A COMPARATIVE ANALYSIS OF THE CYTOTOXICITY OF CYANOTOXINS USING *IN VITRO* (CELL CULTURE) AND *IN VIVO* (MOUSE) ASSAYS

BY

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LIST OF CONTENTS

Acknowledgements..... ii

List of Contents..... iv

List of Figures..... x

List of Tables..... xii

List of Abbreviations..... xiv

Summary..... xvii

CHAPTER 1:

INTRODUCTION..... 1

CHAPTER 2:

LITERATURE REVIEW..... 4

2.1 Introduction..... 4

2.2 Factors affecting bloom formation..... 5

2.2.1 Temperature..... 5

2.2.2 Light and buoyancy..... 6

2.2.3 Phosphorus and nitrogen..... 7

2.3 Toxins produced by cyanobacteria..... 7

2.3.1 Hepatotoxins..... 8

2.3.1.1 Microcystins (MCs)..... 8

2.3.1.2 Nodularins..... 10

2.3.1.3 Mechanism of action of hepatotoxins..... 11

2.3.2 Neurotoxins..... 12

2.3.2.1 Anatoxins and Saxitoxins..... 12

2.3.2.2 Cylindrospermopsin..... 13

2.3.2.3 Lipopolysaccharides (LPS)..... 14

2.4 Mechanism of toxin production..... 14

2.5 Exposure of humans to cyanotoxins..... 16

2.5.1 Introduction..... 16

2.5.2 Exposure through ingestion of water containing cyanobacteria..... 16

2.5.3 Exposure through food contaminated with cyanobacteria..... 17

2.5.4 Exposure through dermal contact with cyanobacteria..... 18

2.5.5 Human clinical cases in South Africa..... 18

2.6 Exposure of animals to cyanotoxins..... 19

2.6.1 Introduction..... 19

2.6.2 Clinical symptoms..... 21

2.6.3 Studies using rat/mice models to establish LD_{50} of cyanotoxins..... 22

2.7 Fate of cyanotoxins in the environment..... 23

2.7.1 Introduction..... 23

2.7.2 Chemical breakdown..... 24

2.7.3 Biodegradation..... 25

2.8 Screening methods for cyanotoxins..... 26

2.8.1 Biological/biochemical screening methods for cyanotoxins..... 26

2.8.2 Physicochemical screening methods for cyanotoxins..... 28

2.9 Guideline values for drinking water quality..... 29

CHAPTER 3:

SAMPLING, IDENTIFICATION AND ENUMERATION OF CYANOBACTERIA..... 31

3.1 Introduction..... 31

3.2 Materials and methods..... 33

3.2.1 Sampling of cyanobacteria..... 33

3.2.2 Identification and enumeration of cyanobacteria..... 35

3.3 Results..... 35

3.4 Discussion..... 38

3.5 Conclusion..... 39

CHAPTER 4:

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)..... 41

4.1 Preparation of extracts from cyanobacterial samples..... 41

4.1.1 Introduction..... 41

4.1.2 Materials and methods..... 42

4.2 ELISA..... 43

4.2.1 Introduction..... 43

4.2.2 Materials and methods..... 44

4.2.3 Statistical analysis..... 45

4.2.4 Results..... 45

4.2.5 Discussion..... 46

4.2.6 Conclusion..... 48

CHAPTER 5:

MOUSE BIOASSAY..... 49

5.1 Introduction..... 49

5.2 Materials and methods..... 51

5.2.1 Intraperitoneal (i.p.) injection of mice with purified MC-LR..... 52

5.2.2 Intraperitoneal (i.p.) injection of mice with cyanobacterial samples..... 52

5.2.3 Disposal of mouse carcasses..... 53

5.3 Results..... 53

5.3.1 Intraperitoneal (i.p.) injection of mice with purified MC-LR..... 53

5.3.2 Intraperitoneal (i.p.) injection of mice with cyanobacterial samples..... 54

5.4 Discussion..... 56

5.5 Conclusion..... 57

CHAPTER 6:

ELECTRON MICROSCOPY..... 58

6.1 Introduction..... 58

6.2 Materials and methods..... 59

6.3 Results..... 59

6.4 Discussion..... 64

6.5 Conclusion..... 65

CHAPTER 7:

***IN VITRO* TOXICITY TESTING OF CYANOTOXINS USING A CATFISH PRIMARY CELL LINE..... 67**

7.1 Introduction..... 67

7.1.1 Non-specific end points..... 69

7.1.2 Liver-specific end points..... 70

7.2 Materials and methods..... 70

7.2.1 Isolation and culturing of primary hepatocytes..... 70

7.2.2 Methyl-thiazol-tetrazolium (MTT) assay..... 71

7.3 Statistical analysis..... 72

7.4 Results..... 72

7.5 Discussion..... 75

7.6 Conclusion..... 77

CHAPTER 8:

PROTEIN PHOSPHATASE INHIBITION ASSAY..... 78

8.1 Introduction..... 78

8.2 Materials and methods..... 79

8.3 Statistical analysis..... 81

8.4 Results..... 81

8.5 Discussion..... 83

8.6 Conclusion..... 84

CHAPTER 9:

GENERAL DISCUSSION..... 85

CHAPTER 10:

CONCLUSION..... 91

CHAPTER 11:

REFERENCES..... 93

LIST OF FIGURES

CHAPTER 2

Fig. 1 – Chemical structure of microcystin-LR (MC-LR)..... 10

Fig. 2 – Chemical structure of nodularin..... 11

CHAPTER 3

Fig. 3 – Morphology of *Microcystis aeruginosa* as shown by light microscopy (A) and scanning electron microscopy (B)..... 37

CHAPTER 6

Fig. 4 – Electron micrograph showing a mouse liver tissue after i.p. injection with saline (control)..... 60

Fig. 5 – Electron micrograph showing a mouse liver tissue after i.p. injection with HBP Dam winter samples..... 61

Fig. 6 – Electron micrograph showing a mouse liver tissue after i.p. injection with HBP Dam winter samples..... 61

Fig. 7 – Electron micrograph showing a mouse liver tissue after i.p. injection with HBP Dam summer samples..... 63

Fig. 8 – Electron micrograph showing a mouse liver tissue after i.p. injection with HBP Dam summer samples..... 63

CHAPTER 7

Fig. 9 – Morphological changes of hepatocytes as shown by light microscope (400 X magnification) after exposure to HBP Dam summer samples..... 73

Fig. 9(A) – 24 h after exposure of hepatocytes to HBP Dam summer samples..... 73

Fig. 9(B) – 72 h after exposure of hepatocytes to HBP Dam summer samples..... 73

Fig. 9(C) – Control hepatocytes (not exposed to toxins) after 72 h..... 73

CHAPTER 8

Fig. 10 – Inhibition curve for MC-LR standards..... 82

LIST OF TABLES

CHAPTER 2

Table 1 – Toxicity of cyanobacterial toxins after i.p. administration to mice..... 23

CHAPTER 3

Table 2 – Identification of cyanobacteria in water samples collected from HBP Dam (winter and summer samples) and KNP..... 38

CHAPTER 4

Table 3 – Toxin concentrations determined by the ELISA on HBP Dam (winter and summer samples) and KNP samples..... 46

CHAPTER 5

Table 4 – Mouse bioassay results after i.p. injection of mice with MC-LR..... 54

Table 5 – Mouse bioassay results after i.p. injection of mice with HBP Dam (winter and summer samples) and KNP samples..... 55

CHAPTER 7

Table 6 – EC_{50} values obtained after exposure of catfish primary hepatocytes to HBP Dam samples (winter and summer), KNP samples and MC-LR..... 75

CHAPTER 8

Table 7 – Toxin concentration determined by PP2A inhibition assay in HBP Dam (winter and summer samples) and KNP samples..... 83

CHAPTER 9

Table 8 – Advantages and disadvantages of the mouse, cytotoxicity (catfish primary hepatocytes), ELISA and PP*i* assays..... 90

LIST OF ABBREVIATIONS

ADDA:	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-dienoic acid
AIDS:	Acquired immune deficiency syndrome
Asp:	Aspartic acid
ATP:	Adenosine-5´-triphosphate
Ca^{2+}:	Calcium ion
CE:	Capillary electrophoresis
CHO-K1:	Chinese hamster ovary
CO_2:	Carbon dioxide
D-Ala:	D-alanine
D-Glu:	D-glutamine
DiFMU:	6,8-difluoro-4-methylumbelliferone
DiFMUP:	6,8-difluoro-4-methylumbelliferyl phosphate
DWAF:	Department of Water Affairs and Forestry
EC_{50}:	The concentration of a chemical estimated to produce a specific effect in 50 % of a population of test species
ELISA:	Enzyme-linked immunosorbent assay
EM:	Electron microscopy
ER:	Endoplasmic reticulum
FCS:	Foetal calf serum
Fe^{2+}:	Ferrous ion

Fe^{3+}:	Ferric ion
GSH:	Glutathione
HBP:	Hartbeespoort
HCl:	Hydrochloric acid
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HIV:	Human immunodeficiency virus
HPLC:	High performance liquid chromatography
IC_{50}:	50 % inhibition concentration
i.p.:	Intraperitoneal
K^+:	Potassium ion
Km:	Kilometer
KNP:	Kruger National Park
LC/MS:	Liquid chromatography/Mass spectrophotometry
LD_{50}:	The dose of a chemical estimated to produce death in 50 % of the treated animals
LDH:	Lactate dehydrogenase
LPS:	Lipopolysaccharides
M. aeruginosa:	*Microcystis aeruginosa*
MC:	Microcystin
MC-LF:	Microcystin-LF (Leucine-Phenylalanine)
MC-LR:	Microcystin-LR (Leucine-Arginine)
MC-LW:	Microcystin-LW (Leucine-Tryptophane)
MC-RR:	Microcystin-RR (Arginine-Arginine)

MC-YR:	Microcystin-YR (Tyosine-Arginine)
Mdha:	*N*-methyldehydroalanine
MTT:	Methyl-thiazol-tetrazoluim
MU:	4-methylumbelliferone
MUP:	4-methylumbelliferyl phosphate
NMR:	Nuclear magnetic resonance
PBS:	Phosphate buffered saline
PDA:	Photodiode array
PP1:	Protein phosphatase type 1
PP2A:	Protein phosphatase type 2A
PP*i*:	Protein phosphatase inhibition
PSP:	Paralytic shellfish poisons
p-NPP:	*p*-nitrophenol phosphate
RTG-2:	Rainbow trout gonad-2
SEM:	Scanning electron microscopy
TEM:	Transmission electron microscopy
TFA:	Trifluoroacetic acid
UV:	Ultraviolet
WHO:	World Health Organization

SUMMARY

A COMPARATIVE ANALYSIS OF THE CYTOTOXICITY OF CYANOTOXINS USING *IN VITRO* (CELL CULTURE) AND *IN VIVO* (MOUSE) ASSAYS

BY

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The main objective of this study was the application and comparison of different assays in assessing toxicity of cyanobacterial samples, and also characterizing toxicity of the field samples. Therefore, toxicity of purified microcystin-LR (MC-LR) and

cyanobacterial samples collected from the Hartbeespoort (HBP) Dam (winter and summer seasons of 2005/2006) and Kruger National Park (KNP) were investigated and compared using the ELISA, mouse bioassay, catfish primary hepatocytes (*in vitro* assay) and protein phosphatase inhibition (PP*i*) assays.

During sampling in the summer season at the HBP Dam, the dam surface was covered with a thick-green layer of cyanobacterial scum and a foul smell coming from the water surface was always present. Only blue-green streaks of cyanobacteria covered the dam surface during the winter season. All HBP Dam samples (winter and summer samples) and KNP samples (Nhlanhanzwani Dam, Mpanama Dam and Sunset Dam) were dominated by *Microcystis aeruginosa* with the exception of Makhohlola Dam samples which were found to have no cyanobacteria.

The World Health Organization (WHO) has proposed a guideline value for human use of 1.0 µg/L (0.001 mg/L) for MC-LR, the most common microcystin (MC) variant, in drinking water (WHO 1998), whereas 2 000 *Microcystis* cells/mL have been recommended as the limit of cyanobacteria in drinking water for animals (DWAF 1996). Cyanotoxin concentrations exceeding the prescribed guideline value were detected in all HBP Dam samples (ELISA results ranging between 3.67 to 86.08 mg/L; PP*i* results ranging between 2.99 to 54.90 mg/L) and KNP samples (ELISA results ranging between 0.1 to 49.41 mg/L; PP*i* results ranging between 0.006 to 10.95 mg/L) using both the ELISA and PP*i* assays.

In the current study, a dose of about 175 µg/kg of purified MC-LR was demonstrated to be lethal in male CD-1 SPF mice. The HBP Dam summer samples and Nhlanganzwani Dam samples were the only cyanobacterial samples that resulted in death (acute toxicity) of mice.

In order to be able to investigate further the *in vivo* effects of cyanotoxins, transmission electron microscopy (TEM) was used to complement results obtained from the *in vivo* assay. Ultrastructural changes of varying degree were observed in livers of mice exposed to both the HBP Dam winter and summer samples. Early stages of hepatocyte to hepatocyte disassociation, slight vesiculation of endoplasmic reticulum (ER) and swollen mitochondria were the most significant ultrastructural changes produced in mouse hepatocyte tissues by the HBP Dam winter samples. The most significant ultrastructural changes produced in mouse hepatocyte tissues by the HBP Dam summer samples were massive hepatic haemorrhage indicated by the appearance of erythrocytes between hepatocytes and the extensive vesiculation of ER.

This is the first time that the African sharptooth catfish primary hepatocyte model has been used to assess the hepatotoxicity of purified MC-LR and cyanotoxin-containing water samples. In this study, the toxicity of cyanobacterial samples and purified MC-LR to cause hepatotoxicity in mice was confirmed *in vitro* using the catfish primary cell line. A comparison among the cyanobacterial samples using EC_{50} showed the following hepatotoxicity trend in the catfish primary cell line: HBP Dam summer samples > Nhlanganzwani Dam samples > HBP Dam winter samples > Mpanama Dam samples >

Sunset Dam samples > Makhohlola Dam samples. The HBP Dam samples were the most hepatotoxic and Makhohlola Dam samples were the least hepatotoxic. The EC_{50} for purified MC-LR using the catfish primary hepatocytes was about 91 nM.

A statistical comparison of the assays used in this study (i.e. ELISA, PP*i*, mouse test and cytotoxicity [catfish primary hepatocyte] assays) was performed based on the Kappa coefficient (K). An almost perfect agreement (K > 0.80) was observed between the mouse test and cytotoxicity assay; mouse test and ELISA; cytotoxicity assay and ELISA; and ELISA and PP*i* assay.

In conclusion, field samples collected during the summer season were found to have very high levels of toxins and a higher degree of toxicity when compared to the winter samples. The cytotoxicity assay using African sharptooth catfish (*Clarias gariepinus*) primary hepatocytes has been shown for the first time to produce results similar to those observed when using the mouse bioassay in assessing cyanobacterial toxicity. Therefore, this primary cell line may be used as a potential alternative to the mouse assay in toxicity testing of cyanotoxins. Three KNP dams (Nhlanganzwani Dam, Mpanama Dam and Sunset Dam) investigated in this study were found to contain *Microcystis aeruginosa*. All four KNP dams (Nhlanganzwani Dam, Mpanama Dam, Makhohlola Dam and Sunset Dam) had cyanotoxin levels above the prescribed guideline value, which is of concern and warrants further investigations to the effects on wildlife in the park.

Future studies will include use of High Performance Liquid Chromatography (HPLC) to investigate the toxin profile of the field samples in order to fully describe the different classes/or types of toxins present in the samples. More validation studies that could give a more comprehensive understanding about the sensitivity of the catfish primary cell line for microcystins will also be undertaken.

CHAPTER 1

INTRODUCTION

In South Africa and other parts of the world, livestock, waterfowl and wildlife have died after drinking water containing heavy blooms of cyanobacteria (Steyn 1945; Soll & Williams 1985; Bell & Codd 1994; 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 1920's, when mass mortalities of thousands of cattle, sheep, horses and rabbits around pans in the south-eastern Transvaal were reported (Steyn 1945; Soll & Williams 1985; Harding & Paxton 2001).

Most incidents of cyanobacterial poisoning in South Africa occur mainly in the Mpumalanga, Gauteng and north-eastern Free State Provinces (Kellerman *et al*. 2005). According to a report published by the Department of Water Affairs and Forestry (DWAF), many South African surface water resources exhibit high nutrient enrichment and eutrophication related problems (DWAF 2002). Increased levels of nitrogen and phosphorus, usually derived from fertilizers, detergents and sewage effluents, are the major causes of excessive growth of cyanobacteria in the aquatic environment (Robarts & Zohary 1984). Bloom formation usually occurs during the warmer months of the year (mid-summer to early autumn), however, outbreaks have also been reported during colder periods (Chorus & Bartram 1999; Kellerman *et al*. 2005).

Most of the available data in the literature indicate that large numbers of livestock and domestic animals die every year in southern Africa after the ingestion of cyanobacteria and their toxins (Steyn 1945; Kellerman *et al*. 2005). Published data on mortality of wildlife in National Parks and private game reserves is scarcer. Soll and Williams (1985) reported the death of three rhinos suspected to have been caused by drinking water containing cyanobacteria at the Barakologadi Game Reserve. In 2005 and early 2007, an unconfirmed number of rhinos died in the Kruger National Park (KNP) after exposure to water containing cyanobacteria (Myburgh 2007, personal communication). It is suspected that a large percentage of wildlife succumb to cyanobacterial poisoning every year but no formal data on mortality is recorded. The one major problem when it comes to the diagnosis of cyanobacterial poisoning in wildlife is that carcasses of these animals are usually found decomposed or partially consumed by scavengers and therefore the cause of death is seldom established. Sometimes the cyanobacterial toxin content of the nearby rivers/pans to which the animals were exposed, would have changed completely thus not reflecting the toxin levels that caused mortality (Soll & Williams 1985).

Even though the outbreaks of cyanobacterial poisoning in South Africa are infrequent, they do pose a potential threat to the health of livestock and wildlife. Given the current status of cyanobacterial blooms in South African water bodies, sensitive and specific monitoring assays for cyanotoxins are required.

The main focus of this study was the application and comparison of different assays in assessing toxicity of field cyanobacterial samples, and also assessing toxicity of field

samples collected during different seasons (summer and winter). Therefore, toxicity of purified microcystin-LR (MC-LR) and cyanobacterial samples collected during summer and winter seasons were compared using the mouse bioassay, catfish primary hepatocytes, Enzyme Linked Immunosorbent Assay (ELISA) and Protein Phosphatase Inhibition (PP*i*) assay.

