Microcystin concentrations in a Nile crocodile (*Crocodylus niloticus*) breeding dam and vertical transmission to eggs

By

Alukhethi Singo: 14437105

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Supervisor: Prof C.J. Botha

Co-supervisor: Prof. J.G. Myburgh

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DEDICATIONS

In memory of my father, **Nditsheni Amos Singo**, you left fingerprints of grace and love in my life, I will forever be grateful to have once had a man like you, selfless, caring and full of support in my whole life and during this project, you and I were looking forward to the graduation! You shall not be forgotten.

DECLARATION

I declare that the dissertation which I hereby submit for the degree MSc at the University of Pretoria is my own work and has not been submitted by me or any other person for a degree at any other University.

Candidate Signature : **Date:**

Supervisor Signature : **Date:**

Co-Supervisor Signature: **Date:**

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LIST OF ABBREVIATIONS

16S rRNA	Bacterial small subunit of ribosomal ribonucleic acid
Abr	Abraxis
Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
BuOH	Butanol
CSIR	The Council for Scientific and Industrial Research
DWAF	Department of Water Affairs
DIC	Deviance Information Criteria
DO	Dissolved oxygen
ELISA	Enzyme-linked immunosorbent assay
EIA	Enzyme immunoassay
GAC	Granular activated charcoal
GST	Glutathione S-transferases
HPDI	Highest Posterior Density Interval
HBP	Hartbeespoort Dam
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LD_{50}	Median lethal dose (dose that kills 50% of treated animals)
MeOH	Methanol
MC	Microcystin
mcyA-J	Gene encoding the microcystin synthetase enzyme complex
MC-LR	Variant leucine and arginine in the positions of X and Z of microcystin
MC-RR	Microcystin variant arginine and arginine in the positions of X and Z of microcystin
MC-YR	Microcystin variant tyrosine and arginine in the positions of X and Z of microcystin
MC-RY	Microcystin variant arginine and tyrosine in the positions of X and Z of microcystin
MC-LA	Microcystin variant leucine and alanine in the positions of X and Z of microcystin
MC-[Dha7]	LR 7-desmethyl variant of MC-LR
MC-[Asp3]	LR 3-desmethyl variant of MC-LR
MCMC	Markov Chain Monte Carlo
Mdhb	N-methyldehydrobutyrin
Mdha	N-methyldehydroalanine

· MO	Month
· Nor	Norwegian
· NIVA	Norwegian Institute for Water Research
· NIV	Norwegian Veterinary Institute
· TADs	Toxin adsorbent disks
· TDS	Total dissolved solids
· SPATT	Solid-phase adsorption toxin tracking
· PP1	Protein phosphatases 1
· PP2	Protein phosphatases 2
· PPIA	Protein phosphatase inhibition assay
· PAC	Powdered activated charcoal
· PSDs	Passive sampling devices
· UN	United Nations
· WHO	World Health Organization

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ABSTRACT

Cyanobacteria or blue green algae are known for their extensive and highly visible blooms in rivers or dams. One of the most important cyanobacteria is *Microcystis aeruginosa* which can synthesize various microcystins that can affect the health of terrestrial and aquatic animals. Commercial Nile crocodile (*Crocodylus niloticus*) farming in South Africa is based on keeping breeders (adult males and females) in big dams on farms (captive-bred approach). Unfortunately, cyanobacterial blooms in the breeder dams are a concern to farm owners, managers and veterinarians. This research project focussed on the monitoring of microcystins in the Hartbeespoort Dam and a crocodile breeding dam over a period of nine months. A commercial, but expensive, Abraxis ELISA kit was compared to a much cheaper and robust Norwegian-developed ELISA to detect microcystins in fresh water. Another objective was to determine if microcystins were present in the contents of crocodile eggs and dead hatchlings.

Water samples were collected monthly from August 2014 to April 2015 at two sites, the Hartbeespoort Dam (control site) and the breeding dam of a commercial Nile crocodile (*Crocodylus niloticus*) farm. In addition, various water quality parameters including nitrate, phosphorous, chlorophyll a, oxygen saturation, pH and total dissolved solids (TDS) were determined to assess eutrophication. During the crocodile hatching season microcystin concentrations in unfertilized eggs, egg-shell membranes and in the yolk and liver of dead hatchlings were determined using liquid chromatography-mass spectrometry (LC-MS).

Water quality parameters showed that there was no significant difference between the two dams' (the Hartbeespoort and the breeding dam) eutrophic state i.e. phosphates, TKN and nitrates; they both seemed to be becoming more eutrophic as the nutrient supply to the dam was increasing. Furthermore, microcystin concentrations during peak summer months were generally higher at the Hartbeespoort Dam compared to the crocodile breeding dam. The two ELISAs as performed on water samples "as is" and following an adsorbent disk/methanol extraction method were positively correlated; however, the

correlation between the two assays was much stronger when using the adsorbent disk/methanol extraction as compared to using water “as is”. Besides dissolved oxygen all the other water quality parameters were not significantly different ($p > 0.05$) between the two sites.

Microcystin concentrations (MC-LR, MC-RR, MC-YR) in the crocodile egg and hatchling samples collected from batches with a good hatching rate (≥ 90%) ranged between 0 - 1.76 ng/g, with the highest concentration in the eggshell membranes. Microcystin concentrations in samples collected from batches with a bad hatching rate (≤ 10%) ranged from 0 – 1.63 ng/g with the highest concentration detected in the hatchling yolk. Although the “tissue” concentration levels were probably underestimated with the extraction method employed for LC-MS as the percentage recovery from spiked samples were very low. Bayesian analysis suggests that the liver, yolk and unfertilized egg all have similar microcystin concentrations, while the membranes have (with moderate to high certainty) higher microcystin concentrations.

In conclusion, when using the Norwegian ELISA it seems as though the use of a resin-containing adsorbent disk followed by methanol extraction is more reliable than analysing water “as is”. Following methanol extraction the results of the two ELISAs were strongly correlated, which suggests that the two ELISAs provide comparable results. There appears to be no difference in microcystin concentrations among good and bad clutches across all tissue types or within a specific tissue type. Vertical transmission of microcystins to the Nile crocodile egg does occur, but due to the small sample size, final conclusion cannot be made if microcystin affects Nile crocodile hatchling mortality and/or hatching of eggs.

Future studies will include a longitudinal study to be done since a single season of breeding is insufficient to conclude that microcystins do not contribute to the low hatching rate in Nile crocodiles.

CHAPTER ONE: INTRODUCTION

Cyanobacteria, previously known as blue-green algae, are considered to be the organisms responsible for the early accumulation of oxygen in the earth's atmosphere (Knoll, 2008). The original name of blue-green algae was based on the fact that they contain a compound called phycocyanin, giving them a slightly blue-green colour in appearance. They are widely distributed in fresh, slightly salty and marine environments, in soil and on moist surfaces. These prokaryotes are an ancient group of organisms that occur all over the world in environments as diverse as the Antarctic soils and volcanic hot springs (often where no other vegetation can exist) (Knoll, 2008). The metabolites synthesized by cyanobacteria, such as microcystins, can cause mortalities in domesticated livestock, wildlife and humans (Stewart *et al.,* 2008). Cyanobacterial blooms can result in a decrease in water quality including reduction of dissolved oxygen (DO) and subsequent death of aquatic animals, aesthetic nuisances (e.g. stenches, foams and crusts, fish tainting, unsightliness), and unpalatable and unsafe drinking water (Paerl 1988; Welch 2002). In South Africa, microcystins have been associated mainly with, but not limited to, *Microcystis aeruginosa,* which have been consistently recorded at the Hartbeespoort and Roodeplaat Dams (DWAF, 2000).

Researchers all over the world have been conducting investigations to determine the dynamics of toxin production (Carmichael, 1992; Carmichael, 1994; Fleming *et al*., 2002; Chorus and Bartram, 1999; Pearl *et al.*, 2001). In Brazil human mortalities were associated with the presence of microcystins in dialysis fluid (Jochimsen *et al*., 1998). Microcystin contaminations have become a significant matter within the agricultural sector (DWAF, 2000).

Reports are available on the effect of microcystin contamination in aquatic vertebrates, mainly fish (Ibelings *et al*., 2005; Xie *et al.,* 2005; Chen *et al*., 2006; Deblois *et al*., 2008; Wilson *et al*., 2008). Several studies have indicated that microcystin has an effect on the early-stage development of aquatic vertebrate animals (Oberemm *et al*., 1997, 1999). Not only does microcystin affect the developing embryos or the early-stage development of aquatic vertebrate animals, it also causes mortalities of aquatic animals, such as wild birds and turtles, which have been reported from around the world, e.g. Kenya (Krienitz *et al*.,

2003; Metcalf *et al*., 2006) and Japan (Matsunaga *et al*., 1999). However, to our knowledge there is no scientific report that documented microcystin concentrations in Nile crocodile eggs or in the tissues of hatchlings.

Microcystins are life threatening to animals and humans and their monitoring in surveillance programmes in fresh water bodies is expensive. It is therefore important to develop an inexpensive, robust, but sensitive and reliable monitoring tool for the detection of these toxins in fresh water bodies. The development of an ELISA by Norwegian researchers for the use in determining cyanotoxin concentrations have been reported (Samdal *et al.,* 2014). Thus, this study was aimed at comparing the sensitivity of this newly developed Norwegian ELISA with the commercially available Abraxis ELISA kit for the monitoring of microcystin in surface water. In addition, the concentration of microcystin LR, RR and YR in Nile crocodile egg contents, as well as in the liver of dead hatchlings was determined. The selected sites for determining microcystin concentrations in surface water was a crocodile breeding dam and a control site, Hartbeespoort Dam, over a nine month period, spanning the summer months of 2014/15.

CHAPTER TWO: LITERATURE REVIEW

2.1 Cyanobacteria in inland waters in South Africa

In 2000, an alert was issued by the Department of Water Affairs and Forestry (DWAF) to the general public, including fishermen and recreational users, warning them about the increase in pollutants in the Hartbeespoort dam. A second report, two years later, indicated the presence of high nutrient levels with eutrophication related problems in many South African surface water resources (DWAF, 2002). The continuation of eutrophication and global climate warming rapidly changes the environment and promote growth of potentially toxic cyanobacteria in inland waters which are important sources of potable water and for recreational activities. *Microcystis aeruginosa* is probably the most important bloom forming organism that synthesizes cyanotoxins in South Africa (Van Ginkel, 2003; Ballot *et al.,* 2014).

Several factors influence the growth of cyanobacteria, such as pH, acidity or the alkalinity, turbidity, cloudiness or purity of the water. Other factors such as temperature of the water (they grow well in warm climates) (McQueen & Lean, 1987), waste water rich in phosphates, nitrogen and nitrogenous compounds can also influence the growth of cyanobacteria and the release of toxins. Cyanobacterial blooms are largely influenced by climatic conditions which are the main reason for seasonal variations in the number of cyanobacteria present in a water body. Since the bloom is highly favoured by high temperatures, in temperate zones where the climate is cold to mild, mass occurrences of cyanobacteria are most dominant during the late summer and early autumn and may last 2-4 months, while in warmer regions with Mediterranean or subtropical climates, the bloom season may start earlier and persist longer (WHO, 1999). About 80 dams were monitored between October 2002 and September 2003 in South Africa and the results revealed that the Hartbeespoort Dam had high levels of eutrophication (DWAF, 2000). The outcomes were not surprising, as the Hartbeespoort Dam is renowned for its poor water quality since the mid twentieth century (Allanson & Gieskes, 1961). The dam is also known for its high concentrations of pollutants (Harding *et al.,* 2004).

High temperatures in summer and an increased level of eutrophication favour cyanobacterial growth, multiplication and toxin release (Reichwaldt & Ghadouani, 2011). Masango and his co-workers (2008) determined the microcystin-LR (MC-LR) concentration in Hartbeespoort Dam during the winter and summer of 2005/2006, using an ELISA assay (Abraxis-Microcystin ELISA kit). They determined higher microcystin concentrations during the summer compared to the winter, where the winter levels were even a 1000 times more than the prescribed guideline value of 1 µg/l (1 ppb).

Microcystis aeruginosa produces hepatotoxic microcystins which have a negative effect on the health of aquatic and terrestrial life, and pose a serious problem for potable water users (Mbukwa *et al.,* 2012). Because of the threat that microcystin poses, the Department of Water Affairs has warned people not to utilise this water owing to the danger microcystins pose (DWAF, 2002).

Hyenstrand and co-workers (1998) determined that cyanobacteria survival in fresh water is also influenced by their ability to live in low carbon dioxide (CO_2) conditions, high pH and their ability to regulate buoyancy through their gas vacuoles. Cyanobacteria have lower half saturation constants (*K*s) for CO_2 compared to other phytoplankton and have the competitive advantage of being able to use both free CO_2 and HCO_3 as a photosynthetic source of carbon (Talling, 1976; Shapiro, 1990). Furthermore, lower CO_2 stimulates increased buoyancy in cyanobacteria (Booker & Walsby, 1981; Klemer *et al*., 1982; Spencer & King, 1985). Cyanobacteria are mostly dominant in conditions of increased pH in environmental water, even though they do not contribute to the increase in pH. Dominance of *M. aeruginosa* over other cyanobacteria in fresh water bodies is associated with low P:N ratios and low NO_3^--N with sufficient NH_4^+-N concentrations (Jansen & Anderson, 1992).

2.2 Microcystins

The most common cyanobacterial toxins are the microcystins (MC) and the related nodularins. These cyclic peptides are synthesized mainly by the cyanobacterial genera *Anabaena, Anabaenopsis, Microcystis, Oscillatoria,* and *Nostoc*. Microcystins are cyclic heptapeptides which are potentially toxic to invertebrates, fish, and mammals at low concentrations (approximately 8 µg/l) (WHO, 1999). The World Health Organisation (WHO) recommends an allowable concentration of total microcystin in the water of 1 µg/l (1 ppb) (WHO, 2004). Total microcystin include both intracellular and extracellular microcystins. Variants of microcystins have been isolated of which the most common is microcystin-LR. Other common microcystin variants include YR, RR and LW. The different variants of microcystin differ with respect to the number of methyl groups and two amino acids within the ring (Fig. 1).

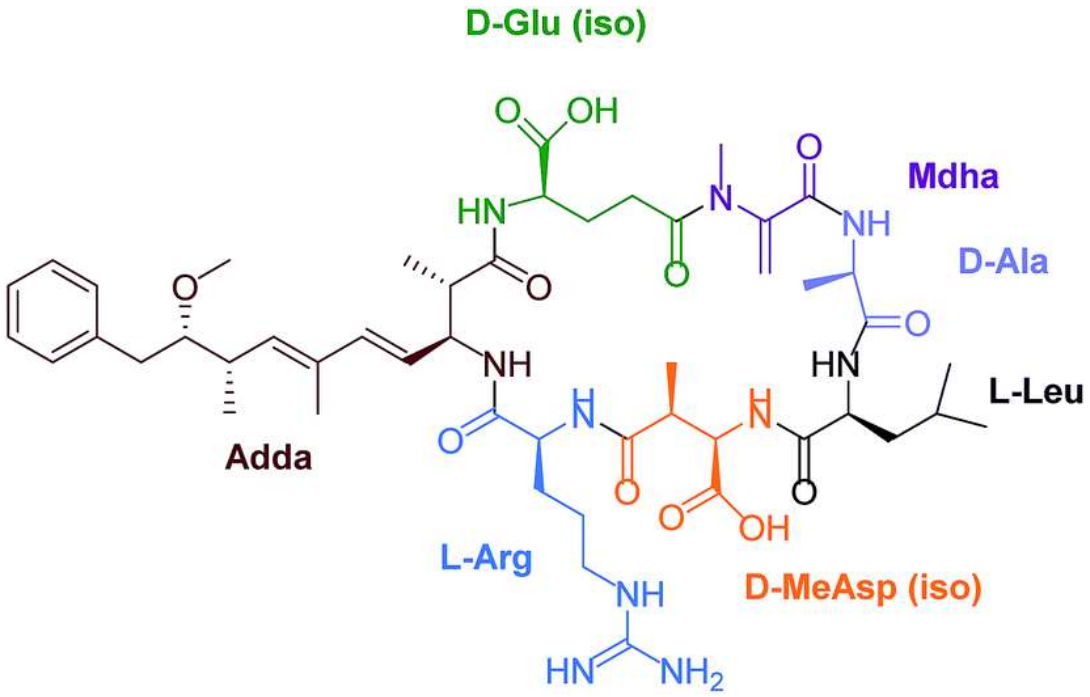

Figure 1: Chemical structure of microcystins (http://www.chemgapedia.de)

Microcystins and nodularins contain a specific amino acid, [Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic], side chain. The toxicity of microcystin is dependent mostly on the Adda-group, and the structural cyclic nature (Hoeger *et al.,* 2007). There are major differences in toxicity as well as in hydrophobic/hydrophilic properties between the variants. Microcystins inhibit protein phosphatases 1 and 2a by forming an irreversible covalent bond (MacKintosh *et al*., 1990). Microcystin functions as a protein phosphatase (PP1/PP2) inhibitor (Runnegar *et al*., 1993; Runnegar *et al*., 1999) causing hyper-phosphorylation resulting in cytoskeletal damage of hepatocytes, and collapsing of liver architecture leading to profuse haemorrhaging into the hepatic parenchyma and cellular necrosis (Carmichael, 1992; Falconer & Yeung, 1992). So far, nearly 80 variants of microcystins have been identified (Dietrich & Hoeger, 2005), which are responsible for mortalities in terrestrial wildlife, livestock (Briand *et al*., 2003) and fish (Landsberg, 2002) globally.

2.3 Purification of fresh water

Different treatment methods are used to control cyanobacterial toxins in potable water. Conventional treatment processes such as coagulation, flocculation, sedimentation and filtration can only remove up to 90% of the total cyanotoxins present if it is contained within healthy cyanobacterial cells (Chow *et al.,* 1999). Dissolved toxins (i.e. toxin that has been released from the cells) must be removed using additional treatments such as powdered activated charcoal (PAC), granular activated charcoal (GAC) and ozone or chlorine (Donati *et al.,* 1993). The doses of oxidant, PAC and the type of activated charcoal required for treatment are dependent on the type of toxin and the water quality.

