Microcystin-LR equivalent concentrations in fish tissue during a post-bloom Microcystis exposure in Loskop Dam, South Africa

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The effects of a decomposing cyanobacteria bloom on water quality and the accumulation of microcystin-LR equivalent toxin in fish at Loskop Dam were studied in May 2012. Enzyme-linked immunosorbent assay [ELISA] was used to confirm the presence of microcystin-LR equivalent in the water and to determine the microcystin (MCYST) concentration in the liver and muscle of fish. The lowest concentration of extracellular MCYST-LR equivalent was recorded in the lacustrine zone, where no cyanobacterial cells were observed, while the highest concentration (3.25 µg l^{-1}), 3.25× higher than World Health Organization standard, was observed in the riverine zone. Extremely high MCYST-LR equivalent concentrations of 1.72 µg MCYST-LReq kg^{-1} in the liver and 0.19 µg kg^{-1} in muscles of *Labeo rosae*, and 2.14 µg MCYST-LReq kg^{-1} in the liver and 0.17 µg kg^{-1} in muscles of *Oreochromis mossambicus*, indicate that the consumption of sufficient fish biomass might cause severe adverse effects in humans. Microscopic analyses of the stomach content of both fish species revealed low numbers of cyanobacterial *Microcystis aeruginosa* cells in comparison to other phytoplankton. The extracellular MCYST-LR equivalent of the decomposing bloom may have played a major role in the high levels observed in the livers of the two fish species. These findings are important for all downstream water users.

Keywords: cyanobacteria, indigenous fish species, *Labeo rosae*, liver, muscle tissue, *Oreochromis mossambicus*

Introduction

Blooms of cyanobacteria alter environmental and economic activities, resulting in a decrease in property values and loss in ecotourism (Oberholster 2009). The cyanotoxin produced by cyanobacteria can affect animals, fish and humans in different ways, depending on the type of toxin. For instance, the potent microcystins (MCYSTs), which are hepatotoxins, may cause liver damage and/or promote tumour growth in animals and humans through inhibition of protein phosphatase 1 and 2A (Mackintosh et al. 1990; Falconer and Humpage 1996). These toxins are highly water-soluble and resistant to boiling, and thus pose a threat to water and food quality if not properly monitored (Falconer and Humpage 2005). More than 80% of the non-covalently bound MCYST in the zooplankton *Bosmina* sp. fed to the sunfish *Lepomis gibbosus* was directly transferred to the sunfish, indicating that free or conjugated MCYST-LR can travel efficiently up the aquatic food web (Smith and Haney 2006).

Smith and Haney (2006) found that cyanobacterial toxins can be biomagnified across the food chain from lower to higher trophic levels, suggesting potential risk posed to human and/or higher vertebrates upon consumption of aquatic biota containing cyanobacteria toxin. Guidelines developed by the World Health Organization (WHO) to minimise the risk for human intoxication is based on toxicological studies in mice and expressed as the tolerable daily intake (TDI) of 0.04 µg microcystin kg^{-1} body weight d^{-1} (Chorus and Bartram 1999). Based on the MCYSTs and their toxicity, the WHO guideline for MCYST-LR equivalent, the most commonly known and most toxic type of MCYST, in drinking water is 1 µg l^{-1} (Falconer et al. 1994). In recent studies (Chen et al. 2009; Zhang et al. 2009a) the presence of MCYSTs was indicated in serum of a chronically exposed human population, fishermen at Lake Chaohu, China, together with signs of hepatocellular damage. Humans usually do not consume high doses of MCYSTs to such an extent that lethal acute effects occur, but are rather exposed to chronic low doses in drinking water. However, only a few studies have tracked the presence of MCYSTs in single or multiple components of aquatic food webs (Kotak et al. 1996; Magalhães et al. 2001, 2003; Babcock-Jackson et al. 2002; Ibelings et al. 2005). Other studies have analysed the uptake of MCYSTs by aquatic organisms in an experimental setting, among others, zooplankton (DeMott 1999; Thostrup and Christoffersen 1999; Rohrlack et al. 2001; Schmidt et al. 2013).

The accumulation of MCYSTs in fish tissues is considered to be the most common and most important potential route of their exposure to humans (Oberholster et al. 2004, 2005). A few ecological studies investigated the environmental role of these toxins on fish. However, from the literature it is evident that the adverse effects of toxic cyanobacteria on

indigenous fish species and their responses to cyanotoxins, mainly on sublethal or chronic exposure situations in Africa, have not been studied. Magalhães et al. (2001) showed that MCYSTs accumulate in the liver and muscle tissue of planktivorous *Tilapia*, particularly during *Microcystis* bloom events. They highlighted that the regular consumption of MCYSTs by fish would exceed the WHO TDI limit and therefore present a potential risk for fish consumers. Because *Tilapia* species are abundant in Africa, and are largely planktivorous and consume cyanobacteria, one might speculate that the consumption of *Tilapia* flesh would pose serious adverse health effects for people consuming it (Bennett and Thorpe 2008).