Specific aims of this study were:

i. To identify the cyanobacteria present in water samples.
ii. To quantify the levels of cyanotoxins present in water samples using available commercial ELISA and PP*i* assay kits.
iii. To investigate the toxicity of cyanotoxins using the mouse bioassay (*in vivo*).
iv. To investigate the hepatotoxicity of cyanotoxins using catfish primary hepatocytes (*in vitro*).
v. To investigate the ultrastructural alterations induced by cyanotoxins in mouse tissues.
vi. To perform a preliminary assessment of cyanobacterial toxicity in the Kruger National Park.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Cyanobacteria, also known as blue-green algae, are Gram-negative photosynthetic prokaryotes which are found in a variety of habitats, colonizing both terrestrial and aquatic biotopes (Briand *et al*. 2003). They only require water, CO_2, inorganic substances and light. Photosynthesis is their principal mode of energy metabolism (Chorus & Bartram 1999).

Cyanobacteria have both beneficial and detrimental properties when judged from a human perspective. They may be used for food or fodder because some strains have a very high content of proteins, vitamins and other essential growth factors, and can also produce pigments of interest. They are also sources for substances of pharmaceutical interest, such as antibiotics (Richmond 1990; Skulberg 1994; Falch *et al*. 1995).

Their extensive growth can create a considerable nuisance for management of inland waters (water supply, recreation, etc) and they may also release substances into the water which may be unpleasant or toxic. The water quality problems caused by dense populations of cyanobacteria are intricate, many and various, and can have a great impact on the economy and health of humans and animals. As a result, the negative aspects of

cyanobacteria have gained research attention and public concern (Chorus & Bartram 1999).

2.2 FACTORS AFFECTING BLOOM FORMATION

The effects of several environmental factors on growth (and toxin production) of cyanobacteria have been studied in batch and continuous cultures (Sivonen 1996). Field studies have also shown that there is a direct correlation between environmental factors and growth of cyanobacteria (Robarts & Zohary 1984; Wicks & Thiel 1990). The environmental factors include temperature, light, phosphorus and nitrogen concentration of water. Each of these factors alone may only partly determine bloom formation and it has been shown that a combination of these factors is responsible for a bloom to develop (Oberholster *et al*. 2004).

2.2.1 Temperature

In general, cyanobacteria prefer warmer conditions for optimal growth (temperatures between 20 ºC and 30 ºC) (Robarts & Zohary 1987). In their study, Robarts and Zohary (1987) found that *Microcystis* was severely limited at temperatures below 15 ºC and were optimal at temperatures around 25 ºC. These optimal temperatures are higher than that for green algae and diatoms and therefore, cyanobacteria can out-compete the other species when subjected to high summer temperature conditions. This also explains why in temperate water bodies most cyanobacteria bloom during summer (Msagati *et al*. 2006).

2.2.2 Light and buoyancy

Like green algae, cyanobacteria contain chlorophyll *a* as a major pigment for harvesting light and conducting photosynthesis. They also contain other pigments such as the phycobiliproteins (e.g. allophycocyanin – blue), phycocyanin (blue) and sometimes phycoerythrine (red). These pigments harvest light in the green, yellow and orange part of the spectrum which is hardly used by other phytoplankton. The phycobiliproteins together with chlorophyll *a* enable cyanobacteria to harvest light energy efficiently and to live in an environment with only green light. Cyanobacteria which form surface blooms seem to have a higher tolerance for high light intensities. High light intensities increase cellular iron intake, since Fe^{3+} seems to be converted to Fe^{2+} by light before it is transported into algal cells which may ultimately be responsible for higher growth rate (Sunda *et al*. 1991; Utkilen & Gjølme 1995). Their maintenance constant is low which means that they require little energy to maintain cell function and structure. As a result of this, cyanobacteria can maintain a relatively higher growth rate than other phytoplankton organisms when light intensities are low (Chorus & Bartram 1999).

Many species of planktonic algae and cyanobacteria have little means of active movement and are therefore, only photosynthetically active when the circulation maintains them in the euphotic zone (i.e. environment where light can penetrate). Cyanobacterial movement within the water column is due to the presence of specialized gas-filled vesicles which gives them a lower density than that of water, making them buoyant (Walsby *et al*. 2006).

2.2.3 Phosphorus and nitrogen

Nutrients such as phosphorus and nitrogen are essential for cyanobacterial growth. Experimental data have shown that cyanobacteria have higher affinity for nitrogen or phosphorus than many other photosynthetic organisms (Metting & Pyne 1986; Kaebernick *et al*. 2001; Villareal & Carpenter 2003). This means that they can out-compete other phytoplanktonic organisms under conditions of phosphorus or nitrogen limitation. In addition to their high affinity, cyanobacteria have a substantial storage capacity for phosphorus enough to perform between two to four cell divisions, which correspond to a 4 to 32 – fold increase in biomass. Low nitrogen to phosphorus ratio has also been observed to favour cyanobacterial blooms (Metting & Pyne 1986; Sivonen 1996; Kaebernick *et al*. 2001; Villareal & Carpenter 2003).

2.3 TOXINS PRODUCED BY CYANOBACTERIA

Cyanobacteria produce a variety of toxins known as cyanotoxins. Cyanotoxins are a diverse group of natural toxins, both from a chemical and toxicological point of view. In spite of their aquatic origin, most of the cyanotoxins that have been identified so far appear to be more hazardous to terrestrial than to aquatic animals (Carmichael 1992; Chorus & Bartram 1999).

Cyanotoxins can be categorized into three broad groups based on their chemical structure, namely, cyclic peptides (microcystins and nodularins); alkaloids (neurotoxins

and cylindrospermopsin); and lipopolysaccharides. Most scientists are more concerned about the cyclic peptide hepatotoxins than the neurotoxic alkaloids or lipopolysaccharides, because the latter are not considered to be widespread, especially in water supplies (Falconer & Humpage 2005).

Cyanobacterial toxins are released when the cells die or disintegrate. This can happen spontaneously in water; after application of copper sulphate to surface water; in the rumen or stomach after ingestion; or as a result of adverse weather conditions. Most often the cyanobacterial cells are ingested with drinking water and the toxins released in the rumen (Kellerman *et al*. 2005).

2.3.1 Hepatotoxins

Globally the most frequently found cyanobacterial toxins in blooms from fresh and brackish waters are the microcystins (MCs) and nodularins belonging to the hepatotoxic cyclic peptides (Carmichael *et al*. 1988; Namikoshi *et al*. 1990).

2.3.1.1 Microcystins (MCs)

Microcystins (MCs) are cyclic heptapeptide toxins (i.e. they contain seven peptide-linked amino acids) produced by members of several cyanobacterial genera including *Microcystis*, *Planktothrix* (*Oscillatoria*), *Anabaena*, *Nostoc*, *Anabaenopsis* and

Hapalosiphon (Carmichael 1992). Nodularins are cyclic pentapeptides produced by *Nodularia* spp and *Microcystis* (Carmichael 1992).

The general structure of MCs is as follows: Cyclo-(D-Ala^{1}-X^{2}-MeAsp^{3}-Z^{4}-Adda^{5}-D-Glu^{6}-Mdha^{7}) where X and Z are variable L-amino acids, D-Ala is D-Alanine, D-Glu is D-Glutamic acid (glutamate), MeAsp^{3} is D-*erythro*-β-methylaspartic acid, Mdha is *N*-methyldehydroalanine, and Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-dienoic acid (Fig. 1). The amino acid Adda is essential for expression of biological activity (Dawson 1998; Falconer & Humpage 2005). The toxins are named according to the two variable L-amino acids at positions X and Z. Currently there are over 60 variants of microcystin, which have been characterized and which differ in their toxicities. The most common MC is microcystin-LR (MC-LR), where the variable L-amino acids are leucine (L) and arginine (R) (Fig. 1) (Dawson 1998; Falconer & Humpage 2005).

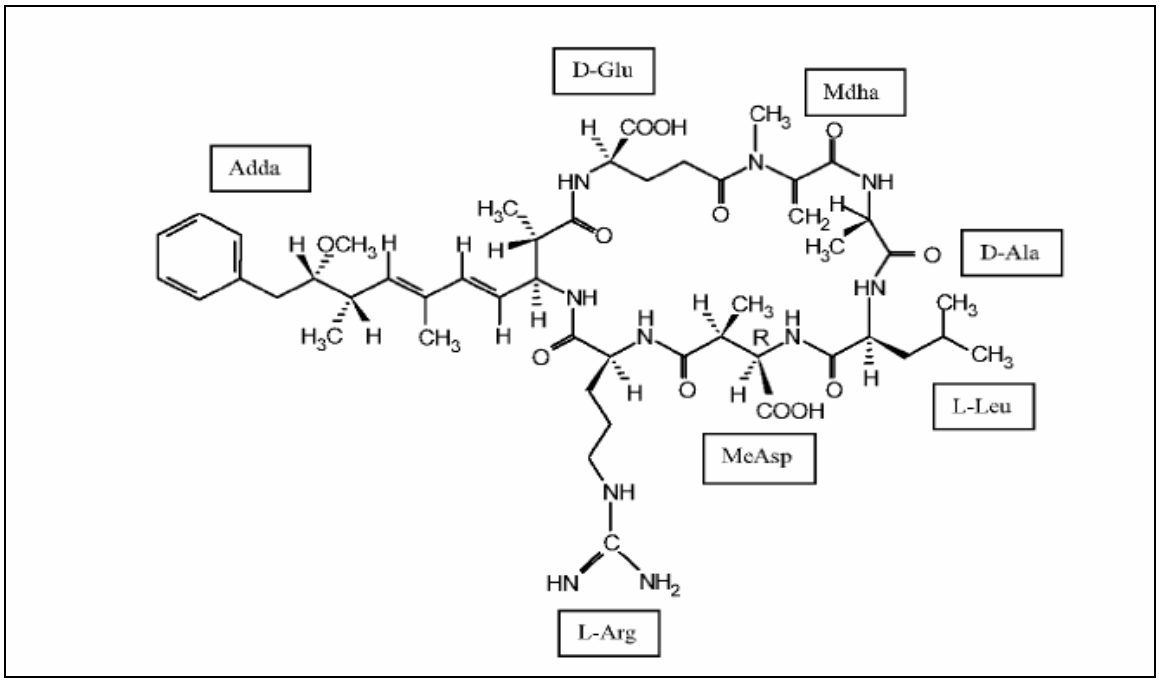


Fig. 1 – Chemical structure of microcystin-LR (MC-LR). D-Ala = D-Alanine; L-Leu = L-Leucine (L-amino acid); MeAsp = D-*erythro*-β-methylaspartic acid; L-Arg = L-Arginine (L-amino acid); Adda = 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-dienoic acid; D-Glu = D-glutamate; Mdha = *N*-methyldehydroalanine (Mankiewicz *et al.* 2003)

2.3.1.2 Nodularins

The cyclic pentapeptide, nodularin, contains amino acids similar to those found in MCs, namely D-*erythro*-β-methylaspartic acid, L-Arginine, Adda, D-glutamic acid and *N*-methyldehydrobutyrine (Fig. 2). Nodularins are structurally similar to MCs and exerts similar toxicities (Carmichael 1992; Falconer & Humpage 2005).

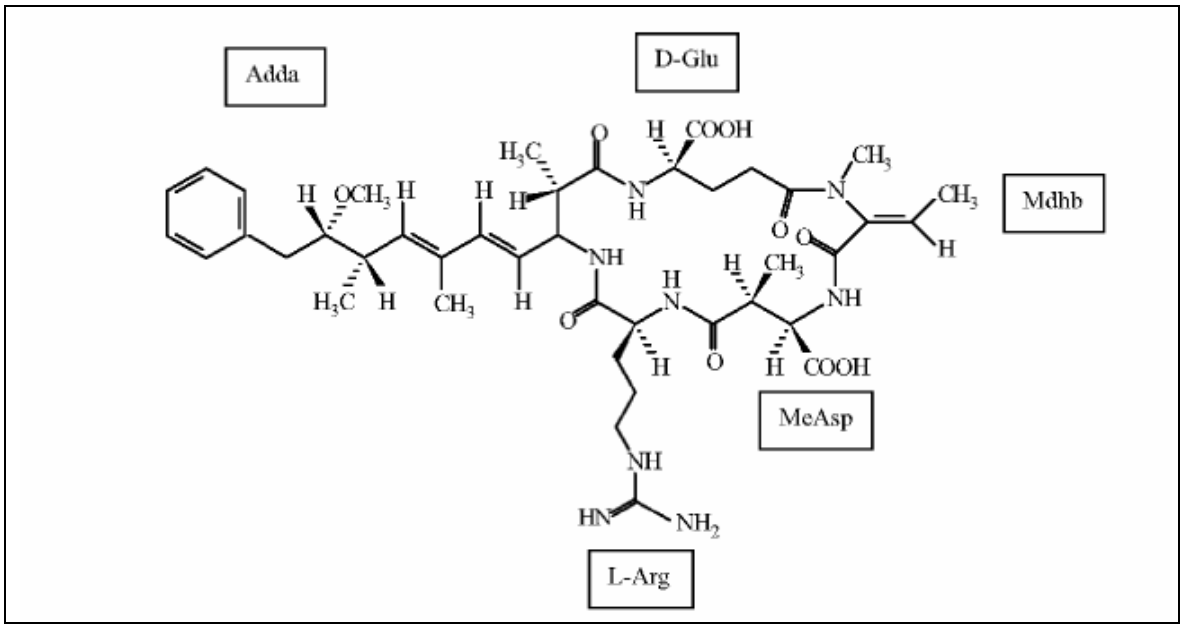


Fig. 2 – Chemical structure of nodularin. MeAsp = D-*erythro*-β-methylaspartic acid; L-Arg = L-Arginine; Adda = 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-dienoic acid; D-Glu = D-glutamate; Mdhb = *N*-methyldehydrobutyrine (Mankiewicz *et al.* 2003)

2.3.1.3 Mechanism of action of hepatotoxins

The hepatotoxic cyclic peptides (MCs and nodularins) are water soluble and most of them are unable to directly penetrate the lipid membranes of animals, plants and bacterial cells. Therefore, to elicit their toxic effects, uptake into cells occurs through carrier-mediated transport systems known as the bile acid transporters. As a result of this, toxicity of MCs and nodularins is restricted to organs expressing the bile acid transporter on their cell membranes, such as the liver (Falconer *et al*. 1981; Eriksson *et al*. 1990; Honkanen *et al*. 1990).

Uptake of the hepatotoxins into liver cells is followed by inhibition of serine/threonine protein phosphatases type 1 and 2A (PP1 and PP2A) enzymes. The consequent protein phosphatase imbalance brings about changes in the actin microfilaments by causing dense aggregation of microfilaments of the cytoskeleton near the centre of the cells. As a result of the loss of cellular support, the parenchymal and sinusoidal endothelial cells are destroyed, leading to massive intrahepatic haemorrhage and hepatic insufficiency. A large increase in liver weight (up to 100 % increase) due to pooling of blood in the liver is observed (Falconer *et al*. 1981; Eriksson *et al*. 1990; Honkanen *et al*. 1990; Carmichael 1992).

2.3.2 Neurotoxins

Three families of cyanobacterial neurotoxins are known, namely, anatoxin-a and homoanatoxin-a; anatoxin-a (s); and saxitoxins (Mahmood & Carmichael 1987; Carmichael 1992). Alkaloids, in general are a broad group of heterocyclic nitrogenous compounds (i.e. they contain ring structures with at least one carbon-nitrogen bond) (Carmichael 1992).

2.3.2.1 Anatoxins and Saxitoxins

Anatoxin-a is produced by *Anabaena flos-aquae*, *Anabaena planktonica*, *Oscillatoria*, *Aphanizomenon* and *Cylindrospermum*. Homoanatoxin-a is an anatoxin-a homologue isolated from *Oscillatoria formosa* strain (Carmichael 1992). The two alkaloids cause

death via a depolarizing blockage of neuromuscular transmission and subsequent respiratory paralysis (Carmichael *et al*. 1975).

Anatoxin-a (s) is produced by *Anabaena flos-aquae* (Matsunaga *et al*. 1989) and induce salivation in mice by which it can be differentiated from other cyanobactarial neurotoxins. It acts as an irreversible anticholinesterase inhibitor (Mahmood & Carmichael 1987; Cook *et al*. 1989).

Saxitoxins, also known as paralytic shellfish poisons (PSPs), were originally isolated from shellfish where they were concentrated from marine dinoflagellates. Saxitoxins have been found in the cyanobacteria *Aphanizomenon flos-aquae*, *Anabaena circinalis*, *Lyngbya wollei* and *Cylindrospermopsis raciborksii* (Mahmood & Carmichael 1986; Humpage *et al*. 1994). These toxins are fast-acting neurotoxins that block sodium channels of excitable membranes, causing death in mammals by respiratory arrest (Carmichael 1992; Sivonen 1996).

2.3.2.2 Cylindrospermopsin

Cylindrospermopsin is an alkaloid hepatotoxin produced mainly by *Cylindrospermopsis raciborskii* (Hawkins *et al*. 1985). In pure form, cylindrospermopsin mainly affects the liver. Studies using cultured rat hepatocytes have shown that cylindrospermopsin inhibits glutathione synthesis (Runnegar *et al*. 1995). Electron microscopic studies on

experimentally poisoned mice showed cylindrospermopsin to be a potent inhibitor of protein synthesis (Terao *et al*. 1994).

2.3.2.3 Lipopolysaccharides (LPS)

Lipopolysaccharides (LPS) also known as irritant toxins are generally found in the outer membrane of the cell wall of Gram-negative bacteria, including cyanobacteria, where they form complexes with proteins and phospholipids. They are pyrogenic and toxic (Weckesser & Drews 1979). It is generally the fatty acid component of the LPS molecule that elicits an irritant allergenic response in humans and mammals. Cyanobacterial LPS are considerably less potent than LPS from pathogenic Gram-negative bacteria, such as *Salmonella* (Chorus & Bartram 1999).

2.4 MECHANISM OF TOXIN PRODUCTION

Cyanobacteria may produce several toxins simultaneously. In general, more than one MC has been characterized from a single cyanobacterial strain. Although many cyanobacterial strains produce several MCs simultaneously, usually only one or two MCs are dominant in any single strain (Sivonen 1996).