2.4 Adverse/toxic effects: humans and animals

Generally, intoxication is acute and there is extensive liver damage, with massive pooling of blood in the liver and haemorrhagic shock (Carmichael, 1994). However, microcystins consumed at low doses over an extended period of time can also induce liver damage (Ueno *et al*.,1996) and can also give rise to the development of chronic gastrointestinal and liver disorders (Falconer, 1996). Subcellularly, microcystins

also cause disruptions of the normal cellular signalling mechanisms (oxidative stress) and mitochondrial changes, both mechanisms are involved in tumour initiation. Besides all the negative effects, microcystins have also been considered as a rich source of natural cytotoxic compounds with the potential to inhibit or prevent the growth of cancerous cells (antineoplastic effects) (Giliane *et al*., 2013).

South African outbreaks of cyanobacterial poisoning involving domesticated animal species have often been recorded (Kellerman, 2005). In the Western Cape the toxicity of the water where cattle and sheep had been drinking was confirmed following intraperitoneal administration to mice. *Microcystis aeruginosa* was the dominant cyanobacterium and the presence of microcystin-LR was demonstrated by high performance liquid chromatography (HPLC) (Van Halderen *et al.,* 1995).

In the Netherlands, (early autumn 2011) three dogs died after they drank water in Amstelmeer. The cyanobacterial scum from the lake contained up to 5.27 µg/l dry weight of microcystin. The vomitus of one of the dogs contained an average 94 µg/g microcystin on a dry weight basis. In both cases, microcystin-LR was the most abundant variant. This finding became one of the first reports of dog mortalities in the Netherlands ascribed to *Microcystis aeruginosa* blooms (Lürling & Faassen, 2013). Microcystin-produced from *Microcystis aeruginosa* strains have also been identified after a wildlife mortality event during 2007 at the Kruger National Park, South Africa (Fig. 2). Water samples that were collected and analysed using ELISA strongly incriminated microcystins as the cause of the wildlife mortality (Masango *et al.,* 2009) (Fig. 2).

In humans acute diarrhoea, due to gastroenteritis, and liver necrosis after exposure to microcystins (Byth, 1980; Turner *et al.,* 1990; Teixeira *et al.,* 1993; Pouria *et al.,* 1998; Annadotter *et al*., 2001) and an increased incidence of primary liver or colorectal cancer after chronic exposure (Yu, 1995; Zhou *et al*., 2002) have been reported. Recently, microcystins have been identified for the first time in the serum (average 0.23 ng MC-LR eq/ml) of chronically exposed humans (fishermen at Lake Chaohu, China) together with indications of hepatocellular damage (Chen *et al*., 2009a).

Recreational users are also at risk as these toxins can cause skin rashes, eye irritation, vomiting, fever as well as pains in muscles and joints. At a haemodialysis centre in Brazil, 50 patients died following exposure to microcystins present in the dialysis fluid (Jochimsen *et al*., 1998). Drinking of contaminated water by humans has also led to intoxication and can even result in death (Giliane *et al*., 2013). Furthermore, the increased incidence of liver and colon cancer has been ascribed to the presence of cyanotoxins in drinking water sources (Giliane *et al*., 2013).

Figure 2: A zebra in the Kruger National Park that died due to microcystin poisoning (Courtesy of J.G. Myburgh, University of Pretoria).

2.5 Adverse effect of microcystins on aquatic species

In recent years, there have been an increasing number of studies to evaluate the effect of microcystins on aquatic vertebrates, with the majority of these studies focusing on fish (Vasconcelos, 1999; Magalhàes *et al*., 2001, 2003; Mohamed *et al*., 2003; Ibelings *et al*., 2005; Xie *et al*., 2005; Chen *et al*., 2006; Deblois *et al*., 2008; Wilson *et al*., 2008). Microcystins are potentially toxic to fish, invertebrates and mammals at a concentration as low as 8 µg/l (Codd *et al*., 2005). Chen and co-workers (2009b) reported the recovery

of microcystins from the eggs of a turtle (*Pelodiscus sinensis*), thereby suggesting the possible vertical transmission of microcystins from adult turtles during egg formation. A toxic effect caused by purified microcystins and an aqueous cyanobacterial extract has also been demonstrated in embryos of zebra fish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*) or chub (*Leuciscus cephalus*) (Oberemm *et al*., 1997; Oberemm *et al*., 1999). Embryotoxic effects include significant mortality, delayed hatching, decreased number of hatchlings, suppression in embryonic development, disturbance of air bladder filling, and significant inhibition of glutathione *S*-transferases (GST) in *Aphanizomenon sp.* and *Planktothrix sp.* (Palikova *et al.,* 2007).

Microcystin-LR (MC-LR) is a potent inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A). Protein phosphatase 1 and PP2A are critical regulators in embryonic development. However, the potential deleterious effects of MC-LR on embryonic development are controversial (Jacquet *et al.,* 2003). Microcystin-LR has been demonstrated to be highly toxic to Japanese medaka (*Oryzias latipes*) embryos, but not for zebra fish or rabbit embryos (Eriksson *et al*., 1990; Runnegar *et al*., 1995). The difference is probably due to membrane impermeability that impairs the transfer of MC-LR into the cytoplasm of zebra fish and rabbit embryos (Eriksson *et al*., 1990; Runnegar *et al*., 1995). Furthermore, cyanobacterial toxins may have adverse effects on fish under field conditions (Wang *et al*., 2005; Rodger *et al*., 1994). Anderson and co-workers (1993) injected fish embryos with microcystins and noticed a consistent display of hepatobiliary abnormalities, such as liver hypertrophy and hepatic haemorrhage in post-hatching juveniles.

Small fish species over the years have been useful both in environmental monitoring and as all-purpose test animals in toxicity and carcinogenicity bioassays (Law, 2001). Particularly zebra fish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*), which can be bred in large numbers, have been used as they have low maintenance and bioassay costs, and a low incidence of tumours. The embryos of these fishes are also excellent models for monitoring aquatic pollution and its toxic effects. Their rapid development and transparent chorion also allow for the study of embryogenesis (Jacquet *et al.,* 2003).

Despite the toxic effect of microcystins, Chen and co-workers (2006) reported that the silver carp (*Hypophthalmichthys molitrix*), which feed on microcystins species, might have developed resistance to the toxins that were recovered from its intestine. This finding suggests that silver carp and bighead fish (*Hypophthalmichthys nobilis*) are probably more resistant to microcystin exposure than other fish species and animals.

When comparing the silver carp with the turtle (*Pelodiscus sinensis*), duck (*Anas platyrhynchos*) and a water bird, the black-crowned night heron (*Nycticorax nycticorax*), although the phytoplanktivorous silver carp directly fed on toxic cyanobacteria and therefore ingested microcystins, they did not accumulate considerably more microcystins in their organs or tissues such as the liver, small intestine, kidney, heart, bile, lungs and the muscles (Chen *et al*., 2006; 2007). In addition, a high concentration of microcystins was also recovered from the gonad, egg yolk and egg white of the night heron and duck, suggesting transmission of microcystin to the night heron and duck embryos. It should be noted that the vitellus sustains both structural (organogenesis) and metabolic processes (energy expenditure) during the early and late development stages of the embryo (Oberemm, 2001; Liu *et al*., 2002). Microcystin induced histopathological modifications of the digestive tract (in particular the pancreas) of medaka fish (*Oryzias latipes*), and have been detected in their newly hatched embryos. MC-LR induced the inhibition of both yolk sac resorption and swim bladder expansion and also caused a strong decrease in the mass and size of the liver of the embryos of the medaka (Huynh-Delermec *et al*., 2005). However, no acute effects of high or environmentally relevant concentrations of MC-LR, -YR, -RR on fish growth and organogenesis have been established during embryonic life or after hatching (Huynh-Delermec *et al*., 2005).

2.6 Monitoring of cyanotoxin concentrations in fresh water bodies

The universal occurrence of toxic cyanobacteria as well as concerns about contamination and potential consequences of exposure to cyanotoxins in recreational and drinking water have prompted the development of numerous methods to detect, identify and quantify toxins and their producers (Kurmayer & Christiansen, 2009; Lawton *et al*., 2010). Comprehensive scientific studies which were conducted on

the extraction and detection of the cyanotoxins, specifically the microcystins, have led to various methods of analysis such as enzyme-linked immunosorbent assay (ELISA) (Rapala *et al*., 2002), protein phosphatase inhibition assays (Li *et al.,* 2004) liquid chromatography (Moreno *et al*., 2004) and capillary electrophoresis (Gago-Martínez *et al*., 2003).

However, the development of an easy-to-use, fast, robust and inexpensive method for the detection of low concentrations of cyanotoxins in fresh water has been prioritised (Xie *et al.,* 2007).

2.6.1 Solid phase adsorption toxin tracking (SPATT)

Mackenzie and co-workers (2004) described a method to monitor cyanobacterial toxins that utilises adsorption to resins. They introduced the notion of algal toxins surveillance by passive binding to solid-phase adsorption toxin tracking (SPATT) devices in marine environments. The SPATT devices consist of polyester mesh bags containing the activated polystyrene divinylbenzene resin and adsorb lipophilic toxins dissolved in sea water. The passive samplers offer an opportunity to sample a series of environmental pollutants over time, imitating the elements of natural uptake (Verhaar *et al*., 1995; Kot-Wasik *et al*., 2007, Rundberget *et al*., 2009).

The SPATT devices provide a more suitable way for time-averaged sampling before or during algal blooms, compared to shellfish or phytoplankton analyses alone (Mackenzie *et al.,* 2004, Rundberget *et al*., 2009). Pedro and co-workers (2013) concluded that 24 hours exposure of passive sampling devices (PSDs) is not enough to adsorb the maximum amount of toxins from the water for analysis. To enhance toxin adsorption; they should at least be placed approximately 1 meter below the water surface (Pedro *et al.,* 2013).

Extracting cyanotoxins from the SPATT devices was much easier than extracting the toxins from shellfish. Sample preparation was rapid and simple, and few interfering compounds were present in the sample extracts. Since the devices adsorb toxins released directly from the algae into the water, the toxin profile is much simpler than the metabolite profile usually present in shellfish. This results in easier assays, fewer

toxins to quantify, and lower detection limits for the targeted toxins. The resin used in the SPATT bags was tested and validated by MacKenzie and co-workers (2004) for a range of algal toxins commonly present in New Zealand namely pectenotoxin-2 (PTX-2), PTX-2 seco acid (PTX-2 SA), yesso toxin (YTX), okadaic acid (OA) and dinophysistoxin-1 (DTX-1).

2.6.2 Toxin adsorbent disks (TADs)

In 2009, Rundberget and co-workers introduced a more advanced method of monitoring algal toxins (Fig. 3) with toxin adsorbent disks (TADs), based on the method described by MacKenzie *et al*. (2004). This is a modification of the SPATT bags by MacKenzie *et al*. (2004), where the preparation of the disks, their deployment, extraction and exposure were refined. The authors concluded that this new design was quick and easy to use. The frames and algal mesh could be washed and re-used. Furthermore, the frames hold the resin in a thin layer that increases the exposure of the resin to the toxins in the water. The TADs are cheap to produce and convenient to use with enzyme-linked immunosorbent assays (ELISAs) and ideal to monitor cyanotoxins (Rundberget *et al*., 2009).

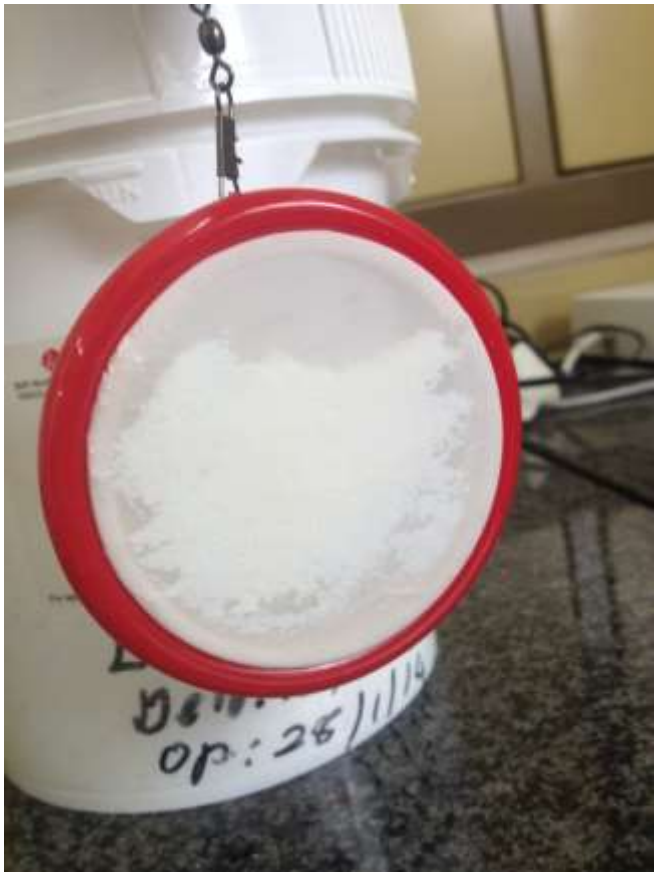

Figure 3: Toxin adsorbent disk (TAD)

2.7 Determination of cyanotoxin concentrations

2.7.1 ELISA (Enzyme-Linked Immunosorbent Assay)

In living organisms immune reactions are triggered by foreign substances (antigens). The one category of immune response is a specific immune response which depends on prior exposure to the foreign substances and recognition of these substances on subsequent exposure to them. Antibodies are specialized proteins which are part of the organism's immune defense mechanism and can specifically combine with the antigen that induced its production (Vander, 1980). In ELISAs, the specific recognition between antigens and antibodies are used in plate-based assays for detection and quantification of foreign substances such as peptides, proteins and hormones. Other names, such as enzyme immunoassay (EIA), are also used to describe the same technology. In an ELISA an antigen must be immobilized to a solid surface and then complexes with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product. In Fig. 4, as illustrated, the antigen can be immobilized to the plate directly or via a "capture" antibody. The enzyme that reacts with the substrate to produce the quantifiable colour reaction can either be conjugated to the primary antibody or a secondary antibody that will bind to the primary antibody.

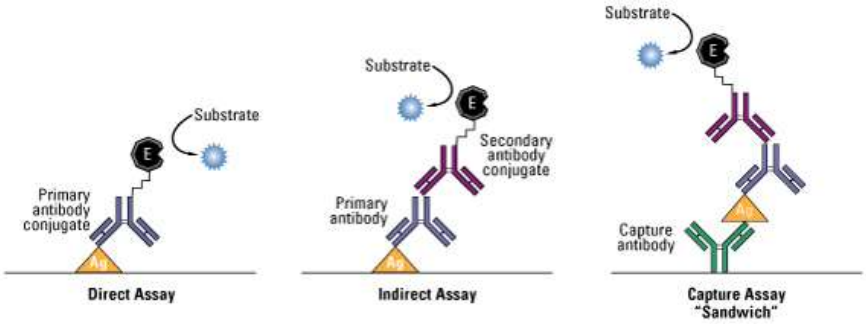

Figure 4: Different non-competitive ELISA configurations

(http://www.piercenet.com/method/overview-elisa)

Competitive ELISAs are based on measuring the competition in binding of the unknown "sample" antigen and the known "labelled" antigen. The assay can also be reversed to measure the competition of labelled and unlabelled antibody to antigen.

In the indirect competitive ELISA the plate is pre-coated with known labelled antigen. The primary antibody and sample antigen are then added simultaneously. With increasing concentrations of sample antigen, less primary antibody will be available to bind to the bound known antigen and thus, a lower signal will be generated once the bound and unbound sample have been removed after washing.

Competitive ELISAs are easier to quantitate and are less influenced by contamination, but require higher accuracy during reagent dispensing and require a pure labelled ligand. In the non-competitive ELISA, dispensing errors of reagents, except the sample, have little effect on the result, and easier to perform, but is more susceptible to cross reactions and non-specific binding (Kemeny, 1991).

ELISAs have been utilized in various disciplines, such as quality control checks in different industries and as a diagnostic tool in medicine, veterinary medicine, plant pathology and toxicology. An ELISA is relatively inexpensive, personnel require minimal training and it is easy to set up and use in the laboratory.

ELISA could be used to determine the presence of cyanotoxins in fresh water bodies. This assay uses antibodies (both polyclonal and monoclonal) against cyanotoxins (Metcalf *et al*., 2000; Zeck *et al*., 2001). It can detect very low concentrations of cyanotoxins in water samples, organisms and tissues (Lawton *et al.*, 2008; Sivonen, 2008). Koreivienė and Belous (2012) in their study concluded that both the ELISA and the colorimetric protein phosphatase inhibition assay (PPIA) have low equipment requirements and allow for the rapid, easy, effective and sensitive detection of total microcystin concentration in samples. The ELISA assay continues to remain the preferred assay in quantifying cyanotoxin content in aquatic systems due to its high sensitivity and ease of analysis (Masango *et al.,* 2008).

2.7.1.1 Abraxis Microcystin-ADDA ELISA

The kit is supplied with plates pre-coated with the known antigen. The kit is also revalidated for reagent batch differences. It can accommodate 5% methanol in the samples. All the reagents are supplied (Abraxis kit product sheet).

Pedro *et al*. (2013) assessed microcystin concentration in fresh water reservoirs in Mozambique. Seven variants of microcystins were detected using the Abraxis ADDA-ELISA kit in combination with LC-MS/MS. MC-LR was the most frequent and abundant variant of microcystin detected in 77% of the samples. The Abraxis-ADDA ELISA exhibited cross reactivity with other cyanobacterial cyclic peptide toxin congeners and it is an expensive screening tool (Pedro *et al*., 2013).

Numerous organic and inorganic compounds commonly present in water samples have been evaluated and found not to interfere with Abraxis-ADDA ELISA. However, due to the high variability of compounds that may be found in water samples, test interferences caused by matrix effects cannot be completely excluded. Samples containing methanol must be diluted to a concentration <5% methanol to avoid matrix effects. The detection limit of this assay, based on MC-LR, is 0.1 µg/l (ppb) and it is a good screening tool for the determination of microcystin concentrations in fresh water bodies (Pedro *et al*., 2013).

2.7.1.2 Norwegian ADDA ELISA

Samdal and co-workers (2014) developed an ELISA method which is much cheaper and robust. Unlike the Abraxis ADDA ELISA kit, the reagents and plate are not ready to use, the plate has to be coated and buffers need to be freshly prepared. The user has to adjust the incubation conditions to compensate for reagent batch differences. The protocol is currently under validation, but the Standard Operating Procedure was supplied.

Both the Abraxis and Norwegian microcystin-ADDA ELISAs are indirect competitive ELISAs for the congener-independent detection of microcystins and nodularins by specific antibodies. The signal generated is inversely proportional to the amount of antigen in the specimen.

