A comparison of in vivo and in vitro assays to assess the toxicity of algal blooms

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Abstract
The toxicity of purified microcystin-LR (MC-LR) and algal material collected during the winter and summer seasons (2005/2006) from the Hartebeespoort dam, South Africa, was investigated using the enzyme-linked immunosorbent assay (ELISA), mouse bioassay,
catfish primary hepatocytes (in vitro assay) and protein phosphatase inhibition (PPi) assays. Microcystis aeruginosa, known producer of microcystins, was the dominant cyanobacteria present in the water samples. Exceptionally high cell numbers per millilitre were observed,
especially with the summer samples (~1.442 × 10^8 cells/ml), indicating a severe algal bloom in the dam. The toxin concentration as detected by ELISA and PPi assay in the winter and summer extracts was at least 1000 times more than the provisional guideline value (1 mg/l) set by the World Health Organization (WHO) for MC-LR in drinking water. Hepatotoxic effects and death of mice were observed after dosing with the summer extracts, while no hepatotoxic effects were observed with winter extracts. The EC_{50} values obtained after exposure of the catfish primary hepatocytes for 72 h to MC-LR, winter and summer extracts was about 0.091, 0.053 and 0.014 mg/l, respectively. Similar toxicity results were obtained when the mouse bioassay and primary hepatocytes were used.

Keywords: Catfish primary hepatocytes; Mouse bioassay; ELISA; Protein phosphatase inhibition; Assay

In South Africa and other parts of the world, livestock, waterfowl, wildlife and game animals have died after drinking water containing heavy blooms of blue-green algae (Steyn, 1945; Soll and Williams, 1985; Bell and Codd, 1994; Harding et al., 1995; Van Halderen et al., 1995; Kellerman et al., 2005). Records of poisoning incidents that can be attributed to cyanobacteria in South Africa date back to the 1920s, when mass mortalities of thousands of cattle, sheep, horses and rabbits around pans in the south-eastern Transvaal were reported (Steyn, 1945; Soll and Williams, 1985; Harding and Paxton, 2001).
According to a report published by the Department of Water Affairs and Forestry (DWAF, 2002), many South African surface water resources exhibit high nutrient enrichments and eutrophication-related problems. About 80 dams were monitored between October 2002 and September 2003 in South Africa (Van Ginkel, 2003). Eleven per cent of the monitored dams were hypertrophic (showing serious water quality problems), 23% were eutrophic (showing increasing signs of water quality problems) and 25% were mesotrophic (showing emerging signs of water quality problems). Microcystin levels detected in the hypertrophic dams ranged from1 to 28 930 mg/l (Wicks and Thiel, 1990; DWAF, 2002; Van Ginkel, 2003). Given the current status of algal blooms in the impoundments of South Africa, sensitive and specific monitoring assays for cyanotoxins are required.
Toxicity testing is important to ensure good water quality for human and animal consumption, and for recreational activities. For many years the mouse bioassay has been used to determine toxicity of blooms (Carmichael, 1992), and to this date, this assay is still routinely used in South Africa. The major advantage of the mouse test is the ability of the animal to provide natural physiological and biochemical functions for toxicological assessment. It also has an advantage of being non-specific; therefore, detecting any toxin, known or unknown, in the algal material. The disadvantage of the mouse test is that it does not have the sensitivity or precision required to be applicable to water samples with concentrations around 1–2 mg/l, the approximate range of the guideline for microcystin-LR (MC-LR) prescribed by the World Health Organization (WHO) (WHO, 1998). For ethical reasons, the mouse test is unsuitable for large scale and routine testing of field samples (Aune and Berg, 1986; Heinze, 1996).
In recent years, in vitro toxicity tests involving the use of cultured cells have been developed to provide a substitute for the mouse bioassay (Storey et al., 1983; Aune and Berg, 1986; Berg and Aune, 1987; Heinze, 1996). The major advantage of using freshly isolated hepatocytes is their ability to maintain the activities of phase I and II drug-metabolizing enzymes, thus allowing various investigations to be performed including determination of metabolic profiles, inhibition and induction effects (Fautrel et al., 1991; Guillouzo, 1998). Another advantage of the primary hepatocytes is that a number of samples can be tested from a single preparation of hepatocytes compared to one mouse required per sample when using the mouse assay. The