Qualitative variation of MCs is most frequently found among strains of *Anabaena* (*Nostoc*) and *Microcystis*. Some *Microcystis* and all *Oscillatoria* and *Nodularia* seem to

produce one major toxin. The same MCs are produced by planktonic *Anabaena*, *Microcystis* and *Oscillatoria* (Sivonen *et al*. 1989; Sivonen *et al*. 1992).

Among neurotoxic strains of *Aphanizomenon* and *Anabaena*, several PSPs are produced simultaneously. Simultaneous neurotoxin and hepatotoxin production has also been observed; the best example is *Anabaena flos-aquae* strain NRC 525-17, which produces anatoxin-a(s) and several MCs (Matsunaga *et al*. 1989; Harada *et al*. 1991). In natural samples which usually contain many strains or more than one toxin-producing species, different combinations of MCs can be found (Chorus & Bartram 1999).

The timing and duration of the bloom season of cyanobacteria depend largely on the climatic conditions of the region; in temperate zones, mass occurrences of cyanobacteria are most prominent during the late summer and early autumn and may last 2-4 months. In regions with more Mediterranean or subtropical climates, the bloom season may start early and persist longer (Kotak *et al*. 1995; Vezie *et al*. 1998). Both toxigenic (toxin producing) and non-toxigenic strains of cyanobacteria often exist together; when grown in the laboratory, particular strains have been found to produce greater amounts of toxins than others (Bolch *et al*. 1997).

2.5 EXPOSURE OF HUMANS TO CYANOTOXINS

2.5.1 Introduction

Water supplies have been associated with gastrointestinal illnesses throughout human history, with cholera, dysentery and typhoid responsible for much human misery and death. Most cases of human injury attributed to cyanobacterial toxins have been studied retrospectively, and complete epidemiological data, especially regarding exposure (number of organisms, type and concentration of cyanotoxins) are rarely available (Jochimsen *et al*. 1998). The epidemiological evidence for human illness due to cyanobacterial toxins therefore needs to be viewed against a background of alternative causes, with bacterial, viral or protozoal infections being the first causes to be investigated (Chorus & Bartram 1999).

Exposure to cyanobacterial toxins is mainly through diet (ingestion) and/or direct contact (swimming, washing) with contaminated waters (Hitzfeld *et al*. 2000; Msagati *et al*. 2006).

2.5.2 Exposure through ingestion of water containing cyanobacteria

The earliest case of gastroenteritis due to cyanobacteria in humans was reported in 1931 in towns along the Ohio River, where low rainfall had caused the development of a large cyanobacterial bloom. The water treatment procedures employed proved to be ineffective

in reducing taste, odour and toxin content of the drinking water. A series of outbreaks of illness were reported during this period, which could not be attributed to infectious agents (Tisdale 1931).

Since then several reports have been published on human poisoning due to cyanobacterial toxins (Lippy & Erb 1976; Byth 1980; Falconer *et al*. 1983; Teixera *et al*. 1993; Yu 1995; Ueno *et al*. 1996; Jochimsen *et al*. 1998; Pouria *et al*. 1998; Azevedo *et al*. 2002).

2.5.3 Exposure through food contaminated with cyanobacteria

Microcystins (MCs) can accumulate in the tissues of fish, particularly in the viscera (such as liver, kidney, etc.) and in shellfish (Falconer *et al*. 1992 (a); Chorus & Bartram 1999). Levels in the tissues depend upon the severity of the bloom in the area where the fish or shellfish are caught or collected. In the case of fish caught from areas where major cyanobacterial blooms occur, consumption of visceral parts of fishes may lead to cyanotoxin exposure (Msagati *et al*. 2006). Moreover, *Spirulina* and other cyanobacterial species used as food supplements, though non-toxic, may lead to exposure of consumers to cyanotoxins if they have been harvested from pools where toxic and non-toxic species of cyanobacteria grow together (Falconer *et al*. 1992 (a)).

These cyanotoxins, especially the hepatotoxins (MCs and nodularins), are known to be resistant to digestion in the gastrointestinal tract of eukaryotes because the peptide bonds linking to the D-amino acids are not susceptible to normal hydrolytic enzymes. They are

highly stable in water and are resistant to boiling and even to irradiation, thereby presenting a high risk to consumers of animal products which have been contaminated (Dawson 1998; Msagati *et al*. 2006).

2.5.4 Exposure through dermal contact with cyanobacteria

Swimming in waters containing toxic blooms of cyanobacteria may expose swimmers to cyanotoxins. Even minor contact with cyanobacteria in recreational water can lead to skin, ear and eye irritation, a condition known as dermatitis or swimmers' itch, and increased likelihood of gastrointestinal symptoms such as vomiting and diarrhoea (Pilotto *et al*. 1997). Individual sensitivity to cyanobacteria in recreational waters varies greatly, because there can be both allergic reactions and direct responses to toxins. Cyanobacterial toxins can cause severe allergic reactions in sensitive individuals (Cohen & Reif 1953).

2.5.5 Human clinical cases in South Africa

According to a report published by the Department of Water Affairs and Forestry (DWAF), many South African surface water resources exhibit nutrient enrichment and eutrophication related problems (DWAF 2002). About 80 dams were monitored between October 2002 and September 2003 in South Africa (Van Ginkel 2003). Eleven percent 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). In spite of the current status of cyanobacterial blooms in water bodies of South Africa, there are no published data on acute/chronic poisoning of humans due to cyanotoxins.

Cases of chronic poisoning in humans could be higher in South Africa, especially in individuals with compromised immune systems (e.g. HIV and AIDS patients), in children and older people. Future studies are needed to describe the relationship between cyanotoxins and the high prevalence of HIV and AIDS in South Africa.

2.6 EXPOSURE OF ANIMALS TO CYANOTOXINS

2.6.1 Introduction

Exposure of animals to cyanobacterial toxins is mainly through ingestion of contaminated waters. Animal deaths from cyanobacterial toxicity have been reported in many parts of the world, including South Africa (Steyn 1945; Bell & Codd 1944; Kellerman *et al*. 2005). Ruminants, especially cattle are more often affected, probably because they do not hesitate to drink water covered with a thick cyanobacterial scum.

Besides the consumption of cyanobacteria from the water, it has been suggested that an additional source of intoxication for terrestrial animals is cyanotoxins that have bioaccumulated in the food chain. For example, freshwater mussels accumulate both

MCs and saxitoxins, and mussels are important food sources for water rats, musk rats and birds (Negri & Jones 1995; Prepas *et al*. 1997).

The first case of livestock mortality was reported in 1878 by Francis from Adelaide, Australia (Hitzfeld *et al*. 2000). Since that time there have been frequent instances of animal poisoning from cyanobacterial blooms around the world (Jackson *et al*. 1984; Odriozola *et al*. 1984; Pybus & Hobson 1986; Mahmood *et al*. 1988; Repavich *et al*. 1990; Gunn *et al*. 1992; Carmichael 1994; Bury *et al*. 1995; Carbis *et al*. 1995; Negri *et al*. 1995; Codd *et al*. 1997; Jochimsen *et al*. 1998; Sahin 2000).

In South Africa, Steyn (1945) described the first cases of cyanobacterial poisoning in 1927 when livestock, rabbits and water birds from the Amersfoort district were affected. Within two years after the completion of the Vaal Dam, livestock deaths were reported from the immediate vicinity and subsequent investigations revealed that *Microcystis* was the cause (Steyn 1945). Since then there have been several reports on deaths of animals due to consumption of water contaminated with cyanobacterial blooms (Soll & Williams 1985; Harding *et al*. 1995; Van Halderen *et al*. 1995; Kellerman *et al*. 2005).

Most cases of animal poisoning worldwide have been caused by the genera *Microcystis*, *Anabaena*, *Aphanizomenon*, *Nodularia*, *Nostoc* and *Oscillatoria*. Although these genera occur in South Africa, poisoning of animals is mainly due to *Microcystis aeruginosa* and *Nodularia spumigena* (Steyn 1945; Soll & Williams 1985; Harding *et al*. 1995; Van Halderen *et al*. 1995; Kellerman *et al*. 2005).

In most cases of animal mortality, post mortem examination revealed evidence of cyanobacterial ingestion as well as characteristic liver pathology. The other main cause of livestock deaths, due to cyanobacterial toxins, is from acute neurotoxicity leading to respiratory failure, with no post mortem indications of organ injury (Gunn *et al*. 1992).

While the reported deaths have usually occurred shortly after the animals have ingested cyanobacterial scums, lasting injury with progressive mortality has also been seen in animals poisoned by *M. aeruginosa*. The characteristic symptoms are those of liver failure with secondary photosensitization, i.e. severe sunburn-like reactions (McBarron & May 1966; Carbis *et al*. 1995).

2.6.2 Clinical symptoms

It appears that acute hepatotoxin poisoning, often causing death in less than one day after exposure is the most common type of poisoning in livestock (Beasley *et al*. 1989; Van Halderen *et al*. 1995; Kellerman *et al*. 2005).

Cattle affected by cyanobacterial hepatotoxins tend to display weakness, reduced general responsiveness, reluctance to move, anorexia, drop in milk production, constipation or diarrhoea, jaundice and sometimes mental derangement (Steyn 1945; Beasley *et al*. 1989; Gunn 1992; Beasley *et al*. 1994). Death may occur from eight hours to a few days after the initial exposure and may be preceded by muscle tremors, periods of forced expiration

and coma. Death from hepatotoxins most often is a result of severe intrahepatic haemorrhage and hypovolemic shock (Beasley *et al*. 1989; Beasley *et al*. 1994).

Poisoning by neurotoxins causes death within minutes to a few hours, depending on species and amount of toxin ingested. Clinical symptoms of neurotoxin intoxication in mice, rats and calves include progression of muscle fasciculation, decreased movements, collapse, exaggerated abdominal breathing, convulsions and sudden death (Steyn 1945; Beasley *et al*. 1989; Kellerman *et al*. 2005). Field cases are usually associated with collapse and sudden death. Death is most probably due to neuromuscular blockade of the muscles of respiration (Beasley *et al*. 1989; Beasley *et al*. 1994).

2.6.3 Studies using rat/mice models to establish LD_{50} of cyanotoxins

Many toxins are more toxic when given by the intraperitoneal (i.p.) route of administration than by the oral route. This difference was evident when LD_{50} values (single dose level that cause death in 50 % of the exposed animals within 7-14 days) were examined for various routes of exposure (Stoner *et al*. 1989; Fawell *et al*. 1999; Yoshida *et al*. 1997). Studies using the i.p. route of administration require less toxins and can be used to indicate relative acute toxicity and may provide information on the mechanism of toxicity (Chorus & Bartram 1999).

The LD_{50} of MCs injected i.p. in mice and rats is in the range of 25-150 µg/kg (Stoner *et al*. 1989; Dawson 1998; Oberholster *et al*. 2004; Msagati *et al*. 2006). The oral LD_{50}

(administered by gavage, i.e. dosing directly into the stomach through the mouth) of MC-LR was found to be 5 000 µg/kg by Fawell *et al*. (1999) in one strain of mice, 10 000 µg/kg by Yoshida *et al*. (1997) in a different strain of mice and much higher in rats by Fawell *et al*. (1999). The LD_{50} values of the different cyanotoxins after i.p. administration to mice are shown in Table 1.

Table 1 – Toxicity of cyanobacterial toxins after i.p. administration to mice

Toxin	LD_{50} (µg/kg)	Organism	Reference
MCs	25-150	*Microcystis* spp.; *Anabaena* spp.; *Oscillatoria* spp.	Stoner *et al*. 1989; Carmichael 1992; Dawson 1998; Msagati *et al*. 2006
Nodularin	50	*N. spumigena*	Carmichael 1992
Anatoxin-a	200-250	*Anabaena* spp.; *Oscillatoria* spp.	Devlin *et al*. 1977; Skulberg *et al*. 1992
Homoanatoxin-a	200-250	*Anabeana* spp.; *Oscillatoria* spp.	Devlin *et al*. 1977; Skulberg *et al*. 1992
Anatoxin-a(s)	20	*A. flos-aquea*	Matsunaga *et al*. 1989
Saxitoxin	10	*A. flos-aquea; A. circinalis; C. raciborskii*	Mahmood & Carmichael 1986
Cylindrospermopsin	2100	*C. raciborskii*	Ohtani *et al*. 1992

2.7 FATE OF CYANOTOXINS IN THE ENVIRONMENT

2.7.1 Introduction

It appears likely that cyanotoxins are produced and contained within the actively growing cyanobacterial cells (i.e. they are intracellular). Release of toxins into the water appears

to occur mostly, if not exclusively, during cell senescence, death and lysis, rather than by continuous excretion (Chorus & Bartram 1999).

In the field, healthy bloom populations produce little extracellular toxins. In lakes and rivers, toxins liberated from cells are rapidly diluted by the large mass of water, especially if mixing of water by wind direction or currents is vigorous. However, the concentration of dissolved toxins may be much higher in ageing or declining blooms (Jones & Orr 1994).

The release of toxins from cells is enhanced by chemical treatment for the eradication of cyanobacteria, especially algicides (either copper-based or organic herbicides). Treatment of a cyanobacterial bloom with copper sulphate, for example, may lead to complete lysis of the bloom population within three days and release of all the toxins into the surrounding water (Berg *et al*. 1987; Kenefick *et al*. 1992; Jones & Orr 1994).

2.7.2 Chemical breakdown

Microcystins (MCs), being cyclic peptides, are extremely stable and resistant to chemical hydrolysis or oxidation at near or neutral pH. Nodularins and MCs remain potent even after boiling. In natural water and in the dark, MCs may persist for months or even years (Jones & Orr 1994). Rapid chemical hydrolysis occurs only under conditions that are unlikely to be attained outside the laboratory, for example, 6.0 M HCl at high temperature (Harada *et al*. 1996).

In full sunlight, MCs undergo slow photochemical breakdown and isomerization, with the reaction rate being enhanced by the presence of water-soluble cell pigments, presumably phycobiliproteins. This breakdown can take as little as two weeks or longer than six weeks, depending on the concentrations of the pigment and toxin (Tsuji *et al*. 1993).

2.7.3 Biodegradation

In spite of their chemical stability and resistance to eukaryotic and many bacterial peptidases, MCs are susceptible to breakdown by aquatic bacteria found naturally in rivers and reservoirs. These bacteria appear to be reasonably common and widespread (Jones *et al*. 1994; Lam *et al*. 1995). Jones and co-workers (1994) isolated a species of aquatic *Sphingomonas* that initiated ring-opening of MC-LR to produce a linear (acyclo-) MC-LR as a transient intermediate. This compound was nearly 200 times less toxic than the parent toxin. The same bacterium, however, did not degrade the closely related cyclic pentapeptide nodularin (Jones *et al*. 1994).

Takenaka and Watanabe (1997) isolated a strain of *Pseudomonas aeruginosa* from a Japanese lake, and showed the degradation of MC by this strain to occur by attack on the Adda side chain of MC. The beta-subgroup of *Proteobacteria* has also been found to be capable of degrading MCs and nodularins (Lahti *et al*. 1997). Little work has been undertaken on the biodegradation of anatoxins, saxitoxins or cylindrospermopsin (Rapala *et al*. 1994).

2.8 SCREENING METHODS FOR CYANOTOXINS

Two methods are generally used in the detection and identification of cyanobacterial toxins, namely, biological or biochemical screening assays and physicochemical methods. These methods differ in terms of principles of detection, information they provide and simplicity/complexity of the set-up. Selection of techniques depends on the availability of facilities and expertise as well as the type of information required. However, specificity and sensitivity of these techniques are important criteria that can be used to select the most reliable method (Chorus & Bartram 1999; Msagati *et al*. 2006).

2.8.1 Biological/biochemical screening methods for cyanotoxins

Biological or biochemical screening methods commonly used for cyanobacterial toxins include the mouse bioassay, *in vitro* (primary and continuous cell lines) assays, Enzyme Linked Immunosorbent Assay (ELISA) and Protein Phosphatase Inhibition (PP*i*) assay.

The major advantage of the mouse assay 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 suspected sample. 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 1.0-2.0 µg/L, the approximate guideline range for MC-LR prescribed by the World Health Organization (WHO 1998). For ethical reasons, the mouse test is

unsuitable for large-scale and routine testing of field samples (Aune & 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 & Berg 1986; Berg & Aune 1987; Heinze 1996). The major advantage of using freshly isolated hepatocytes is their ability to maintain the activities of phase I and II 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 large number of samples can be tested from a single preparation of hepatocytes (from one animal) compared to one mouse required per sample when using the mouse assay. The disadvantage associated with the use of primary hepatocytes is their limited effective period, after which a decline in the activity of phase I and II enzymes is observed (Fautrel *et al*. 1991; Guillouzo 1998).

Previous studies have shown that the liver is the target organ for cyanotoxins (Falconer *et al*. 1981; Eriksson *et al*. 1990; Honkanen *et al*. 1990), and freshly isolated primary hepatocytes seem to be an ideal tool for *in vitro* toxicity testing of extracts from cyanobacteria (Aune & Berg 1986). The bile acid transporter has been proposed as a possible explanation for the specificity of MCs on hepatocytes (Eriksson *et al*. 1990). This transporter plays a crucial role in transporting cyanotoxins across cell membranes

into cells whereby they induce cytotoxic response. Only cells expressing the bile acid transporter on their membranes are affected by the cyanotoxins (Eriksson *et al*. 1990).

Both the ELISA and PP*i* assays are sensitive methods used for detecting MCs. The major advantage of using the ELISA and PP*i* assays is that they both have a detection limit lower than the proposed guideline of 1.0 µg/L set by the World Health Organization (WHO) 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 MC toxins (Mountfort *et al*. 2005). The disadvantage of the PP*i* 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).

2.8.2 Physicochemical screening methods for cyanotoxins

These are analytical methods which use physicochemical properties of cyanobacterial hepatotoxins such as the presence of UV chromophores within their structures, molecular weights, and their reactivities due to specific functional groups present in their structures. Among the methods used are High Performance Liquid Chromatography (HPLC), Liquid Chromatography/Mass Spectrophotometry (LC/MS), Capillary Electrophoresis (CE) and Nuclear Magnetic Resonance (NMR) (Meriluoto 1997). High Performance Liquid Chromatography and LC/MS are the most commonly used analytical systems for the detection of MCs (Gathercole & Thiel 1987; Lawton *et al*. 1994; Bateman *et al*. 1995; Meriluoto *et al*. 1998).