2.8 Other methods of analysis

2.8.1 Liquid Chromatography/Mass Spectrometry (LC-MS)

Liquid Chromatography/Mass Spectrometry (LC-MS) is an integration of liquid chromatography and mass spectrometry. A mass spectrometer is typically composed of three major parts: ion source, mass analyser and detector. While the ion source converts sample molecules into ions, the mass analyser revolves these ions either in a time-of-flight tube or in an electromagnetic field before they are measured by the detector (Winding *et al.,* 1996). This technique can be used for both screening and confirmation, allowing identifying unambiguously the single compounds, at ppb or sub-ppb levels (Lawton & Edwards, 2008).

2.8.2 Radioimmunoassay

Radioimmunoassay's (RIA) were used before ELISA and were the only way of conducting an immunoassay. RIA use radioactively labelled antigens or antibodies. The radioactivity provides the signal which indicates whether a specific antigen or antibody is present in the sample. RIA was first described in a scientific paper by Yalow and Berson published in 1960. The use of radioactivity unfortunately has negative effects on the health of personnel and a safer and alternative way was sought.

2.9 The Nile crocodile (*Crocodylus niloticus*) egg

Crocodiles are sexually dimorphic and adult males are larger than the females. Copulation takes place in water. All crocodiles lay hard-shelled eggs, which may weigh between 80-160 grams. The female lays 12-48 eggs per nest (clutch) which depends on her age, size and species. The Nile crocodile (*Crocodylus niloticus*) digs a hole in the ground and refills it with dirt after the eggs have been deposited. The incubation period takes about 55 to 100 days. The gender of the developing embryo is determined by the temperature during the incubation period. Until hatching occurs, the female remains close to the nest to protect the eggs from predators (Wermuth & Ross, 2014).

2.9.1 Development of the egg

Crocodilian eggs share many general structural, biochemical and developmental properties with their avian counterparts (Packard *et al*., 1977; Ferguson, 1982). This similarity, along with their large yolk and calcium carbonate-shelled egg, provides for comparison of yolk structures between crocodiles and birds.

Although when fully formed, the average weight of a large egg of a hen is 57 g which is less than what the crocodilian egg weighs. The hen Yolk (ovum) components make up 32% of the egg's weight and are formed by the liver and transported to the ovary via the blood stream, while the albumen (egg white) components make up 58% of the egg's weight and are formed by the growing follicles and oviduct (Kan & Petz, 2000; United States Department of Agriculture, 2000; Coutts & Wilson, 2007). The shell of a hen makes up 10% of the egg's weight and is the last to be formed in the process of egg formation (Kan & Petz, 2000; United States Department of Agriculture, 2000; Coutts & Wilson, 2007).

The hen's egg is formed gradually over a period of 25 hours and many organs and systems contribute to the various substances that become part of the egg (Coutts & Wilson, 2007). During the crocodile egg formation which is similar to the hen's egg formation as shown in Table 1, the yolk material is transported from the liver, where it is synthesized, to the ovary via the blood. A small dose of non-toxic dye administered to a pre-laying female crocodile will stain all yolk lipids transported through the follicle wall

on the day of dosing (Grau, 1976). In the case of crocodile egg formation, where the female lives in water that is contaminated with cyanobacteria, it is possible that microcystins may be incorporated in the egg content.

Table 1: Maternal organs that form the different components of a crocodile egg (Jambalang, 2011).

ORGAN OF FORMATION	EGG COMPONENT
Liver	Yolk (Protein & Albumen)
Oviduct (Magnum)	Albumen
Shell Gland (between Isthmus & Vagina)	Shell
Shell Gland	Cuticle

2.10 Justification

The recently developed Norwegian ELISA has not been used to monitor microcystin concentrations in fresh water bodies in Africa. This project was designed to evaluate and contribute to the validation of the Norwegian ELISA under African conditions.

At the crocodile farm (Le Croc), there has been a decrease in the hatching rate of crocodile eggs. Although eggs are been laid, there is a poor hatchability. In 2013, the hatching rate was 62% which was an improvement when compared to 48% in 2012. Le Croc farm considers a hatching rate of 75% and above as an excellent rate, and 62% to 65% as a fair hatching rate and anything below 61% as a poor hatching rate.

This study investigated if poor hatchability at Le Croc can be attributed to the presence of cyanobacteria, as a consequence of eutrophication of the water in the crocodile breeding dam, and vertical transmission

of microcystins to the crocodile eggs during egg formation. Based on the study that has been reported by Chen *et al*., (2009b), it is possible that microcystins will be recovered from the crocodilian eggs.

2.11 Aims

1. To compare the sensitivity and accuracy of the Norwegian ELISA to the commercial Abraxis ELISA kit.
2. To determine the difference in nitrates and total phosphate concentrations, total dissolved solids (TDS), oxygen saturation, pH and chlorophyll A levels of the water in the crocodile breeding dam and the Hartbeespoort Dam.
3. To determine the presence of microcystin in the contents of unfertilized crocodile eggs, the egg-shell membranes that remained after hatching and the yolk and liver of dead hatchlings.

CHAPTER 3: MATERIALS AND METHODS

3.1 Research site

The study was conducted in the North-West Province, South Africa at two different sites:

(i) Hartbeespoort Dam - situated in the North-West Province of South Africa. It lies in a valley to the south of the Magaliesberg range and north of the Witwatersrand range, about 35 kilometres west of Pretoria. The Hartbeespoort Dam was originally planned as a water supply reservoir for Pretoria and Johannesburg, but after completion was mainly used for irrigation and recreation (Cochrane, 1987; Swanepoel *et al.,* 2008). A map designating the location of Hartbeespoort Dam and the crocodile breeding dam is presented in Fig. 5. Figures 6 and 7 indicate the sample collection sites at Hartbeespoort Dam (control site).

(ii) The Crocodile farm, Le Croc, is situated in the North-West Province of South Africa. It is found in the area of Sanddrift, 20 kilometres north of the town called Brits (Fig. 5). Le Croc is the trading name for the crocodile tannery, breeding farm (Fig. 8) and guesthouse operations. In 2007, a tannery was established and fitted out with the latest technological equipment. During 2008 Le Croc commenced the production of high quality leather with its team of professional staff. Today, Le Croc's leather is well received and respected by the top fashion houses of the world.

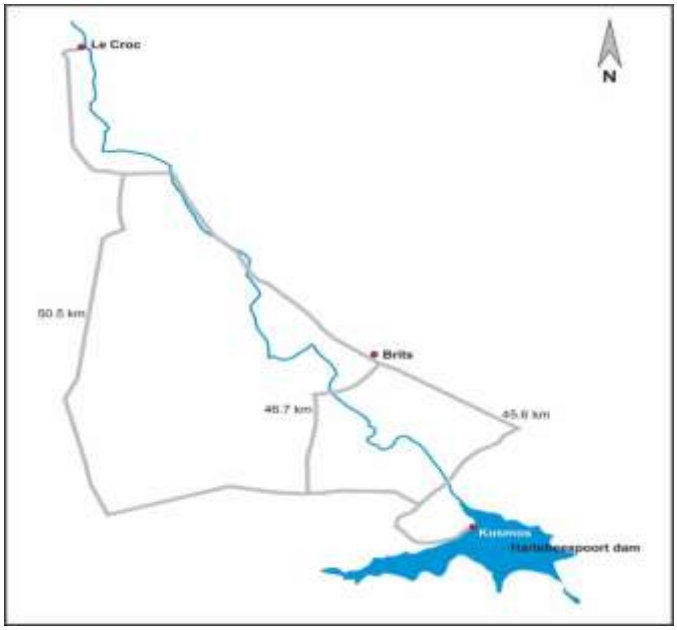

Figure 5: Locations of the Hartbeespoort Dam and the crocodile breeding dam, North-West Province, South Africa.

Figure 6 (left) & 7 (right): Collection site at the Cosmos Marina, Hartbeespoort Dam

Figure 8: Crocodile breeding dam, Le Croc, Brits, North-West Province, South Africa. The green colour of the water is due to cyanobacteria.

The water used in the crocodile breeding dam and tannery is obtained from the Crocodile River bordering the farm. The breeding dam houses more than four hundred adult crocodiles. Fig. 9 shows an adult crocodile covered with a thick layer of blue-green algae. The water is changed only once a year as this is a big dam, but the dam is topped up every week due to evaporation. The algal bloom due to eutrophication of the breeding dam is a big concern for the farm owner and Le Croc management team (Stefan van As, Personal communication, 2014).

Figure 9: Crocodile covered with blue-green algae (Myburgh 2009)

3.2 Water samples

3.2.1 Collection

Samples were collected during the day between 12:00 – 14:00 from the two collection site, Le Croc breeding dam and the Hartbeespoort Dam. For the water samples at the Le Croc breeding dam, a bucket attached to a rope was used to collect a sub-surface water sample which was lowered from the bridge spanning the dam, this was done for safety reasons, and at the Hartbeespoort Dam; a bucket was used not attached to the rope to also collect the sub-surface water sample. The water was scooped twice using a clean bucket to stir the dam water before the actual sample was collected from the two sites. A total volume of eight litres of water sample was collected into light protected glass and plastic bottles. Samples were immediately transferred to a cooler box containing ice blocks.

Samples were collected as follows:

Sample A - Five litres of water sample was collected into plastic bottles for microcystin ELISA analysis.

Sample B - One litre was collected in plastic bottles for chemical analysis.

Sample C - Two litre water samples were collected in a glass bottle for chlorophyll A determination.

Water samples were collected according to the *Condensed Laboratory Methods for Monitoring Phytoplankton, including Cyanobacteria, in South African Freshwaters* (WRC, 2008).

Samples collected were submitted to the Council of Scientific and Industrial Research (CSIR) for nitrate, total phosphate and chlorophyll A analysis. Additional samples were further processed (methanol extraction) and analysed to determine the microcystin concentration by the two ELISA methods in the analytical laboratory of the Department of Paraclinical Sciences at Onderstepoort and by LC-MS at the National Horse Racing Authority (NHRA) laboratories.

Measurements of water pH, conductivity (K), temperature (T °C) and dissolved oxygen (DO) were carried out at the sampling sites using a portable Hach HQ40d meter. The following water quality parameters were determined at the time of water collection at the two sampling sites: pH, total dissolved solids (TDS) and oxygen saturation (DO).

Water samples were collected monthly from August 2014 to April 2015 at the Hartbeespoort Dam (Cosmos Marina) (Figs. 6 & 7) and the Le Croc crocodile breeding dam (Fig. 8). The Hartbeespoort Dam is known for its high eutrophication level and *Microcystis aeruginosa* blooms. However, the crocodile breeding dam has never been monitored for cyanotoxins.

3.3. Water sample processing

3.3.1 Microcystin extraction

Five litres containing water samples were frozen at -20°C, thawed three times and sonicated for 5 min to break the cells in order to release intracellular microcystin. After sonication, samples were filtered through nylon net (90-µm mesh size) as indicated in Fig.10**,** to remove impurities. One litre of the sample was added to 3 g of activated Dialon HP-20 resin (Sigma-Aldrich). The resin was activated by soaking in 100% methanol for 15 min, followed by soaking three times for 5 min using distilled water respectively, according to the manufacturer's (Supelco) instructions. The resin and samples were shaken overnight for 19 h on a shaker (model 202, Labotec) as indicated in Fig. 11, to allow sufficient contact time for the microcystins to be adsorbed to the resin.

Figure 10 (left): Filtering of sonicated sample through nylon net (90-µm mesh size) to remove impurities.

Figure 11 (right): Water samples with added 3 g of HP20 resin on a shaker overnight for 19 h.

The resin (Fig. 12) was then quantitatively transferred to a 25 ml Varian Bond-elute reservoir fitted with non-absorbent cotton wool and washed free of salts using 30–50 ml deionized water. Excess water was removed from the column by using a plunger. Ten ml methanol was added to the column and the resin was stirred gently, and left to stand for 15 min. The column was then eluted slowly (0.5–1 drop/s) and when finished, the process was repeated with another 10 ml methanol. Finally, an additional 3 ml methanol was pushed through to flush the remaining microcystin from the column. The eluted extract was kept in the freezer at -20°C in 20 ml screw cap glass tubes (Rundberget *et al.,* 2009).

Figure 12: Methanol extraction process of microcystin from resin quantitatively transferred into a 25 ml Varian Bond-elute reservoir fitted with non-absorbent cotton wool.

3.3.2 Analysis

The Abraxis and Norwegian ELISAs were used at the Onderstepoort laboratory for analysis. For the Norwegian ELISA, analysis was started with varying dilutions. Starting dilutions for the Abraxis ELISA was made depending on the level of the microcystin determined by the Norwegian ELISA.

Sample A: A five ml sample of the methanol extract was sent to the NHRA laboratory for analysis using liquid chromatography–mass spectrometry (LC-MS). The remaining of the methanol samples were used for microcystin analysis in the Toxicology Laboratory at Onderstepoort. Both ELISA's were also performed on water collected and following freezing, thawing and sonication ("as is").

Sample B: A one litre water sample was collected in plastic bottles and sent to the Council of Scientific and Industrial Research (CSIR) in Pretoria, South Africa within eight hours of collection for total phosphate and nitrate analysis.

Sample C: A two litre water sample was collected in a glass bottle and submitted to the CSIR in Pretoria, South Africa within eight hours of collection for chlorophyll A analysis.

3.3.2.1 Control – spiking of water

A blind experiment was performed in the laboratory where samples were also sent to the Onderstepoort Veterinary Institute (ARC-OVI) for comparison purposes. Mill Q water was spiked with three different concentration of microcystin LR (Abraxis). The spiked Milli Q water samples were analysed to determine concentrations using both the Abraxis ELISA and the Norwegian developed ELISA. Water samples were also sent to the reference laboratory (ARC-OVI) for analysis using the Abraxis ELISA. The results of the three analyses, the Abraxis and Norwegian ELISA's and the ARC-OVI analysed by Abraxis too were compared and the mean of the three pairs of observation of the spiked samples shows that there is no significant difference for the matrix effect for the three sets of samples, p=0.92.

3.3.2.2 Cyanotoxins analysis

Cyanotoxins concentrations in water were analysed using the two different ELISA methods i.e. the commercial Abraxis ADDA ELISA kit and developed Norwegian ELISA.

3.3.2.2.1 Abraxis ELISA

The assay was performed following the manufacturer's instructions provided on the package insert. Analysis of the samples was conducted in duplicate. The methanol extracted samples were diluted to ensure that the concentration of the methanol did not exceed 5% to avoid false positive results. Absorbance was read at 450 nm using a microplate reader (Biotek Synergy GEN 5) within 15 min after the addition of the "stop solution" to quench the reaction.

3.3.2.2.2 Norwegian ELISA

The ELISA plate was pre-coated overnight with the coating antigen supplied. The reagents were prepared according to the protocol provided by the Norwegian scientists. Sample analysis was performed as described by the Norwegian standard operating procedure (SOP, Norwegian Veterinary Institute, Oslo, Norway). The extracts were diluted to contain less than 10% methanol. Absorbance was read at 450 nm using a Biotek Synergy (GEN 5) microplate reader within 15 min after the addition of the "stop solution" (10% H_2SO_4).

3.4. Crocodile egg and hatchling samples

Le Croc's crocodile hatching season (The incubation period is 76 days) lasts from late November until early February. Sample collection was undertaken during the peak of the hatching season, December 2014 to January 2015. Samples were collected from good clutches (≥90% hatching rate) and called "good batches", and bad clutches (≤10% hatching rate) and called "bad batches". Five (5) to ten (10) samples were collected from each clutch during each sampling trip (5 trips) that was made depending on the hatching rate of the eggs. Samples from 53 clutches were collected. The following samples were collected, 55 unfertilized eggs, 188 egg-shell membranes of hatched eggs, 78 egg yolk of dead hatchlings and 78 livers of dead hatchlings and one control liver collected from the abattoir after the crocodile was routinely slaughtered.

3.4.1 Dead hatchling's liver and yolk

The liver was carefully removed from dead hatchlings using a scalpel (Fig. 13 – 16). The length of the liver ranged between 1-2 centimetres with a weight ranging from 0.5 to 2 grams. A large number of hatchlings died soon after hatching and as the complete resorptions of the yolk into the abdomen has not yet occur, the yolk was also sampled. The collected samples were stored on ice in a cooler box and transported back to the laboratory.

Figure 13 (top left): Removal of the liver as part of sample collection.

Figure 14 (top, right): "Dead-in-egg" crocodile hatchling.

Figure 15 & 16 (bottom pictures): Crocodile hatchlings that died shortly after the hatching process.

3.4.2 Unfertilized egg yolk

The unfertilized eggs depicted in Fig. 17 were carefully cracked open to collect the egg yolk into a plastic container using a pair of scissors. The egg yolk was weighed before being placed on ice in a cooler box.

Figure 17: Unfertilized crocodile eggs.

3.4.3 Egg-shell membranes of hatched eggs

The egg membranes were extracted from the inside of the shell of hatched eggs using a disposable fork as indicated in Fig. 19. The egg-shell membranes were placed into a plastic container. The mass of all the collected membranes were also weighed using a weighing balance before they were pooled and stored on ice in a cooler box.

Figure 18 (left): Hatched crocodile eggs.

Figure 19 (right): Removal of the remainder of the egg-shell membranes from hatched eggs using a plastic fork.

3.5 Crocodile sample processing and analysis

The weight and length of the dead hatchlings were measured as indicated in Fig. 20. The liver, egg membrane and yolk samples were transported on ice in a cooler box to the laboratory where the samples were immediately frozen at −20°C.

Figure 20: Measurement of the body length of a crocodile hatchling.

3.5.1 Crocodile egg and hatchling sample processing

Later the samples were thawed and categorized into three groups for analysis based on the season of collection (early, middle and late collection); the samples were pooled and homogenized in a glass beaker using a PRO200 homogenizer. The crocodile egg and hatchling samples were sent to the National Horse Racing Authority (NHRA) laboratory for LC-MS analysis to determine microcystin concentrations.

3.5.2 LC-MS analysis of water, crocodile egg and hatchling samples

After processing of the water, tissue and egg samples in the laboratory, samples were sent to the National Horse Racing Authority (NHRA) laboratory for analysis. The microcystin concentrations (MC-LR, MC-RR, and MC-YR) in the organ samples were analysed using LC-MS.

3.5.2.1 Chemicals and equipment

HPLC grade methanol, acetonitrile and hexane were obtained from Burdick and Jackson, USA. Ammonium acetate (>98%) and formic acid (98-100%) were obtained from Sigma-Aldrich Chemie, Germany. Clean-up C18 (6cc, 500mg) solid phase extraction (SPE) cartridges from UCT, USA.