Loskop Dam is considered to be eutrophic to hypertrophic due to pollution by mankind, such as nutrient enrichment by partially treated or untreated sewage water in the upper river catchment, causing ongoing degradation of water quality resulting in the loss of aquatic biota (Oberholster and Botha 2011). Water enriched with nutrients may lead to several water quality discrepancies that may arise from the accumulation of nuisance growth of cyanobacteria and filamentous algae (van Ginkel 2004; Oberholster et al. 2012). The effects of blooms of these algae are the depletion in dissolved oxygen, decline in fish and zooplankton population, and excessive production of cyanotoxins.

The aim of this study was to determine MCYST-LR equivalent concentrations in selected fish tissues at Loskop Dam during a decomposing *Microcystis* bloom.

Materials and methods

Study site

Loskop Dam (25°26′57.05″ S, 29°19′44.36″ E) is a eutrophic to hypertrophic dam in Mpumalanga province, South Africa, with its main water supply coming from the upper Olifants River catchment (Figure 1), which flows through a confined 10 km gorge before entering Loskop Dam.

The reservoir has a surface area of 24.27 km^2 and a volume of 374.3 $\times$ 10^3 m^3 at full supply capacity, and was designed primarily to supply water for irrigated agriculture downstream of the dam wall (DWAF 2004). The total catchment area that drains into Loskop Dam is 11.464 km^2. Over the past 15 years Loskop Dam has had a history of isolated incidents of fish mortality (Oberholster et al. 2010). These incidents have escalated during the past five years and are linked with crocodile mortalities and a population decline from ±80 animals to a total of six in 2008 (Oberholster et al. 2010). The *Microcystis* bloom started to develop in November 2011 (early summer) in the riverine zone of Loskop Dam when the cell count was 1.1 $\times$ 10^1 cells l^{-1}. During late January 2012 the bloom reached a cell count of 1.3 $\times$ 10^7 cells l^{-1}, after which the cell counts started to decline reaching 1.5 $\times$ 10^2 cells l^{-1} in May 2012 (autumn).

Physico-chemical parameters

One sampling site was selected in each of the three zones of the dam (Figure 1). Dissolved oxygen, water temperature, pH and electrical conductivity values were measured *in situ* at the water surface at each site, using a Hach sensION™ 156 portable multiparameter instrument (Loveland, USA). Water samples were taken with a Van Dorn sampler and filtered through 0.45 µm pore size Whatman GF/filters after which it was stored in polyethylene bottles (1 litre) that had been pre-rinsed with dilute sulphuric acid (pH 2.0) for analysis of dissolved nutrients. All analyses were carried out according to standard methods (APHA, AWWA and WEF 1992). Chlorophyll *a* (Chl *a*) was extracted from lyophilised Whatman GF filters using N,N-dimethylformamide for 2 h at room temperature and measured spectrophotometrically at 647 nm and 664 nm according to Porra et al. (1989). Water transparency was measured with a 25 cm Secchi disc.

Phytoplankton sampling

At each site, water samples were collected at 1 and 3 m using a Van Dorn sampler and pooled to form one integrated sample. One litre of the integrated sample was preserved in the field with Lugol's solution for phytoplankton identification, while the rest was used for chemical analyses and determination of MCYST-LR equivalent toxicity. The water samples were kept cool and in the dark during the 3 h period of transfer from the field to the laboratory. All algal identifications were done with a compound microscope at 1 250× magnification, after van Vuuren et al. (2006) and Taylor et al. (2007). Strip counts were made until at least 300 individuals of each of the dominant phytoplankton species had been counted. The relative abundance of phytoplankton taxa at each sampling site was categorised according to Hörnström (1999).

ELISA assays of water

For the determination of extracellar MCYST-LR equivalent concentrations at the three sampling sites, one litre of the integrated water sample was poured gently through a 934 AH glass fibre filter in the field, after which the filtered water was stored on dry ice and returned to the laboratory for toxin analysis. The filtered water was applied directly to the enzyme-linked immunosorbent assay (ELISA) using PowerWave HT microplate spectrophotometer (Biotek, Winooski, USA) in the laboratory to determine extracellular MCYST-LR equivalent levels using the ELISA assay (Mitsoura et al. 2013). The ELISA assay was conducted with a Quanti-Kit for microcystins (EnviroLogix, USA).

