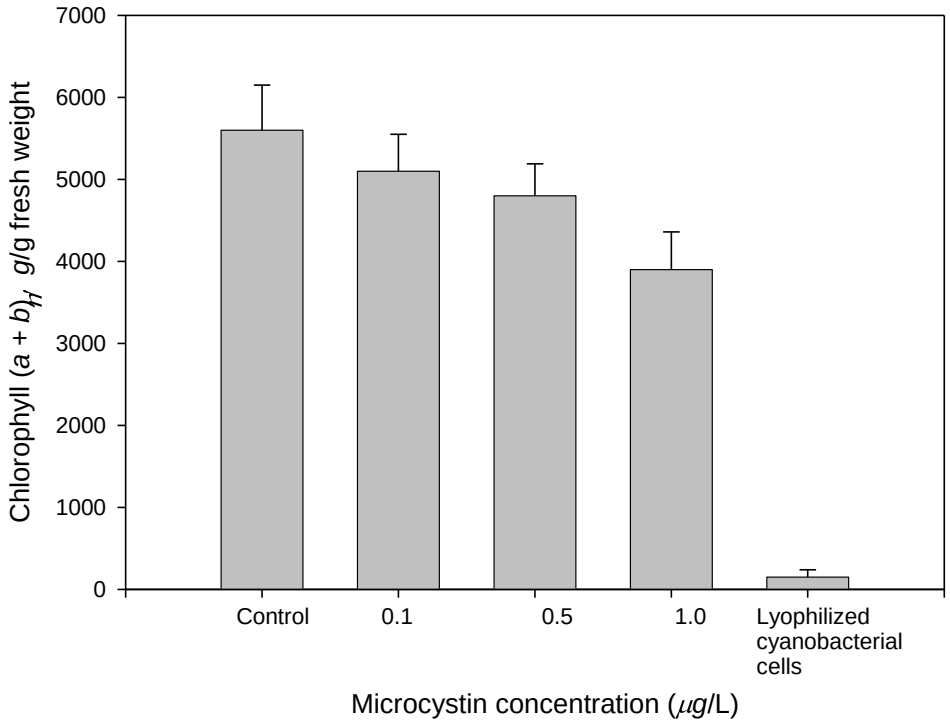

Figure 2. Chlorophyll (chl *a* and chl *b*) content of *S. punctata* fronds (ten plants) after 5 days of incubation with different concentrations of microcystin-LR and a crude extract from microcystis strain UP053.

between the decrease of chlorophyll (*a* + *b*) µg/g fresh weight and the increase in synthetic MC-LR concentrations from 0.1 to 1.0 µg/L (Figure 2). Distinctive chlorosis of *S. punctata* fronds were observed at day 5, in the plants exposed to the lyophilized crude extract from *Microcystis* (UP051) cells at a microcystin-LR level of 46.8 µg/L as measured by ELISA.

P. americana, T. molitar, G. bimaculatus and *A. salina* bioassays

No mortalities of *P. americana* and *T. molitar* were observed within the first 48 h after exposure to any of the synthetic MC-LR concentrations (Figure 3). However, DNA fragmentation occurred within this time period after injection of these specimens with the crude extract from the lyophilized *M. aeruginosa* (UP051) cells (total MC-LR level = 46.8 µg/L) (Figure 4). Although no mortalities within 48 h was observed in test specimens of *T. molitar* after injection with crude extract from the *Microcystis* (UP051) cells, a positive correlation ($r = 0.7611$; $p \leq 0.05$) exists between the 30% mortality rate of *P. americana* after exposure to the *Microcystis* crude extract (46.8 µg/L MC-LR) over the same period (Figures 3 and 4).