GM200 Knife Mill (Retsch GmbH, Germany), IKA Ultraturrex homogeniser, Zymark Turbovap.

3.5.2.2 Extraction method

3.5.2.2.1 Preparation of the calibration curve

A combined standard was prepared in methanol containing 1000 ng/ml of each of the microcystins. Serial dilutions were made to obtain a range between 500 and 62.5 ng/ml.

3.5.2.2.2 Water samples

At the NHRA, water samples were weighed to determine the exact volume and evaporated under nitrogen until dry. Samples were dissolved in 200 µl methanol. An aliquot of each sample (20µl) was diluted to

total volume of 520 µl and analysed. Samples that exceeded the calibration range were diluted further and analysed.

3.5.2.2.3 Tissue samples

Tissue samples were extracted according to Bruno and co-workers (2009). Quality control (QC) for each sample was prepared for each tissue type from the samples supplied. An aliquot of the milled samples and QC's were weighed and homogenized in 10 ml 5% methanol in acetonitrile. The samples were centrifuged, the supernatant transferred to a clean tube and the pellet re-extracted. The combined supernatant contained a lot of visible fat and a 10 ml hexane wash was introduced to remove excess fat. The supernatant were then evaporated under nitrogen. Dried samples were dissolved in 1 ml methanol, 4 ml water was added and it was applied to the SPE columns which were conditioned with 2 ml methanol followed by 2 ml water. Columns were washed with 5 ml methanol, followed by 5 ml 1% formic acid in methanol. Elutes were dried under nitrogen and reconstituted in 200 µl methanol and analysed.

3.5.3 LC-MS analysis

3.5.3.1 LC conditions

HPLC was an Agilent 1260 Infinity instrument (Agilent, California, USA) with an XSelect CSH C18 column 150 × 2.1 mm, 5µm particle size (Waters Corporation, USA). The column temperature was 40°C and the flow rate 350 µl/min. The mobile phase was a gradient of water to acetonitrile, both containing 5mM ammonium acetate and 1.0% formic acid. The linier gradient started at 2 min from 2% to 98% acetonitrile at 20 min, kept at 98% to 26 min, followed by a return to initial conditions at 28 min. The column was allowed to recondition until 35 min when the next injection started.

3.5.3.2 HRMS Conditions

Mass spectrometric analysis employed a Thermo Fisher QExactive high resolution mass spectrometer (HRMS) (Thermo Fisher, USA) controlled by Xcalibur software. The scan range was from 100 to 1100

m/z units with a resolution of 140 000 in positive mode. The AGC target was set to $3x10^{-6}$ and the maximum injection time set to 200 msec. The instrument interface was an Ion Max API source fitted with a HESI-II probe. Tune file settings were: heater temperature at 400°C, capillary temperature at 250°C, the sheath gas flow rate at 50, the aux gas flow rate at 10 and sweep gas was switched off. The source voltage in positive ion mode was 3.5 kV and the S-lens was set at 55 V. Quantitative analyses were performed using Xcalibur Quanbrowser software.

3.6 Quantification and statistical analysis

Abraxis ELISA data was analysed using the 4-parameter logistic fitting with Excel Solver for Microcystin ELISA Version 20060924 (ELISA Software) supplied with the Abraxis kit and the Norwegian ELISA data was analysed with the software programme provided by the Norwegian researchers (Norwegian Veterinary Institute in Oslo, Norway).

Statistical analysis for correlation and significance of the Abraxis ELISA, Norwegian ELISA, LC-MS as well as the results of the water quality parameters were analysed using ANOVA, Student's *t*-test, Pearson product-moment correlation (*r*) and Bayesian statistical evaluation.

CHAPTER 4: RESULTS AND DISCUSSION

4.1. Comparison between the Norwegian and the Abraxis ELISA's

When water samples were analysed for microcystin concentrations (Table 2) there were differences between the Norwegian and the Abraxis ELISA's. In general, when analysing the water samples "as is" the Abraxis ELISA concentrations were higher when compared to the Norwegian ELISA. Where microcystin concentrations in water samples were very low, the buffers which were added to avoid the matrix effect when using the Norwegian ELISA, diluted the samples even further. However, after methanol extraction, the Norwegian ELISA appeared to be more sensitive and the microcystin concentrations were similar as those measured with the Abraxis ELISA (Table 2).

Table 2: Microcystin concentrations in water samples collected from the Hartbeespoort dam and Le Croc as analysed by the Norwegian and Abraxis ELISA's as well as LC-MS (µg/l)

Month	Water "as is"				MeOH					
	Hartbeespoort		Le Croc		Hartbeespoort			Le Croc		
	Norwegian*	Abraxis#	Norwegian	Abraxis	Norwegian+	Abraxis#	LC-MS†	Norwegian	Abraxis	LC-MS
Aug 2014	<0.15	0.14	0.19	4.18	0.12	0.15	0.01	2.59	2.92	0.65
Sept 2014	3.85	0.187	0.19	7.56	0.07	0.20	0.01	3.78	4.15	0.59
Oct 2014	<0.15	1.795	0.17	8.51	0.86	1.31	0.18	0.47	1.56	0.12
Nov 2014	0.19	1.066	<0.15	1.98	0.17	0.29	0.20	0.09	0.40	0
Dec 2014	18.65	83.75	<0.15	1.09	473	43.71	86.35	0.07	0.24	0.01
Jan 2015	6.8	422.07	2.15	9.26	>195	362.76	368.79	5.53	3.25	2.62
Feb 2015	2.02	5.89	1.14	4.34	8.44	7.51	3.53	9.35	7.66	5.08
Mar 2015	12.92	36.26	7.83	45.61	0.47	114.42	76.86	15.79	13.92	8.27
Apr 2015	7.23	8.07	7.25	150.72	4	11.89	1.24	9.2	9.74	4.09

*LOD for water "as is" = 0.15 µg/l; #LOD = 0.10 µg/l; +LOD for methanol = 0.04; †LC-MS = only MC-LR, MC-YR, MC-RR

When comparing the three methods of analysis using methanol extracts the values were mostly within the same range, although the LC-MS analysis only reflected the concentrations of 3 microcystin variants (MC-LR, MC-YR, and MC-RR). Nevertheless, the microcystin concentrations determined with the two ELISA's were consistently higher. A possible explanation is that the ELISA's will also detect other microcystin variants and even nodularins (Fischer *et al.,* 2001).

4.2 Comparing the two sampling sites

Initially, during the months of August – October 2014 the microcystin concentrations in the crocodile breeding dam as determined by both ELISAs (Table 2) was higher than the Hartbeespoort Dam. This, however, changed around December 2014 when the Hartbeespoort Dam contained higher concentrations. This coincides with warmer climatic conditions and rainfall with subsequent run-off of nutrients into the dam. It should also be noted that the breeding dam was emptied and refilled during early January 2015, which reduced the cyanobacterial content and microcystin concentrations.

4.3. Correlation between different sample processing techniques

We determined the sample Pearson product-moment correlation coefficient (*r*) between pairs of microcystin measurement methods in R (R Core Team, 2012) and Just another Gibbs sampler (JAGS) (Plummer, 2003), using robust Bayesian parameter estimation (Kruschke, 2013). We modelled paired microcystin measurements comparing ELISAs (Norwegian versus Abraxis, within an extraction method), comparing non-extraction and extraction methods (water "as is" versus adsorbent disk/methanol extraction), and comparing liquid chromatography-mass spectrometry analysis and the two ELISAs (Abraxis and Norwegian) after adsorbent disk/methanol extraction. All paired measurements were matched by site and month. Given the log-transformed sample data, we estimated the posterior distributions on the parameters of a bivariate *t*-distribution from which the sample data were likely to have been drawn.

The two ELISAs produced log-transformed microcystin measurements that were positively correlated; however, the correlation between the two assays was much stronger when using methanol extraction than when using water “as is” (Figure 21). The log-transformed LC-MS microcystin measurements and the log-transformed methanol-extracted Abraxis or Norwegian microcystin ELISA measurements were also highly (positively) correlated (Figure 22). When using water “as is” measurements from both the Abraxis and Norwegian ELISAs, this correlation was much weaker (Figure 23). Within a given method (Abraxis or Norwegian ELISA), the correlation between water “as is” and adsorbent disk/methanol-extracted measurements was strong (positive) for the Abraxis method, but considerably weaker for the Norwegian method (Figure 24).

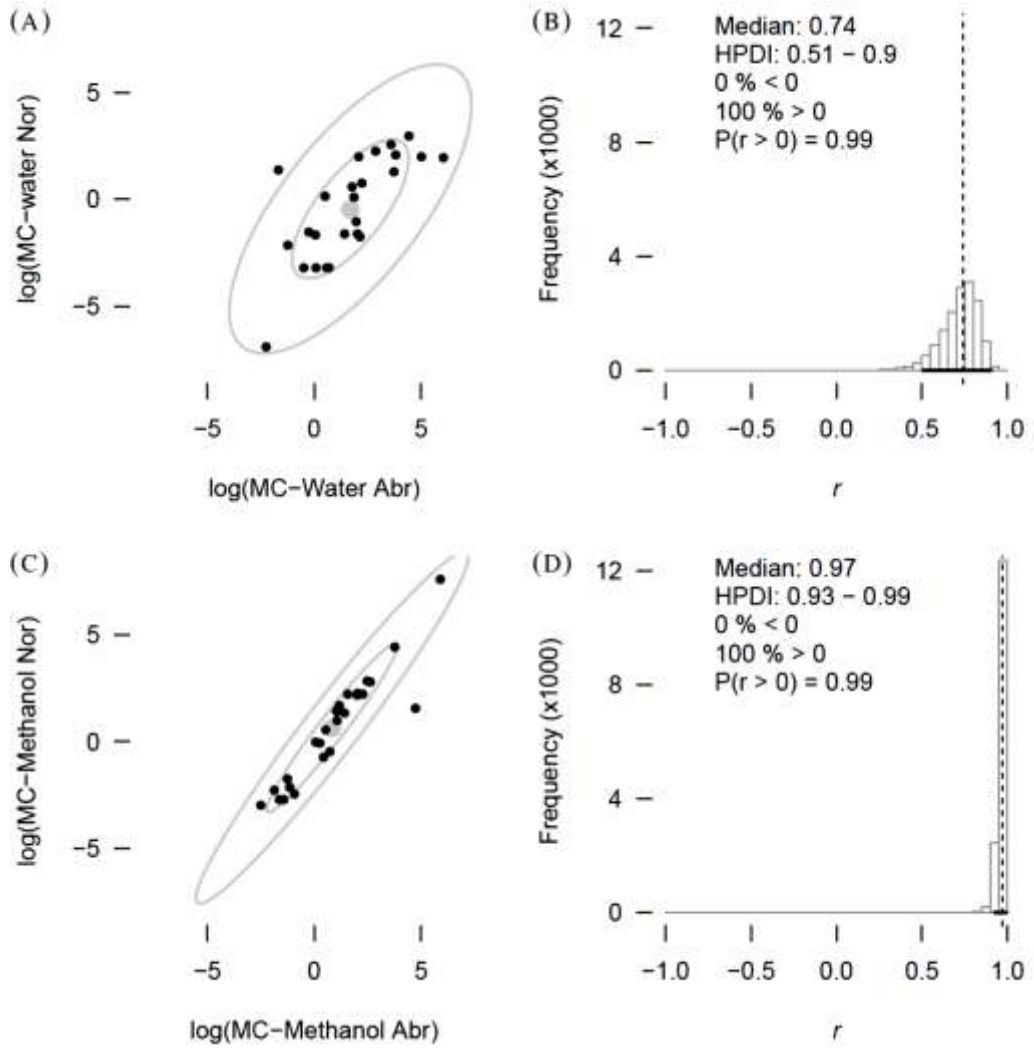

Figure 21: Correlation between log-transformed microcystin (MC) concentrations measured using the Abraxis (Abr) and Norwegian (Nor) ELISAs. Two processing techniques were compared: water "as is" (A and B) and adsorbent disk/methanol (C and D). (A and C) Model fit of the estimated bivariate *t*-distribution (ellipses covering 50% and 95% of the distribution) relative to the log-transformed raw data. (B and D) Bayesian posterior distribution on the sample Pearson product-moment correlation coefficient (*r*). The highest posterior density interval (HPDI) is depicted with the thick horizontal line (B and D).

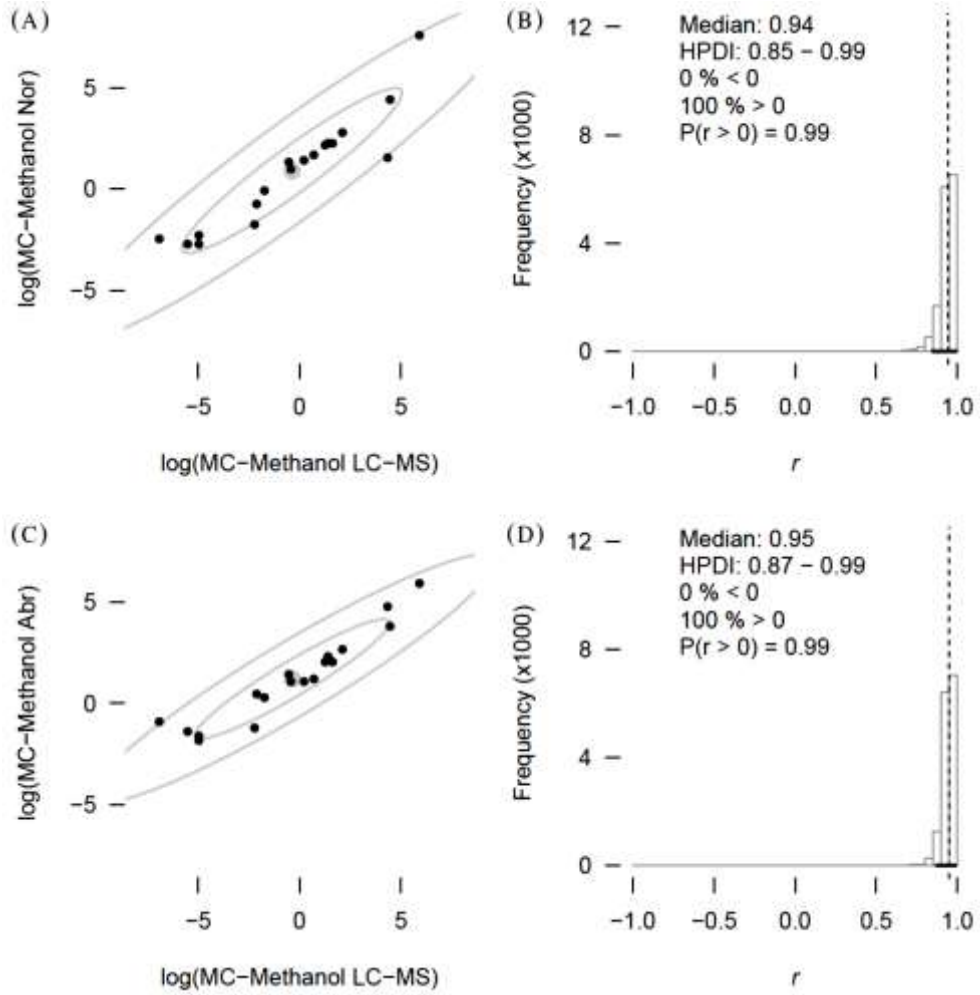

Figure 22: Correlation of log-transformed microcystin (MC) concentrations measured via adsorbent disk/methanol extraction between (A and B) Norwegian ELISA (Nor) and liquid chromatography-mass spectrometry (LC-MS) and between (C and D) Abraxis ELISA (Abr) and liquid chromatography-mass spectrometry. (A and C) Model fit of the estimated bivariate *t*-distribution (ellipses covering 50% and 95% of the distribution) relative to the log-transformed raw data. (B and D) Bayesian posterior distribution on the sample Pearson product-moment correlation coefficient (*r*). The highest posterior density interval (HPDI) is depicted with the thick horizontal line (B and D).

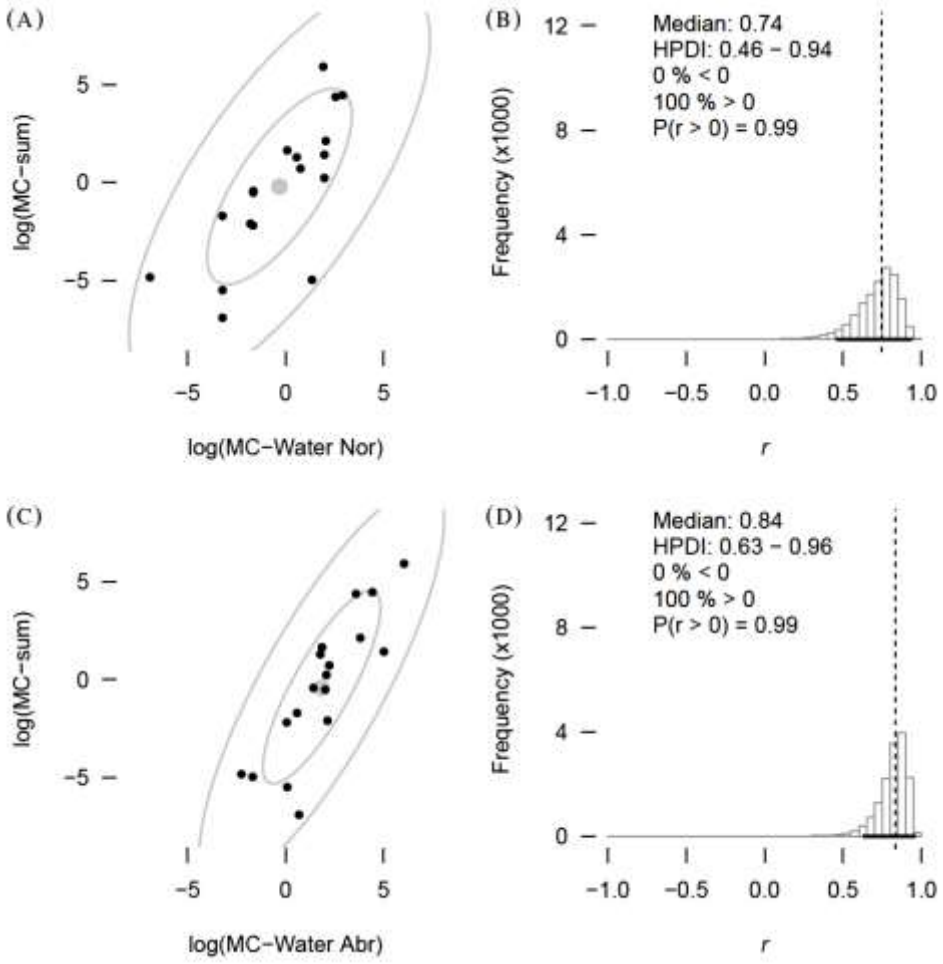

Figure 23: Correlation between water "as is" and adsorbent disk/methanol extraction via the Norwegian ELISA (A and B) and the Abraxis ELISA (C and D). (A and C) Model fit of the estimated bivariate *t*-distribution (ellipses covering 50% and 95% of the distribution) relative to the log-transformed raw data. (B and D) Bayesian posterior distribution on the sample Pearson product-moment correlation coefficient (*r*). The highest posterior density interval (HPDI) is depicted with the thick horizontal line (B and D).