ELISA assays of liver and muscle

Twenty *Labeo rosae* and six *Oreochromis mossambicus* were caught using 120 mm stretched mesh gillnets set near each sampling site in May 2012. These fish species were chosen due to their omnivorous feeding habits. From each fish caught, length and body weight were recorded and the fish was then sacrificed by severing the spinal cord just behind the head, before dissection of the different tissues. Tissues were kept frozen at −80 °C until the extraction procedure was undertaken to determine MCYST-LR equivalent.

MCYSTs were extracted from the tissues of fish with 100% methanol after Magalhães et al. (2001), stirred overnight at room temperature and then centrifuged at 1 300 $\times$ *g* for 15 min in a Universal 32 centrifuge. The supernatant was collected and stored overnight at 4 °C. The supernatant was transferred into new Eppendorf tubes and kept on ice until protein concentration was determined using

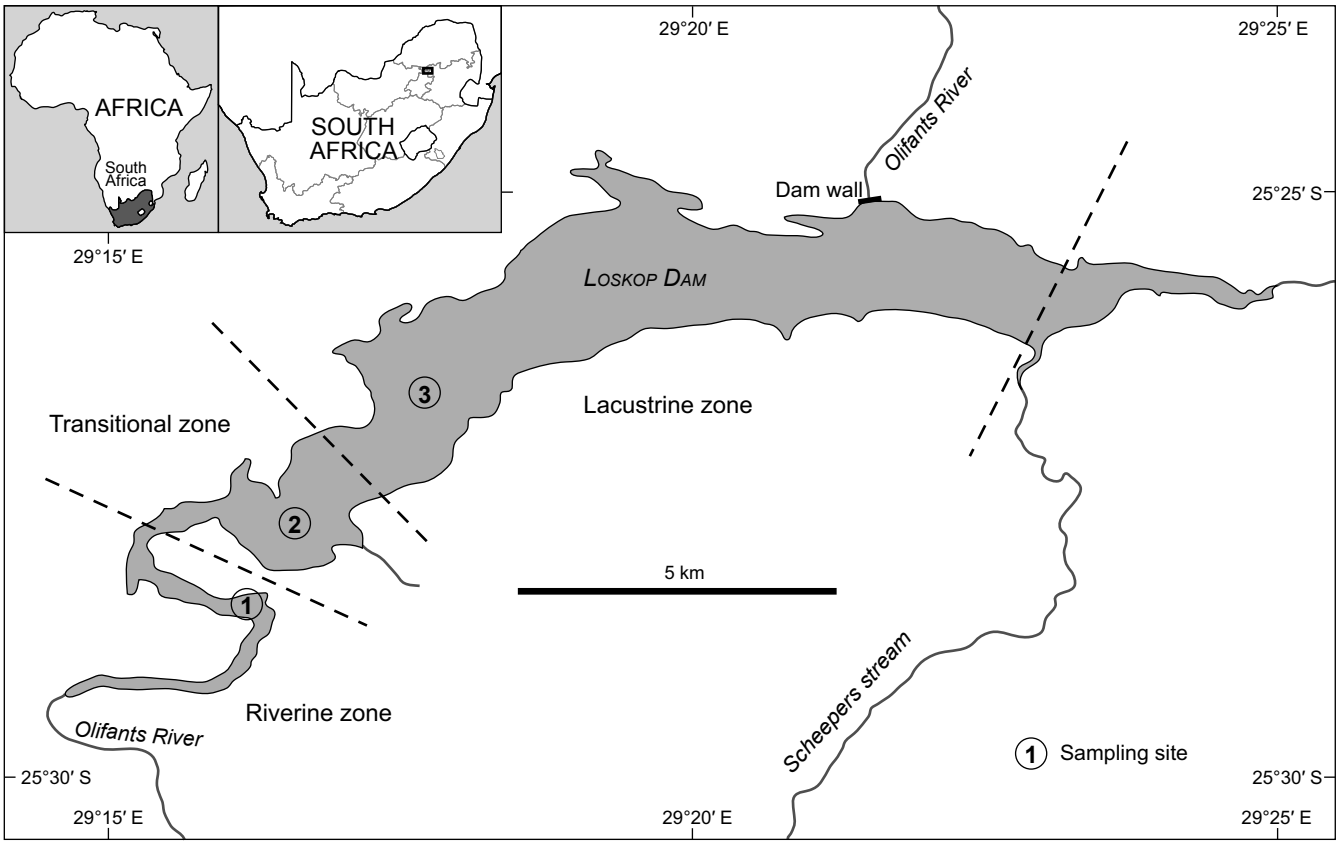

Figure 1: Map of Loskop Dam, Mpumalanga province, South Africa, showing locations of sampling sites (modified after Oberholster et al. 2010)

Nanodrop (Nanodrop ND-1000). One mg of protein from each sample (liver or muscle) was used. These samples were analysed by the ELISA method using microcystin plate kits (Envirologix Inc.®). Each sample was analysed in triplicate and MCYST values were expressed as MCYST-LR equivalents (Magalhães et al. 2001).