Typical HPLC analysis uses a reverse-phase C18 silica column with separation achieved over a gradient of water and acetonitrile, both containing 0.05 % triflouroacetic acid (TFA). A photodiode array (PDA) UV detector is used to collect spectral data between 200-300 nm (Lawton *et al*. 1994; Moollan *et al*. 1996; Harada *et al*. 1997; Ikawa *et al*. 1999; Spoof *et al*. 2001).

When further confirmation and identification of MCs is required, the LC/MS is very useful as it enables the simultaneous separation and identification of MCs in a mixture (Kondo *et al*. 1992; Poon *et al*. 1993; Mountfort *et al*. 2005).

These analytical methods have the disadvantage of being expensive and, of the large range of congeners known; only a small range of standards is available. The methods also require highly skilled personnel and pre-concentration of water samples if the 1.0 µg/L detection limits are to be achieved (Mountfort *et al*. 2005).

2.9 Guideline values for drinking water quality

A provisional guideline value of 1.0 µg/L has been adopted for human use by the World Health Organization (WHO) for MC-LR (WHO 1998), whereas 2 000 *Microcystis* cells/ml have been recommended as the limit of cyanobacteria in drinking water for animals (DWAF 1996). As is standard practice, an average adult body weight of 60 kg and an average water intake for adults of 2.0 L per day was used. In water containing cyanobacterial cells, this guideline value should be applied to the total cell-bound and

extracellular MCs. Exceeding the provisional guideline value of 1.0 µg/L for MC-LR can be tolerated. This may occur if, for example, discontinuation of exposure is expected in the near future due to implementation of measures to eliminate cyanotoxins from drinking water or cyanobacteria from the water resource. In such instances, it may be appropriate that information is communicated to the public, and especially to particularly susceptible sub-populations such as patients with HIV-AIDS, liver diseases, parents of infants, or dialysis patients (Falconer *et al*. 1994; Chorus & Bartram 1999).

CHAPTER 3

SAMPLING, IDENTIFICATION AND ENUMERATION OF CYANOBACTERIA

3.1 INTRODUCTION

Waters suspected of containing toxic cyanobacteria are normally collected at points where the accumulation of cyanobacteria is likely to affect both humans and livestock, or at drinking water reservoirs. The process of selecting the location and number of sampling sites is also an important factor that may influence the decision of where to collect samples (Msagati *et al*. 2006).

Caution and attention must be taken when working with cyanobacteria, particularly when they are highly concentrated in scums. It is advisable to always treat all blooms as highly toxic. Contact with water should be minimized during sampling and gloves and rubber boots should be worn because cyanobacteria might contain toxins and could also have a high allergic potential (Cohen & Reif 1953; Pilotto *et al*. 1997).

Sample collection and storage procedures differ depending on the type of analysis to be carried out. The three principal categories of analysis usually performed are: nutrient analyses (phosphorus and nitrogen); cyanobacterial identification and quantification; cyanotoxin analysis, e.g. toxicity testing and analysis of cell-bound and dissolved toxins (Bartram & Ballance 1996).

Two different types of samples can be taken from surface waters. A grab sample is the simplest type, which is defined as a discrete volume of water taken at a selected location, depth and time. In contrast, composite or integrated samples are made up of several sub-samples from different parts of the water body (Bartram & Ballance 1996).

Some of the observations that should be made while sampling for cyanobacteria in the field include:

- Presence of scums
- Weather conditions on the day of sampling and if available, an indication of conditions over the previous 24 hours
- Determining water temperature and dissolved oxygen
- Determining whether the bottom of the lake/river is clearly visible at approximately 30 cm depth along the shore line
- Noting if any cyanobacteria can be seen as blue-green streaks on the surface or as accumulations in bays and along shorelines
- Noting whether blue-green scums affect large parts of the water surface (Bartram & Ballance 1996).

Microscopic examination of a bloom sample is very useful even when accurate enumeration is not being carried out. The information obtained regarding the cyanobacteria detected can provide an instant alert that harmful cyanotoxins may be present (Ballantine 1953).

Most cyanobacteria can be readily distinguished from other phytoplankton and particles under the microscope by their morphological features at magnification of 200-1000 times. When identifying cyanobacteria, it is preferable to give only the genus name, especially if differentiation between species by microscopy is due to a lack of: current general taxonomic knowledge; locally available expertise; or characteristic features of the specimens to be identified (Komárek & Anagnostidis 1986).

Microscopic enumeration of cyanobacterial cells, filaments or colonies has the advantage of directly assessing the presence of potentially toxic organisms. Little equipment in addition to a microscope is required. The method may be rather time consuming, ranging from a few minutes to several hours per sample, depending upon the accuracy required and the number of species to be differentiated (Box 1981).

3.2 MATERIALS AND METHODS

3.2.1 Sampling of cyanobacteria

Cyanobacterial samples were collected from the Hartbeespoort (HBP) Dam, South Africa, following a standard operating procedure prepared by the Department of Water Affairs and Forestry (DWAF 2004). The HBP Dam is a 20 km^2 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 its

close proximity to the Pretoria and Johannesburg Metropolitan areas. About 80 % of the water from this dam is used for irrigation (NWPG 2005).

Scums were sampled near the dam wall using 1.0 L bottles. Wide-necked bottles (Merck, Guateng, South Africa) were used to collect samples from the upper layer of the scum on the water surface. Collected samples were labeled appropriately and stored in a cooler box during transportation to the laboratory. In the laboratory, samples were aliquoted and stored at -20 ºC prior to analysis. Cyanobacterial samples were collected from July 2005 to July 2006 (i.e. July 2005, October 2005, March 2006 and July 2006). Samples collected in July 2005 and July 2006 were designated as winter samples, whereas samples collected in October 2005 and March 2006 were designated as summer samples.

Cyanobacterial samples were also collected from the Kruger National Park (KNP) and sent to our Toxicology laboratory by the KNP personnel. Cyanobacterial samples were collected from four man-made dams in the KNP, namely, Nhlanganzwani Dam (25º13’52”S; 31º58’27”E) with samples showing severe visible green discolouration; Mpanama Dam (25º19’03”S; 31º58’20”E) with samples showing moderate visible green discolouration; Makhohlola Dam (25º25’18”S; 31º18’31”E) with samples showing no visible green discolouration; and Sunset Dam (25º07’00”S; 31º54’43”E) with samples showing moderate visible discolouration. Two samples were collected from each KNP dam.

3.2.2 Identification and enumeration of cyanobacteria

Samples used for the identification and enumeration of cyanobacteria were preserved with 2.0 % formaldehyde. Identification and enumeration was carried out by the Centre for Environmental Management (University of the Free State) based on distinct morphological features of the cyanobacteria using light microscopy at magnification of 200-1000 times. Scanning electron microscopy was carried out by the Electron Microscope Unit personnel at the Faculty of Veterinary Science, University of Pretoria. A Philips CM10 transmission electron microscope operated at 80 kV was used to view the processed samples.

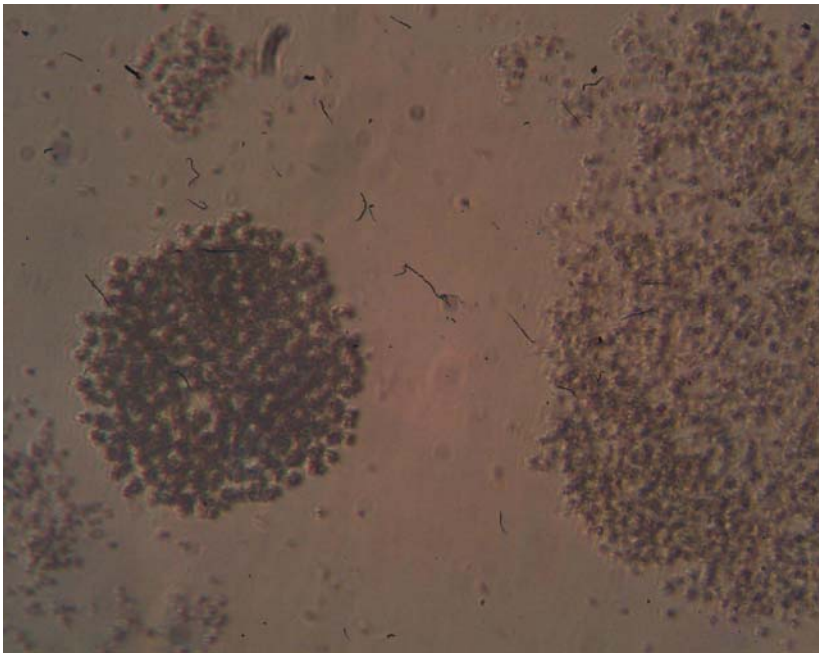
3.3 RESULTS

During the summer season (October 2005 and March 2006), the water surface near the HBP Dam wall was completely covered with cyanobacterial scums and blue-green streaks were observed on the remainder of the water surface. A foul 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; only a few of the blue-green streaks were observed during this season.

Microscopic observation revealed the presence of *M. aeruginosa* and *Planktothrix* sp. as the most dominant cyanobacteria in water samples collected during winter and summer

seasons in the HBP Dam (Fig. 3). The number of *M. aeruginosa* cells per milliliter in the summer samples was very high (±1.44 X 10^8 cells/ml) when compared to the winter samples (±2.67 X 10^6 cells/ml) as shown in Table 2. Large colonies (±400-800 µm in diameter) of *M. aeruginosa* with each colony consisting of ±1 000 to 5 000 cells were observed in winter and summer samples. The KNP samples were also dominated by *M. aeruginosa* with the exception of samples collected from Makhohlola Dam (Table 2). Even though cyanobacterial enumeration was not carried out on the KNP samples, it could be safely deduced that the samples contained large numbers of cells due to turbidity of the samples.

A)



B)

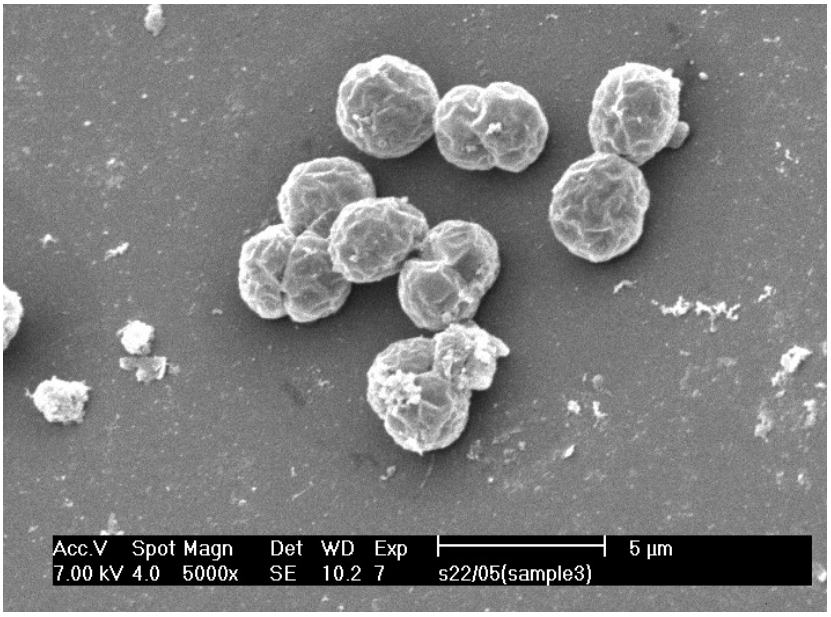


Fig. 3 – Morphology of *Microcystis aeruginosa* as shown by light microscopy (400 X magnification) (A), and scanning electron microscopy (B)

Table 2 – Identification of cyanobacteria in water samples collected from HBP Dam (winter and summer samples) and KNP

Samples	Cyanobacterial species	Number of cells per millilitre (cells/ml)
HBP Dam winter samples (n = 6)	*Microcystis aeruginosa*	$2.67 \times 10^6 \pm 1.77 \times 10^6$
	Planktothrix sp.	$2.92 \times 10^5 \pm 1.91 \times 10^5$
	Nitzschia sp.	$4.33 \times 10^3 \pm 1.15 \times 10^3$
HBP Dam summer samples (n = 6)	*Microcystis aeruginosa*	$1.44 \times 10^8 \pm 3.48 \times 10^7$
	Planktothrix sp.	$2.45 \times 10^5 \pm 7.58 \times 10^4$
	Melosira sp.	$4.17 \times 10^4 \pm 5.77 \times 10^3$
KNP samples: Nhlanganzwani Dam (n = 2)	*Microcystis aeruginosa*	*ND
Mpanama Dam (n = 2)	*Microcystis aeruginosa*	*ND
Makhohlola Dam (n = 2)	None	*ND
Sunset Dam (n = 2)	*Microcystis aeruginosa*	*ND

*ND = Cyanobacterial enumeration was not done

3.4 DISCUSSION

Microcystis aeruginosa, known producer of MCs and nodularins (Carmichael, 1992; Dawson, 1998), and *Planktothrix* sp., known producer of MCs 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 HBP Dam, South Africa (Table 2). *Microcystis aeruginosa* was also found to be the most dominant cyanobacteria in samples collected from KNP (Table 2). Previous data reported *M. aeruginosa* as the most dominant cyanobacteria in the HBP Dam throughout the year

(Robarts & Zohary 1984; Wicks & Thiel 1990). The exceptionally high cell numbers per milliliter observed, especially with the summer samples, indicate a severe cyanobacterial bloom in the HBP Dam. Robarts and Zohary (1984) found the exceedingly high levels of nitrogen and phosphorus to be the most important contributing factors in eutrophication of the dam. When cyanobacteria die, they release their intracellular toxins into the surrounding water. Toxins released by these cyanobacteria have been implicated in acute and chronic cases of animal and human poisoning (Steyn 1945; Lippy & Erb 1976; Byth 1980; Falconer *et al*. 1983; Soll & Williams 1985; Harding & Paxton 2001). The MCs in drinking water have also been cited as one of the factors contributing to the elevated levels of primary liver cancer in some areas in China (Lehman 2007). The HBP Dam residents and animals drinking water from the dam for longer period are at high risk of developing chronic liver damage.

The foul smell which was noted during the summer season from the HBP Dam was probably due to the taste and odour compounds, geosmin and 2-methyl isoborneol (2-MIB), which are normally produced by cyanobacterial genera such as *Microcystis*, *Planktothrix* and *Anabeana* (Perrson 1983; Kenefick *et al*. 1992).

3.5 CONCLUSION

The Centre for Environmental Management, University of the Free State (2006) described the current eutrophication status in the HBP Dam following the identification and enumeration of the samples, by stating that ‘The very high cell numbers per millilitre

is the highest we have ever seen and probably presents the upper limit of cyanobacterial growth in a natural system'.

CHAPTER 4

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

4.1 PREPARATION OF EXTRACTS FROM CYANOBACTERIAL SAMPLES

4.1.1 INTRODUCTION

Since water samples frequently contain low concentrations of either live cells or released toxins, concentration of toxins is advisable prior to screening or testing, in order to bring the concentration up to the sensitivity range of the detection procedures (Lawton *et al*. 1994). The technique used for this include: (A) freeze-drying or air-drying of water and cyanobacterial samples, followed by resuspension in physiological saline or phosphate-buffered saline (pH 7.5, 0.05 M) at 200 mg in 10 mL; (B) concentration by boiling; (C) freeze-thawing; or (D) in the case of filtered water, passage of the water through acetonitrile or methanol-activated C18 reverse-phase cartridges followed by methanol elution of toxins, drying and re-solution in physiological saline (Lawton *et al*. 1994; Harada *et al*. 1997; Meriluoto 1997; Fastner *et al*. 1998; Vasa *et al*. 2004).

When the test material is a freshly sampled bloom with live cyanobacterial colonies, with little evidence of dead cells, a concentrate is most easily achieved by centrifuging the cyanobacterial cells into a pellet, and carrying out toxicity testing on the pellet. The pellet is resuspended in physiological saline (1.0 g in 10 mL) and ultrasonicated to disrupt the

cells. *Microcystis* cells may, however, be very resistant to sonication, and may require repeated freeze-thawing for disruption (Falconer 1993).

Samples prepared by freeze-drying or by sonication should be passed through a bacterial filter or held in a boiling water bath for 10 minutes for sterilization in order to prevent any possibility of bacterial infection (Falconer 1993). *Microcystis* and *Nodularia* toxins are heat resistant, as well as anatoxin-a from *Anabaena*; however, anatoxin-a(s) is highly labile and will be destroyed by boiling (Mahmood & Carmichael 1986).

4.1.2 MATERIALS AND METHODS

Cyanobacterial samples used in this study were extracted following the method described by Falconer (1993). Using the VirTris Benchtop SLC freeze-dryer (SP Industries), condenser temperature at -40 ºC, the cyanobacterial samples were freeze-dried. Freeze-dried samples were then weighed and resuspended in physiological saline at 200 mg in 10 mL. Resuspended samples were held in a boiling water bath for 10 minutes for sterilization in order to prevent any possibility of bacterial infection in the mice. Extracted samples were stored at -20 ºC until analyzed.

4.2 ELISA

4.2.1 INTRODUCTION

Many researchers have developed immunoassays for MCs using monoclonal (Kfir *et al*. 1986; Nagata *et al*. 1995; Ueno *et al*. 1996) and polyclonal (Chu *et al*. 1989; An & Carmichael 1994; McDermott *et al*. 1995) antibodies. However, all of these antibodies were raised against specific MC congeners, and the assays therefore tend to be sensitive to a relatively narrow range of MC analogues. Such assays are not ideal for screening because of the possibility of false negatives in the presence of toxic congeners to which the assay is insensitive (Fischer *et al*. 2001).

In their study, Fischer and co-workers (2001) developed a competitive indirect ELISA for the detection of MCs and nodularins using polyclonal antibodies raised against ADDA-haptens. The unusual β-amino acid ADDA is present in most (>80 %) of the known toxic penta- and heptapetide toxin congeners. This ELISA technique allows detection and quantification of numerous MC congeners, including nodularin, in drinking water at levels well below those proposed by the WHO, without any sample preparation or preconcentration steps. The integrative nature of ELISA sums the total MC content of the water, presenting the analyst with a value representing the total concentration of MC and nodularin hepatotoxins (Fischer *et al*. 2001).

Commercially, polyclonal ELISA kits are available for MCs. The antibodies are fixed to the walls of the wells of a microtitre plate. The first step involves binding of the calibrators (non-toxic MC-LR surrogates), a negative control and the samples to the antibodies in the wells. This is followed by the addition of a MC-enzyme conjugate which binds the remaining antibodies. After thorough rinsing, the concentration of the bound enzyme is measured colorimetrically in an ELISA plate reader.