disadvantage associated with the use of primary hepatocytes is their limited survival period, after which a decline in the activity of phase I and II enzymes is observed (Fautrel et al., 1991; Guillouzo, 1998).
Both the enzyme-linked immunosorbent assay (ELISA) and protein phosphatase inhibition assays are sensitive methods used for detecting microcystins. The major advantage of using the ELISA and protein phosphatase inhibition assays is that they both have a detection limit lower than theWHO proposed guideline of 1 mg/l and they can be performed without sample pre-concentration. The disadvantage of using ELISA is that it does not give any indication of which congeners are present in a mixture of microcystin toxins (Mountfort et al., 2005). The disadvantage of the protein phosphatase inhibition assay is that it responds to a wide variety of non-cyanobacterial toxins and metabolites including okadaic acid, tautomycin and calyculin A (Metcalf et al., 2001).
In the current study, toxicity of MC-LR and algal samples collected during summer and winter seasons were compared using the mouse bioassay, catfish primary hepatocytes, ELISA and protein phosphatase inhibition assays.

2. Materials and methods

2.1. Collection of cyanobacterial samples

Cyanobacterial samples were collected from the Hartebeespoort dam, South Africa, following a standard operating procedure prepared by the Department of Water Affairs and Forestry (DWAF, 2004). Hartebeespoort dam is a 20 km2 water reservoir located about 50 km west of Pretoria. All inhabitants around the dam and large settlements downstream use purified dam water for drinking. Recreational use is intensive due to the close proximity of the Pretoria and Johannesburg Metropolitan areas. About 80% of water from this dam is used for irrigation (NWPG, 2005).
Scums were sampled near the dam wall using 1 l glass bottles. Wide-necked bottles were used to collect samples from the upper layer of the scum on the water surface. Collected samples were labelled appropriately and stored in a cooler box during transportation to the laboratory. Samples were collected from July 2005 to July 2006 (i.e. July 2005, October 2005, March 2006 and July 2006). During the summer season (October 2005 and March 2006), the water surface near the dam wall was covered completely with blue-green scums and blue-green streaks were observed on the remainder of the water surface. An unpleasant smell coming from the water surface of the dam was also noted during this season. Only blue-green streaks were observed near the dam wall during the winter season (July 2005 and July 2006). Although the rest of the water surface appeared clear, a few of the blue-green streaks were observed. In the laboratory, samples were aliquoted and stored at -20 °C prior to analysis. Two per cent formaldehyde was added to some of the aliquoted samples that were sent for identification and enumeration of cyanobacteria.

2.2. Identification and enumeration of cyanobacteria

Samples used for the identification and enumeration of cyanobacteria were preserved with 2% formaldehyde. Identification and enumeration was based on distinct morphological features of the cyanobacteria using light microscopy at a magnification of 200–1000 times (Chorus and Bartram, 1999). Gentle ultrasonication was used to disintegrate colonial cells
to allow easy counting of the individual cells in the colonies (Chorus and Bartram, 1999).

2.3. Preparation of extracts from cyanobacterial scums

Algal samples were extracted following the method described by Falconer (1993). Using the VirTris Benchtop SLC freezedryer (SP Industries), condenser temperature at -40 °C, the algal samples were freeze-dried for about 24h for a 50-ml sample. Freeze-dried samples were then weighed and resuspended in physiological saline at 200mg in 10 ml. Re-suspended samples were held in a boiling water bath for 10 min for sterilization in order to prevent any possibility of bacterial infection to the mice. Extracted samples were stored at -20 °C until analysed.

