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Full Length Research Paper

Occurrence of microcystins in freshwater bodies in Southern Mozambique

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Many countries have started to monitor cyanobacterial cell densities and microcystin concentrations in raw water sources and recreational water due to human and animal poisonings and the results of toxicological studies, which have shown the adverse effects of microcystins to some mammals. Mozambique has no reports of intoxications or deaths of animals or human by cyanobacterial toxins. However, blooms of cyanobacteria have been reported in some aquatic ecosystems. The aim of this study was to detect and quantify the microcystins extracted in water samples from Pequenos Libombos Dam, Nhambavale Lake and Chòkwé Irrigation Channel, all located in southern Mozambique using LC-MS. The Microcystins MC-LR, -YR and –RR were detected in the three sampling areas. MC-LR was found in higher concentrations than the other two variants. The highest total concentration of microcystins detected was 7.89 µg/L in water samples from Nhambavale Lake. Pequenos Libombos Dam revealed concentrations of MC-LR at below quantifiable levels. Based on these results, it is recommended that drinking water supplies in Mozambique are monitored and that the risks of human and animal intoxication by cyanotoxins are assessed and managed.

Keywords: Mozambique, LC-MS, MC-LR, Microcystins.

INTRODUCTION

Mozambique is a developing country located in the southeast coast of Africa (10° 30'-26° 52'S and 40° 50'-30° - 31' E). The country covers a total land area of 800,000 km2 and has a wide range of freshwater bodies used as sources of drinking water. Few studies have focused on cyanobacteria and their toxin in Mozambique (Pedro *et al.* 2011; Mussagy *et al.* 2006; Bojcevska and Jergil 2003). Given the limited resources of the country, problems of aquatic pollution have not received the proper attention. Toxic cyanobacterial blooms are commonly found in fresh, brackish and marine water. A survey in freshwater has shown that in average, approximately 60% of the blooms in freshwater contain toxins of cyanobacteria (Sivonen 2007). The most common cyanobacterial toxins (cyanotoxins) are hepatotoxins (microcystins and nodularins), neurotoxins (anatoxin-a, anatoxin-a(S) and saxitoxins) and citotoxins (cylindrospermopsins) (Sivonen 2007). Microcystins are the most prevalent cyanobacterial hepatotoxins in freshwater and are most frequently produced by strains of the genera *Anabaena, Microcystis, and Planktothrix* (formerly *Oscillatoria*) (Sivonen and Jones 1999).

Abbreviations: MC, Microcystin; **PSDs**, Passive sampling devices; **LC-MS**, Liquid chromatography-mass spectrometry; **ELISA**, Enzyme linked immunosorbent assay; **PL**, Pequenos Libombos Dam; **NL**, Nhambavale lake; **CH**, Chòkwé Irrigation Channel.

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Moreover, the involvement of *Anabaenopsis, Hapalosiphon,* and *Nostoc* have been less frequently reported (Dittmann and Börner 2005; Vaitomaa *et al.* 2003; Lyra *et al*. 2001; Hummert *et al.* 2001). The microcystins are composed of five common amino acids and pairs of L-amino acids as variants. The most common ones are methyl aspartic acid, alanine, N-methyldehydro-alanine, glutamic acid, and a unique amino acid called Adda (3-amino-9- methoxy-2,6,8-trimethyl-10- phenyldeca-4,6 dienoic acid) (Takino and Kyono 2000).

More than 80 different analogues of microcystins have been described from natural blooms or laboratory cultures of cyanobacteria (Krüger *et al*. 2009; Welker and van Döhren 2006), and most of these differ in the structures of the amino acid residues in positions 2, 3, 4 and 7 of the cyclic peptides (Eckart 1994). Microcystins contain N-methyldehydroalanine (Mdha) or Dehydroalanine (Dha) at unit 7 (Kaya *et al.* 2001). MC-LR as well as all other variants of microcystins has tumor promotion activity (Nishiwaki-Matsushima *et al.* 1992), however there is no evidence of cancer-related activities in the case of [Dhb^7] microcystins (Kaya *et al.* 2001).