The concentrations of MCYST-LR equivalent were expressed in nanogrammes (ng) of MCYST-LR equivalent per gramme of liver and muscle tissue. These data were compared with concentrations from rat tissues of the WHO provisional standards.

Stomach contents

The stomach was dissected from each fish specimen and the stomach content of each fish was fixed with 4% formaldehyde for microscopic examination of phytoplankton content and abundance according to Oberholster et al. (2012).

Statistical analysis

Subsequent multiple comparisons were done with the Tukey *post hoc* test at $p < 0.05$ to compare different mean phytoplankton species abundances between sampling sites. All statistical calculations were done with the statistical package SPSS v 21.0 (SPSS Inc., Chicago). The Berger–Parker dominance index (Berger and Parker 1970) was used to estimate the dominance of organisms at each sampling site.

Results

Physical and chemical parameters

The more acidic pH value (6.49) of the water at Site 3 in the lacustrine zone of Loskop Dam differed notably from the alkaline values of 9.76 recorded in the riverine zone and of 8.64 in the transitional zone of the dam during May 2012 (Table 1). Nutrient ratios of TN to TP were 1:13 at Site 1, 1:28 at Site 2, and 1:26 at Site 3, indicating that the area within the riverine zone was eutrophic according to the Forsberg and Ryding (1980) index. Surface water electrical conductivity values varied between 613 µS cm^{-1} at Site 1 and 446 µS cm^{-1} at Site 3 (Table 1). The average surface water temperature was 22.5 °C, while Secchi disc measurements fluctuated between 0.50 m at the riverine zone sampling site and 2.63 m at Site 3 in the lacustrine zone. The transitional and lacustrine zones were found to be oligotrophic, with more or less the same concentration of total phosphates, whereas the riverine zone was hypertrophic with 0.2 mg l^{-1} of phosphorus (Table 1) and 0.15 mg l^{-1} of chlorophyll *a* (Figure 2).

Phytoplankton assemblage

The dominant diatom species at Site 1 in the riverine zone (Berger–Parker index 0.213) was *Melosira varians* (Agardh) with an average biovolume of 4.2 mm^3 l^{-1}, while the cyanobacterium *Microcystis aeruginosa* (Kützing) was present at a much higher biovolume on 13 mm^3 l^{-1}. The

Table 1: Physico-chemical water quality in the three zones of Loskop Dam in May 2012

Parameter	Riverine	Transitional	Lacustrine
pH	9.76	8.64	6.49
Conductivity (µS cm^{-1})	613	463	446
Dissolved oxygen (%)	26.5	23.3	21.3
Secchi disc (m)	0.50	0.81	2.63
TDS	371.8	266.5	271.05
Bicarbonate (mg l^{-1})	40	37	32
Carbonate (mg l^{-1})	30	24	28
TN (mg l^{-1})	2.6	1.4	1.3
NH_4 (mg l^{-1})	0.24	0.1	0.21
NO_3 (mg l^{-1})	0.66	0.8	0.60
TP (mg l^{-1})	0.2	0.05	0.03
Silica (mg l^{-1})	0.2	3.4	3.9

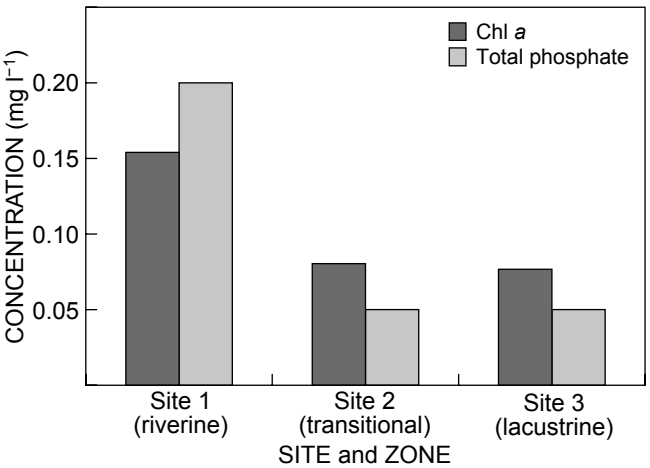

Figure 2: Chlorophyll *a* (Chl *a*) content and total phosphate at three sites in Loskop Dam in May 2012

dominant (Berger–Parker index 0.463) cyanobacterium *M. aeruginosa* at Site 1 was totally absent at Site 3 and therefore was not a contributing factor to the extracellular MCYST-LR equivalent concentrations in the water column at Site 3 (Figure 3). The highest concentration of extracellular MCYST-LR equivalent, 3.25 µg l^{-1}, was observed at Site 1, this being 3.25 times higher than the WHO standard, but was not significantly ($p > 0.05$) higher in comparison to that at Sites 2 and 3.