4.2.2 MATERIALS AND METHODS

A commercial ABRAXIS – MC ELISA kit (Aqualytic Environmental and Laboratory Services, Gauteng, South Africa) was used. The ELISA is an indirect-competitive ELISA used for the quantitative analysis of MCs and nodularins. All extracted field samples (as prepared in Chapter 4.1.2) were diluted as they were showing concentrations higher than 5.0 µg/L. The HBP Dam winter samples were diluted 1/10 000 in physiological saline and HBP Dam summer samples were diluted 1/50 000. The KNP samples were diluted as follows: Nhlanganzwani Dam samples 1/50 000; Mpanama Dam samples 1/5 000; Sunset Dam samples 1/200; and Makhohlola Dam samples 1/20. After addition of the standard solutions and cyanobacterial samples (50 µL) into the wells of the microtitre plate, an antibody solution (50 µL) [ABRAXIS] was added to the wells. The microtitre plate was incubated for 90 min at room temperature. At the end of the incubation period, wells of the plate were washed three times using the washing buffer solution (ABRAXIS). An enzyme conjugate solution (100 µL) [ABRAXIS] was added to each well; the microtitre plate was incubated for 30 min at room temperature. After another washing step, a

substrate solution (100 µL) [ABRAXIS] was added to individual wells followed by incubation for 20-30 min at room temperature. At the end of the incubation period, a stop solution (50 µL) [ABRAXIS] was added and absorbance was measured at 450 nm using a microplate ELISA spectrophotometer (Bio-Tek µQuant, A. D. P., South Africa). Each test was done in triplicate. A standard curve was constructed using MC-LR at concentrations of 0; 0.15; 0.4; 0.75; 1.0; 2.0; 5.0 µg/L (GraphPad Prism version 4 software) and concentrations of the samples were determined from this standard curve (log-log transformation of data).

4.2.3 Statistical analysis

The results are reported as mean ± SD from each winter and summer season. 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.

4.2.4 RESULTS

The cyanotoxin concentrations as detected by ELISA in HBP Dam winter and summer samples are shown in Table 3. According to the MC ELISA, which cross-reacts with MC-LR, MC-RR, MC-LW, MC-YR, MC-LF and nodularin, the HBP Dam winter samples contained toxins belonging to this group with concentrations of about 3.67 mg/L. The HBP Dam summer samples contained toxins belonging to this group, with much higher toxin concentrations of about 86.08 mg/L. The concentration of toxins detected in

the KNP samples was in the range of 0.1 to 49.41 mg/L, with Nhlanganzwani Dam samples (49.41 mg/L) showing the highest concentration and Makhohlola Dam samples (0.10 mg/L) showing the lowest toxin concentration (Table 3).

Table 3 – Toxin concentrations determined by the ELISA on HBP Dam (winter and summer samples) and KNP samples

Cyanobacterial samples	Mean toxin concentration (mg/L)
HBP Dam winter samples (n = 6)	3.67 ± 2.83
HBP Dam summer samples (n = 6)	86.08 ± 18.07
KNP samples:	
Nhlanganzwani Dam (n = 2)	49.41 ± 0.98
Mpanama Dam (n = 2)	1.56 ± 0.07
Makhohlola Dam (n = 2)	0.10 ± 0.004
Sunset Dam (n = 2)	0.25 ± 0.003

4.2.5 DISCUSSION

The World Health Organization (WHO) has proposed a guideline value for human use of 1.0 µg/L (0.001 mg/L) for MC-LR, the most common MC variant, in drinking water (WHO 1998), whereas 2 000 *Microcystis* cells/mL have been recommended as the limit of cyanobacteria in drinking water for animals (DWAF 1996). This is the reason why sensitive, rapid and simple tests suitable for routine monitoring purposes are needed. ELISAs using either polyclonal or monoclonal antibodies for MCs are highly specific,

sensitive and quick methods to detect MCs and nodularins (Rapala *et al*. 2002). The ELISA assay used in this study is stoichiometrically based, reacting with toxins which have the ADDA moiety. It therefore detects most of the known toxic penta- and heptapeptide toxin congeners (Fischer *et al*. 2001).

The toxin concentrations of HBP Dam winter and summer samples as detected by the ELISA assay in this study was at least 1 000 times more than the prescribed guideline value, with summer samples showing much higher cyanotoxins concentrations than the winter samples (Table 3). Toxin concentrations of the KNP samples were also found to be higher than the prescribed guideline value (Table 3). It is interesting to note that in the Makhohlola Dam samples no cyanobacteria were identified (Chapter 3.3), but when determining the toxin levels using the ELISA, the samples were found to contain cyanotoxins at levels above the prescribed guideline value. This could be attributed to the ability of MCs to remain stable and continue to persist in water for longer periods (up to months) after being released from dying cyanobacterial cells (Jones & Orr 1994). This means that long after a cyanobacterial bloom has disappeared MCs tend to remain in the water, hence correct measures should be taken to ensure that the water is safe for human and animal consumption and for recreational purposes. The very high levels of MCs detected in the HBP Dam during this study, correlate with previous results reported by other investigators (Wicks & Thiel 1990; Van Ginkel *et al*. 2001; DWAF 2002; Van Ginkel 2003).

4.2.6 CONCLUSION

The absence of visible cyanobacterial cells does not mean the absence of cyanotoxins (MCs), as these toxins tend to persist for longer periods in water bodies long after a cyanobacterial bloom has disappeared. Toxicity of a cyanobacterial bloom depends on whether the bloom contains toxic or non-toxic strains of cyanobacteria. Therefore, it is very important to treat all cyanobacterial blooms as toxic.

The consistent high levels of cyanotoxins through the years, is of concern as it evident that no strategies are in place to deal with the problem of cyanobacteria in the HBP Dam.

CHAPTER 5

MOUSE BIOASSAY

5.1 INTRODUCTION

In the mouse bioassay, the majority of routine testing of cyanobacterial toxicity is done using Swiss Albino mice of 25-30 g weight, usually males, since there is a sex difference to MC toxicity (males are more sensitive than females) (Falconer *et al*. 1988).

This assay (mouse bioassay) is used primarily to determine the toxicity of bloom material (i.e. it is generally used in a qualitative manner to determine a cyanobacterial bloom as 'toxic' or 'non-toxic'), from the toxic response, the identity of the class of toxin can be determined (Msagati *et al*. 2006).

Toxicity is tested by i.p. injection of 0.1-1.0 mL of cyanobacterial material into mice followed by 24 h observation. The observation period is extended to between 2-7 days where cylindrospermopsin is suspected (Chorus & Bartram 1999). At the end of the observation period all animals are sacrificed and a complete post mortem examination is done. The observed symptoms and the results of the post mortem are used to determine which cyanotoxin is present. However, where more than one type of cyanotoxin is present, the more rapid-acting toxins may mask other symptoms (Falconer 1993).

After i.p. injection, the clinical symptoms of MC and nodularin toxicity in mice are identical. The animals become progressively more pale due to blood loss from the circulation, and die between 15 min and 4 h after injection from circulatory failure. Autopsy usually shows extensive haemorrhage and swelling of the liver with minor signs of damage to other tissues (Falconer *et al*. 1981). Animals subjected to a non-lethal dose show a dose-dependent darkening or congestion of the liver, which on histological examination demonstrates sinusoidal breakdown and infiltration of erythrocytes into areas of disorganized hepatocytes (Falconer *et al*. 1981).

Cylindrospermopsin attacks a wide range of tissues once injection into mice, causing progressive organ necrosis over a number of days. Death may be due to a combination of renal and hepatic failure over 2-7 days (Hawkins *et al*. 1997).

A lethal i.p. injection of the neurotoxin, anatoxin-a, causes neuromuscular symptoms within 5 min, and death usually follows within 15 min. Prior to death the mouse shows gasping breathing and leaping movements, and death is sudden (Carmichael 1992).

Post-mortem examination of mice after i.p. injection with a neurotoxic sample shows no visible tissue injury. Thus, it is easy to differentiate highly neurotoxic from highly hepatotoxic cyanobacterial samples by mouse assay. Where both types of toxicity are present in the same sample, the time of death is less if lethal neurotoxin is present, even when the liver damage is seen post-mortem. Low levels of neurotoxicity cannot, however, be detected *in vivo* in the presence of a lethal hepatotoxin (Falconer 1993).

Mouse assays have the advantage of being non-specific, and therefore toxicity, known or unknown in the bloom material, can be detected. Although this bioassay provides a measure of the total toxicity within a few hours, it does not have the sensitivity or precision required to be applicable to water samples. It is not practical to use it for water samples with concentrations around 1.0-2.0 µg/L, the approximate range of the guideline for MCs set by WHO (Nagata *et al*. 1997). Another limitation of this assay is the ethical one, that is, the number of mice required would be impractical and unacceptable, and moreover it is not permitted in some countries unless a license is issued (Falconer 1993).

5.2 MATERIALS AND METHODS

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

Ethical approval was obtained from the OVI Animal Ethics Committee (reference number 15/10 P001). All experiments were carried out under strict conditions in the presence of a qualified veterinarian.

5.2.1 Intraperitoneal (i.p.) injection of mice with purified MC-LR

Toxicity of purified MC-LR (Sigma-Aldrich, Gauteng, South Africa) was tested using the mouse bioassay according to the method described by Aune and Berg (1986). One milliliter of MC-LR concentrations (0.001; 0.005; 0.01; 0.025; 0.05; 0.1; 3.13; 6.25; 12.5; 25 and 50 mg/L) were injected i.p. into pre-weighed mice using a 1.0 mL tuberculin syringe and a 21 G needle. Control mice were injected i.p. with 1.0 mL saline solution. Three animals were used for each concentration of MC-LR and the control.

5.2.2 Intraperitoneal (i.p.) injection of mice with cyanobacterial samples

Pre-weighed mice were also injected i.p. with 1.0 mL of cyanobacterial sample solutions (HBP Dam winter and summer samples; Nhlanganzwani Dam samples) containing equivalent final concentrations per mouse of 20 mg suspended cyanobacterial matter (500-667 mg/kg). At least six mice were used for each winter and summer samples and two mice were used for the Nhlanganzwani Dam samples. Four control mice were injected i.p. with 1.0 mL saline solution. All mice were observed for 24 h after i.p. injection. During the 24 h observation period, signs of poisoning and survival times were recorded. At the end of the observation period all surviving animals were sacrificed and a post-mortem examination of tissue injury was performed on all the experimental animals. Animals were sacrificed by injecting with sodium pentabarbitone (Euthapent or Euthanaze 200 mg) into the peritoneal cavity at the correct dosage according to the size of the mouse. Livers were removed from the mice and weighed. The liver weight/body

mass percentage was determined. Death with the characteristic enlarged liver (liver weight/body mass % > 7) was used as the measure of hepatotoxicity (Heresztyn & Nicholson 2001).

5.2.3 Disposal of mouse carcasses

After completion of the experiment, mouse carcasses were discarded into biohazard plastic bags and incinerated on the premises of OVI.

5.3 RESULTS

5.3.1 Intraperitoneal (i.p.) injection of mice with purified MC-LR

Table 4 shows the results of toxicity observed with the different concentrations of purified MC-LR after i.p. injection in mice. The MC-LR concentrations ranging from 0.001 to 3.13 mg/L (0.03 to 91 µg MC-LR/kg body weight) did not induce any observed hepatotoxic effects in mice and no death 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 death within 2 h were observed in mice i.p. injected with MC-LR concentrations ranging from 6.25 to 50 mg/L (182 to 1458 µg MC-LR/kg body weight). When they were examined macroscopically, the livers were found to be very dark in colour (congested) due to haemorrhaging and also larger when compared to the control livers. Therefore, the

minimum concentration of purified MC-LR that induced hepatotoxicity and death in male CD-1 SPF mice was estimated to be about 6.0 mg/L (175 µg MC-LR/kg body weight).

Table 4 – Mouse bioassay results after i.p injection of mice with MC-LR

MC-LR (mg/L)	Dose (µg MC-LR/kg body weight)	Liver weight/body mass %	Symptoms of hepatotoxicity	Survival time (h)
0.001 (n = 3)	0.03	5.55 ± 0.35	No	24
0.005 (n = 3)	0.15	5.10 ± 0.28	No	24
0.01 (n = 3)	0.29	5.85 ± 0.64	No	24
0.025 (n = 3)	7.30	5.40 ± 0.42	No	24
0.05 (n = 3)	1.46	5.95 ± 1.20	No	24
0.1 (n = 3)	2.90	6.25 ± 0.07	No	24
3.13 (n = 3)	91.20	6.70 ± 0.85	No	24
6.25 (n = 3)	182.30	8.45 ± 0.21	Yes	2
12.5 (n = 3)	364.60	9.40 ± 0.42	Yes	< 1
25 (n = 3)	729.20	9.80 ± 0.28	Yes	< 1
50 (n = 3)	1458.40	9.85 ± 0.78	Yes	< 1
Control (n = 3)	0	6.25 ± 0.64	No	24

5.3.2 Intraperitoneal (i.p.) injection of mice with cyanobacterial samples

Toxicity of HBP Dam winter and summer samples after i.p. injection in mice is shown in Table 5. Hepatotoxic effects and death of mice were observed within 1 h after i.p. injection with the summer samples. On macroscopic examination the livers were found to be very dark in colour (congested) and larger due to haemorrhaging. Mice i.p. injected with the winter samples did not show any observed hepatotoxic effects and the livers appeared normal when they were examined. Nhlanganzwani Dam samples were the only KNP samples tested for toxicity in mice because of their high toxin levels (49.41 mg/L or

1458 µg MC-LR/kg body weight). Because the toxin concentration of the other KNP samples (Mpanama Dam, Makhohlola Dam and Sunset Dam) was below 6.0 mg/L (175 µg MC-LR/kg body weight) toxicity of these samples was not tested in mice. Hepatotoxic effects and death of mice were observed within 1 h after i.p. injection with the Nhlanganzwani Dam samples (Table 5).

Table 5 – Mouse bioassay results after i.p injection of mice with HBP Dam (winter and summer samples) and KNP samples

Cyanobacterial samples	Toxin concentration detected by ELISA (mg/L)	Dose (µg MC-LR/kg body weight)	Liver weight/body mass %	Symptoms of hepatotoxicity	Survival time (h)
HBP Dam winter samples (n = 6)	± 3.77	110	6.67 ± 0.41	No	24
HBP Dam summer samples (n = 6)	± 86.08	2511	9.12 ± 0.70	Yes	< 1
KNP samples: Nhlanganzwani (n = 2)	± 49.41	1441	9.47 ± 0.78	Yes	< 1
Mpanama	± 1.56	NT	NT	NT	NT
Makhohlola	± 0.10	NT	NT	NT	NT
Sunset	± 0.25	NT	NT	NT	NT
Control (n = 4)	0	0	6.65 ± 0.92	No	24

NT = Not tested

5.4 DISCUSSION

The mouse bioassay is generally used in a qualitative manner to determine if a cyanobacterial bloom is toxic or non-toxic and also to identify the class of toxins present in the water samples based on the observed clinical symptoms (Msagati *et al*. 2006). The clinical symptoms observed in mice after they were injected with the cyanobacterial samples, especially the HBP Dam summer and Nhlanganzwani Dam samples, strongly suggested the presence of hepatotoxins (symptoms: dark-coloured appearance and enlargement of liver) in the samples (Table 5).

The HBP Dam winter samples were found to be non-lethal to the mice but this does not mean that these samples are safe, because they contained MC levels above the prescribed guideline value (1.0 µg/L) set by WHO.

Microcystins have been shown to have a wide range of LD_{50} (25-150 µg/kg) when injected i.p. into mice (Stoner *et al*. 1989; Dawson 1998; Oberholster *et al*. 2004; Msagati *et al*. 2006). In their study, Fawell and co-workers (1999) found 50-158 µg/kg MC-LR to be the LD_{50} range after i.p. injection in Cr1:CD-1(ICR) BR strain of mice. They also found the mouse to be a more sensitive model in detecting MCs than the rat. In the current study, a dose of about 175 µg/kg of purified MC-LR was demonstrated to be lethal in mice. This value, even though it is slightly higher, is consistent with the LD_{50} values reported in literature (Fawell *et al*. 1999). The observed slight increase in the lethal dose could be due to the difference in strains of mice used. The majority of the

LD_{50} values obtained in literature were established using Balb C mice, whereas in this study CD-1 SPF mice were used.

Cyanobacterial hepatotoxins have been shown to cause rapid death in mice due to intrahepatic haemorrhage which leads 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 i.p. injection of mice with the HBP Dam summer samples and Nhlanganzwani Dam samples in the current study.

5.5 CONCLUSION

Although there are ethical constraints to the use of the mouse test, it still remains the one test that is able to detect toxicity of all the cyanotoxins in water samples, irrespective of whether the toxins are known or unknown. The major disadvantage of the mouse test is that it is not sensitive enough to detect MC-LR toxicity at doses below 90 µg/kg, as shown in this study.

CHAPTER 6

ELECTRON MICROSCOPY

6.1 INTRODUCTION

Electron microscopy (EM) is primarily used to investigate ultrastructural changes of organelles, such as the mitochondria, nucleus, endoplasmic reticulum (ER), etc., after exposure to toxic substances.

Using both scanning electron microscopy (SEM) and transmission electron microscopy (TEM), hepatic ultrastructural changes following exposure of rats and mice to *M. aeruginosa* toxins have been reported (Dabholkar & Carmichael 1987; Miura *et al*. 1989; Hooser *et al*. 1990; Hermansky *et al*. 1993).

Sequential SEM and TEM of mouse liver following i.p. administration of an aqueous *M. aeruginosa* extract showed progressive disappearance of the space of Disse, damage to hepatocyte membranes, and necrotic changes in the hepatocyte cytoplasm (Falconer *et al*. 1983).

Marked differences in the response of mice and rats to MC-LR were noted by Hooser *et al*. (1989). In mice, massive, centrilobular to midzonal haemorrhage and death occur within 60 and 90 minutes. In rats, although hepatic necrosis and haemorrhage occur

within 60 minutes, the haemorrhage is not as severe as in mice, and rats survive 20 to 32 hours (Hooser *et al*. 1989).