2.4. Enzyme-linked immunosorbent assay (ELISA)

A commercial ABRAXIS-Microcystin ELISA kit (Aqualytic Environmental and Laboratory Services, Gauteng, South Africa) was used. The ELISA is an indirect-competitive method used for the quantitative analysis of microcystins and nodularins. All field extracts were diluted as they were showing concentrations higher than 5 µg/l. All winter extracts were diluted 1/10 000 in physiological saline and summer extracts were diluted 1/50 000. After addition of the standard solutions and algal extracts (50 µl) into the wells of the microtitre plate, an antibody solution (50 µl) was added to the wells. The microtitre plate was incubated for 90min at room temperature. At the end of the incubation period, wells of the plate were washed three times using the washing buffer solution. An enzyme conjugate solution (100 µl) was added to each well; the microtitre plate was incubated for 30min at room temperature. After another washing step, a substrate solution (100 µl) was

added to individual wells followed by incubation for 20–30min at room temperature. At the end of the incubation period, a stop solution (50 µl) was added and absorbance was measured at 450nm using a microplate ELISA spectrophotometer (Bio-Tek µQuant, A. D. P., South Africa). Each test was carried out in triplicate. A standard curve was constructed using MC-LR at concentrations of 0, 0.15, 0.4, 0.75, 1, 2 and 5 µg/l (GraphPad Prism software), and concentrations of the extracts were determined from this standard curve.

2.5. Animals

Adult male mice (CD-1 SPF strain) weighing between 30 and 40 g were purchased from Onderstepoort Biological Products (OBP), Gauteng, South Africa. The animals were housed individually in the Laboratory Animal Facility with vermiculite as bedding material and were provided with pellet food (OBP, Gauteng, South Africa) and water ad libitum. Temperature
was maintained between 21 and 23 °C.

2.6. Mouse bioassay

Toxicity of purified MC-LR (Sigma-Aldrich, Gauteng, South Africa) and cyanobacterial extracts were tested using the mouse bioassay according to the method described by Aune and Berg (1986). One millilitre of MC-LR concentrations (0.001, 0.005, 0.01, 0.025, 0.05, 0.1, 3.125, 6.25, 12.5, 25 and 50 mg/l) was injected intraperitoneally (i.p.) into mice. Three animals
were used for each concentration of MC-LR.
Mice were injected i.p. with 1ml of winter and summer extract solutions containing equivalent final concentrations per mouse of 20mg suspended blue-green algal matter (500–667 mg/kg). At least six mice were used for each winter and summer extract. Control mice were injected i.p. with 1ml saline solution. All mice were observed for 24 h after i.p. injection. During the 24h observation period, signs of poisoning and survival times were recorded. At the end of the observation period, all surviving animals were sacrificed and post-mortem examination of tissue injury was performed on all the experimental animals. Mortality with the characteristic enlarged liver (liver weight/body mass % 47) was used as the measure of hepatotoxicity (Heresztyn and Nicholson, 2001).

2.7. Isolation and culturing of primary hepatocytes

Primary hepatocytes were isolated from the African sharptooth catfish (*Clarias gariepinus*) using a modified two-step collagenase in situ perfusion method optimized in our Toxicology Tissue Culture Laboratory (Naicker et al., 2007). Briefly, the first perfusion was performed in situ with a HEPES buffer, pH 7.5, 20mM (Highveld Biologicals, Gauteng, South Africa) at a flow rate of 0.5 ml/min. Final perfusion was performed for 12min at a flow rate of 5 ml/min with a perfusion solution containing collagenase, type IV (Sigma- Aldrich, Gauteng, South Africa). The yield of cells was about 3_10^6 cells/ml. Cells were plated in MatriGel based membrane matrix (BD Biosciences) coated 96-well plates (50 000 cells/well) and maintained at 16 1C in a 5% CO2 humidified atmosphere for 24h to allow cells to attach to the wells.