The higher cell counts of *M. aeruginosa* at Site 1 correlated significantly ($p < 0.05$) with the higher total phosphorus concentrations on 0.2 µg l^{-1} at this site (Table 1). The diatom *Craticula cuspidata* (Kützing) also occurred at a low biovolume (1.9 mm^3 l^{-1}) at Site 1 (Table 2). The latter is a good indicator of eutrophic water conditions and may tolerate critical to very heavy pollution, according to Taylor et al. (2007).

The dominant phytoplankton species that formed a bloom in the transitional and main basin zones of Loskop Dam throughout the year was the larger and slower-growing species *Ceratium hirundinella* (Müller). During May 2012 it had an average biovolume of 14 mm^3 l^{-1}, which correlated positively ($p < 0.05$) with the higher average Si

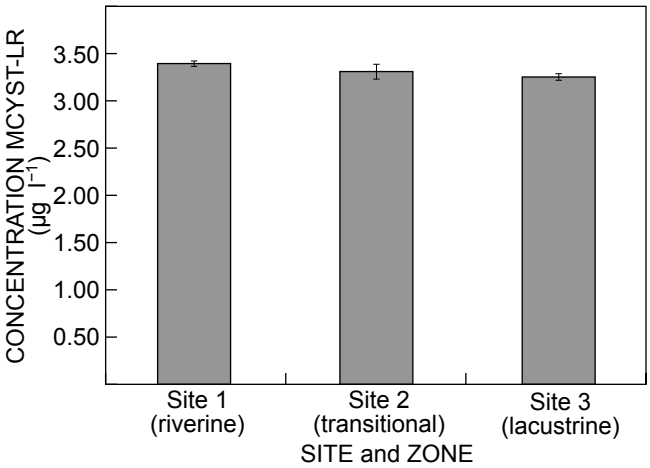

Figure 3: Extracellular microcystin-LR concentrations in water column at three sites in Loskop Dam. Error bars indicate SD; $p > 0.05$

concentrations of 3.4 and 3.9 mg l^{-1} in the water column at Sites 2 and 3, in comparison with lower concentrations of 0.2 mg l^{-1} at Site 1 in the riverine zone (Tables 1 and 2).

Comparative studies of fish

The total body weight and body length of *L. rosae* and *O. mossambicus* are summarised in Figure 4. The overall total number of fish for the study was 26. Most *L. rosae* were smaller in weight, with a minimum mass of 708 g and length of 37 cm in comparison to *O. mossambicus* with a minimum mass of 1 437.9 g and length of 39.2 cm. Of the 26 fish sampled nine *L. rosae* were females and 11 males, whilst no male *O. mossambicus* were recorded. Males were bigger than females with respect to weight and length.

Stomach contents of both species were analysed microscopically to determine their main diet (Figure 5). Very little (<5% of total stomach content) cyanobaterial cells (*Microcystis aeruginosa*) was consumed orally in comparison to *Ceratium hirundinella* that made up 25–53% of the total stomach content of the two different fish species.

Detection of microcystin-LR equivalent in fish

Of the 26 fish examined more than 80% had average MCYST-LR equivalent concentrations above the WHO TDI level of 0.04 µg kg^{-1} body weight d^{-1} (Chorus and Bartram 1999), i.e. 1.72 µg MCYST-LReq kg^{-1} in liver tissue and 0.19 µg MCYST-LReq kg^{-1} in muscle tissue for *L. rosae*, and 2.14 µg MCYST-LReq kg^{-1} in liver tissue and 0.17 µg MCYST-LReq kg^{-1} in muscle tissue for *O. mossambicus*, which means that the daily consumption of fish by humans could be higher than the safety value. All *L. rosae* had significantly lower concentration in the liver as compared to *O. mossambicus* caught from the same site (Figure 6).