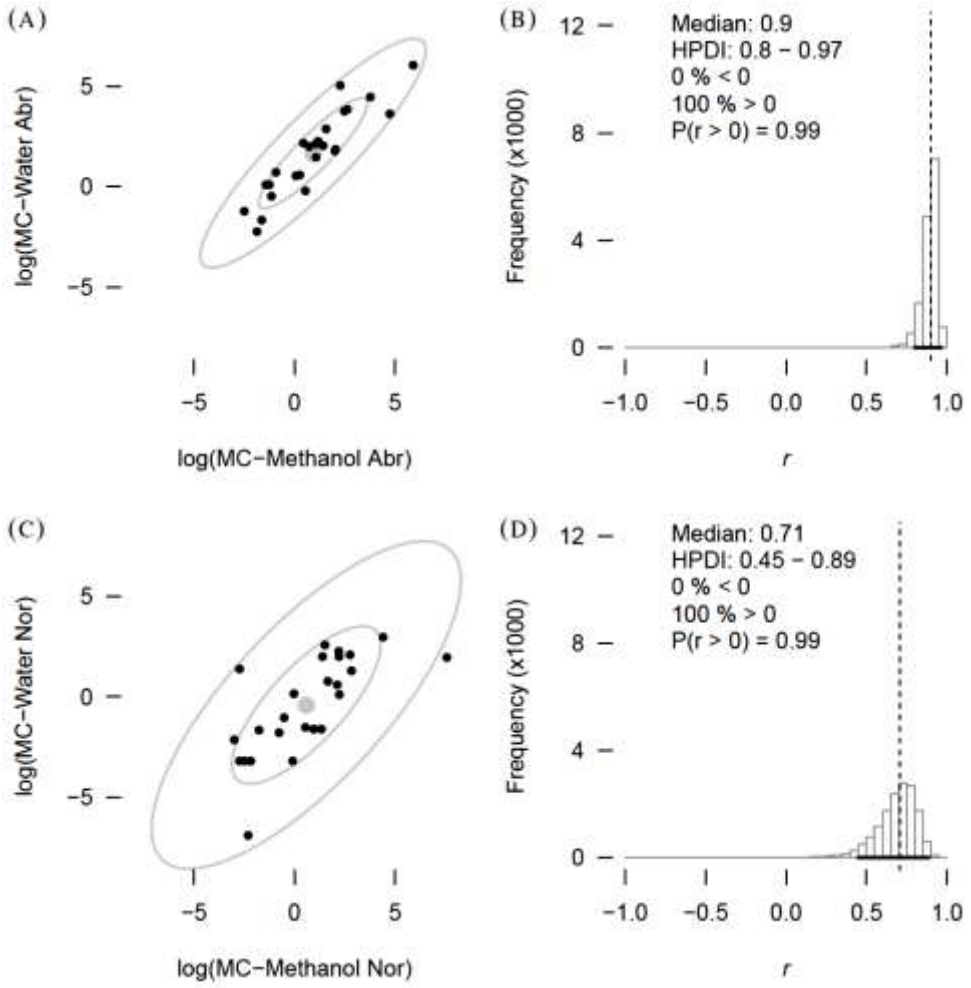

Figure 24: Correlation between water "as is" and adsorbent disk/methanol-extracted log-transformed microcystin (MC) concentrations measured using (A and B) Abraxis ELISA (Abr) and (C and D) Norwegian ELISA (Nor). (A and C) Model fit of the estimated bivariate *t*-distribution (ellipses covering 50% and 95% of the distribution) relative to the log-transformed raw data. (B and D) Bayesian posterior distribution on the sample Pearson product-moment correlation coefficient (*r*). The highest posterior density interval (HPDI) is depicted with the thick horizontal line (B and D).

4.4. Microcystin concentrations during sample collection period

Microcystin concentrations were plotted per month for the two sites, the Hartbeespoort Dam and the crocodile farm downstream of the dam (Table 3). The water in the crocodile breeding dam is supplied by the Hartbeespoort Dam. The relative change (x-fold) between concentrations in the dam and downstream at the crocodile farm was determined (Table 3).

The concentrations of microcystin (RR, YR, and LR) at both sites for the longitudinal samples (August 2014 through to April 2015) follow approximately log-normal distribution. The concentrations for each of the microcystins during lowest (nadir) months were close to or at the lower limits of detection for both sites (Table 3). Months with median values for the three microcystin variants indicated an increase in RR concentrations at the crocodile farm relative to the dam, while the values for YR and LR were higher for the dam relative to the farm, respectively (Table 3). For peak months, all three microcystin variants indicated higher values at the dam relative to the farm (Table 3). The microcystin concentrations (RR, YR and LR) during the lowest and median months were similar when comparing between sites, but the concentrations during peak months were generally one or two orders of magnitude higher at the Hartbeespoort Dam relative to the crocodile farm (Table 3). The nadirs in the three microcystins occurred at approximately the same time of year within a site, but the lowest at the crocodile farm (early wet season) lagged those at the dam (late dry season) by approximately two months (Table 3; Fig. 25). Peaks in the RR concentrations occurred during the same month (late wet season) at both sites, while peaks in the YR and LR concentrations occurred earlier at the dam (middle wet season) than at the farm (late wet season) (Table 3; Fig. 25).

Table 3: Microcystin (MC) concentrations (RR, YR, LR, and their sum, estimated in ng/g) and relative increases (x-fold) at the Hartbeespoort Dam (HBP) and a crocodile breeding dam (LC) downstream of the dam from water samples collected each month (mo) between August 2014 and April 2015.

MC	Site	Nadir	(mo)	Median	x-fold	Peak	x-fold	(mo)
RR	HBP	0.00	10	0.35		13.89	+4.06	3
	LC	0.00	12	0.49	+0.40	2.75		3
YR	HBP	0.00	9	0.06	+4.33	157.69	+80.20	1
	LC	0.00	11	0.01		1.94		2
LR	HBP	0.00	9	0.35	+1.31	204.04	+54.70	1
	LC	0.00	11	0.15		3.66		3
Sum	HBP	0.01	9	1.24	+0.92	368.78	+43.61	1
	LC	0.00	11	0.65		8.27		3

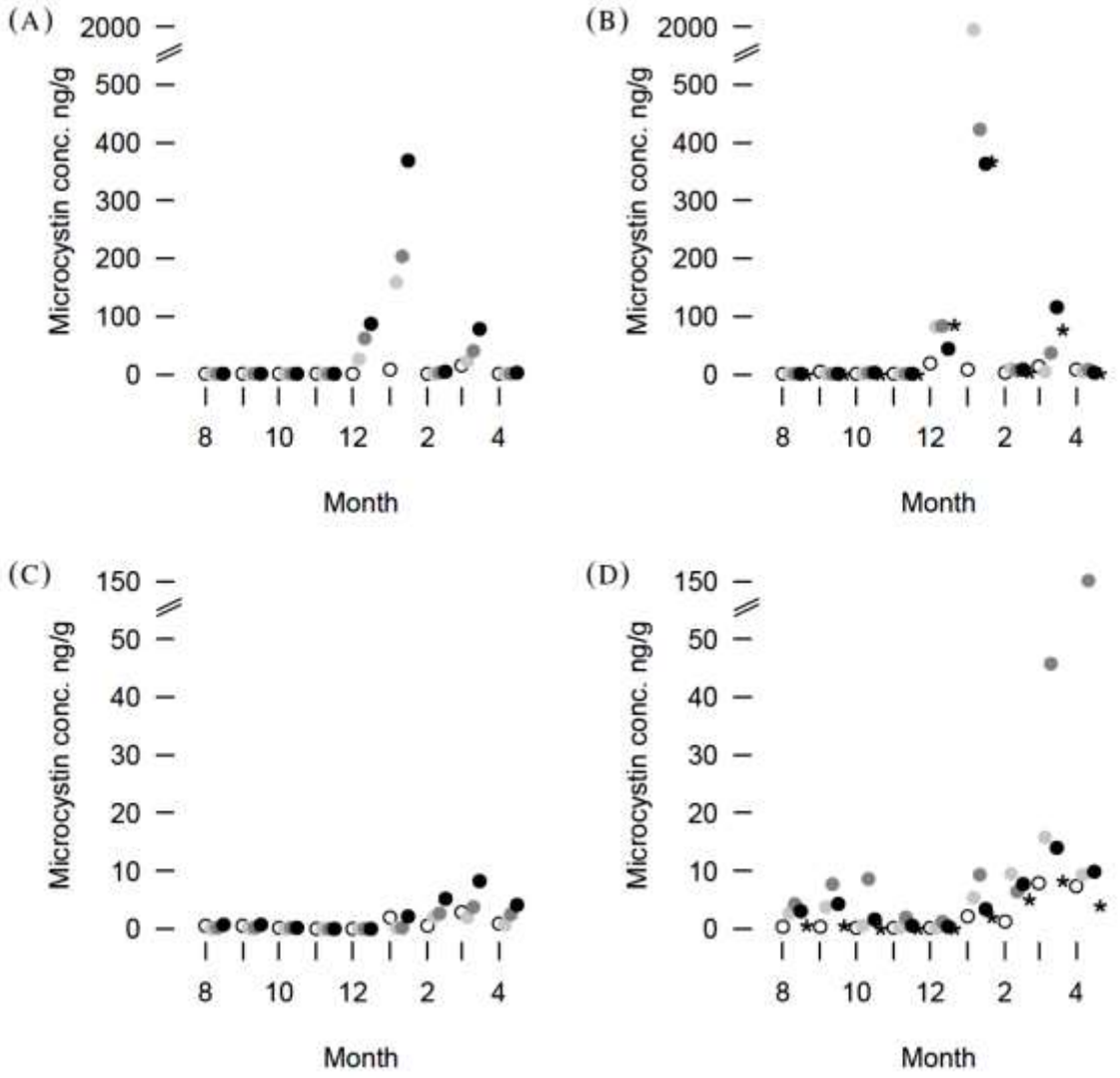

Figure 25: Microcystin concentrations by month (August 2014 through April 2015) from water samples analysed "as is" (A and C: open dots, RR; light grey dots, YR; grey dots, LR; black dots, sum of RR, YR, LR) and adsorbent disk/methanol [(B and D: open dots, water "as is" Norwegian ELISA; light grey dots, methanol Norwegian ELISA; grey dots, water "as is" Abraxis ELISA; black dots, methanol Abraxis ELISA; asterisk, methanol (LC-MS)] at the Hartbeespoort dam (A and B) and a crocodile breeding dam downstream of the dam (C and D).

4.5 Dead hatchling liver and yolk, egg-shell membranes and unfertilized eggs

Pooled liver and egg samples were only analysed using LC-MS. A liver sample of a slaughtered crocodile and other samples were spiked with microcystins (MC-LR, MC-RR and MC-YR) (Abraxis) and were also analysed by the LC-MS to determine the percentage recovery of the microcystins. Table 4 shows all tissue per period of collection. Microcystin concentrations (MC-LR, MC-RR, MC-YR) in the crocodile egg and hatchling samples collected from batches with a good hatching rate ranged between 0 - 1.76 ng/g where the egg-shell membranes had the highest concentration. Microcystin concentrations in samples collected from batches with a bad hatching rate ranged from 0 – 1.63 ng/g with the highest concentration detected in the hatchling yolk. However, it should be noted that the percentage recovery of spiked samples were low (Table 5). Spiked samples were also extracted according to the method described by Bruno and co-workers (2009).

Table 4: Microcystin (sum of MC-LR, MC-RR and MC-YR) concentrations (ng/g wet mass) in pooled crocodile hatchling liver and yolk, egg-shell membranes and unfertilized eggs collected during various stages of the hatching process.

Sample type	Early		Middle		Late	
	Good	Bad	Good	Bad	Good	Bad
Egg-shell membrane	1.76 (n=10)	0.531 (n=10)	0.192 (n=10)	0.31 (n=10)	0.734 (n=10)	0.559 (n=9)
Unfertilized egg	0.165 (n=4)	0 (n=5)	0 (n=5)	0. 044 (n=5)	0.047 (n=5)	0.058 (n=5)
Hatchling liver	0.426 (n=2)	0 (n=5)	0.553 (n=4)	1.268 (n=5)	0 (n=5)	0 (n=5)
Hatchling yolk	0.167 (n=2)	1.626 (n=3)	0.18 (n=4)	1.484 (n=5)	0.149 (n=5)	0 (n=5)

Table 5: Percentage recovery of spiked samples

Sample type	Microcystin LR	Microcystin RR	Microcystin YR
Egg-shell membrane	7.0%	5.8%	4.9%
Unfertilized eggs	16.1%	14.5%	11.3%
Hatchling liver	26.9%	4.4%	15.0%
Hatchling yolk	33.5%	15.2%	15.6%

The combined microcystin data (MC-LR, MC-YR, and MC-RR) approximately followed a lognormal distribution thus, for all analyses we log-transformed the data. For the Bayesian analyses, we conducted six different analyses of variance. For each analysis, we estimated the posterior distribution for the parameters of the model using Markov Chain Monte Carlo (MCMC) methods using R2jags (Su and Yajima, 2015), JAGS (Plummer, 2003), and R (R Core Team, 2015).

Of the six Bayesian models that we ran, the tissue type model (differences among tissue types irrespective of survivorship group) was the best model (Figure 26). The tissue type model suggests that the liver, yolk and unfertilized egg tissues all have similar microcystin concentrations, while the membranes have (with moderate to high certainty) higher microcystin concentrations (Figure 26). The (back transformed) effect size for the membrane is 6.7 ng/g.

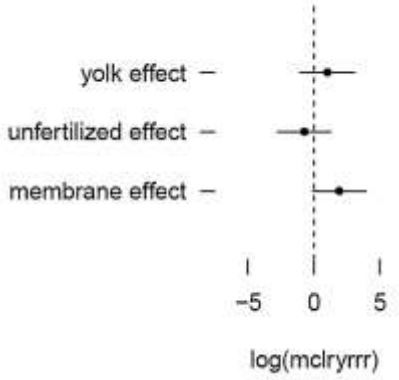

Figure 26: Median and Bayesian 95% credible interval for the size of the effect (log transformed) of survivorship group alone in explaining variability in microcystin concentrations in Nile crocodiles.

The posterior distributions on the survivorship groups (Fig. 27) bear out the similarity in microcystin concentrations between the two groups, as seen in the model. Posterior distributions on the tissue types (Fig. 28) illustrate the high variability in both the membrane and yolk samples, from both the good and bad survivorship groups (Fig. 29).

Figure 27: Medians and Bayesian 95% credible intervals for the survivorship groups (back-transformed).

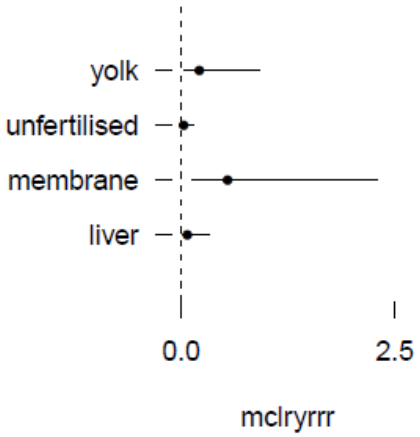

Figure 28: Medians and Bayesian 95% credible intervals for the tissue types (back-transformed).

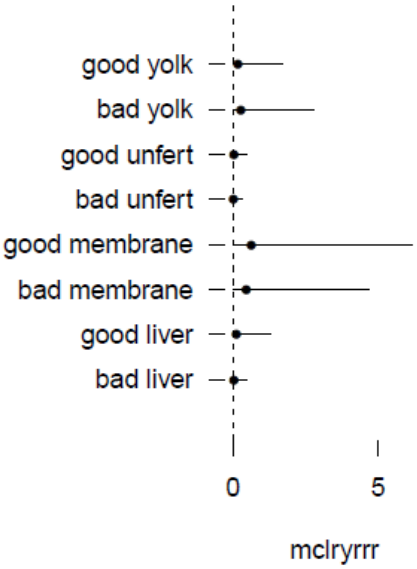

Figure 29: Medians and Bayesian 95% credible intervals for the survivorship groups and tissue types combined (back-transformed).

4.6 Water quality parameters

4.6.1 Nitrate

The monthly nitrate concentrations in the Hartbeespoort Dam ranged from 0.22- 6.2 mg/l with a mean of 3.06 mg/l (±2.21) and in the crocodile breeding dam it ranged between 1.6 - 7 mg/l with a mean of 3.9 mg/l (±2.27 mg/l). When the two concentrations were subjected to statistical test, a Student's *t*-test at a 95% confidence interval, p>0.05, thus there was no significant difference between the two dams.