Microcystins inhibit eukaryotic serine/threonine protein phosphatase 1 and 2A, resulting in excess hosphorilation of cytoskeletal filaments. This may cause massive hepatic hemorrhage and death (Znachor *et al*. 2006; Runnegar *et al.* 1991; Romanowska-Duda *et al.* 2002; Honkanen *et al.* 1990; MacKintosh *et al.* 1990; Carmichael 1994; Vaitomaa *et al*. 2003). Although there are several potential routes of exposure for microcystins (oral consumption, inhalation, or skin absorption), the most common is ingestion of water (Znachor *et al*. 2006). It is also noteworthy that microcystins are very stable and may accumulate in the food chain (Watanabe *et al.* 1992). A wide range of wild animals and birds have been affected, with consequences ranging from non-fatal (inhibition of invertebrate feeding, delayed fish egg-hatching) to fatal (mass mortalities of birds, wild and domestic animals and humans) (Metcalf and Codd 2004; Bourke and Hawes 1983; Skulberg *et al.* 1984; Carmichael and Falconer 1993; Ressom *et al.* 1994; Jochimsen *et al.* 1998).

For effective management of cyanobacterial hazards to human health, it is necessary with a basic understanding of the properties, the behaviour in natural ecosystems and the environmental conditions which support the growth of certain species (WHO 1999). Cyanobacterial growth in water bodies is stimulated by nitrogen- and phosphorus- containing nutrients, warm temperatures, stagnant water and intense sunlight (Gobler *et al.* 2007). A high cyanobacteria biomass contributes to aesthetic problems, impairment of recreational use (because of scum and unpleasant odor), affects the taste of treated drinking water and more importantly it poses a serious health risk (Znachor *et al*. 2006; Rantala *et al.* 2006; Sivonen and Jones 1999; Pan *et al*. 2002). Identification and quantification of cyanobacteria in water resources is the principal component of cyanotoxin monitoring programmes and can provide an effective early warning system for the development of potentially toxic blooms. The World Health Organization (WHO) has set a provisional guideline of 1 µg/L of total microcystins in drinking water (WHO 2008) and for recreational water there are provisional guidelines of 20000 cells /ml and 100000 cells/ml resulting in low or moderate probabilities of adverse health effects, respectively (Falconer *et al*. 1999; Foulds *et al.* 2002). Ueno *et al.* (1996) have advocated for even stricter guideline value for microcystin LR of 0.1 µg/L due to the potential liver damage since rural communities may be consuming surface water containing cyanotoxins at low level over a period of time. For the risk assessment studies, it is important not only to know the contents of total microcystins but also the presence and relative proportion of each of the microcystin analogue in water collections. (Codd *et al*. 1999).

Many countries in Africa have reported cases of intoxication and deaths of animal that may have been caused by cyanobacterial toxins. In South Africa, *Microcystis aeruginosa* was reported as the most common specie of cyanobacteria causing death of livestock (Oberholster *et al.* 2004). Frequent mass mortalities of lesser flamingos have been reported in Kenya Rift Valley (Ballot *et al.* 2004; Ndetei and Munhandiki 2005) and Tanzania (Nonga *et al.* 2011). Cyanobacterial toxicosis was considered the most probable cause of mass mortalities based on the evidence of traces of toxin in the liver of the flamingos. In 1984 massive fish mortality was observed in the Nyanza Gulf of Lake Victoria in Kenya. This fish mortality coincided with the occurrence of cyanobacteria (Ochumba 1990).

Mozambique has no reports of intoxication or mortality on animal or human populations due to cyanobacterial toxins. However, species of cyanobacteria and microcystins have been reported in some aquatic ecosystems by Polymerase Chain reaction (PCR), microscopic observation, Liquid Chromatography coupled to Mass Spectrometry (LC-MS) and Enzyme Linked Immunosorbent Assay (ELISA) (Pedro *et al*. 2011; Mussagy *et al*. 2006; Bojcevska and Jergil 2003). The aim of this study was to investigate the presence of microcystins in water samples collected in the selected water bodies in southern Mozambique using approach LC-MS.