There was a significant difference ($p < 0.05$) in MCYST-LR equivalent concentration found in liver and muscle tissue between the two fish species but no significant differences ($p > 0.73$) between the sexes (result not shown). High concentrations of MCYST-LR equivalent in the liver and muscle tissue from both species were detected and

Table 2: Algal community compositions at 1 and 3 m depths at three sites in Loskop Dam during a decomposed cyanobacterial bloom in May 2012. + = rare (≤50 cells l^{-1}); ++ = scarce (51–250 cells l^{-1}); +++ = common (251–1 000 cells l^{-1}); ++++ = abundant (1 001– 5000 cells l^{-1}); and +++++ = predominant (≥5 001 cells l^{-1})

Phylum/Class	Major species	Site 1		Site 2		Site 3	
		1 m	3 m	1 m	3 m	1 m	3 m
Cryptophyta							
Cryptophyceae	*Cryptomonas marsonii* (Ehrenberg)	+	+				
Chrysophyta							
Chrysophyceae	*Dinobryon divergens* (Ehrenberg)	+	+				
Bacillariophyta							
Bacillariophyceae	*Aulacoseira muzzanensis* (Meister)	+					
	Diatoma vulgaris (Bory)	++					
	Craticula cuspidata (Kützing)	+		+	+		
	Fragilaria ulna (Nitzch)	+				+	+
	Pinnularia viridis (Nitzch)	+					
	Synedra acus (Kützing)						
	Melosira varians (Agardh)	++	++		++		
	Fragilaria capucina (Desmaziéres)	+		+			
	Gyrosigma rautenbachiae (Cholnoky)	+					
	Aulacoseira granulata (Ehrenberg)	++	+		+	+	
	Nitzschia palea (Kützing)	+					
	Staurosira elliptica (Schumann)				+		+
	Asterionella formosa (Hassall)	+			+		
	Fragilaria crotonensis (Kitton)			++++	++++	++	+
Dinophyta							
Dinophyceae	*Peridium africana* (Ehrenberg)	+	+	+++	+++	+	
	Ceratium hirundinella (Müller)			+++++	+++++	++	+
Chlorophyta							
Chlorophyceae	*Closterium lineatum* (Nitzch)	+				+	+
	Pediastrum duplex (Meyer)	+		+			
	Cosmarium pseudo praemorsum (Corda)					++	++
	Oocystis rupestris (Braun)					++	
	Scenedesmus quadricauda (Meyer)	+	+			+	+
	Cladophora glomerata (Kützing)	+	+				
	Spirogyra reinhardi (Kützing)	+					
Euglenophyta							
Euglenophyceae	*Trachelomonas intermedia* (Ehrenberg)	++	+	++		+	+
	Phacus pleuronectes (Dujardin)				+		
	Euglena sociabilis (Bold)				+		
Cyanophyta							
Cyanophyceae	*Microcystis aeruginosa* (Kützing)	+++	+++	+			
	Oscillatoria tenuis (Vaucher)	+		+			

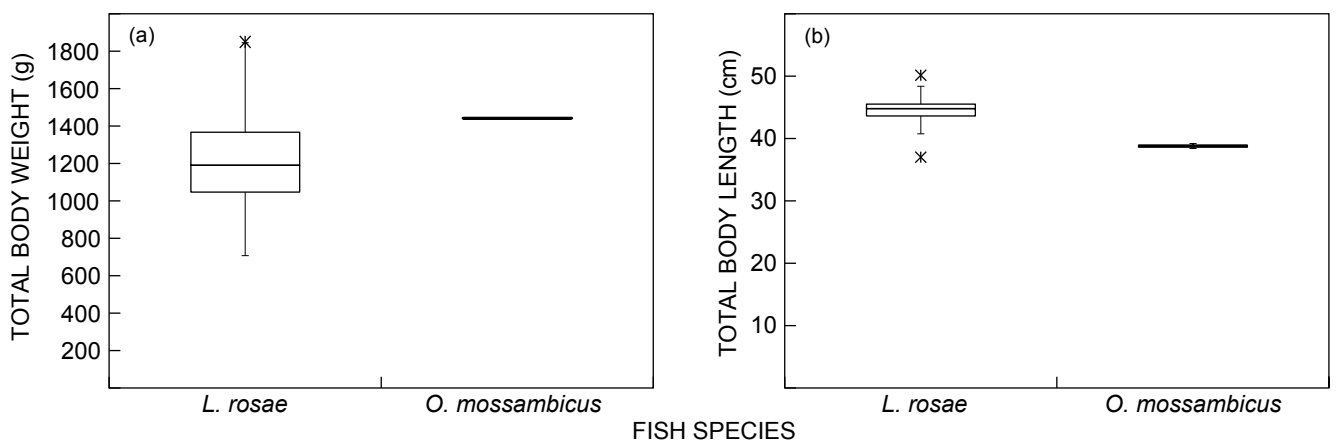

Figure 4: Box plots of (a) weight and (b) total length of *Labeo rosae* and *Oreochromis mossambicus* in Loskop Dam in May 2012. Error bars indicate SD; stars indicate outliers; $p < 0.05$