6.2 MATERIALS AND METHODS

Processing of the mouse livers for electron microscopy was carried out by the Electron Microscope Unit personnel at the Faculty of Veterinary Science, University of Pretoria. After i.p. administration of mice with HBP Dam samples (winter and summer samples) [Chapter 5.2 – mice i.p. injected with HBP Dam winter samples were euthanazed after 24 h; mice i.p. injected with HBP Dam summer samples died within 1 h], liver lobes were immediately removed, weighed and cut into small pieces (±1.0 mm^3) and fixed in 2.5 % gluteraldehyde in Millonig's buffer and then post-fixed in 1.0 % osmium tetroxide in Millonig's buffer. The liver tissues were then processed using standard techniques for TEM. Ultra-thin sections were stained with uranyl acetate and lead citrate and viewed in a Philips CM10 transmission electron microscope operated at 80 kV. The method has been modified from Glauert (1980).

6.3 RESULTS

No ultrastructural changes were seen in the control livers; the hepatocytes were characterized by large prominent nuclei, distinct nucleoli and numerous mitochondria in close proximity to the endoplasmic reticulum (ER) (Fig. 4). Representative electron micrographs of hepatocytes from a mouse injected i.p. with the HBP Dam winter samples

are shown in Fig. 5 and 6. Death was not observed in mice injected i.p. with the HBP Dam winter samples (±109 µg/kg). Early stages of disassociation between the hepatocytes were observed, however, no erythrocytes were seen in the mouse hepatocyte tissue (Fig. 5). The hepatocytes were binucleated, possibly undergoing cell division. A number of electron dense mitochondria and lipid droplets were also observed in the hepatocyte.

Slight vesiculation of ER was observed and the ER appeared to have retained their long, tubular profiles arranged in parallel arrays or stacks (Fig. 6). Ribosomes attached to the ER could be seen upon closer observation of the organelles. Electron dense and slightly swollen mitochondria were also observed.

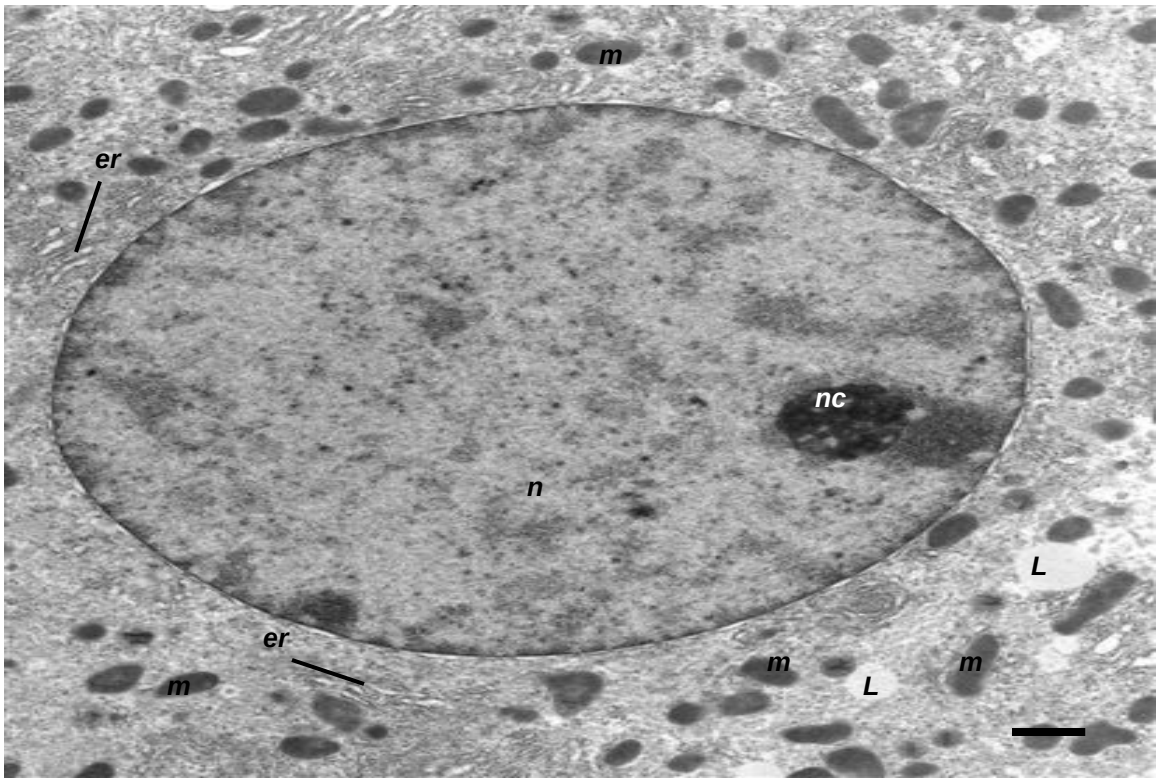


Fig. 4 – Electron micrograph showing a mouse liver tissue after i.p. injection with saline (control). Normal nucleus (*n*) containing nucleolus (*nc*); electron dense mitochondria (*m*); endoplasmic reticulum (*er*); Lipid droplets (*L*) . bar = 1 µm

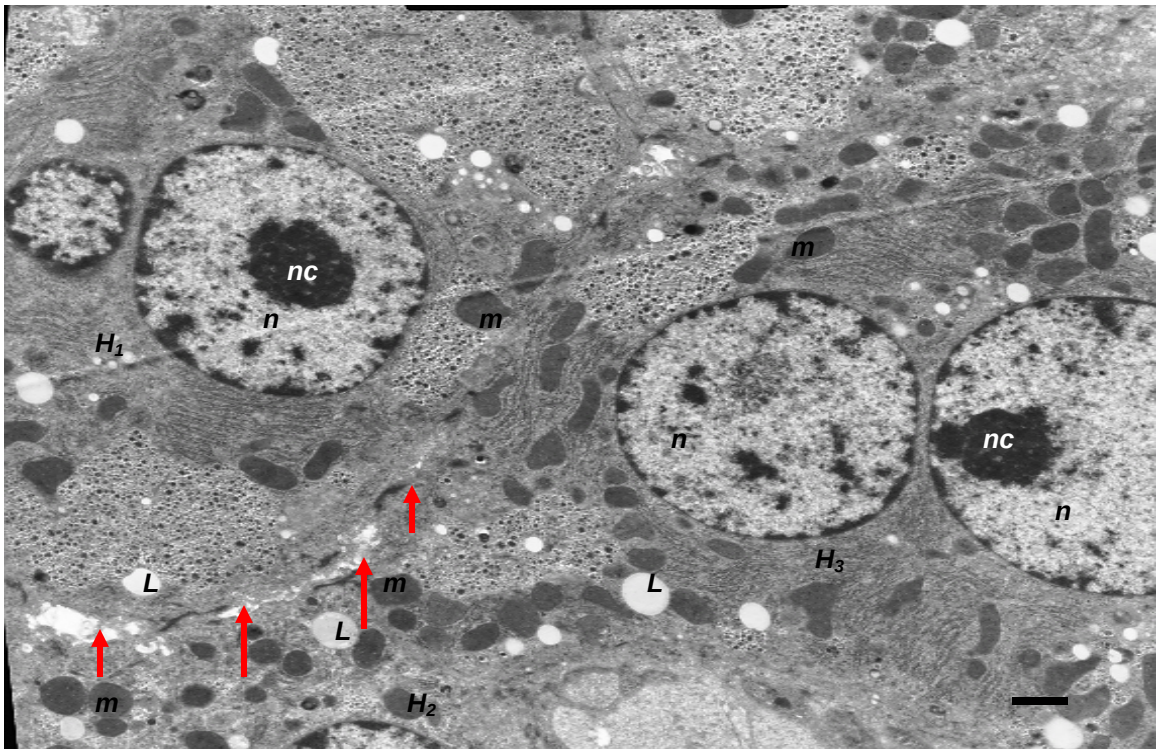


Fig. 5 – Electron micrograph showing a mouse liver tissue after i.p. injection with HBP Dam winter samples (109 µg/kg). Early stages of hepatocyte (H_1) to hepatocyte (H_2) disassociation was observed (*arrows*). Lipid droplets (*L*); mitochondria (*m*); nucleus (*n*); nucleolus (*nc*). bar = 1 µm

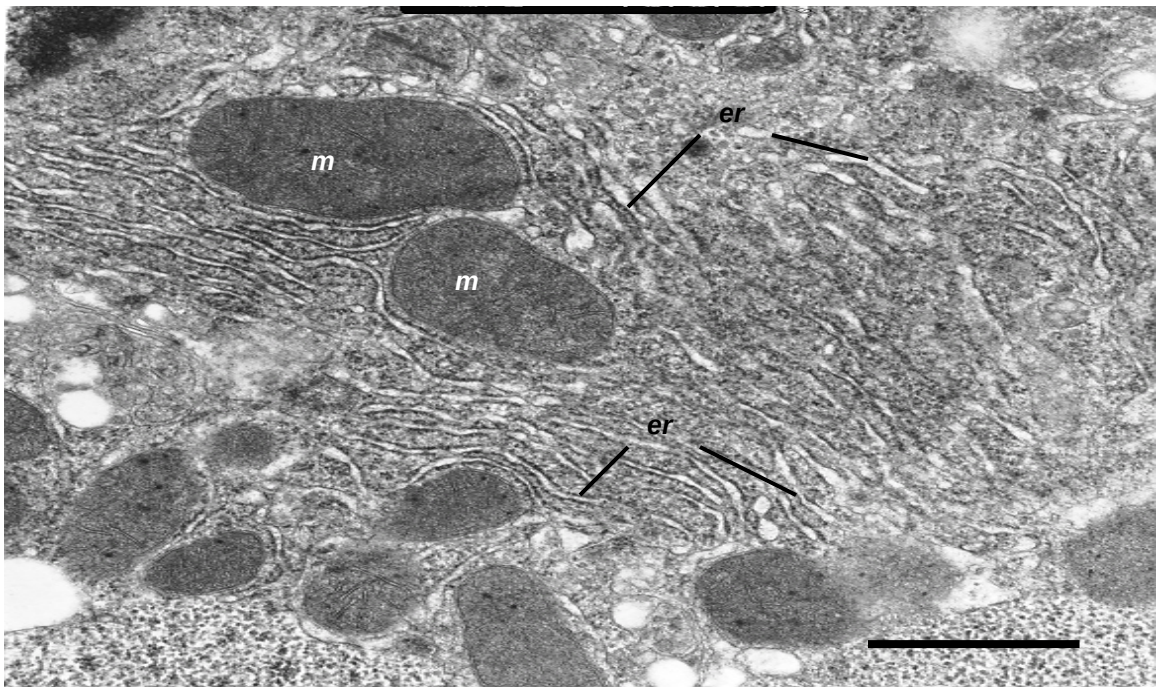


Fig. 6 – Electron micrograph showing a mouse liver tissue after i.p. injection with HBP Dam winter samples (109 µg/kg). Slightly swollen mitochondria (*m*); slightly vesiculated ER (*er*) studded with ribosomes. bar = 1 µm

Representative electron micrographs of hepatoctes from a mouse injected i.p. with the HBP Dam summer samples are shown in Fig. 7 and 8. Death was observed within 1 h after i.p. injection of mice with the HBP Dam summer samples (±2 510 µg/kg). The TEM revealed a loss of symmetry in the distribution of cellular organelles within the mouse hepatocyte tissue. The most striking ultrastructural change observed was increased appearance of erythrocytes within the mouse hepatocyte tissue which is indicative of a severe hepatic haemorrhage (Fig. 7).

The most striking ultrastructural change shown in Fig. 8 is the extensive vesiculation of ER found in the mouse hepatocyte tissue. However, the ER on the upper side of the micrograph appeared to have retained its long, tubular profiles arranged in parallel arrays or stacks (Fig. 8). A number of vesicles seemed to have lost their ribosomes from their membranes and appeared to be smooth surfaced. Electron dense and slightly swollen mitochondria were also seen. Some mitochondria were surrounded by ER cisterna. The nucleus containing nucleoli and surrounded by the nuclear membrane appeared to have been unaffected by the cyanobacterial toxins.

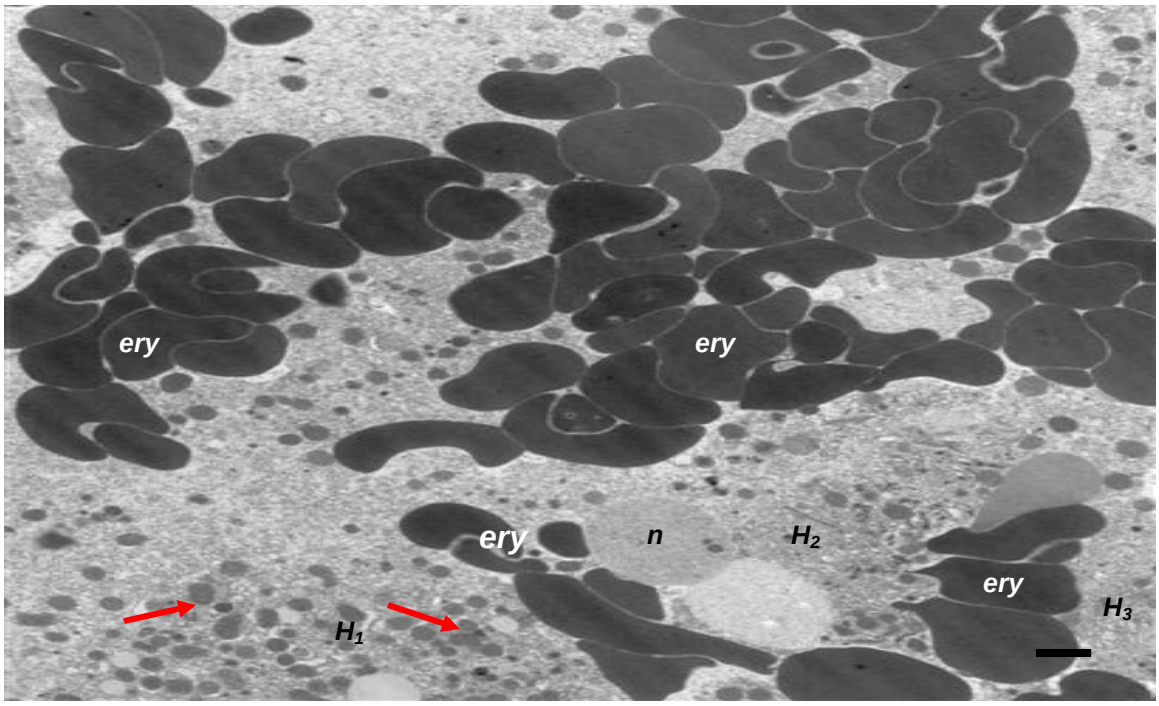


Fig. 7 – Electron micrograph showing a mouse liver tissue after i.p. injection with HBP Dam summer samples (2 510 µg/kg). Increased number of erythrocytes (*ery*) between hepatocytes H_1, H_2, H_3 was observed. A number of electron dense mitochondria (*arrows*) were visible; nucleus (*n*) containing nucleolus. bar = 2 µm

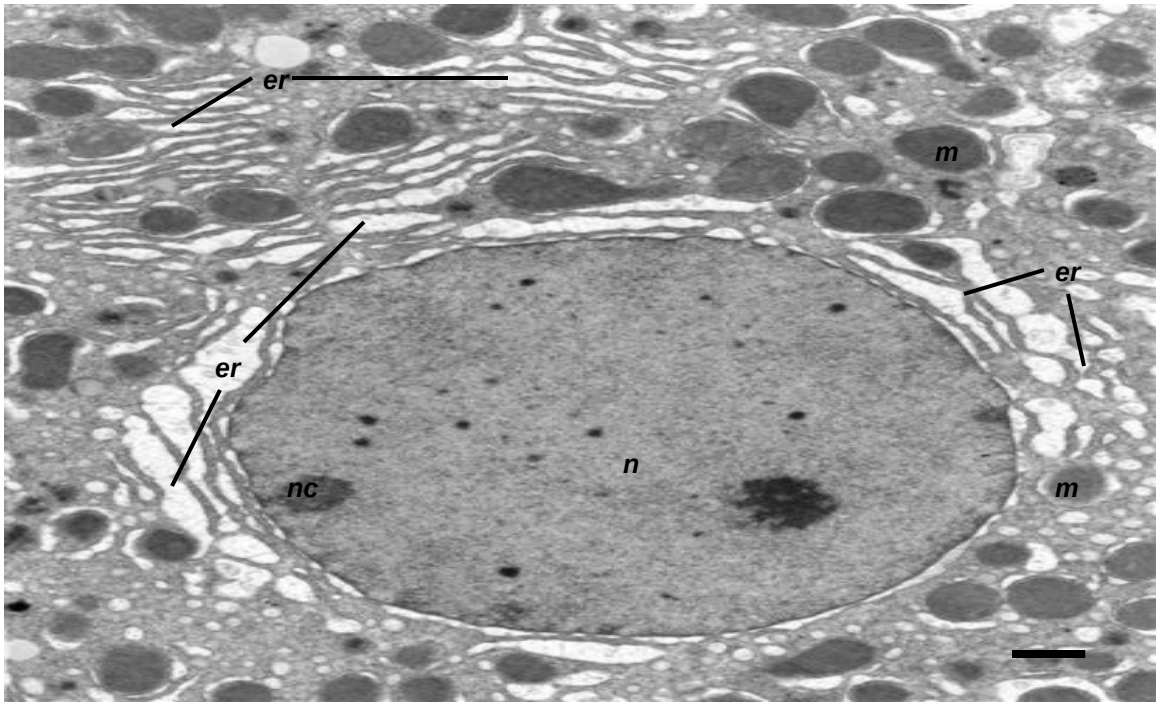


Fig. 8 – Electron micrograph showing a mouse liver tissue after i.p. injection with HBP Dam summer samples (2 510 µg/kg). Extensive vesiculation of ER (*er*) was observed in the hepatocyte. Nucleus (*n*) appeared intact; nucleolus (*nc*); electron dense and slightly swollen mitochondria (*m*). bar = 1 µm

6.4 DISCUSSION

Early stages of hepatocyte to hepatocyte disassociation, slight vesiculation of ER and swollen mitochondria were the most significant ultrastructural changes produced in mouse hepatocyte tissues by the HBP Dam winter samples at approximately 109 µg/kg dose (Fig. 6). The absence of erythrocytes in the mouse hepatocyte tissue could be due to the fact that the disassociation between the hepatocytes was mild and in the early stages. The most significant ultrastructural changes produced in mouse hepatocyte tissues by the HBP Dam summer samples at approximately 2 510 µg/kg dose, were the massive hepatic haemorrhage indicated by the appearance of erythrocytes between hepatocytes (Fig. 7) and the extensive vesiculation of ER (Fig. 8).