2.8. Methyl-thiazol-tetrazolium (MTT) assay

Assessment of cell viability was carried out using a modified method of Mosmann (1983) based on methyl-thiazol-tetrazolium (MTT) (Sigma-Aldrich, Gauteng, South Africa). After attaching to the wells, the catfish primary hepatocytes were exposed to summer and winter extracts (100 µl of extracts added to 100 µl of M199 culture medium supplemented with
10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml fungizone). Control wells were prepared by adding 200 µl of culture medium. The catfish hepatocytes were also exposed to purified MC-LR (0.75×10^{-3}, 1×10^{-3}, 2×10^{-3}, 5×10^{-3}, 1×10^{-2}, 2.5×10^{-2}, 5×10^{-2} and 1×10^{-1} mg/l). After exposure, cellswere incubated at 16 °C in a 5% CO_2 humidified
atmosphere for 72 h. At the end of the incubation period, 10 ml of MTT (5mg/ml in PBS) was added into each well and the cells were further incubated for 4h. The formation of colour (formazan) was measured with a microtitre plate spectrophotometer (Bio-Tek µQuant, A. D. P., South Africa) at 570nm. Cell viability was estimated as the percentage absorbance of
sample relative to control.

2.9. Protein phosphatase inhibition (PPi2A) assay

A commercial RediPlate 96 EnzChek Serine/Threonine Phosphatase assay kit (Scientific Group, South Africa, product of Molecular Probes, Inc.) and protein phosphatase type 2A (PP2A) enzyme (Whitehead Scientific, South Africa, product of Promega) were used. PP2A comprises the 36–38kDa catalytic subunit isolated from human red blood cells and was

supplied in lots of 25U, 1U being defined as the amount of enzyme required to hydrolyse 1nmol of pNPP/min at 30 1C under the specified assay conditions (Promega Technical Bulletin). The principle of the assay is that the PP2A enzyme specifically removes phosphate groups from a fluorogenic substrate, DiFMUP (Molecular Probes), to produce DiFMU that is measured by the fluorometer. In the presence of an inhibitor (MC-LR or algal extracts), the activity of PP2A is inhibited, therefore resulting in reduced production of DiFMU.

All winter extracts were diluted 1/10 000 and summer extracts were diluted 1/50 000. After adding MC-LR (80 μl) or algal extracts (80 μl) and the enzyme (20 μl) to the wells, the microtitre plate was incubated at 37 °C and fluorescence was measured over a period of 90 min at 355/460nm using a Fluoroskan Ascent FL fluorescent microplate reader (product of Thermo Electron Corporation, supplied by AEC-Amersham, South Africa). Controls consisted of assays with the samples or standards replaced by the reaction buffer. Blanks consisted of assays in which all components were present except the enzyme.

Sample concentrations were determined from a standard fitted curve generated using MC-LR at concentrations of 10^{-7}, 10^{-5}, 10^{-3}, 10^{-2}, 10^{-1}, 0.5, 1, 10 and 100 μg/l. The microcystin inhibition (calibration) curve was plotted as the percentage activity of PP2A relative to the control, versus microcystin concentration, where PP2A activity(%)

$$= \frac{\text{Absorbance}_{\text{standard}} - \text{Absorbance}_{\text{blank}}}{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{blank}}} \times 100.$$

In order to more precisely determine sample concentrations, only the linear region of the calibration curve, i.e. the region between 20% and 80% activity, was used for quantification.

2.10. Statistical analysis

The results are reported as mean7SD from individual determinations with at least three replicates. Statistical differences were analysed by unpaired t-test using the GraphPad Prism version 4 software (GraphPad Software Inc, San Diego, CA). Values of $P<0.05$ were regarded as significant. Kappa coefficient (Cohens Kappa) was also used to determine the degree of agreement between the assays (mouse bioassay and cytotoxicity (catfish primary hepatoctes) assay, and ELISA and PPi2A assay) used in this study. The Kappa coefficient (K) is defined as an indication of the degree of agreement for nominal or categorical data (i.e. K40.80, almost perfect agreement; K = 0.60–0.80, substantial agreement; K = 0.40–0.60, moderate agreement; K = 0.20–0.40, fair agreement; K<0.20, slight agreement).