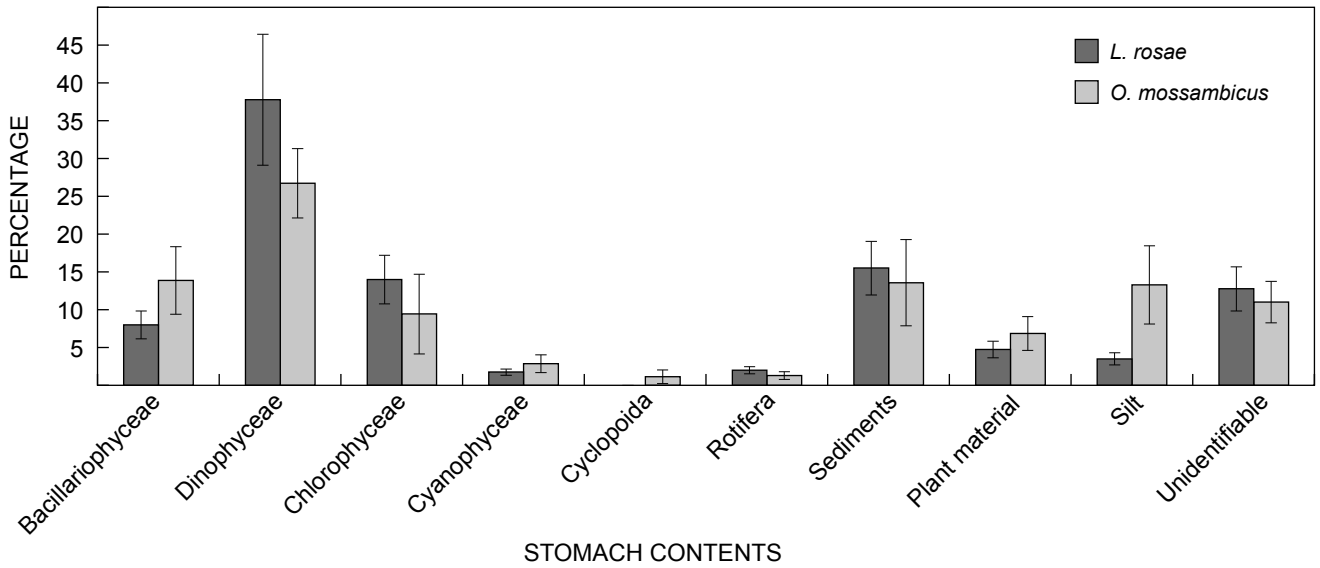

Figure 5: Biotic and abiotic stomach contents of *Labeo rosae* and *Oreochromis mossambicu*s from Loskop Dam in May 2012. Error bars indicate SD; $p < 0.05$

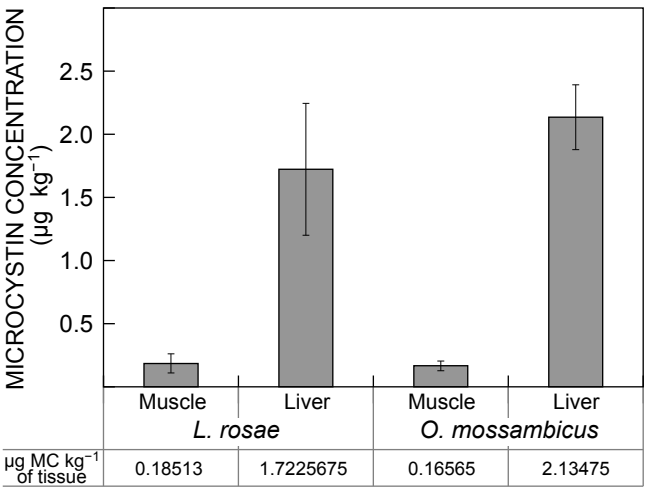

Figure 6: Mean microcystin-LR concentrations in muscle and liver tissue of *Labeo rosae* and *Oreochromis mossambicus* from Loskop Dam in May 2012. Error bars indicate SD; $p < 0.05$

consumption of adequate fish biomass (<0.04 µg kg^{-1} body) of these tissues might cause severe acute effects.