Hooser *et al*. (1989) showed that the lethal doses (LD_{50} = ±120 µg/kg) of purified MC injected i.p. in rats caused histologic lesions (i.e. disassociation, degeneration, necrosis and rounding of centrilobular hepatocytes) that began 20 to 30 min post-administration. In another study, where rats were injected i.p. with a lethal dose of MC-LR (160 µg/kg), Hooser *et al*. (1990) found that hepatocyte to hepatocyte disassociation, loss of hepatocyte microvilli, and hepatocyte plasma membrane invagination seen ultrastructurally were all compatible with structural changes in the cells caused by alterations in actin filaments. These ultrstructural changes suggest progressive and irreversible hepatocellular damage following treatment of mice with 100 µg/kg MC-LR (Hermansky *et al*.1993).

Hermansky *et al*. (1993) showed that a 100 µg/kg dose of MC-LR resulted in altered distribution of hepatocellular organelles, increase in the amount of intracellular glycogen, increased appearance of erythrocytes within the mouse hepatocyte tissue and loss of uniform distribution of mitochondria within 60 min after treatment of mice.

Early stages of hepatocyte to hepatocyte disassociation, increased presence of erythrocytes within the mouse hepatocyte tissue, extensive vesiculation of ER and swollen mitochondria observed in this study at doses of cyanotoxins ranging between 109 to 2 510 µg/kg, are changes which are in agreement with changes observed by other investigators in mice and rats administered with MC-LR (Hermansky *et al*. 1993; Dabholkar & Carmichael 1987). Dabholkar & Carmichael (1987) attributed the increased appearance of erythrocytes in the mouse hepatocyte tissue to widening and disassociation of gaps between adjacent hepatocytes, thus suggesting the loss of some desmosomes (tight junctions). When the hepatocytes disaggregate from each other under the effect of *Microcystis* toxins, hepatic haemorrhage follows. This hepatic haemorrhage leads to a fall in blood pressure and eventual death of mice by hypovolemic shock.

6.5 Conclusion

Ultrastructural changes induced by cyanotoxins after i.p. injection in mice, were clearly shown by TEM. This study demonstrates that TEM is an effective tool that can be used to observe disruption to internal architecture of the hepatocytes as a result of exposure to

high concentrations of cyanotoxins and may be used in pathological profiles of animals dying due to ingestion of water contaminated with cyanobacterial blooms.

CHAPTER 7

IN VITRO TOXICITY TESTING OF CYANOTOXINS USING A CATFISH PRIMARY CELL LINE

7.1 INTRODUCTION

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 & Berg 1986; Berg & Aune 1987; Heinze 1996). Among the *in vitro* liver preparations recognized (i.e. tissue slices, isolated perfused organs and isolated hepatocytes); the most widely used model is isolated hepatocytes (Guillouzo 1998).

The major advantage of using primary hepatocytes is that a number of chemicals/samples can be tested from a single preparation of hepatocytes compared to one mouse required per sample when using the mouse assay. Another advantage of primary hepatocytes is their ability to maintain the activities of phase I and II enzymes, thus allowing various investigations to be performed including determination of kinetic parameters, metabolic profiles, inhibition and induction effects, and drug-drug interactions in culture (Fautrel *et al*. 1991; Guillouzo 1998).

The disadvantage associated with the use of primary hepatocytes is their limited biological activity period of approximately seven days, after which a decline in the activity of the phase I and II enzymes is observed (Fautrel *et al*. 1991; Guillouzo 1998).

Howard and co-workers (1967) were the first to describe an enzymatic technique in which primary hepatocytes were prepared using a buffered medium containing dissociating enzymes, namely hyaluronidase and collagenase. The two-step collagenase perfusion technique modified by Seglen (1975) has gained popularity in isolating primary hepatocytes. The first step involves perfusion of the liver *in situ* with a calcium-free buffer followed by circulation of a calcium-supplemented buffer containing collagenase enzyme. The two-step collagenase perfusion technique has been successfully applied to other livers of various species including humans, fish, birds, frogs, pigs and rabbits (Maslansky & Williams 1982; Chenery *et al*. 1987; Guillouzo 1998; Naicker *et al*. 2007).

Freshly isolated primary hepatocytes were first used by Aune and Berg (1986) in investigating the toxicity of cyanotoxins. Previous studies have shown that the liver is the target organ for cyanotoxins (Falconer *et al*. 1981; Eriksson *et al*. 1990; Honkanen *et al*. 1990), and freshly isolated primary hepatocytes seem to be an ideal tool for *in vitro* toxicity testing of extracts from cyanobacteria (Aune & Berg 1986).

A wide array of morphologic and biochemical markers are available for obtaining information at cellular and molecular levels to detect and measure chemically-induced perturbations. These include parameters that estimate plasma membrane integrity and

subcellular effects, parameters that assess irreversible cytotoxicity (cell death) and reflect reversible changes, or non-specific and liver-specific end points (Fautrel *et al*. 1991; Guillouzo 1998).

7.1.1 Non-specific end points

Non-specific end points are usually used to estimate irreversible cellular damage. The choice of a cytotoxicity indicator depends on a number of factors that include correspondence with the lesion being induced *in vivo*, reliability, reproducibility and sensitivity, convenience, cost and species specificity (Storey *et al*. 1983; Fautrel *et al*. 1991). Non-specific end points for *in vitro* cytotoxicity evaluation include the following:

i. Light microscopy: which can be used to investigate changes in cell shape, nuclear and cytoplasm; accumulation of vacuoles; formation of lipid droplets and blebs; cell attachment and detachment (cell count).

ii. Electron microscopy: which can be used to investigate alterations of organelles, such as mitochondria, etc.

iii. Plasma membrane integrity: which can be investigated by determining proteins or DNA content, enzyme leakage (LDH); by performing neutral red uptake, or trypan blue exclusion tests.

iv. Alterations of cell permeability: which can be investigated by determining leakage of ions and small molecules (K^+ concentration; Ca^{2+} flux).

v. Metabolic parameters: which can be investigated by performing 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) test; or

determining ATP content, lactate/pyruvate ratio, glutathione (GSH) content, lipid peroxidation (Fautrel *et al*. 1991; Guillouzo 1998).

7.1.2 Liver-specific end points

A number of markers representative of the various liver-specific functions can also serve as indicators of functional disturbances in isolated hepatocytes exposed to toxic substances. The most widely tested markers include neosynthesis of liver-specific plasma proteins such as albumin and transferin and acute phase proteins, glyconeogenesis, glycogen synthesis, urea synthesis, lipoprotein synthesis, induction or inhibition of specific cytochrome P-450 (CYP) enzymes (Santone & Acosta 1982; Goethals *et al*. 1984; Padgham & Paine 1993).

7.2 MATERIALS AND METHODS

7.2.1 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, 20 mM (Highveld Biologicals, Gauteng, South Africa) at a flow rate of 0.5 mL/min. Final perfusion was performed for 12 min at a flow rate of 5.0 mL/min with a perfusion solution containing

collagenase, Type IV (Sigma-Aldrich, Gauteng, South Africa). The yield of cells was about 3.0 X 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 ºC in a 5.0 % CO_2 incubator for 24 h to allow cells to attach to the wells.

7.2.2 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 HBP Dam samples (summer and winter samples) and KNP (Nhlanganzwani Dam, Mpanama Dam, Makhohlola Dam and Sunset Dam) samples. Primary hepatocytes were exposed to serial dilutions of cyanobacteria, ranging from 100 % to 3.13 % in culture media (M199 culture media 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; 1.0; 2.0; 5.0; 10; 25; 50; 100 µg/l). After exposure, cells were incubated at 16 ºC in a 5.0 % CO_2 incubator for 72 h. At the end of the incubation period, 10 µL of MTT (5.0 mg/mL in PBS) was added into each well and the cells were further incubated for 4 h. The formation of colour (formazan) was measured with a microtitre plate spectrophotometer (Bio-Tek µQuant, A. D. P., South Africa) at 570 nm. Cell viability was estimated as the percentage absorbance of sample relative to control.

7.3 Statistical analysis

The results are reported as mean ± SD from each winter and summer season. 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.

7.4 RESULTS

Successful isolation and culturing of the African sharptooth catfish primary hepatocytes were achieved. Within 24 h before exposure to toxins, the cells were firmly attached to the Matrigel coated plates and appeared round and intact. Morphological changes of the primary hepatocytes after exposure to HBP Dam summer samples (2 510 µg/kg), monitored during the 72 h incubation period, are shown in Fig. 9A and 9B. Progressive damage of the hepatocytes was observed during the incubation period. After 24 h of incubation, some of the cells had surface blebs, appeared irregular and demonstrated disruption of the plasma membrane, and detached from the Matrigel coated substrate (Fig. 9A). After 72 h of incubation, essentially all hepatocytes appeared irregular, disrupted and flattened (Fig. 9B). Morphological changes of the hepatocytes after exposure to HBP Dam winter samples (109 µg/kg) were less severe (results not shown). Most of the control hepatocytes (not exposed to the toxins) remained round and intact after 72 h incubation (Fig. 9C).

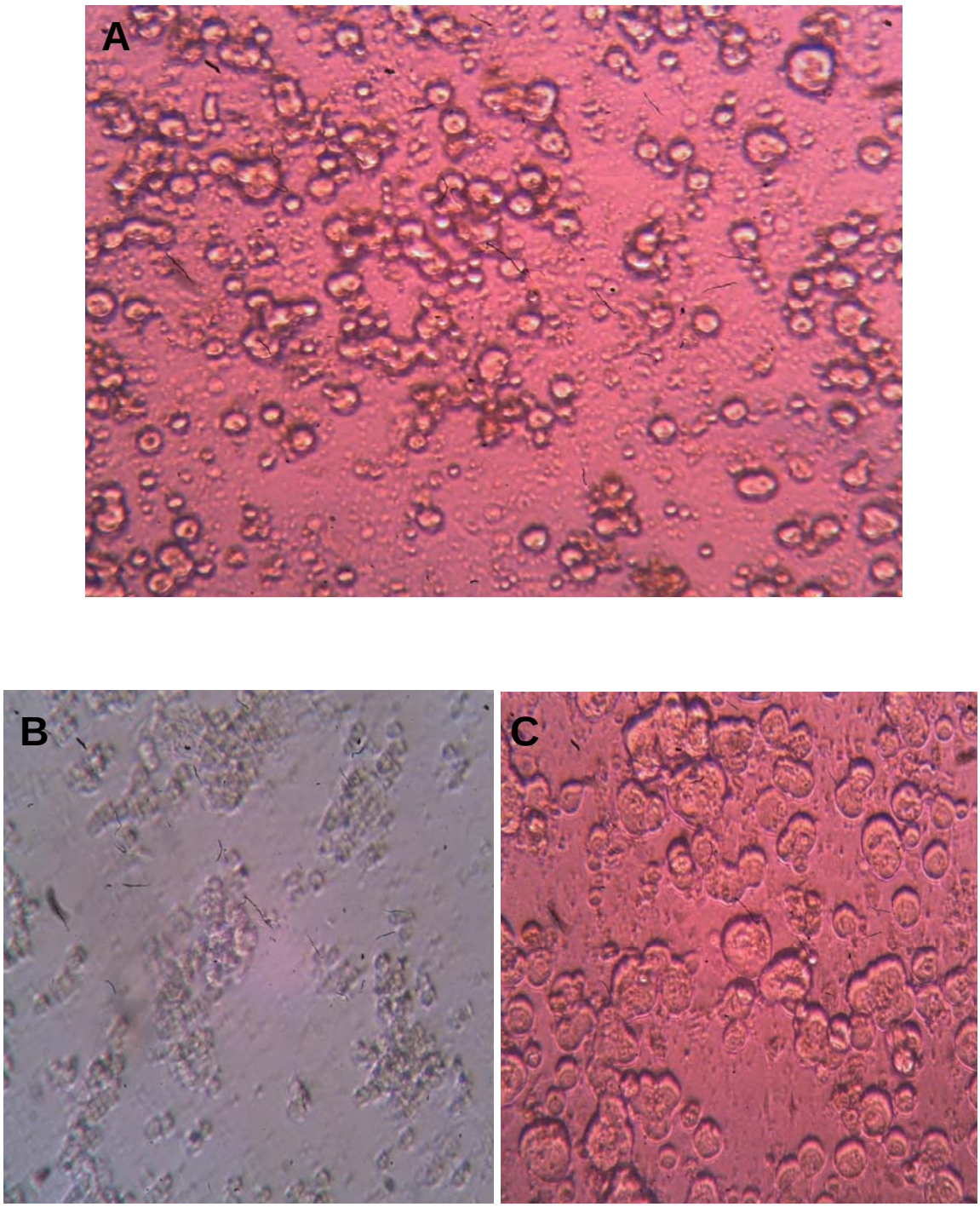


Fig. 9 – Morphological changes of hepatocytes as shown by light microscope (400 X magnification) after exposure to HBP Dam summer samples (2 510 µg/kg). (A) 24 h after exposure of hepatocytes to HBP Dam summer samples; (B) 72 h after exposure of hepatocytes to HBP Dam summer samples; (C) Control hepatocytes (not exposed to toxins) after 72 h

Viability of the catfish primary hepatocytes after exposure to purified MC-LR, HBP Dam samples (summer and winter) and KNP (Nhlanganzwani Dam, Mpanama Dam, Makhohlola Dam and Sunset Dam) samples was investigated based on the mitochondrial succinate dehydrogenase activities of the hepatocytes (MTT assay). 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, HBP Dam and KNP samples was expressed in terms of the EC_{50} values. An EC_{50} is defined as the exposure concentration of a cyanobacterial material that is expected to cause cytotoxicity in 50 % of the cell population. The EC_{50} values obtained after exposure of catfish primary hepatocytes for 72 h to the cyanotoxin-contaminated water samples are shown in Table 6. The EC_{50} obtained for the HBP Dam summer samples was about 47 %, and 77 % for the HBP Dam winter samples. The EC_{50} values obtained for the KNP samples are as follows: 54 % - Nhlanganzwani Dam; 84 % - Mpanama Dam; 95 % - Makhohlola Dam and 93 % - Sunset Dam samples. An EC_{50} of 91 nM was obtained after exposure of cells to purified MC-LR

Table 6 – EC_{50} values obtained after exposure of catfish primary hepatocytes to HBP Dam samples (winter and summer), KNP samples and MC-LR

Cyanobacterial samples	**EC_{50} (%)**
HBP Dam winter samples (n = 6)	77.33 ± 7.77
HBP Dam summer samples (n = 6)	47.67 ± 6.43
KNP samples:	
Nhlanganzwani Dam (n = 2)	54.67 ± 8.02
Mpanama Dam (n = 2)	84.33 ± 6.11
Makhohlola Dam (n = 2)	95.33 ± 4.04
Sunset Dam (n = 2)	93.30 ± 3.22
Purified toxin standard	**EC_{50} (nM)**
MC-LR	91.33 ± 3.22

7.5 DISCUSSION

In recent years, *in vitro* toxicity tests involving cultured cells have been developed to provide a substitute for the mouse bioassay (Seglen 1975; Aune & Berg 1986; Heinze 1996). Previous investigators have focused on the cytotoxic effects of purified MCs in primary hepatocytes (Aune & Berg 1986; Heinze 1996; Boaru *et al*. 2006) and continuous cell lines (Chong *et al*. 2000; Pichardo *et al*. 2005). Data reporting on the cytotoxic effects of field samples using primary hepatocytes is scarce.

In this study, the ability of cyanobacterial samples and purified MC-LR to cause hepatotoxicity in mice was confirmed, *in vitro*, using African sharptooth catfish primary hepatocytes. A comparison among the cyanobacterial samples using EC_{50} showed the following hepatotoxicity trend in the catfish primary hepatocytes: HBP Dam summer samples > Nhlanganzwani Dam samples > HBP Dam winter samples > Mpanama Dam samples > Sunset Dam samples > Makhohlola Dam samples. The HBP Dam samples were the most hepatotoxic and Makhohlola Dam samples were the least hepatotoxic. The EC_{50} for purified MC-LR using the catfish primary hepatocytes was about 91 nM.

Other investigators have demonstrated the successful use of mammalian and rainbow trout primary hepatocytes in determining cytotoxicity of the cyanotoxins (Aune & Berg 1986; Heinze 1996; Boaru *et al*. 2006). Bouaïcha and Maatouk (2004) observed an EC_{50} of 48 nM in rat primary hepatocytes exposed to MC-LR for 24 h, and Fladmark *et al* (1998) found morphologically an EC_{50} of 400 nM after exposure to MC-LR. Our results (EC_{50} of about 91 nM obtained for MC-LR using the catfish primary hepatocytes) confirm the findings made by Fladmark *et al.* (1998) that fish primary hepatocytes are less sensitive than rat primary hepatocytes for MC-LR; however, fish primary hepatocytes are more sensitive than continuous cell lines. Fastner *et al*. (1995) and Chong *et al*. (2000) observed no toxicity in the Chinese hamster ovary (CHO-K1) and Rainbow trout gonad-2 (RTG-2) cells exposed to MC-LR.

Cellular changes observed in the catfish primary hepatocytes after exposure to cyanobacterial samples in the current study using light microscopy are in agreement with

changes observed by Aune and Berg (1986) in rat primary hepatocytes after exposure to cyanobacterial samples. These authors observed a time-dependent increase of cellular damage at concentrations of cyanobacterial samples ranging from 0.65 to 5.0 mg/mL.

The bile acid transporter has been proposed as a possible explanation for the specificity of MCs on hepatocytes (Eriksson *et al*. 1990). This transporter plays a crucial role in transporting cyanotoxins across cell membranes into cells whereby they induce cytotoxic response. Only cells expressing the bile acid transporter on their membranes are affected by the cyanotoxins (Eriksson *et al*. 1990). Changes in cell shape and morphology induced by MCs appear to have been caused by alterations in the hepatocytes cytoskeleton. These cytoskeletal changes are probably consequences of toxin-induced inhibition of protein phosphatase resulting in phosphorylation of cytoskeletal proteins (Falconer *et al*. 1992 (b)).

7.6 CONCLUSION

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 other fish primary hepatocytes. Because of their sensitivity and ability to function similar to the liver *in vivo*, primary hepatocytes provide an attractive alternative to the mouse assay in toxicity testing of cyanotoxins. Future studies to validate the use of this hepatocyte model in MC-LR toxicity is planned.