3. Results

3.1. Identification and enumeration of cyanobacteria

Microscopic observation revealed the presence of Microcystis *aeruginosa* and *Planktothrix* sp. as the most dominant cyanobacteria in water samples collected during the winter and summer seasons (Table 1). The number of *M. aeruginosa* cells per millilitre in the summer samples (~1.442~10^8 cells/ml) was very high when compared to the winter samples (~2.667~10^6 cells/ml). Winter and summer samples were dominated by the unicellular form (95%) of *M. aeruginosa*. Large colonies (~400~800 μm, diameter) of *M. aeruginosa* with each colony consisting of ~1000–5000 cells were observed in winter and summer samples and this constituted about 5% of the total number of cells.

3.2. ELISA

The ELISA assay used in this study is stoichiometrically based, reacting with toxins that have the ADDA moiety. It therefore detects most of the known toxic penta- and heptapeptide toxin congeners (Fischer et al., 2001). The cyanotoxin concentrations as detected by ELISA in winter and summer extracts are shown in Table 2. According to the microcystin ELISA, which cross-reacts with MC-LR, MC-RR, MC-LW, MC-YR, MC-LF and nodularin, the winter extracts contained toxins belonging to this group with concentrations of about 3.673 mg/l. The summer extracts contained toxins belonging to this group as well, with much higher toxin concentrations of about 86.083 mg/l.

3.3. Mouse bioassay

Table 3 shows the results of toxicity observed with the different concentrations of purified MC-LR after i.p. injection in mice. MC-LR concentrations ranging from 0.001 to 3.125 mg/l did not induce any observed hepatotoxic or neurotoxic effects in mice and no mortality was observed with these concentrations. When the livers were removed for macroscopic examination they were found to be unaffected. However, hepatotoxic effects (liver weight/body mass % >7) accompanied by mortality within 2h were observed in mice dosed with MC-LR concentrations ranging from 6.25 to 50mg/l. When they were examined macroscopically, the livers were found to be very dark in colour due to haemorrhaging and also larger when compared to the control

livers. Therefore, the minimum concentration of purified MC-LR that induced hepatotoxicity and mortality in male CD-1 SPF mice was estimated to be about 6mg/l.
Toxicity of winter and summer extracts after i.p. injection in mice is shown in Table 4. Hepatotoxic effects and mortality of mice were observed within 2h after dosing with the summer extracts. Symptoms typical for neurotoxin intoxication, namely reduced activity, spasms, respiratory difficulties and paralysis of the hind legs, were also observed on mice dosed with the summer extracts. On macroscopic examination, the livers were found to be very dark in colour (haemorrhaging) and larger. Mice dosed with the winter extracts did not show any observed hepatotoxic or neurotoxic effects and the livers appeared normal when they were examined.

3.4. Toxicity to catfish primary hepatocytes

Viability of the catfish primary hepatocytes after exposure to purified MC-LR, summer and winter extracts was investigated based on the mitochondrial succinate dehydrogenase activities of the hepatocytes. A decline in formazan crystals indicates a decrease in the activity of the dehydrogenase enzyme and cellular metabolism, hence a reduction in cell viability. The cytotoxic response of the catfish primary hepatocytes after exposure to purified MC-LR, summer and winter algal extracts was expressed in terms of the EC_{50} values. An EC_{50} is defined as the exposure concentration of an algal material that is expected to cause cytotoxicity in 50% of the cell population. EC50 values obtained after exposure of catfish primary hepatocytes for 72h to the cyanotoxins are shown in Table 5. The EC50 obtained for the summer extracts was about 0.014 and 0.053 mg/l for the winter extracts. 0.091 mg/l was the EC50 obtained after exposure of the cells to purified MC-LR.

3.5. Protein phosphatase inhibition (PPi2A) assay

The calibration (inhibition) curve for MC-LR standards is shown in Fig. 1. An IC_{50}, defined as the concentration of MCLR required for 50% inhibition, determined from the calibration curve was 0.22 µg/l. The portion of the curve between 20% and 80% activity, which is relatively linear and normally used for quantification, defines the working range (0.01–1.0 µg/l) of
the assay. Higher concentrations of the field samples were determined by dilution of the samples (winter extracts 1/ 10 000 and summer extracts 1/50 000 dilution). Toxin concentrations determined by the PP2A inhibition assay on winter and summer extracts are shown in Table 2. Concentration of the winter extracts was about 2.993 mg/l; a higher concentration of about 54.900 mg/l was observed with the summer extracts. Complete inhibition in the activity of PP2A enzyme was observed with undiluted winter and summer extracts.