The MCYST-LReq concentrations in liver and muscle tissue for *L. rosae* were 43.6 times and 4.6 times higher, respectively, than the WHO guidelines. The MCYST-LReq concentrations in liver and muscle tissue for *O. mossambicus* were 53.4 times and 4.1 times higher than WHO guidelines. The low concentrations of cyanobacteria cells detected in the stomach content of both fish species may have played a minor role, and the bioaccumulation of cyanotoxin MCYST-LR equivalent in the fish organs may have occurred through the intake of water containing extracellular MCYST-LR equivalent, or from consuming prey that had indigested toxic cyanobacterial cells.

Discussion

High MCYST concentrations pose a health hazard to aquatic life, humans and wildlife (Oberholster et al. 2009). This suggests that biomagnification of MCYSTs in aquatic food chains may be another important factor, suggesting a potential risk for higher trophic level species. Epidemiological investigations showed that the consumption of water contaminated with MCYSTs poses many adverse health effects such as the promotion of liver cancer, gastrointestinal diseases and spleen diseases as well as DNA fragmentation (Zhou et al. 2002; Oberholster et al. 2004). It was evident that fish were exposed to MCYSTs in the water column, even though the intake of cyanobacteria cells was minimal via ingestion (Figure 5). However, it cannot be ruled out that exposure to MCYSTs may have been contributed by prey such as zooplankton that had fed on cyanobacteria (Malbrouck and Kestemont 2006). In the present study, zooplankton was detected only in very low numbers in the stomach content of both fish species.

The concentrations of MCYST-LR equivalent were much higher in the liver tissue than in the muscle tissue and could possibly be related to the functions of the different tissues. The liver serves as a detoxifying organ of the body and therefore it is exposed to high loads of different toxins. The liver also contains more fatty tissue, a target tissue for MCYSTs, than muscle tissue.

These higher MCYST-LR equivalent concentrations in fish tissues correspond with high concentrations of extracellular MCYST-LR equivalent recorded in the water column, suggesting that toxin accumulation may be either through bioconcentration and/or biomagnification. This is supported by the results from the stomach content analysis where *M. aeruginosa* was present in very low numbers. Significant concentrations of MCYSTs were found in the water and in both fish species. Cyanotoxins could be accumulated in

animals through the food chain, causing health problems even to humans (Vasconcelos 2006). Nonetheless, *Microcystis* blooms can be highly localised (Andersen 1997, 2004) and it is likely these fish were exposed to high extracellular concentrations of MCYSTs at Sites 2 and 3 where few or no cyanobacterial cells were observed in the water column. In the present study we did not correlate the internal MCYST-LR equivalent concentrations of the cyanobacterial cells of the bloom with the extracellular MCYST-LR equivalent concentrations, since few or no cyanobacterial cells were observed in the water column at Sites 2 and 3, where 70% of the fish under study were captured.

Regarding the risk to humans, estimated daily intake (EDI) estimation would be necessary before the measured concentrations of MCYSTs in the fish tissue samples could be compared to the WHO TDI provisional guidelines. At the levels found in *L. rosae* in our study, the calculated EDI in liver was 46.6 times higher, and in muscle 4.6 times higher, than the advisable TDI value on 0.04 µg kg^{-1} of body weight. In *O. mossambicus* from the current study the calculated EDI in liver was 53.4 times higher, and in muscle 4.1 times higher, than the advisable TDI value. Therefore, both fish species from Loskop Dam could possibly pose a health hazard and they are thus not safe for human consumption. In addition, according to Soares et al. (2004), cyanobacterial toxins could still be found in fish muscle several days after the end of a toxic bloom.

Only the uptake of MCYSTs from aquatic organisms was used to evaluate exposure risk of humans to MCYSTs in Loskop Dam. A possible human health hazard that needs further investigation is the influence of extracellular concentrations of MCYST-LR equivalent on the irrigated crops produced on the second largest irrigation scheme in South Africa downstream of Loskop Dam. Secondary metabolites can accumulate in the tissue of plants irrigated with contaminated water, as previously highlighted by Codd (1999) who stated that single cells of *M. aeruginosa* and the hepatotoxin MCYST were retained on salad lettuce after being sprayed with irrigation water containing MCYST-producing cyanobacteria. Therefore, extracellular chronic toxic effects to humans from exposure through drinking water or contaminated food are probable, especially if there is long-term frequent exposure (Magalhães et al. 2001, 2003; Zhang et al. 2009b).

Acknowledgements — The study was funded by the National Research Foundation (NRF; TTK2006062100013); Council for Scientific and Industrial Research; Department of Genetics, University of Stellenbosch; and the Department of Biodiversity, University of Limpopo. We thank the Belgian Vlaamse Interuniversitaire Raad University Development Cooperation funding programme for additional funding. Tshepiso Ramalepe and Charles Nyalungu, University of Limpopo, are thanked for assistance during fieldwork.

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