In the cases of the *G. bimaculatus* and *A. salina* bioassays a 48 h LD_{50} was established at 5.0 µg/L MC-LR. However, within the first 24 h the highest mortalities were recorded for *G. bimaculatus* and *A. salina* at MC-LR concentrations of 5.0 µg/L with mortality rates of 20 and 30%, respectively. Moreover, the vast majority of mortalities of these 2 organisms occurred after exposure to the crude *Microcystis* extract and correlated positively ($r = 0.9615$; $p \leq 0.05$), with mortality rates of 80 and 90% respectively at 48 h. Genomic DNA fragmentation patterns were visible within 48 h after exposure of *G. bimaculatus* test specimens to 1.0 µg/L of MC-LR, as compared to the maxi-mum allowable concentration of MC-LR in human drinking water (WHO, 1996). Genomic DNA breakdown was also visible within the first 48 h after exposure of *A. salina* and *T. molitar* test specimens to synthetic MC-LR concentration of 5.0 µg/L (Figure 4). In both these experiments, the average DNA strand lengths were larger than the controls, while *A. salina* showed a large zone of smaller DNA fragments which migrated further than larger fragments (Figure 4).

DISCUSSION

In rural areas of South Africa the choice of supply of drinking water is limited and when the water is of doubtful quality, the only choice may be bottled water, which is financially out of reach for the majority of the population. Furthermore, children are more vulnerable to drinking water contaminated with low levels of cyanotoxins since