4. Discussion

Toxicity of purified MC-LR has been studied extensively using biological or biochemical and physiological assays. This study reports on the toxicity of field algal material using biological or biochemical assays and shows the significance of toxicity in algal material, also observed with purified toxins, and the seasonal toxicity. *Microcystis aeruginosa*, known producer of microcystins and nodularins (Carmichael, 1992; Dawson, 1998), and *Planktothrix*
sp., known producer of microcystins and anatoxin-a (Carmichael, 1992; Dawson, 1998), were the most dominant cyanobacteria identified in water samples collected during winter and summer seasons of 2005/2006 from the Hartebeespoort dam, South Africa (Table 1).
The provisional guideline value set by the WHO for MC-LR in drinking water is 0.001 mg/l (WHO, 1998). The toxin concentrations of winter and summer extracts as detected by the ELISA assay was at least 1000 times more than the prescribed guideline value, with summer extracts showing much higher cyanotoxin concentrations than the winter extracts (Table 2). The very high levels of microcystins detected in the Hartebeespoort dam during this study correlate with previous results reported by other investigators (Wicks and Thiel, 1990; Van Ginkel et al., 2001; DWAF, 2002; Van Ginkel, 2003). The observed differences in toxicities for summer and winter extracts (Table 2) could have been caused by the presence of nutrients such as phosphorus and nitrogen in high levels during the summer season compared to the winter season. Other investigators have demonstrated the positive correlation between the presence of high levels of nutrients (phosphorus and nitrogen) and algal toxicity (Wicks and Thiel, 1990; Lehman, 2007). Robarts and Zohary (1984) found the exceedingly high levels of phosphorus and nitrogen to be the most important contributing factors in eutrophication of the Hartbeespoort Dam. In addition to the presence of high levels of nutrients,Wicks and Thiel (1990) also suggested that during the summer seasons Hartbeespoort Dam is dominated by the toxic strains of *M. aeruginosa*, whereas during winter seasons the dam is dominated by the non-toxic strains.

Microcystins and nodularins inhibit serine and threonine phosphatase (type 1 and 2A) enzymes responsible for the dephosphorylation of intracellular phosphoproteins (MacKintosh et al., 1990). The degree of inhibition of these enzymes can therefore be used as a measure of toxicity/toxin concentration. The IC_{50} for MC-LR (0.22 µg/l) obtained in this study (Fig. 1) is in agreement with IC_{50} obtained by Heresztyn and Nicholson (2001) for MC-LR (0.3 µg/l). Toxin concentration of the summer extracts was shown to be higher than the winter extract concentrations (Table 2). Compared to the ELISA, concentrations obtained using the PP2A inhibition assay were much lower. Fischer et al. (2001) also state that the ELISA gave higher values than the protein phosphatase assay, but had a five-fold better sensitivity. Results obtained from a study by An and Carmichael (1994) demonstrated that among different microcystins, the concentration of congener producing 50% inhibition of protein phosphatase differed considerably and in some cases virtually no inhibition was observed. However, in the ELISA assay cross-reactivity was observed with these congeners using polyclonal antibodies (An and Carmichael, 1994). The differences in the mechanism and response between the protein phosphatase and ELISA assays may explain the different concentrations observed with the two assays used in this study. The ELISA is primarily used to estimate the total amount of toxin present and the PP2A assay is used to estimate total toxicity (Mountfort et al., 2005).