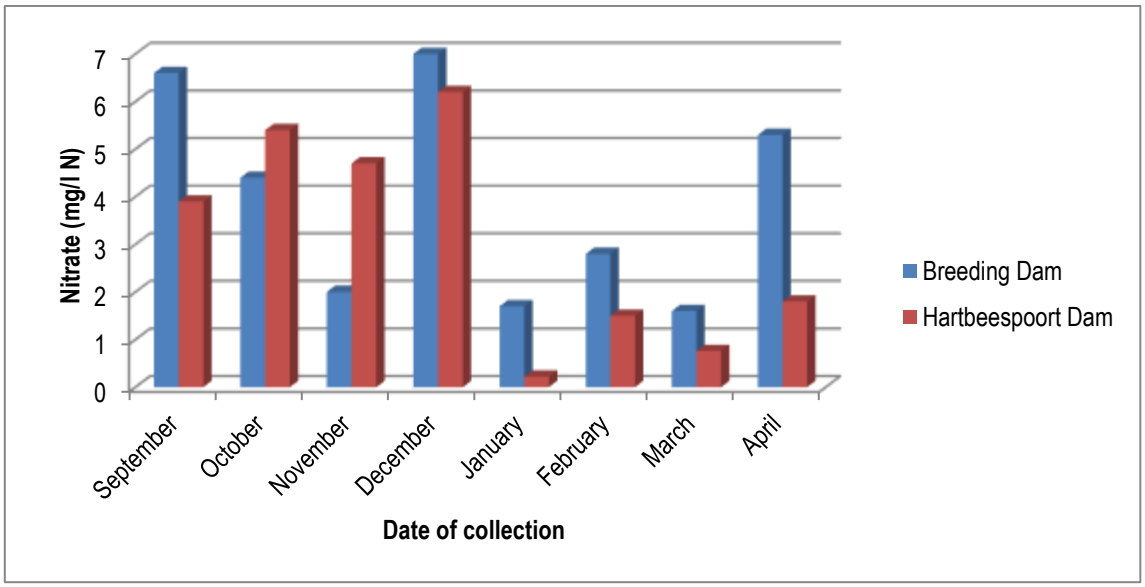

Figure 30: Monthly nitrate concentrations at the two sampling sites.

4.6.2 Total Kjeldahl Nitrogen

Total Kjeldahl Nitrogen (TKN) ranged from 4.2 - 14 mg/l with a mean of 8.7 mg/l (±3.55) over a period of 8 months in the breeding dam and from below the detectable limit to 11 mg/l with a mean of 3.5 mg/l (±3.54) in the Hartbeespoort Dam. Student's *t*-test was done to test for statistical difference between the dams, at 95% confidence interval, the $p>0.05$, thus there was no significant difference between the two dams.

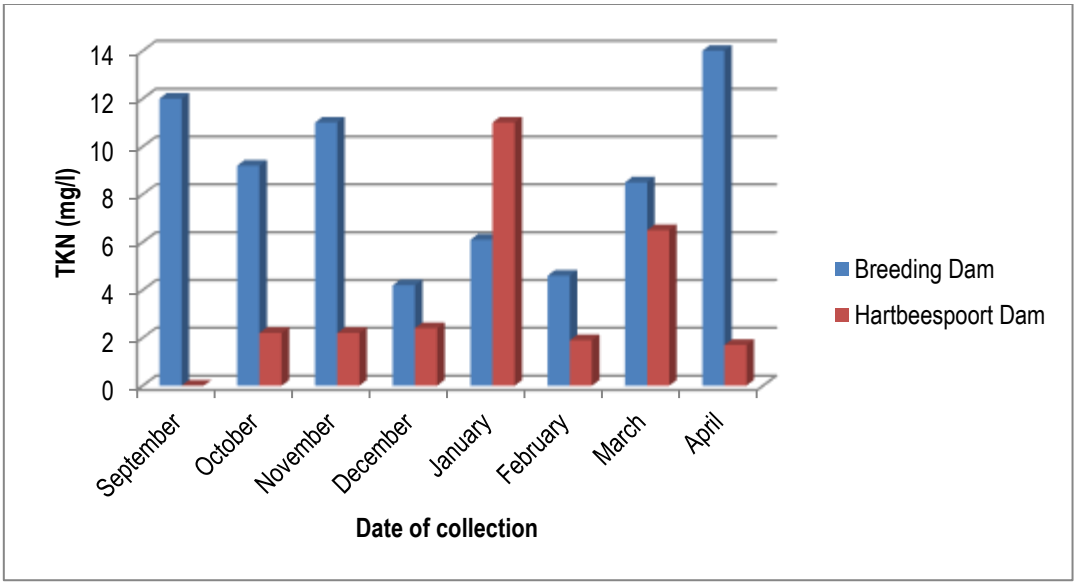

Figure 31: Monthly total Kjeldahl nitrogen concentrations at the two sampling sites.

4.6.3 Total phosphorous

Total phosphorous in the crocodile breeding dam ranged between 1.3 - 3.1 mg/l with a mean of 1.96 mg/l (±0.70 mg/l) and it was 0.26 - 1.5 mg/l over the collection period in the Hartbeespoort dam with a mean of 0.65 mg/l (±0.38). Student's *t*-test showed that $p>0.05$, and there was no significant difference between the two dams in total phosphorus concentration.

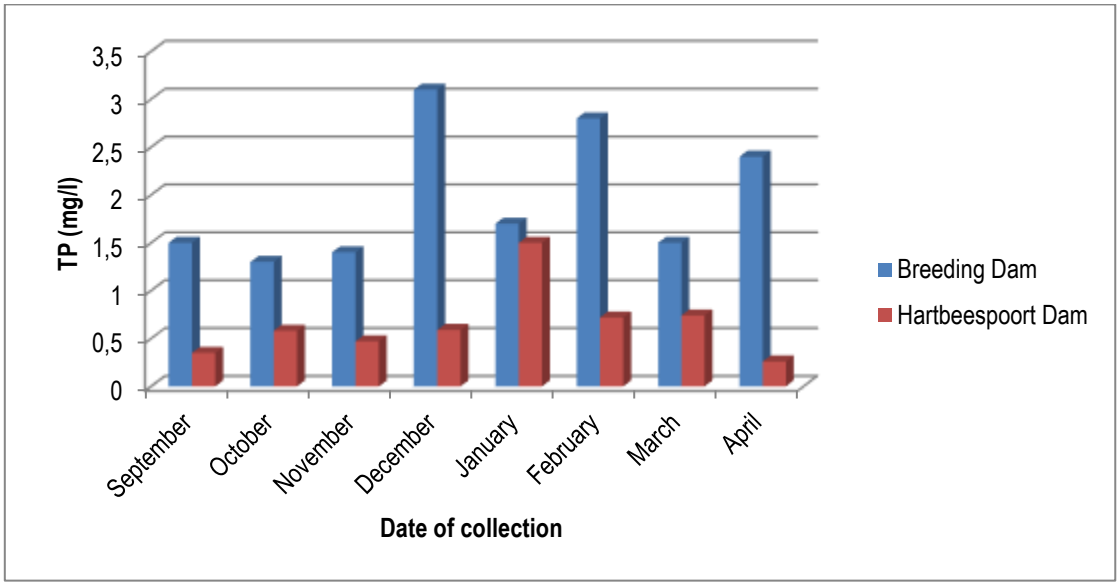

Figure 32: Monthly total phosphorous concentrations at the two sampling sites.

4.6.4 pH value

The pH in the crocodile breeding dam ranged from 8.81 - 10.64 with a mean of 9.7 (±0.95) and 7.18 - 10.15 with a mean of 8.9 (±0.61) in the Hartbeespoort Dam over the eight months period of collection. When subjected to Student's *t*-test, the $p>0.05$, there was no statistical difference between the pH of the two dams.

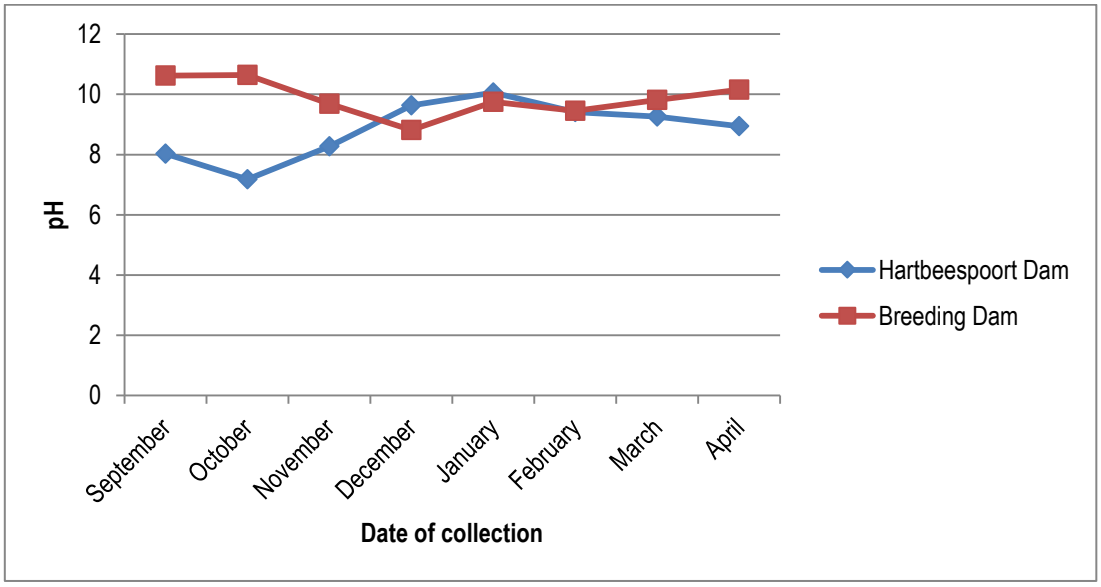

Figure 33: Monthly water pH readings at the two sampling sites.

4.6.5 Chlorophyll A

Chlorophyll A concentration in the crocodile breeding dam ranged between 237.3 - 1091.37 µg/l with a mean of 628.27 µg/l (±286.88) and it ranged between 2.41 - 1578.69 µg/l in the Hartbeespoort Dam with a mean of 447.4 µg/l (±587.05) over the months of collection. Chlorophyll A concentration showed no statistical difference between the two dams, $p>0.05$, using Student's *t*-test when each sampling site results combined.

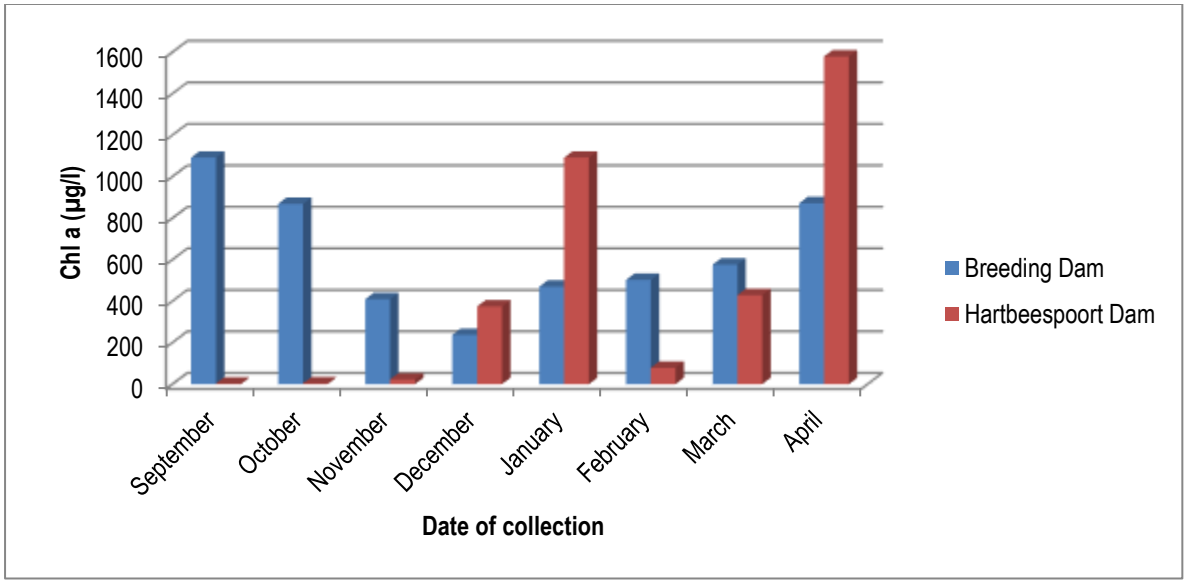

Figure 34: Monthly Chlorophyll A concentrations at the two sampling sites.

4.6.6 Conductivity

Conductivity in the crocodile breeding dam ranged from 613 µS/cm to 890 µS/cm between water sample collections in August 2014 and April 2015 with a mean of 762.67 µS/cm (±105.37). In the Hartbeespoort Dam, conductivity ranged between 481 µS/cm to 619 µS/cm between water sample collections in August 2014 and April 2015 with a mean of 553.78 µS/cm (±61.42). The $p>0.05$ and there was no statistical difference between the conductivity readings of the two dams.

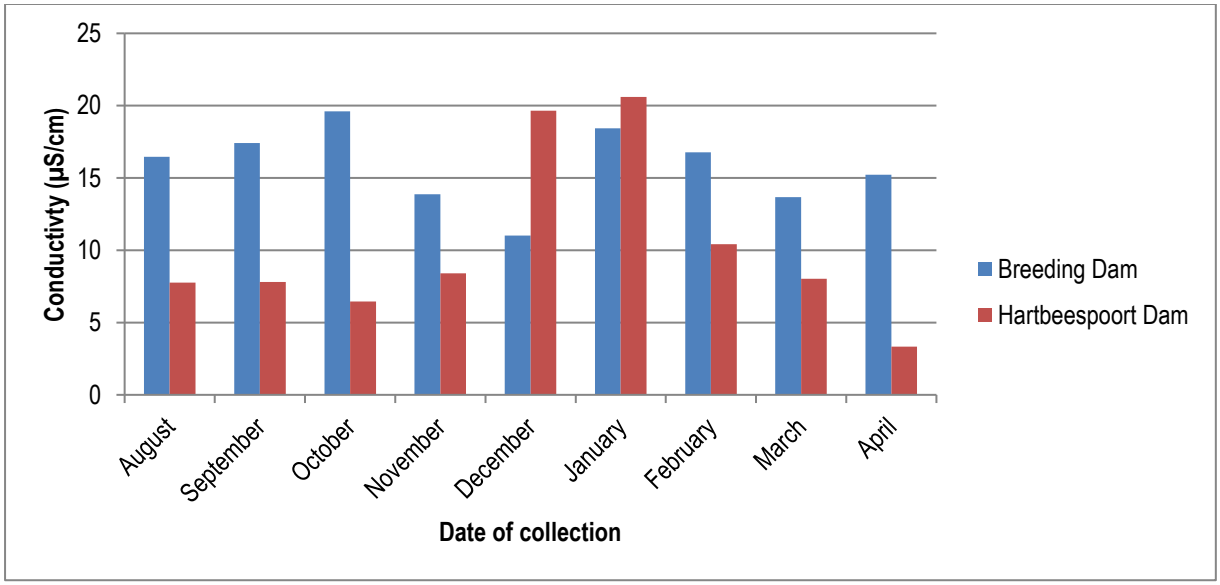

Figure 35: Monthly conductivity readings at the two sampling sites.

4.6.7 Dissolved Oxygen

Dissolved oxygen ranged between 16.46 -19.60 mg/l with a mean of 18.03 mg/l (±2.66) in the breeding dam and the average in the Hartbeespoort Dam it was 3.34 - 20.61 mg/l with a mean of 10.27 mg/l (±5.90). When the readings from the two dams were subjected to a Student's *t*-test for statistical differences, $p<0.05$, thus there was a significant difference in the concentration of the dissolved oxygen between the two dams.

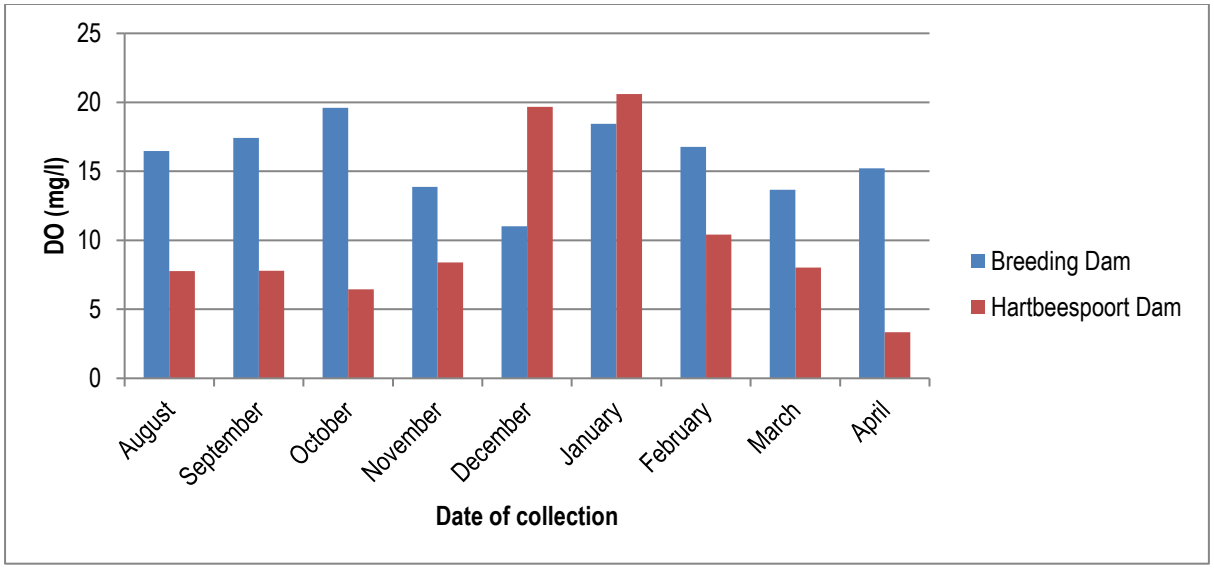

Figure 36: Monthly dissolved oxygen concentrations at the two sampling sites.

4.6.8 Temperature

There was an increase of the water temperature from the first month of sampling in the crocodile breeding dam. Temperature increased from 18.4°C in August 2014 to a high of 31.4°C in February 2015. Then there was a drop in the water temperature in March and April (23.5°C). The mean temperature over the 8 months was 25.53°C (±3.90). In the Hartbeespoort Dam, the water temperature increased from 16.5°C in August 2014 to 31.3°C in February 2015, although there were slight fluctuations between the months. The water temperature dropped to 24°C in April 2015 with a mean temperature over the period of 25.03°C (±4.81). When the temperature readings from the two dams were subjected to a Student's *t*-test for statistical difference test, $p>0.05$, and there was no significant difference in the water temperature between the two dams.