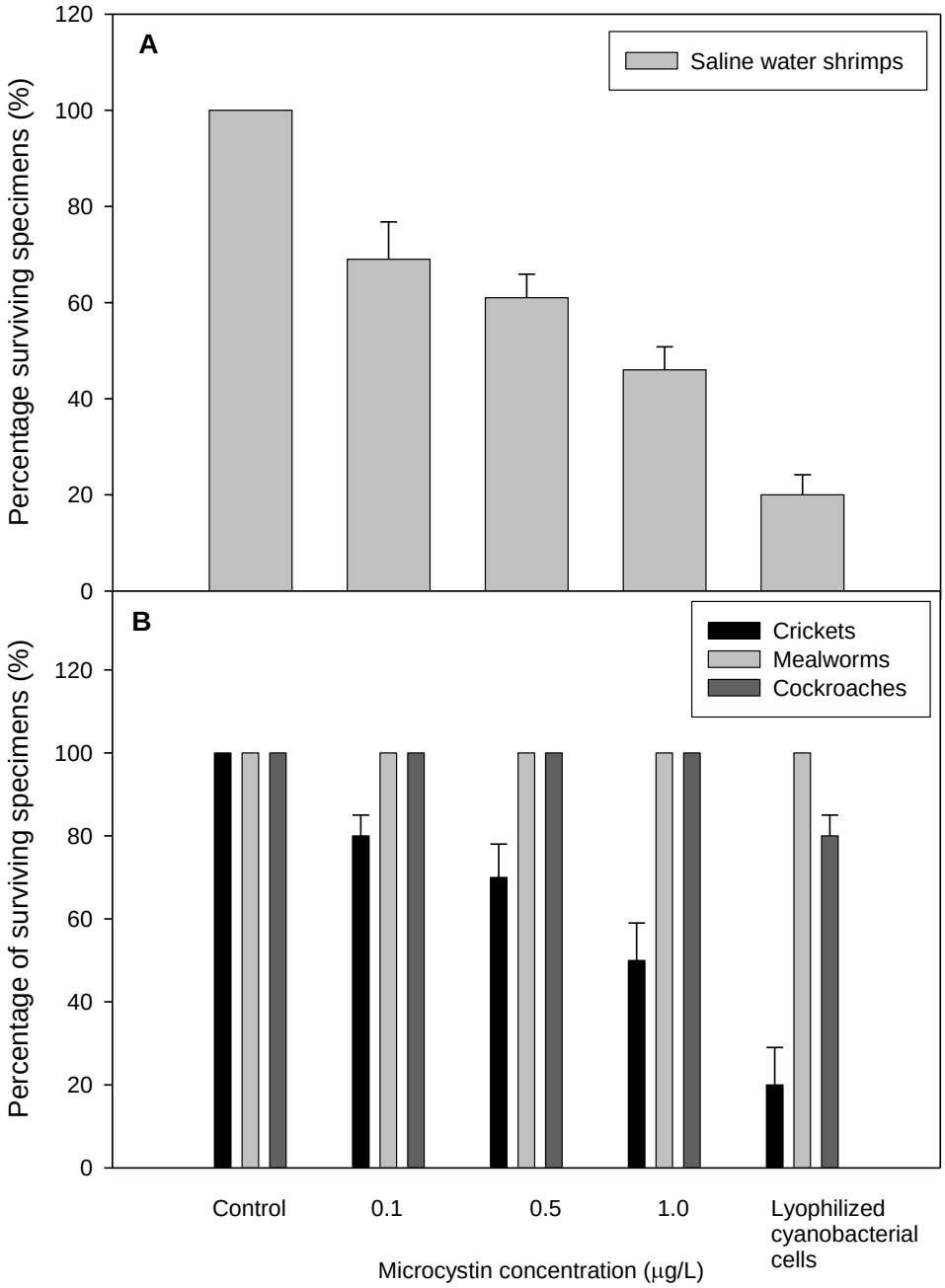

Figure 3. Percentage surviving organisms after 48 h of exposure to different concentrations of microcystin-LR and a lyophilized crude extract. (A) Brine water shrimps; (B) crickets, mealworms and cockroaches.

they drink more water per unit body weight per day (WHO, 1996).

It could be postulated that chronic exposure to low levels of cyanotoxins by people who live in rural areas with suppressed immune systems due to the HIV/AIDS epidemic and its close relationship with other communicable and poverty related diseases such as tuberculosis will have serious social and economic consequences in the near future for society at large. Doyle (1991) estimates that there are roughly a thousand new cases of HIV/AIDS every day in South Africa. Furthermore, common symptoms of cyanotoxin poisoning (that is, diarrhoea, vomiting, stomach pains) are also the symptoms of gastrointestinal illness caused by bacteria, viral and protozoan infection in South Africa and are thus never linked to possible cyanotoxin poisoning (Falconer, 2005). For children under 5 years of age, diarrhoea is the third most important cause of death after HIV/AIDS and low

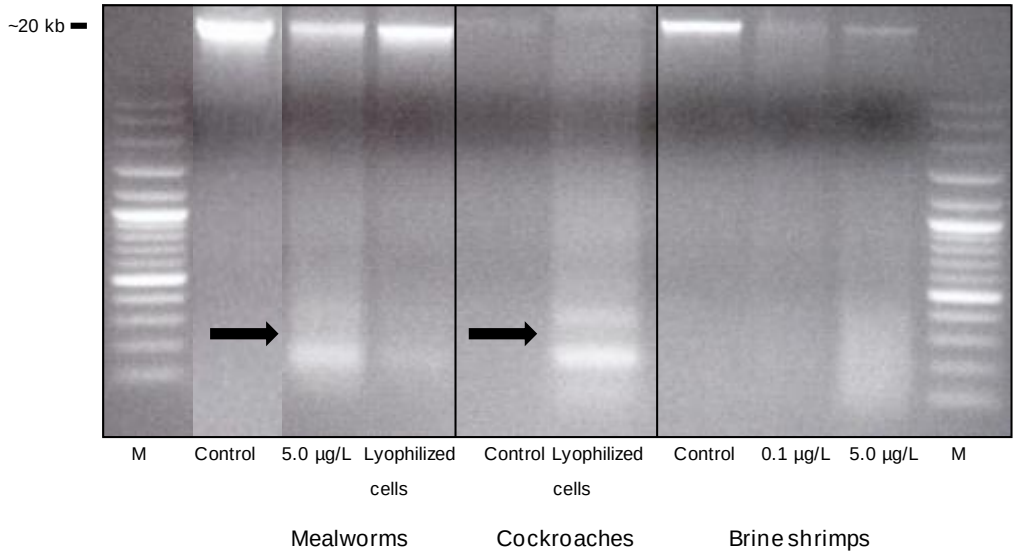

Figure 4. DNA fragmentation patterns and visible genomic DNA breakdown after exposure of invertebrate test specimens to different concentrations of microcystin-LR and a lyophilized crude biotoxin extract. Arrows indicates DNA fragmentation and/or DNA breakdown. M = 100 bp ladder (O'RangeRular™ 100 bp DNA Ladder).

birth weight, representing ~10% of all deaths in this age group in South Africa (Bradshaw et al., 2003). These facts illustrating the need for affordable and reliable water quality tests that can be used as an alert system for the presence of cyanotoxin in drinking water of rural areas.