The mouse bioassay is generally used in a qualitative manner to determine if a bloom is toxic or non-toxic and also used to identify the class of toxins present in the water samples based on the observed clinical symptoms (Msagati et al., 2006). The clinical symptoms in mice observed after dosing with the field algal extracts, especially the summer extracts, strongly suggested the presence of hepatotoxins (symptoms: dark-coloured appearance and enlargement of liver) and neurotoxins (symptoms: reduced activity, spasms, respiratory difficulties and paralysis of the hind legs) in the extracts (Table 3). The ELISA results presented in the current study only reflected the hepatotoxin (microcystin and nodularin) concentrations, which could be an underestimation of the total toxin concentration, as other toxin groups such as the neurotoxins could have been present in the water samples. This was also supported by the identification of neurotoxin-producing cyanobacteria (Planktothrix sp.) in the water samples. Therefore, the death of mice observed with the summer extracts cannot be assigned to one single toxin but appears to have been caused by several toxins acting together. HPLC can be used as a tool in the future to investigate the toxin profile of the field extracts in order to describe the different classes of toxins present in the extracts.

The winter samples were found to be non-toxic in mice. The minimum concentration of purified MC-LR that induced hepatotoxicity and mortality within 2h in male CD-1 SPF mice was estimated to be about 6mg/l. MC-LR concentrations below 6 mg/l were determined to be non-toxic as no symptoms of hepatotoxicity and mortality were observed with these concentrations. Cyanobacterial hepatotoxins have been shown to cause rapid death in mice due to intrahepatic haemorrhage which lead to the increase in liver weight (Hermansky et al., 1993; Fawell et al., 1999), this explains the increase in liver weight (liver weight/body mass% >7) that was observed after treatment of mice with the summer extracts in the current study.

The potency of algal extracts to cause toxicity in mice was confirmed *in vitro* using the catfish primary hepatocytes. In the current study, viability of the catfish hepatocytes was shown to be affected significantly by exposure to both the winter and summer extracts. The EC_{50} values obtained after exposure of the catfish primary hepatocytes for 72h to MC-LR, winter and summer extracts was about 0.091, 0.053 and 0.014 mg/l, respectively (Table 5). Fastner et al. (1995) found EC50 values of 0.2 mg/l in rat primary hepatocytes after exposure to MC-LR for 4h and 4.5 mg/l after exposure to MC-RR for 4h. The different EC_{50} values obtained with the catfish primary hepatocytes and rat primary hepatocytes could be due to the differences in exposure times of these primary hepatocytes to the cyanotoxins. Other investigators have demonstrated the successful use of mammalian and rainbow trout primary hepatocytes in determining cytotoxicity of the cyanotoxins (Aune and Berg, 1986; Heinze, 1996; Boaru et al., 2006). Because of their sensitivity and their ability to function similar to the liver *in vivo*, isolated primary hepatocytes provide an attractive alternative to the mouse assay in toxicity testing of cyanotoxins.

The cytotoxic assay involving use of the African catfish (*Clarias gariepinus*) primary hepatocytes has been shown in this study to produce results similar to those observed when using the mouse bioassay in assessing bloom toxicity (K = 1.00). The ELISA assay continues to remain the preferred assay in quantifying cyanotoxin content in aquatic systems due to its ability to detect most of the known toxic penta- and heptapeptide toxins.

5. Conclusions

For the first time, the African sharptooth catfish primary hepatocyte model has been exposed to purified MC-LR and cyanotoxin-containing water samples. Results obtained with the catfish primary hepatocytes in the current study are consistent with results obtained using the mouse assay. Therefore, this primary cell line may be used as a potential alternative to the mouse assay in toxicity testing of cyanotoxins, thus significantly reducing the use of animals when assessing bloom toxicity. Future studies to validate the use of this hepatocyte model in microcystin toxicity testing are planned. The ELISA assay continues to remain the preferred assay in quantifying cyanotoxin content in aquatic systems due to its high sensitivity and ease of analysis.

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Table 1 *– Cyanobacteria identified in water samples collected during winter and summer seasons*

Sample	Cyanobacteria Species	Number of cells per millilitre (cells/ml)
Winter Samples	Microcystis *aeruginosa*	2.667×10^{6}71.756×10^{6}
	Planktothrix sp.	2.917×10^{5}71.909×10^{5}
	Nitzschia sp.	4.333×10^{3}71.154×10^{3}
Summer Samples	Microcystis *aeruginosa*	1.442×10^{8}73.484×10^{7}
	Plantothrix sp.	2.450×10^{5}77.583×10^{4}
	Melosira sp.	4.167×10^{4}75.774×10^{3}

Cell number/ml of winter samples represent the average determined from water samples collected during July 2005 and July 2006. Cell number/ml of summer samples represent the average determined from water samples collected during October 2005 and March 2006. Winter and summer samples were dominated by the unicellular form (95%) of *M. aeruginosa* (po0.05).