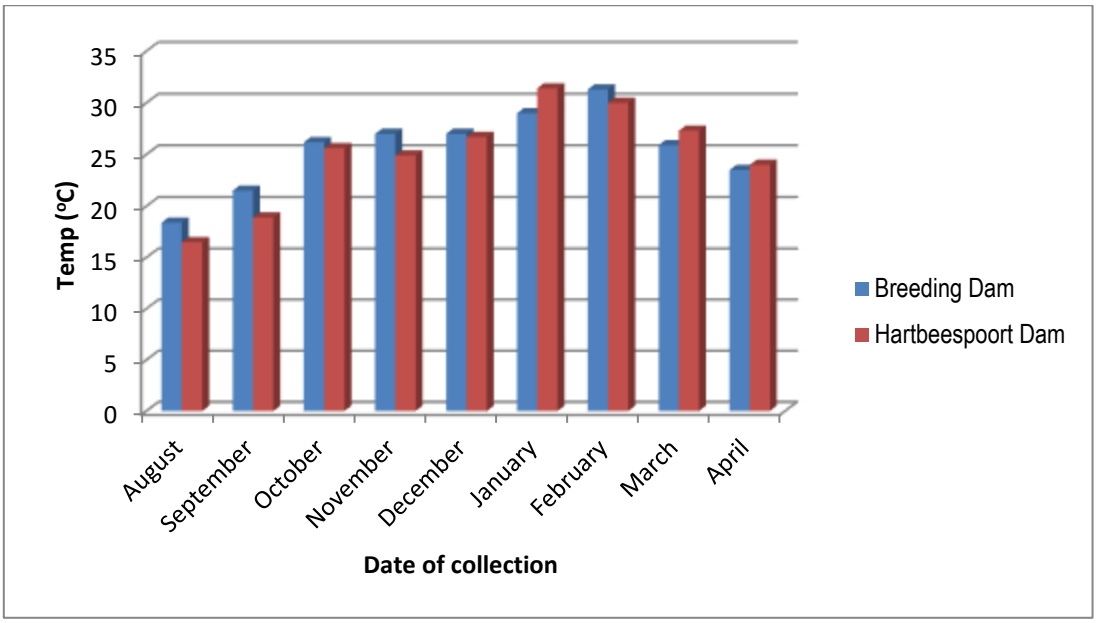

Figure 37: Monthly water temperature readings at the two sampling sites.

CHAPTER 5: GENERAL DISCUSSION, CONCLUSSIONS AND RECOMMENDATION

5.1 Correlation between different water sample processing techniques

This study compared the Norwegian developed ELISA and the commercial Abraxis ELISA by monitoring the cyanotoxin concentrations in two dams. The concentrations as determined by the Norwegian ELISA and Abraxis ELISA were strongly correlated when analysing water samples extracted by using a resin in an adsorption disk followed by methanol elution. When comparing the two ELISAs using water "as is" the correlation was much weaker.

Overall, it seems as though the use of an adsorbent disk followed by methanol extraction is more reliable than water analysed "as is" when using the Norwegian ELISA. Following methanol extraction the results of the two ELISA methods were strongly correlated which suggests that the two ELISAs provide comparable results. It appears as though the sum of the microcystin (MC-LR, MC-RR, MC-YR) concentrations as determined by LC-MS analysis are approximately in the same order as the ELISA results. However, the ELISA results were consistently higher, most probably because they can detect other microcystin variants and even nodularins as well.

5.2 Microcystin concentrations in Nile crocodile eggs and hatchlings

High concentrations of microcystins were recorded in the crocodile breeding dam and the control dam (Hartbeespoort Dam). The results also indicate that there is a vertical transmission of microcystins from the female crocodile to the eggs, which could be detected in "dead-in-egg" hatchling liver and yolk. However, no conclusion could be drawn as to whether microcystin does have a negative effect on the hatchability of crocodile eggs and hatchling survivorship. Previous studies using zebra fish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*) or chub (*Leuciscus cephalus*) confirmed that microcystins do have an effect on the egg hatching rate of these various fish species (Oberemm *et al*., 1997; Oberemm *et al*., 1999). Another study conducted by Palikova and co-workers (2007) showed that the purified microcystin

and an aqueous cyanobacterial extract have embryotoxic effects which included significant mortality, delayed hatching, decreased number of hatchings, suppression in embryonic development, disturbance of air bladder filling and significant inhibition of glutathione *S*-transferases (GST) in *Aphanizomenon* sp. and *Planktothrix* sp.

The data suggests that there are differences in microcystin concentrations among tissue types, with moderate to high certainty. A slightly higher microcystin concentration in egg-shell membranes relative to the other tissue types (i.e. hatchling liver and yolk and unfertilized eggs) was detected. The egg-shell membrane does influence hatching success since it is an outer covering offering protection to the yolk and probably helps in offering support and protection to the yolk or the albumen. However, if microcystins were able to penetrate through the egg membranes into the yolk it might affect the hatchability of crocodilian eggs, to which from the current study, microcystins was found in the hatchlings liver and yolk samples. It is not possible to determine from this study whether the estimated effect is biologically significant. There appears to be no difference in microcystin concentrations among good and bad batches across all tissue types or within a specific tissue type. The tissue levels were probably underestimated with the extraction method employed for LC-MS as the percentage recovery from spiked samples were very low. The data suggests that vertical transmission of microcystins to the Nile crocodile egg does not play a significant role in the hatchability of eggs and survivorship of the hatchlings.

One problem with this study design is the low sample size (i.e. three samples per survivorship x tissue group, one each from the early, middle and late incubation periods). Although samples reflect pooled samples from a larger sampling population, the variability within some of the groups remained very high and this combined with the low sample size reduces the available power to detect differences. Presumably, if there were enough groups from which pooled samples were obtained, the survivorship within a group could be used as a quantitative explanatory variable.

5.3 Water quality parameters

When the nutrient supply to the system as a whole is increased i.e. phosphates, TKN and nitrates, the dams become eutrophic. In the Le Croc dam, the concentration of nutrients was high in the months of September and December compared to the Hartbeespoort Dam which was very low in the month of September, but high in the month of December. This could be due to the fact that the Le Croc dam is a small dam with stagnant water and a large number of crocodiles feeding, defecating and urinating inside the dam. This surely contributed to the enrichment of the breeding dam resulting in a great increase of phytoplankton in the waterbody. Enrichment with phosphorous (P) is usually a precursor to cyanobacterial bloom formation. There are many examples where increased P loading has caused a progression from a clear, less productive state to one that is more turbid and productive and favours nuisance cyanobacterial blooms (Paerl, 1988). Much higher P concentrations in the two dams during the period of sampling probably drove conditions that favoured cyanobacterial blooms. Nitrogen (Total Kjeldahl Nitrogen (TKN) which measures a combination of organic N, ammonia and ammonium) and nitrogenous compounds can also influences the growth of cyanobacteria and the release of toxins.

5.4 Conclusions

In conclusion, when using the Norwegian ELISA it seems as though the use of a resin-containing adsorbent disk followed by methanol extraction is more reliable than analysing water “as is”. Following methanol extraction the results of the two ELISAs were strongly correlated, which suggests that the two ELISAs provide comparable results. From the current study, unfortunately, the data and analysis suggest only one primary conclusion — that there are differences in microcystin concentrations among tissue types, with moderate to high certainty. There appears to be no difference in microcystin concentrations among good and bad survivorship groups, across all tissue types or within a given tissue type. Based on the small sample size, final conclusion cannot be made although these data suggested that microcystin may not play a significant role in the Nile crocodile hatchling mortality.

5.5 Recommendations

In the next crocodile hatchling study, I would recommend that a longitudinal study be done since a single season of breeding is insufficient to conclude that microcystins do not contribute to the low hatching rate in Nile crocodiles. Different breeding dams should be used as a control and the sample size need to be increased. A qualified statistician needs to be consulted first before sample collection to establish a feasible sample size for statistical analysis purposes. A different and more effective method of extraction of microcystin from tissue samples need to be used to avoid low concentration recovery of the toxins from tissue samples. Several other microcystins could also be included in the analysis and the possible extraction of bound microcystins as well as ELISA analysis on tissue samples could be considered. In addition, a dose-response study could be conducted where increasing concentrations of microcystin can be injected in crocodile eggs in a controlled experiment to determine the maximum tolerance level for the developing embryo.

CHAPTER 6: REFERENCES

Allanson, B.R. (1961). Investigations into the ecology of polluted inland waters in the Transvaal. *Hydrobiologia,* 18(1–2), 77-94.

Andersen, R.J., Luu, H.A., Chen, D.Z., Holmes, C.F., Kent, M.L., Le Blanc, M. and Williams, D.E. (1993). Chemical and biological evidence links microcystins to salmon 'netpen liver disease'. *Toxicon,* 31(10), 1315-1323.

Annadotter, H., Cronberg, G., Lawton, L., Hansson, H., Göthe, U. and Skulberg, O. (2001). An extensive outbreak of gastroenteritis associated with the toxic cyanobacterium *Planktothrix agardhii* (Oscillatoriales, Cyanophyceae) in Scania, South Sweden. *Cyanotoxins, occurrence, causes, consequences*. Berlin, Springer, 200-208.

Ballot, A., Sandvik, M., Rundberget, T., Botha, C.J. and Miles, C.O. (2014). Diversity of cyanobacteria and cyanotoxins in Hartbeespoort Dam, South Africa. *Marine and Freshwater Research,* 65(2), 175-189.

Booker, M. and Walsby, A. (1981). Bloom formation and stratification by planktonic blue - green algae in an experimental water column. *British Phycological Journal,* 16(4), 411-421.

Briand, J., Jacquet, S., Bernard, C. and Humbert, J. (2003). Health hazards for terrestrial vertebrates from toxic cyanobacteria in surface water ecosystems. *Veterinary Research,* 34(4), 361-377.

Bruno, M., Melchiorre, S., Messineo, V., Volpi, F., Di Corcia, A., Aragonac, I., Guglielmone, G., Di Paolod, C., Cennid, M., Ferranti, P., and Gallo, P. (2009). Microcystin detection in contaminated fish from Italian lakes using ELISA immunoassays and LC-MS/MS analysis. Handbook on Cyanobacteria ISBN: 978-1-60741-092-8.

Byth, S. (1980). Palm Island mystery disease. *The Medical Journal of Australia,* 2(1), 40-42.

Carmichael, W.W. (1992). A status report on planktonic Cyanobacteria (Blue-Green Algae) and their Toxins. EPA/600/R-92/079, United States Environmental Protection Agency, Cincinnati, OH.

Carmichael, W.W. (1994). The toxins of cyanobacteria. *Scientific American,* 270, 78 – 86.

Chen, J., Xie, P., Zhang, D., Ke, Z. and Yang, H. (2006). In situ studies on the bioaccumulation of microcystins in the phytoplanktivorous silver carp (*Hypophthalmichthys molitrix*) stocked in Lake Taihu with dense toxic *Microcystis* blooms. *Aquaculture,* 261(3), 1026-1038.

Chen, J., Xie, P., Zhang, D. and Lei, H. (2007). In situ studies on the distribution patterns and dynamics of microcystins in a bio manipulation fish–bighead carp (*Aristichthys nobilis*). *Environmental Pollution,* 147(1), 150-157.

Chen, J., Xie, P., Li, L. and Xu, J. (2009a). First identification of the hepatotoxic microcystins in the serum of a chronically exposed human population together with indication of hepatocellular damage. *Toxicological Sciences: An official Journal of the Society of Toxicology,* 108(1), 81-89.

Chen, J., Zhang, D., Xie, P., Wang, Q. and Ma. Z. (2009b). Simultaneous determination of microcystin contaminations in various vertebrates (fish, turtle, duck and water bird) from a large eutrophic Chinese lake, Lake Taihu, with toxic *Microcystis* blooms. *Science of the Total Environment,* 407, 3317-3322.

Chorus, E.I. and Bartram, J. (1999). Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. WHO; Geneva, Switzerland.

Chow, C.W., Drikas, M., House, J., Burch, M.D. and Velzeboer, R.M. (1999). The impact of conventional water treatment processes on cells of the cyanobacterium *Microcystis aeruginosa*. *Water Research,* 33(15), 3253-3262.

Cochrane, K. (1987). The biomass and yield of the dominant fish species in Hartbeespoort Dam, South Africa. *Hydrobiologia,* 146(1), 89-96.

Codd, G.A., Morrison, L.F. and Metcalf, J.S. (2005). Cyanobacterial toxins: risk management for health protection. *Toxicology and Applied Pharmacology,* 203(3), 264-272.

Coutts, J. A. and Wilson, G. C. (2007). Introduction: Quality Control, In: Optimum Egg Quality – A Practical Approach. 5M Publishing, Sheffield, UK.

Cutts, J.A., Wilson, G.C. and Fernández, S. (2007). Optimum egg quality: a practical approach, 5M Publishing.

Deblois, C.P., Aranda-Rodriguez, R., Giani, A. and Bird, D.F. (2008). Microcystin accumulation in liver and muscle of tilapia in two large Brazilian hydroelectric reservoirs. *Toxicon,* 51(3), 435-448.

Department Of Water Affairs and Forestry (DWAF). (2002). National Eutrophication Monitoring Programme. South African National Water Quality Monitoring Programmes Series, Implementation Manual.

Department Of Water Affairs and Forestry (DWAF). (2004). Sampling protocol for toxic algae. Water Resource Quality Monitoring 3, 8 – 9.

Dietrich, D. and Hoeger, S. (2005). Guidance values for microcystins in water and cyanobacterial supplement products (blue-green algal supplements): a reasonable or misguided approach? *Toxicology and Applied Pharmacology,* 203(3), 273-289.

Donati, C., Drikas, M., Hayes, R. and Newcombe, G. (1993). Adsorption of microcystin-LR by powdered activated carbon. *Water-Melbourne Then Artarmon,* 20(3), 25-28.

Falconer, I.R. (1996). Potential impact on human health of toxic cyanobacteria. *Phycologia,* 35(6), 6-11.

Ferguson, M.W. (1982). The structure and composition of the eggshell and embryonic membranes of *Alligator mississippiensis*. *The Transactions of the Zoological Society of London,* 36(2), 99-152.

Ferguson, M.W. (1985). Reproductive biology and embryology of the crocodilians. *Biology of the Reptilia,* 14, 329-491.

Fischer, W. J., Garthwaite, I., Miles, C.O., Ross, K.M., Aggen, J.B., Chamberlin, A.R., Towers, N.A., and Dietrich, D.R. (2001). Congener-Independent Immunoassay for Microcystins and Nodularins. *Environmental Science and Technology*. 35, 4849-4858.

Fleming, L.E., Rivero, Burns, C. J., Williams, C. J., Bean, A., Shea, K.A. and Stinn, J. (2002). Blue green algal (cyanobacterial) toxins, surface drinking water, and liver cancer in Florida. *Harmful Algae*, 1, 157–168.

Gago-Martínez, A., Pineiro, N., Aguete, E., Vaquero, E., Nogueiras, M., Leao, J., Rodrıguez-Vazquez, J. and Dabek-Zlotorzynska, E. (2003). Further improvements in the application of high-performance liquid chromatography, capillary electrophoresis and capillary electrochromatography to the analysis of algal toxins in the aquatic environment. *Journal of Chromatography A,* 992(1), 159-168.

Giliane, Z., and Eduardo, C.O. (2013). Cyanobacteria and Cyanotoxins: From Impacts on Aquatic Ecosystems and Human Health to Anticarcinogenic Effects. *Toxins,* 5(10), 1896-1917.

Grau, C. (1976). Ring structure of avian egg yolk. *Poultry Science,* 55(4), 1418-1422.

Harding, W., Thornton, J., Steyn, G., Panuska, J. and Morrison, I. (2004). Hartbeespoort Dam Remediation Project (Phase 1). Action Plan (Volume I) Final Report. Department of Agriculture, Conservation, Environment and Tourism Project 58/2003. 158 pp.

Hoger, S.J., Schmid, D., Blom, J.F., Ernst, B. and Dietrich, D.R. (2007). Analytical and functional characterization of microcystins [Asp3] MC-RR and [Asp3, Dhb7] MC-RR: consequences for risk assessment? *Environmental Science and Technology,* 41(7), 2609-2616.

Huynh-Delerme, C., Edery, M., Huet, H., Puiseux-Dao, S., Bernard, C., Fontaine, J., Crespeau, F. and De Luze, A. (2005). Microcystin-LR and embryo–larval development of medaka fish, *Oryzias latipes*. I. Effects on the digestive tract and associated systems. *Toxicon,* 46(1), 16-23.

Hyenstrand, P., Blomqvist, P. and Pettersson, A. (1998). Factors determining cyanobacterial success in aquatic systems: a literature review. *Archiv für Hydrobiologie Special Issues Advanced Limnology* 51, 41-62.

Ibelings, B.W., Bruning, K., De Jonge, J., Wolfstein, K., Pires, L.D., Postma, J. and Burger, T. (2005). Distribution of microcystins in a lake foodweb: no evidence for bio magnification. *Microbial Ecology,* 49(4), 487-500.

Jacquet, C., Thermes, V., De Luze, A., Puiseux-Dao, S., Bernard, C., Joly, J., Bourrat, F. and Edery, M. (2004). Effects of microcystin-LR on development of medaka fish embryos (*Oryzias latipes*). *Toxicon,* 43(2), 141-147.

Jansen, H.S. and Anderson, F.O. (1992). Importance of temperature, nitrate and Ph for phosphate release from aerobic sediments of four shallow, eutrophic lakes. *Limnology and Oceanography,* 37 (3), 577.

Jambalang, A.R. (2012). The development and validation of a bacteriological screening test for antimicrobial residues in eggs. PhD thesis. University of Pretoria, South Africa.

Jochimsen, E.M., Carmichael, W.W., An, J., Cardo, D.M., Cookson, S.T., Holmes, C.E., Antunes, M.B., de Melo Filho, Djalma A, Lyra, T.M. and Barreto, V.S.T. (1998). Liver failure and death after exposure to microcystins at a haemodialysis center in Brazil. *New England Journal of Medicine,* 338(13), 873-878.

Kan, C.A. and Petz, M. (2000). Residues of veterinary drugs in eggs and their distribution between yolk and white. *Journal of Agricultural and Food Chemistry,* 48(12), 6397-6403.

Kellerman, T.S., Coetzer, J.A.W., Naude´, T.W. and Botha, C.J. (2005). Plant poisoning and mycotoxicoses of livestock in Southern Africa. 2nd ed.: Oxford University Press, Cape Town.

Kemeny, D. (1991). Practical guide to ELISA. Pergamon Press.

Klemer, A.R., Feuillade, J. and Feuillade, M. (1982). Cyanobacterial blooms: carbon and nitrogen limitation have opposite effects on the buoyancy of oscillatoria. *Science (New York, N.Y.),* 215, 1629-1631.

Knoll, A.H. (2008). Cyanobacteria and earth history. *The Cyanobacteria: Molecular Biology, Genomics, and Evolution,* 484.

Koreivienė, J. and Belous, O. (2012). Methods for cyanotoxins detection. *Botanica Lithuanica,* 18(1), 58-65.