At present the commonest and most reliable screening test for cyanobacterial toxicity, the mouse bioassay, is not always feasible in South Africa. Since standardized laboratory facilities with specific rating, as required by law, is not financially viable for testing by water purification plants in rural areas. Furthermore, mouse bioassays can only detect toxin in concentrated samples of cyanobacteria and is not sensitive enough to detect toxins released into a water body or tap water.

While an abundance of potentially useful assays seems to be available, the primary impediment to their widespread use is the lack of a demonstrated link between effects at the sub-organism level with effects on growth at the individual level and with higher levels of biological organization. The use of aquatic plants in water quality assessment has been common for years as *in situ* bioindicators (Pflugmacher, 2002). The sensitivity of various plant and animal tests have been compared on numerous occasions and found to be chemical and species specific (Shigeoka et al., 1988; Oberholster et al., 2005). Duckweeds are common to South Africa and are usually floating species unattached to the substrate. Their small size, ease of culture and rapid reproductive rate (doubling time 1-4 days) are attributes that have led to their use in this battery of bioassays. *Lemna minor* and *Lemna gibba* are the most commonly tested species used in numerous studies in first world countries as indicator macrophyte species (Marwood et al., 2001). No clear trend exists to the relative sensitivities of the different duckweed species and the sensitivities of the duckweeds relative to faunal species. However, there appears to be little difference in the sensitivities of *L. minor* and *L. gibba*, based on the results of Cowgill et al. (1991). In this study, no clear differences in sensitivity of *S. punctata* to MC-LR where observed if compared to a study by Romanowska-Duda and Tarczynska (2002) on *S. oligorrhiza*. Preliminary investigations by Weiss and Liebert (1998) reported a significant reduction in growth of *L. minor* in co-culture with a toxic strain of *M. aeruginosa* Kützing. They also observed a significant decrease of chl *a* and chl *b* ratios in *L. minor* after exposure to 5 mg/L microcytin-RR for 6 days (Weiss et al., 2000).

Romanowska-Duka and Tarczynska (2002), on the other hand observed a clear reduction in frond mass and a change in chlorophyll (chl *a* and chl *b*) ratios after 24 h incubation of *S. oligorrhiza* in MC-LR at concentrations of 0.2 and 0.1 µg/L. These observations were concurrent with our findings with *S. punctata* after exposure to synthetic MC-LR at a low concentration of 0.1 µg/L. These findings were also supported by Pflugmacher (2000) who showed that after plants were exposed to synthetic MC-LR concentration of 0.5 µg/L and higher, a sharp increase in chl *b* was observed while chl *a* concentrations µg/g fresh weight on the other hand decreased. These changes of pigment pattern were also observed in *S. punctata* plants in this study after plants were exposed to different concentrations of MC-LR. This phenomenon was possibly due to damage to the plant photosystems, since chl *a* which is a component of peripheral antenna complexes may have been altered by the presence of MC-LR (Anderson, 1986). Furthermore, Pflugmacher

(2000) suggested that the greater reduction of chl *a* and the increase of chl *b* was a sign of stress in plants and could be related to exposure to the pure MC-LR. Although chl *a* and chl *b* contents were good indicators of macrophytes exposure to toxic MC-LR, it is not a feasible option to use in the case of rural water treatment plants in South Africa, since few if not any of these treatment plants have the equipment or the ability to do these analyses. Therefore, we concluded from data generated in this study that reduction in root growth and frond mass as bioindicators, might be a better option to detected low concentrations of MC-LR in drinking water. The measurements of these characteristics of macrophytes were effective and simple and no sophisticated instruments were needed. Precisely how the MC-LR influences the entire metabolic growth processes of *S. punctata* plants cannot be determined from the experiments presented here. In this study we also observed significant chlorosis in *S. punctata* fronds exposed to the high concentration (46.8 µg/L) of microcystins from the crude extract of cyanobacterial strain UP051. These observations were also clearly visible from the chlorophyll data indicating that the microcystin extract act as a phytotoxic agent.