CHAPTER 8

PROTEIN PHOSPHATASE INHIBITION ASSAY

8.1 INTRODUCTION

The protein phosphatase inhibition (PP*i*) assay is a sensitive screening method for MCs and nodularins which uses the biochemical activity of these toxins. The MCs and nodularins inhibit serine and threonine phosphatase (type 1 and 2A) enzymes responsible for the dephosphorylation of intracellular phosphoproteins. The extent of inhibition of these enzymes is related to the hepatotoxicity of these compounds (MacKintosh *et al*. 1990). Protein phosphatase (PP) assays are sufficiently sensitive to detect MCs below the WHO guideline value (1.0 µg/L) without the need for sample pre-concentration and perform well in a variety of sample matrices (Heresztyn & Nicholson 2001; Rapala *et al*. 2002).

The basis of this assay is the measurement of phosphatase release from a phosphorylated protein substrate in the presence of a phosphatase enzyme preparation and an inhibitor such as MC (Heresztyn & Nicholson 2001). The PP*i* assays include the use of ^{32}P radiolabelled substrates (Lambert *et al*. 1994; Lam *et al*. 1995) or chromogenic substrate *p*-nitrophenol phosphate (*p*-NPP) (An & Carmichael 1994; Ash *et al*. 1995).

The major draw back with the ^{32}P radiolabeled assay is that the ^{32}P isotope has a short half-life, and radiolabelled proteins for the assay have to be prepared on a regular basis using reasonably sophisticated procedures (Heresztyn & Nicholson 2001). Another disadvantage of the PP*i* 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).

Recently, Bouaïcha *et al*. (2002) employed an alternative version of the assay using the fluorogenic substrates 4-methylumbelliferyl phosphate (MUP) and 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) to detect MCs in drinking water. In this assay, the activity of protein phosphatase type 2A (PP2A) enzyme is indicated by formation of the fluorescent compounds 4-methylumbelliferone (MU) from MUP and 6,8-difluoro-4-methylumberlliferone (DiFMU) from DiFMUP. These substrates provided a 2-fold increase in assay sensitivity, and more reproducible measurements of toxicity were achieved compared with the chromogenic substrate *p*-NPP (Bouaïcha *et al*. 2002).

8.2 MATERIALS AND METHODS

A commercial RediPlate 96 EnzChek Serine/Threonine Phosphatase assay kit (Scientific Group, South Africa, product of Molecular Probes, Inc) and PP2A enzyme (Whitehead Scientific, South Africa, product of Promega) were used. The PP2A comprises the 36-38 kDa catalytic subunit isolated from human red blood cells and was supplied in lots of 25

units, 1.0 unit being defined as the amount of enzyme required to hydrolyse 1.0 nmol of *p*-NPP/min at 30 ºC 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 cyanobacterial samples), the activity of PP2A is inhibited, therefore resulting in reduced production of DiFMU. HBP Dam winter samples were diluted 1/10 000 and HBP Dam summer samples were diluted 1/50 000. The KNP Dam samples were diluted as follows: Nhlanganzwani Dam samples 1/50 000; Mpanama Dam samples 1/5 000; Sunset Dam samples 1/200; and Makhohlola Dam samples 1/20. After adding MC-LR (80 µL) or cyanobacterial samples (80 µL) [as prepared in Chapter 4.1.2] and the enzyme (20 µL) to the wells, the microtitre plate was incubated at 37 ºC and fluorescence was measured over a period of 90 min at 355/460 nm 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; 1.0; 10^{1}; 10^{2} µg/L. The MC inhibition (calibration) curve was plotted as percentage activity of PP2A relative to the control, versus MC concentration, where

$$\text{PP2A activity (\%)} = \frac{\text{Absorbance}_{\text{standard}} - \text{Absorbance}_{\text{blank}}}{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{blank}}} \times 100$$

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. Each test was done in triplicate.

8.3 Statistical analysis

The results are reported as mean ± SD from each winter and summer season. 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.

8.4 RESULTS

The calibration (inhibition) curve for MC-LR standards is shown in Fig. 10. An IC_{50}, defined as the concentration of MC-LR 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 (HBP Dam winter samples 1/10 000; HBP Dam summer samples 1/50 000; Nhlanganzwani Dam samples 1/50 000; Mpanama Dam samples 1/5 000; Makhohlola Dam samples 1/20; Sunset Dam samples 1/200). Toxin concentrations

determined by the PP2A inhibition assay on HBP Dam samples and KNP Dam samples are shown in Table 7. Concentration of the HBP Dam winter samples was about 2.99 mg/L while a higher concentration of about 54.90 mg/L was observed with the HBP Dam summer samples. Nhlanganzwani Dam samples were the only samples from KNP that exhibited high toxin concentrations (10.95 mg/L). Low toxin concentrations in the range of 0.006 to 0.05 mg/L were observed with the other KNP samples. Complete inhibition in the activity of PP2A enzyme was observed with undiluted HBP Dam winter and summer samples and also with the Nhlanganzwani Dam samples.

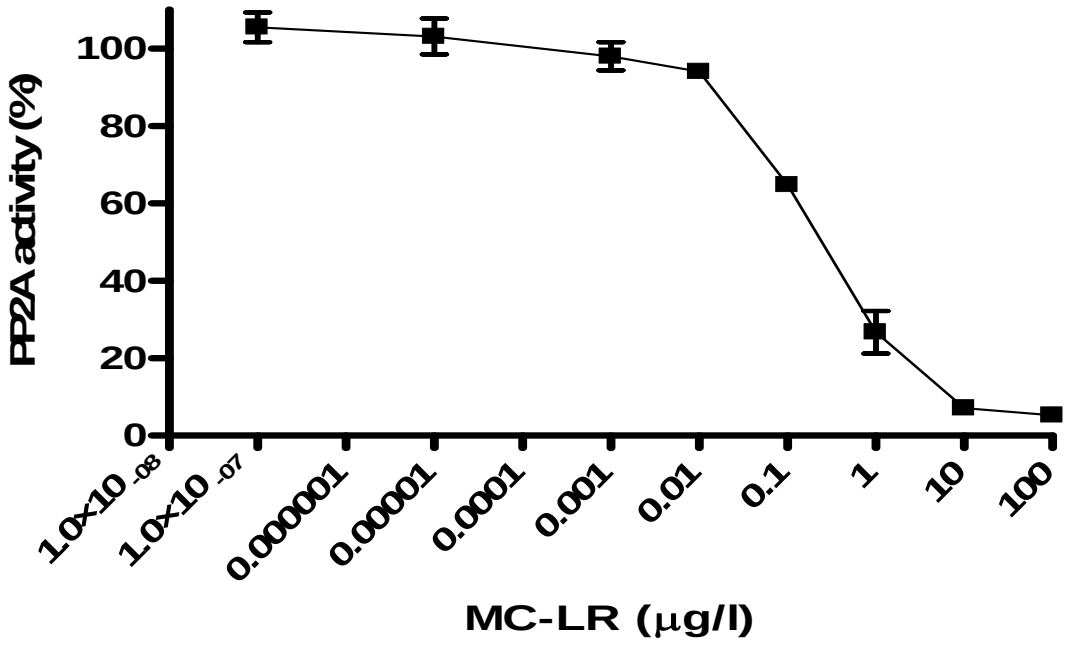


Fig. 10 – Inhibition curve for MC-LR standards. The linear region of the calibration curve (i.e. the region between 20 and 80 % activity) was used to determine sample concentrations. Each concentration was tested in triplicate

Table 7 – Toxin concentration determined by PP2A inhibition assay in HBP Dam (winter and summer samples) and KNP samples

Cyanobacterial samples	Toxin concentration (mg/L)
HBP Dam winter samples (n = 6)	2.99 ± 1.59
HBP Dam summer samples (n = 6)	54.90 ± 4.77
KNP samples:	
Nhlanganzwani Dam (n = 2)	10.95 ± 1.58
Mpanama Dam (n = 2)	0.03 ± 0.01
Makhohlola Dam (n = 2)	0.006 ± 0.004
Sunset Dam (n = 2)	0.05 ± 0.005

8.5 DISCUSSION

Microcystins (MCs) 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 and/or toxin concentration. Data published by Honkanen and co-workers (1994) indicated that PP2A is approximately 50 times more sensitive than PP1 in its inhibition by MCs, as a result only the inhibition of PP2A activity was investigated in this study.

The IC_{50} for MC-LR (0.22 µg/L) obtained in this study (Fig. 10) is in agreement with IC_{50} obtained by Heresztyn and Nicholson (2001) for MC-LR (0.3 µg/L). Toxin

concentrations of the HBP Dam summer samples and Nhlanganzwani Dam samples were shown to be higher than toxin concentrations of the other water samples investigated in this study, i.e. HBP Dam summer samples > Nhlanganzwani Dam samples > HBP Dam winter samples > Sunset Dam samples > Mpanama Dam samples > Makhohlola Dam samples. Toxin concentrations of all the water samples investigated in this study were above the prescribed guideline value set by the WHO (1.0 µg/L).

8.6 CONCLUSION

Protein phosphatase inhibition (PP*i*) assay is sufficiently sensitive to detect MCs below the WHO guideline value (1.0 µg/L) without the need for sample pre-concentration. However, the protein phosphatase enzyme is susceptible to inhibition by other toxins that may be present in the water sample. Therefore, this assay is prone to false positives of MC-LR.

CHAPTER 9

GENERAL DISCUSSION

Microcystins (MCs) are distributed in drinking and recreational waters worldwide (Carmichael 1994), as a result there is a high probability that animals and human populations are exposed to these toxins on an acute and chronic basis. With the exception of a few reports of fatalities in humans and animals following exposure to cyanotoxins (Steyn 1945; Falconer *et al*. 1983; Jochimsen *et al*. 1998; Pouria *et al*. 1998; Sahin 2000; Azevedo *et al*. 2002; Kellerman *et al*. 2005), much of the toxicities associated with MC exposure are undocumented.

There are several methods available for the detection of MCs, including the classical mouse bioassay. More sensitive methods have been developed in recent years using advanced techniques for the detection of MCs in water samples. These methods include: ELISA; PP*i* assay; *in vitro* (primary and continuous cell lines) assay; HPLC and LC/MS. A suitable method would be one that is able to detect MCs below 1.0 µg/L (i.e. prescribed guideline value set by WHO) without sample pre-concentration, and it must also be simple and easy to apply in routine analysis of MCs. Very few studies have been done to compare these methods using environmental samples.

The HBP Dam is one of the dams in South Africa that has the longest history of eutrophication – related problems caused mainly by inputs into the dam from the

surrounding industrial, agricultural and human activities. Because of its unique wildlife, the KNP plays an important role in attracting tourists to South Africa, thus contributing significantly to the economy of the country. Continuous assessment of the KNP Dams for the presence of cyanobacteria and their toxins is important in ensuring that correct measures are taken by the authorities in order to prevent the wildlife from being exposed to water bodies containing toxic cyanobacteria.

During sampling in the summer season at the HBP Dam, the dam surface was covered with a thick-green layer of cyanobacterial scums and a foul smell coming from the water surface was noted. Only blue-green streaks of cyanobacteria covered the dam surface during the winter season. All HBP Dam samples and KNP samples were dominated by *M. aeruginosa* with the exception of Makhohlola Dam samples which were found to have no cyanobacteria.

The World Health Organization (WHO) has proposed a guideline value of 1.0 µg/L (0.001 mg/L) for MC-LR, the most common MC variant, in drinking water (WHO 1998), whereas 2 000 *Microcystis* cells/ml have been recommended as the limit of cyanobacteria in drinking water for animals (DWAF 1996). Cyanotoxin concentrations exceeding the prescribed guideline value were determined in all HBP Dam samples and KNP samples using both the ELISA and PP*i* assays. An and Carmichael (1994) demonstrated that the PP*i* assay was not able to detect all MC variants that were investigated in their study, but when the ELISA was used it was able to detect all the MC variants even those that were not detected by the PP*i* assay. The polyclonal antibodies used in the ELISA assay were

able to cross react with all of the MC variants that were investigated (An & Carmichael 1994). The differences in the mechanism and response between the PP*i* 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 PP*i* assay is used to estimate total toxicity (Mountfort *et al*. 2005).

It is interesting to note that the Makhohlola Dam samples contained cyanotoxins at levels above the set prescribed guideline value even though no cyanobacteria were identified in these samples. This shows that cyanotoxins are able to persist in water even after the bloom has disappeared.

Mouse assays have the advantage of being non-specific, and therefore able to detect any cyanobacterial toxin, known or unknown, in a water sample (Chorus & Bartram 1999). Microcystins (MCs) have been shown to have a wide range of LD_{50} (25-150 µg/kg) when injected i.p. into mice (Stoner *et al*. 1989; Dawson 1998; Oberholster *et al*. 2004; Msagati *et al*. 2006). Sensitivity of mice for MCs may be influenced by factors such as the strain and sex of mice (Falconer *et al*. 1988). In the current study, a dose of about 175 µg MC-LR/kg was demonstrated to be lethal in mice. The HBP Dam summer samples and Nhlanganzwani Dam samples were the only cyanobacterial samples that resulted in death (acute toxicity) of mice. Chronic exposure to low levels of cyanotoxins has been found to cause primary liver cancer (Ueno *et al*. 1996), therefore, long term exposure to HBP Dam winter samples, Mpanama Dam samples, Sunset Dam samples and Makhohlola Dam samples could cause liver pathology.

In order to be able to investigate further the *in vivo* effects of cyanotoxins, TEM was used to complement results obtained from *in vivo* assays. Ultrastructural changes of varying degree were observed in livers of mice exposed to both the HBP Dam winter and summer samples. Early stages of hepatocyte to hepatocyte disassociation, slight vesiculation of ER and swollen mitochondria were the most significant ultrastructural changes produced in mouse hepatocyte tissues by the HBP Dam winter samples at a dose of approximately 109 µg/kg. The most significant ultrastructural changes produced in mouse hepatocyte tissues by the HBP Dam summer samples at approximately 2 510 µg/kg dose, were massive hepatic haemorrhage indicated by the appearance of erythrocytes between hepatocytes and the extensive vesiculation of ER. These changes were also observed by other investigators after exposure of mice to MC-LR (100-120 µg/kg) (Hermansky *et al*. 1993; Dabholkar & Carmichael 1987). The ultrastructural changes observed with the HBP Dam winter samples (109 µg/kg) confirms the fact that exposure to low levels of cyanotoxins (i.e. levels which do not cause acute poisoning) for longer periods could lead to chronic damage of the liver.

In recent years, *in vitro* toxicity tests involving cultured cells have been developed to provide a substitute for the mouse bioassay (Seglen 1975; Aune & Berg 1986; Heinze 1996). Other investigators have demonstrated the successful use of mammalian and rainbow trout primary hepatocytes in determining cytotoxicity of the cyanotoxins (Aune & Berg 1986; Heinze 1996; Boaru *et al*. 2006).

This is the first time that the African sharptooth catfish primary hepatocyte model has been exposed to purified MC-LR and cyanotoxin-containing water samples. In this *in vitro* study, it was confirmed that the catfish primary hepatocytes are very sensitive to purified MC-LR and water samples containing cyanobacteria and could be used as a diagnostic tool. A comparison between the cyanobacterial samples using EC_{50} showed the following hepatotoxicity trend in the catfish primary cell line: HBP Dam summer samples > Nhlanganzwani Dam samples > HBP Dam winter samples > Mpanama Dam samples > Sunset Dam samples > Makhohlola Dam samples. The HBP Dam samples were the most hepatotoxic and Makhohlola Dam samples were the least hepatotoxic. The EC_{50} for purified MC-LR using the catfish primary hepatocytes was about 91 nM.

A statistical comparison of the assays used in this study (i.e. ELISA, PP*i*, mouse test and cytotoxicity [catfish primary hepatocyte] assays) was performed based on the Kappa coefficient. The Kappa coefficient (K) is defined as an indication of the degree of agreement for nominal or categorical data (i.e. K > 0.80, 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). An almost perfect agreement (K > 0.80) was observed between the mouse test and cytotoxicity assay; mouse test and ELISA; cytotoxicity assay and ELISA; and ELISA and PP*i* assay.

In the current study, consistent results were obtained using the ELISA, PP*i*, mouse and cytotoxicity assays. Even though these assays were applied successfully, they do have

some advantages and disadvantages. Table 8 shows the summary of advantages and disadvantages of these assays.

Table 8 – Advantages and disadvantages of the mouse, cytotoxicity (catfish primary hepatocytes), ELISA and PP*i* assays

Assay	Advantages	Disadvantages	Duration of assay
Mouse assay	Biological test system which is used to directly demonstrate the presence of known and unknown toxins	Test entails severe suffering of animals Requires ethical approval One animal is required per test sample Not sensitive enough to detect MCs below 1.0 µg/l	24 h
Cytotoxicity (catfish primary hepatocytes)	Functions similar to liver *in vivo* A number of samples can be tested from a single preparation of hepatocytes	Short i*n vitro* lifespan Loses of *in vivo* properties Hepatocytes need to be isolated for every test	5 days
ELISA	Does not require pre-concentration of samples Has a detection limit of 0.1 µg/L	Does not indicate which variant of MC is present in a sample	2.5 h
PP*i* assay	Does not require sample pre-concentration Has a detection limit of 0.01 µg/L	Responds to variety of non-cyanobacterial toxins and metabolites	90 min

CHAPTER 10

CONCLUSION

The following conclusions were made from the current study:

(1) Field samples collected during the summer season were found to have very high levels of toxins and also higher degree of toxicity when compared to the winter samples. The seasonal toxicity that was observed could be due to the presence of high numbers of toxin-producing cyanobacteria during the summer season.

(2) 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.

(3) The cytotoxicity assay involving use of the African catfish (*Clarias gariepinus*) primary hepatocytes has been shown for the first time to produce results similar to those observed when using the mouse bioassay in assessing bloom toxicity. 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.

(4) Our preliminary assessment of the KNP dams show the four dams (Nhlanganzwani Dam, Mpanama Dam, Makhohlola Dam and Sunset Dam) investigated in this study to contain *M. aeruginosa* and cyanotoxin levels above the prescribed guideline value. Nhlanganzwani Dam appears to be the most affected dam by the cyanobacterial bloom.

Future studies will include use of HPLC to investigate the toxin profile of the field samples in order to fully describe the different classes/or types of toxins present in the samples. More validation studies using the catfish primary hepatocyte cell line will also be undertaken, to obtain a more comprehensive understanding about the sensitivity of this cell line to MC-LR. A more comprehensive study will also be undertaken in the KNP in order to fully monitor and assess the toxicity of cyanobacteria and to investigate the extent to which cyanobacteria affect wildlife in the park.

CHAPTER 11

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