Table 2 *– Mean toxin concentrations determined byoxin concentrations determined by ELISA and PPi2A assays on winter and summer extracts*

Algal extract	ELISA (mg/l)	PPi2A (mg/l)
Winter extracts	3.67372.832	2.99371.598
Summer extracts	86.083718.074	54.90074.769

Winter extracts were diluted 1/10 000 and summer extracts were diluted 1/50 000.
Six tests were performed for each winter and summer extract.
Each test was carried out in triplicate (po0.05).

Table 3 *– Mouse bioassay results after i.p injection of mice with MC-LR*

MC-LR (mg/l)	Liver weight/body mass %	Symptoms of hepatotoxicity	Symptoms of neurotoxicity	Survival time (h)
0.001	5.55070.354	No	No	24
0.005	5.10070.283	No	No	24
0.01	5.85070.636	No	No	24
0.025	5.40070.424	No	No	24
0.05	5.95071.202	No	No	24
0.1	6.25070.071	No	No	24
3.125	6.70070.849	No	No	24
6.25	8.45070.212	Yes	No	2
12.5	9.40070.424	Yes	No	<1
25	9.80070.283	Yes	No	<1
50	9.85070.778	Yes	No	<1
Control	6.25070.636	No	No	24

Toxicity of different concentrations of purified MC-LR after i.p. injection in CD-1 SPF mice. Three mice were used for each MC-LR concentration. Control mice were i.p. injected with saline solution. Mortality with the characteristic enlarged liver (liver weight/body mass % >7) was used as the measure of hepatotoxicity.

Table 4 *– Mouse bioassay results after i.p injection of mice with winter and summer extracts*

Algal extract	Toxin concentration (mg/l)	Liver weight/body mass%	Symptoms of hepatotoxicity	Symptoms of neurotoxicity	Survival time (h)
Winter	73.767	6.66770.408	No	No	24
Summer	786.083	9.11770.703	Yes	Yes	<1
Control	NT	6.65070.919	No	No	24

Toxicity of winter and summer extracts after i.p. injection in CD-1 SPF mice. Six mice were used for each winter and summer extract. Control mice were i.p. injected with saline solution. Mortality with the characteristic enlarged liver (liver weight/body mass % 47) was used as the measure of hepatotoxicity. Symptoms of neurotoxicity included reduced activity, spasms, respiratory difficulties and paralysis of the hind legs. NT ¼ not tested.

Table 5 *– EC_{50} values obtained after exposure of catfish primary hepatocytes for 72h to purified microcystin-LR (MC-LR), winter and summer algal extracts*

Algal extract	EC_{50} (mg/l)
MC-LR	0.091±0.023
Winter extracts	0.053±0.018
Summer extracts	0.014±0.005
Each algal sample was tested on nine culture wells ($p<0.05$).	

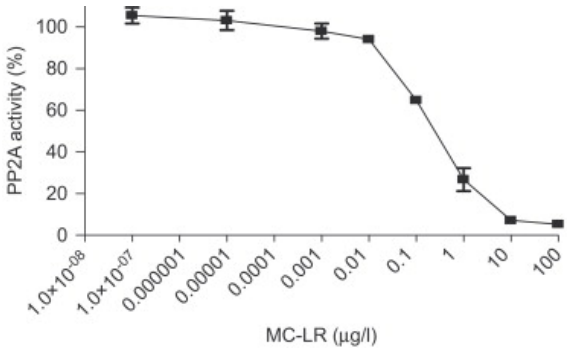

Fig. 1 *– Inhibition curve for microcystin-LR standards. The linear region of the calibration curve (i.e. the region between 20% and 80% activity) was used to determine sample concentrations. Each concentration was tested in three replicates.*