PCD with the distinct morphological hallmark of DNA laddering are indicative of basic physiological processes that occur in organisms under various stresses e.g. exposure to low levels of toxic compounds (Buckner et al., 2000). Hence, Gorczyca et al. (1994) reported that the time window during which the morphological changes occur was relatively short (30 min to 2 h) and thus many cells during the early stages of apoptosis may escape detection, while genomic DNA laddering occurs at later stages as observed in this study. Arends et al. (1990) reported in their study that the morphological changes were not always characteristic enough to be a sure marker of apoptotic cell death, while extensive DNA degrading on the other hand appears to be a quite specific marker of apoptosis as observed in the insects and the brine shrimp in this study (Arends et al., 1990).

Although several reports on microcystin toxicity and aquatic invertebrates exist, little is known about microcystin toxicity and insects (Penaloza et al., 1990). A report by Kiviranta et al. (1992), on the larval stages of the yellow fever mosquito, *Aedes aegypti*, immersed in a solution of cyanobacterial toxin showed that microcystin-RR (variable L-amino acids arginine and arginine) gave a 48 h LD_{50} value of 14.9 µg/ml, whereas adult female *Culex pipiens* mosquitoes gave a LD_{50} of 500 µg/Kg microcystin-LR, when injected intrathoracically (Turell and Middlebrook, 1988). Compared to i.p. LD_{50} values in rats and mice that ranges from 20 to 800 µg/Kg (Kungsuwan et al., 1988; Runnegar et al., 1991; Sivonen and Jones, 1999), insects are more sensitive than mice or rats. In our study the 48 h LD_{50} for *G. bimaculatus* was 263 µg/Kg, suggesting that the common cricket was more sensitive than the *C. pipiens* mosquito.

A plausible explanation for the detection of DNA fragmentation but no mortalities of the *T. molitar* test specimens within the first 48 h after exposure to crude extract of the cyanobacterial strain UP051-may be the effective DNA repair mechanisms in these organisms. It seems from the data generated in this study that the DNA strand breaks followed by the cleavage of DNA into oligonucleosome-sized fragments were repaired even though a high concentration of injected cyanobacterial toxin was present in their body cavity. However, no sublethal responses were observed in these insects, even when the experiment was extended to 5 days. Therefore we conclude from the data generated in this study that DNA fragmentation is not always associated with mortalities in the case of different insect species exposed to microcystins. However the use of apoptosis as end point for insects exposed to MC-LR is not feasible in the rural South African context, since water treatment plants lack proper infrastructure and necessary expertise to interpret DNA data generated.

Conclusion

Unfortunately, not all biomarkers in this study were sensitive enough to response to MC–LR at the WHO guideline level of 1.0 µg/L for drinking water. From the obtained results we recommend the use of reduction in root growth and frond mass of *S. punctata* as toxin bioindicators, since these responses are rapid and an outcome can be established within 24 h after incubation at low concentrations of MC-LR. Although ease of culturing of American cockroaches and yellow mealworms make them ideal organisms to include in a battery of bioassays, their 48 h LD_{50} non-response to low levels of microcystin exclude them from such a rapid sensitive bioassay. The shortfall of the common cricket and brine shrimp assays were that a 48 h LD_{50} were not establish at 1.0 µg/L although their calculated MC-LR LD_{50} values for body weight were within the range of mice bioassays observed in previous studies. Thus, they deserve further evaluation as alternatives bioassays which will act as a screening tool for the presence of cyanobacterial toxin. It should be emphasized that there are still a great deal of research needed in this area before a standar-dized battery of bioassays could be used as an alter-native technique to the mouse bioassays. Finally, as a cost saving device and to reduce health risks, the 24 h bioassays using *S. punctata* and *G. bimaculatus* may be implemented in rural water treatment plants as an early warning system, provided that when positive cyanotoxin test results are obtained, the water is send to an analytic laboratory for confirmation of the presence of cyanotoxins.

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