Kot-Wasik, A., Zabiegała, B., Urbanowicz, M., Dominiak, E., Wasik, A. and Namieśnik, J. (2007). Advances in passive sampling in environmental studies. *Analytica Chimica Acta,* 602(2), 141-163.

Krienitz, L., Ballot, A., Kotut, K., Wiegand, C., Putz, S., Metcalf, J.S., Codd, G.A. and Pflugmacher, S. (2003). Contribution of hot spring cyanobacteria to the mysterious deaths of Lesser Flamingos at Lake Bogoria, Kenya. *FEMS Microbiology Ecology,* 43(2), 141-148.

Kruschke, J.K. (2013). Bayesian estimation supersedes the t-test., *Journal of Experimental Psychology*: *General,* 142, 573.

Kurmayer, R., and Christiansen, G. (2009). The genetic basis of toxin production in Cyanobacteria. *Freshwater Reviews*, 2, 31-50

Landsberg, J.H. (2002). The effects of harmful algal blooms on aquatic organisms. *Reviews in Fisheries Science,* 10(2), 113-390.

Law, J.M. (2001). Mechanistic considerations in small fish carcinogenicity testing. *ILAR Journal / National Research Council, Institute of Laboratory Animal Resources,* 42(4), 274-284.

Lawton, L.A. and Edwards, C. (2008). Conventional laboratory methods for cyanotoxins, in: Hudnell, KH (Ed.), Cyanobacterial harmful algal blooms: state of the science and research needs. *Springer*, New York, USA, 513-537.

Lawton, l. A., Chambers, H., Edwards, C., Nwaopara, A. A., Healy, M. (2010). Rapid detection of microcystins in cells and water. *Toxicon,* 55, 973-978.

Liu, Y., Song, L., Li, X. and Liu, T. (2002). The toxic effects of microcystin-LR on embryo-larval and juvenile development of loach, *Misguruns mizolepis Gunthe*. *Toxicon,* 40(4), 395-399.

Lürling, M. & Faassen, E.J. (2013). Dog poisonings associated with a *Microcystis aeruginosa* bloom in the Netherlands. *Toxins,* 5(3), 556-567.

MacKenzie, L., Beuzenberg, V., Holland, P., McNabb, P. and Selwood, A. (2004). Solid phase adsorption toxin tracking (SPATT): a new monitoring tool that simulates the biotoxin contamination of filter feeding bivalves. *Toxicon,* 44(8), 901-918.

MacKintosh, C., Beattie, K.A., Klumpp, S., Cohen, P. and Codd, G.A. (1990). Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Letters,* 264(2), 187-192.

Masango, M., Myburgh, J., Botha, C., Labuschagne, L. and Naicker, D. (2008). A comparison of in vivo and in vitro assays to assess the toxicity of algal blooms. *Water Research,* 42(13), 3241-3248.

Masango, M.G., Myburgh, J.G., Labuschagne, L., Govender, D., Bengis, R.G. and Naicker, D. (2009). Assessment of Microcystins bloom toxicity associated with wildlife mortality in the Kruger National Park, South Africa. *Journal of Wildlife Diseases,* 46(1), 95-102.

Matsunaga, H., Harada, K., Senma, M., Ito, Y., Yasuda, N., Ushida, S. and Kimura, Y. (1999). Possible cause of unnatural mass death of wild birds in a pond in Nishinomiya, Japan: sudden appearance of toxic cyanobacteria. *Natural Toxins,* 7(2), 81-84.

Mbukwa, E.A., Msagati, T.A. and Mamba, B.B. (2012). Quantitative variations of intracellular microcystin-LR,-RR and-YR in samples collected from four locations in Hartbeespoort Dam in North West Province (South Africa) during the 2010/2011 summer season. *International Journal of Environmental Research and Public Health,* 9(10), 3484-3505.

McQueen, D. & Lean, D. (1987). Influence of water temperature and nitrogen to phosphorus ratios on the dominance of blue-green algae in Lake St. George, Ontario. *Canadian Journal of Fisheries and Aquatic Sciences,* 44(3), 598-604.

Metcalf, J.S., Morrison, L., Krienitz, L., Ballot, A., Krause, E., Kotut, K., Puetz, S., Wiegand, C., Pflugmacher, S. and Codd, G. (2006). Analysis of the cyanotoxins anatoxin-a and microcystins in Lesser Flamingo feathers. *Toxicological & Environmental Chemistry,* 88(1), 159-167.

Metcalf, J., Bell, S. and Codd, G. (2000). Production of novel polyclonal antibodies against the cyanobacterial toxin microcystin-LR and their application for the detection and quantification of microcystins and nodularin. *Water research,* 34(10), 2761-2769.

Mohamed, Z.A., Carmichael, W.W. and Hussein, A.A. (2003). Estimation of microcystins in the freshwater fish *Oreochromis niloticus* in an Egyptian fish farm containing a Microcystis bloom. *Environmental Toxicology,* 18(2), 137-141.

Moreno, I.M., Pereira, P., Franca, S. and Camean, A. (2004). Toxic cyanobacteria strains isolated from blooms in the Guadiana River (south-western Spain). *Biological Research,* 37(3), 405-417.

Oberemm, A., Fastner, J. and Steinberg, C.E. (1997). Effects of microcystin-LR and cyanobacterial crude extracts on embryo-larval development of zebra fish (*Danio rerio*). *Water Research,* 31(11), 2918-2921.

Oberemm, A., Becker, J., Codd, G. and Steinberg, C. (1999). Effects of cyanobacterial toxins and aqueous crude extracts of cyanobacteria on the development of fish and amphibians. *Environmental Toxicology,* 14(1), 77-88.

Oberemm, A. (2001). Effects of cyanotoxins on early life stages of fish and amphibians. *Cyanotoxins—Occurrence, Causes, Consequences. Springer, Berlin,* 240-248.

Packard, G.C., Tracy, C.R. and Roth, J.J. (1977). The physiological ecology of reptilian eggs, embryos and the evolution of viviparity within the class Reptilia. *Biological Reviews,* 52(1), 71-105.

Paerl, H.W. (1988). Nuisance phytoplankton blooms in coastal, estuarine, and inland waters1. *Limnology and Oceanography,* 33(4part2), 823-843.

Paerl, H.W. (1996). A comparison of cyanobacterial bloom dynamics in freshwater, estuarine and marine environments. *Phycologia,* 35(6S), 25-35.

Paerl, H.W., Fulton, R.S., Moisander, P.H. and Dyble, J. (2001). Harmful freshwater algal blooms, with an emphasis on cyanobacteria. *The Scientific World Journal, Volume* 176-113.

Palíková, M., Krejčí, R., Hilscherová, K., Babica, P., Navrátil, S., Kopp, R. and Bláha, L. (2007). Effect of different cyanobacterial biomasses and their fractions with variable microcystin content on embryonal development of carp (*Cyprinus carpio L.*). *Aquatic Toxicology,* 81(3), 312-318.

Pedro, O., Correia, D., Lie, E., Skåre, J.U., Leão, J., Neves, L., Sandvik, M. and Berdal, K.G. (2013a). Polymerase chain reaction (PCR) detection of the predominant microcystin-producing genotype of cyanobacteria in Mozambican lakes. *African Journal of Biotechnology,* 10(83), 19299-19308.

Pedro, O., Lie, E., Correia, D., Neves, L., Skaare, J.U., Sandvik, M. and Berdal, K.G. (2013b). Quantification of microcystin-producing microcystis in freshwater bodies in the Southern Mozambique using quantitative real time polymerase chain reaction. *African Journal of Biotechnology,* 12(30), 4850-4857.

Pedro, O.C.N. (2013). Molecular and chemical methods as tools to evaluate cyanobacterial toxins in Mozambique. PhD thesis. Norwegian School of Veterinary Science, Norway,Oslo.

Plummer, M. (2003). Jags: A program for analysis of Bayesian graphical models using Gibbs sampling, in: Proceedings of the 3rd International Workshop on Distributed Statistical Computing. 20–22.

Pouria, S., de Andrade, A., Barbosa, J., Cavalcanti, R., Barreto, V., Ward, C., Preiser, W., Poon, G.K., Neild, G. and Codd, G. (1998). Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. *The Lancet,* 352(9121), 21-26.

R Core Team. (2012). R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna. URL http://www.R-project.org/.

Rapala, J., Erkomaa, K., Kukkonen, J., Sivonen, K. and Lahti, K. (2002). Detection of microcystins with protein phosphatase inhibition assay, high-performance liquid chromatography–UV detection and enzyme-linked immunosorbent assay: Comparison of methods. *Analytica Chimica Acta,* 466(2), 213-231.

Reichwaldt, E.S. & Ghadouani, A. (2012). Effects of rainfall patterns on toxic cyanobacterial blooms in a changing climate: between simplistic scenarios and complex dynamics. *Water Research,* 46(5), 1372-1393.

Rodger, H., Turnbull, T. and Richards, R. (1994). Myopathy and pancreas disease in salmon: a retrospective study in Scotland. *Veterinary Records,* 135(10), 234-235.

Rundberget, T., Gustad, E., Samdal, I.A., Sandvik, M. and Miles, C.O. (2009). A convenient and cost-effective method for monitoring marine algal toxins with passive samplers. *Toxicon,* 53(5), 543-550.

Runnegar, M.T., Kong, S. and Berndt, N. (1993). Protein phosphatase inhibition and in vivo hepatotoxicity of microcystins. *The American Journal of Physiology,* 265, 224-231.

Runnegar, M., Berndt, N. and Kaplowitz, N. (1995). Microcystin uptake and inhibition of protein phosphatases: effects of chemoprotectants and self-inhibition in relation to known hepatic transporters. *Toxicology and Applied Pharmacology,* 134(2), 264-272.

Runnegar, M., Seward, D.J., Ballatori, N., Crawford, J.M. and Boyer, J.L. (1999). Hepatic toxicity and persistence of ser/thr protein phosphatase inhibition by microcystin in the little skate Raja erinacea. *Toxicology and Applied Pharmacology,* 161(1), 40-49.

Samdal, I.A., Ballot, A., Løvberg, K.E., and Miles, C.O. (2014). Multihapten Approach Leading to a Sensitive ELISA with Broad Cross-Reactivity to Microcystins and Nodularin. Environmental Science and Toxicology. 48, 8035-8043.

Su, Y.S., Yajima, M. (2015). R2jags: Using R to Run 'JAGS', URL http://CRAN.R-project.org/package=R2jags, r package version 0, 5-7.

Shapiro, J. (1990). Current beliefs regarding dominance by blue-greens: the case for the importance of CO_2 and pH. *Verhandlungen des Internationalen Verein Limnologie*, 24, 38–54.

Spencer, C.N. & King, D.L. (1985). Interactions between light, NH_4, and CO_2 in buoyancy regulation of anabaena flos-aquae (cyanophyceae). *Journal of Phycology,* 21(2), 194-199.

Stewart, I., Seawright, A.A., and Shaw, G.R. (2008). Cyanobacterial poisoning in livestock, wild mammals and birds – an overview. In: Hudnell, H.K. (ed), Cyanobacterial Harmful Algal Blooms State of the Science and Research Needs. Springer.

Swanepoel, A., Du Preez, H., Schoeman, C., Janse van Vuuren, S & Sundram, A. (2008). Condensed laboratory methods for monitoring phytoplankton, including Cyanobacteria, in South African freshwaters. WRC Report TT323/08. Water Research Commission, Pretoria, South Africa, 108.

Talling, J. (1976). The depletion of carbon dioxide from lake water by phytoplankton. *The Journal of Ecology,* 79-121.

Teixeira, M., Costa, M., Carvalho, V., Pereira, M. and Hage, E. (1993). Gastroenteritis epidemic in the area of the Itaparica Dam, Bahia, Brazil. *Bulletin of PAHO,* 27(3), 244-253.

Turner, P.C., Gammie, A.J., Hollinrake, K. and Codd, G.A. (1990). Pneumonia associated with contact with cyanobacteria. *BMJ (Clinical Research ed.),* 300(6737), 1440-1441.

Ueno, Y., Nagata, S., Tsutsumi, T., Hasegawa, A., Watanabe, M.F., Park, H.D., Chen, G.C., Chen, G. and Yu, S.Z. (1996). Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. *Carcinogenesis,* 17(6), 1317-1321.

United States Department of Agriculture (USDA), 2000. United States Standards, Grades, and Weights Classes for Shell Eggs AMS 56.
http://www.ams.usda.gov/AMSv1.0/getfile?dDocName=STELDEV3004376. Accessed 20/02/2015.

Van Vuuren, S.J., Swanepoel, A., du Preez, H., Schoeman, C., Sundram, A. (2008). Condensed Laboratory Methods for Monitoring Phytoplankton, including Cyanobacteria, in South African Freshwaters. Water Research Council.

Van Ginkel, C. (2004). A national survey of the incidence of cyanobacterial blooms and toxin production in major impoundments. *Published by: Directorate: Resource Quality Services, Department of water Affairs and Forestry. Private Bag X313, Pretoria, 0001, South Africa.*

Van Halderen, A., Harding, W.R., Wessels, J.C., Schneider, D.J., Heine, E.W.P., Van Der Merwe, J. & Fourie, J.M. (1995). Cyanobacterial (blue-green algae) poisoning of livestock in the Western Cape Province of South Africa. *Journal of the South African Veterinary Association*, 66(4), 260 – 264.

Vander, A.J. & Luciano, D. (1980). Human physiology: the mechanisms of body function.

Vasconcelos, V. (1999). Cyanobacterial toxins in Portugal: effects on aquatic animals and risk for human health. *Brazilian Journal of Medical and Biological Research,* 32(3), 249-254.

Verhaar, H.J., Busser, F.J. and Hermens, J.L. (1995). Surrogate parameter for the baseline toxicity content of contaminated water: simulating the bio concentration of mixtures of pollutants and counting molecules. *Environmental Science & Technology,* 29(3), 726-734.

Water Research Commission (2008.) Condensed Laboratory Methods for Monitoring Phytoplankton, including Cyanobacteria, in South African Freshwaters. Report number: TT323/08

Wang, P., Chien, M., Wu, F., Chou, H., Lee, S. (2005). Inhibition of embryonic development by microcystin-LR in zebrafish, *Danio Rerio*. *Toxicon* 45 (3), 303 – 308.

Welch, E.B. (2002). Ecological Effects of Waste Water*:* Applied limnology and pollutant effects, CRC Press.

Wermuth, H., F, and Ross, J., P. 2014. Encyclopaedia Britannica.

Wilson, A.E., Gossiaux, D.C., Höök, T.O., Berry, J.P., Landrum, P.F., Dyble, J. and Guildford, S.J. (2008). Evaluation of the human health threat associated with the hepatotoxin microcystin in the muscle and liver tissues of yellow perch (*Perca flavescens*). *Canadian Journal of Fisheries and Aquatic Sciences,* 65(7), 1487-1497.

Winding, W., Phalp, J. M., Payne, A. W. (1996). A Noise and Background Reduction Method for Component Detection in Liquid Chromatography/Mass Spectrometry. *Analytical Chemistry*, 68, 3602-3606.

World Health Organization. (1999). Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management. E & FN Spon, Editor: Chorus I and Bartram J.

World Health Organization. (2004). Guidelines for drinking-water quality: recommendations, World Health Organization. Third edition. Volume 1: Recommendations.

Xie, L., Xie, P., Guo, L., Li, L., Miyabara, Y. and Park, H. (2005). Organ distribution and bioaccumulation of microcystins in freshwater fish at different trophic levels from the eutrophic Lake Chaohu, China. *Environmental Toxicology,* 20(3), 293-300

Xie, L. & Park, H. (2007). Determination of microcystins in fish tissues using HPLC with a rapid and efficient solid phase extraction. *Aquaculture,* 271(1), 530-536.

YU, S. (1995). Primary prevention of hepatocellular carcinoma. *Journal of Gastroenterology and Hepatology,* 10(6), 674-682.

Yalow, R.S., and Berson, S.A. (1960). Immunoassay of endogenous plasma insulin in man. *Journal for Clinical Investigation* 39, 1157–1175.

Zeck, A., Weller, M.G., Bursill, D. and Niessner, R. (2001). Generic microcystin immunoassay based on monoclonal antibodies against Adda. *Analyst,* 126(11), 2002-2007.

Zhou, B., Xiao, J.F., Tuli, L. and Ressom, H.W. (2012). LC-MS-based metabolomics. *Molecular BioSystems,* 8(2), 470-481.

http://www.quora.com/Compared-to-humans-which-animals-have-uniquely-different-physiology-and-what-purpose-does-that-serve. Accessed date 2014/09/17.

http://www.fao.org/docrep/006/t0226e/t0226e19.htm Forestry Department. Accessed date 2014/10/02.

http://www.chemgapedia.de viewed 19 August 2015.

http://www.gmotesting.com/testing-options/immuno-analysis/elisa.aspx viewed 21 May 2014.

http://www.lecroc.co.za/about-us.html viewed 20 April 2014.

http://www.abraxiskits.com/ accessed 21 January-2014.

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25 June 2014

University of Pretoria

Faculty of Veterinary Science
Private Bag X04
Onderstepoort
0110

Tel: +27 12 529 8000
Fax: +27 12 529 8300

Prof CJ Botha
Department Paraclinical Sciences
(christo.botha@up.ac.za)

Dear Prof Botha

PROTOCOL V050/14: COMPARISON BETWEEN NORWEGIAN AND ABRAXIS MICROCYSTINS-ADDA ELISAs – *A Singo*

I am pleased to inform you that the abovementioned protocol was **registered** by the Research Committee as project V050/14.

Kindly note that, if there are animal ethical issues involved in the project, the protocol needs to be approved by the Animal Ethics Committee before you may commence with the project. Once AEC approval is granted, the project will finally be approved by the Research Committee.

Kind regards

NIESJE TROMP
SECRETARY: RESEARCH COMMITTEE

Copy: Ms A Singo, Researcher (alukatelove@gmail.com)
Ms Elmarie Mostert, Animal Ethics Committee (elmarie.mostert@up.ac.za , aec@up.ac.za)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Presence of Microcystin in Dead Nile Crocodile hatchlings and eggs**
PROJECT NUMBER	**V017-15**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Ms. AA Singo**

STUDENT NUMBER (where applicable)	**144 371 05**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Dead hatchlings and eggs**	
NUMBER OF ANIMALS	**To be reported**	
Approval period to use animals for research/testing purposes		**1 April 2015 - 1 April 2016**
SUPERVISOR	**Prof. CJ Botha**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	20 April 2015
CHAIRMAN: UP Animal Ethics Committee	Signature	

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