MATERIAL AND METHODS

Study area

This study was carried out in three different sampling areas located in south of Mozambique: Pequenos

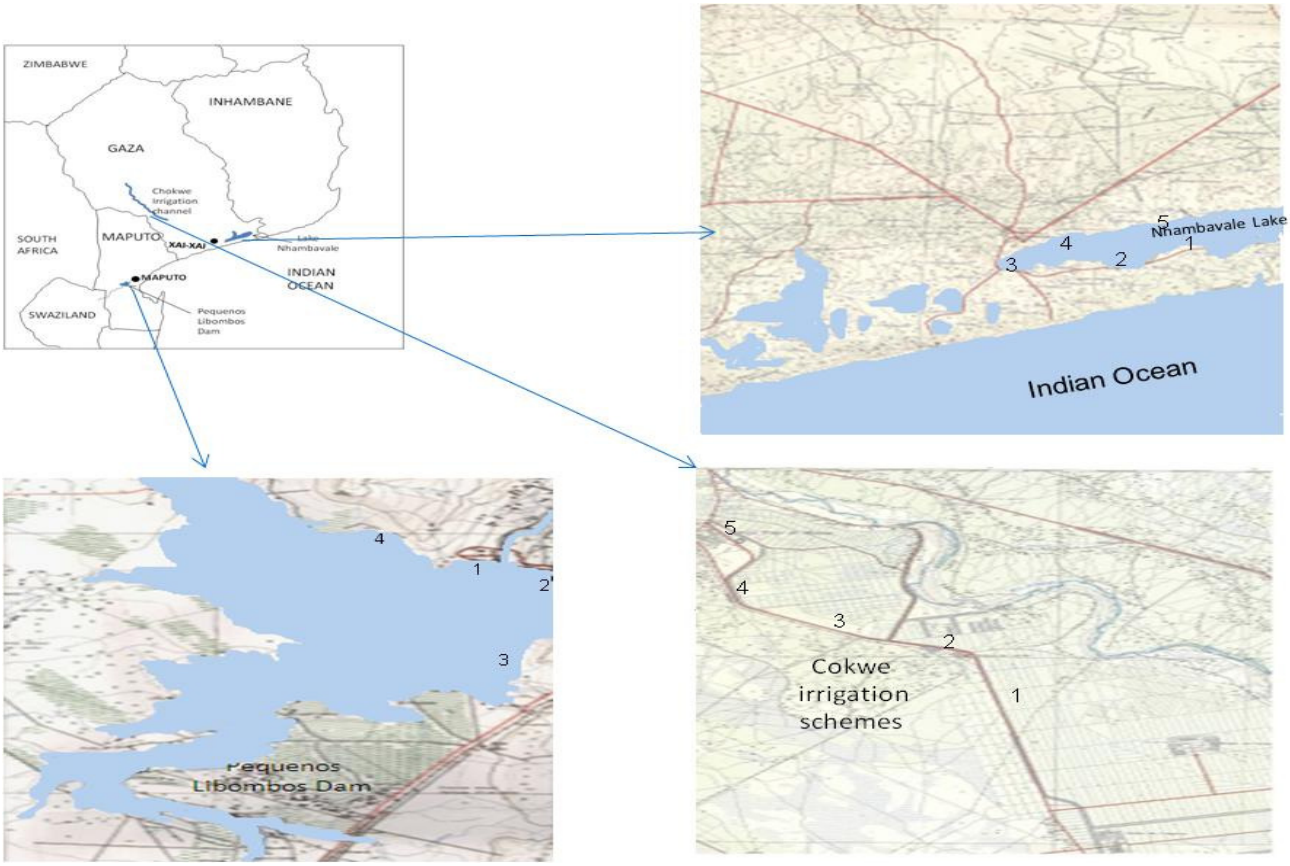

Figure 1. Map of south of Mozambique showing the three study areas: Pequenos Libombos Dam, Chòkwé Irrigation Channels and Nhambavale Lake. The numbers 1 to 5 indicate the sampling stations at each area.

Pibombos dam (PL), Nhambavale lake (NL) and Chòkwé Irrigation Channel (CH). Pequenos Libombos Dam is a manmade impoundment located 35 km west of Maputo and is the main drinking-water supplier to Maputo, the capital of Mozambique, and also used for fishing. Nhambavale Lake is located in North of Gaza province in Chidenguele region and is also used for drinking, fishing and recreation. A Chokwé irrigation Channel, also located in Gaza Province, is used mainly as source of water for irrigation, but also as a source of drinking water. Water samples were collected in three to five sampling stations at each sampling area (Figure 1). The difference in number of sampling stations was due to the limited accessibility of the sampling areas. The samples used here have been previously analyzed by PCR (Pedro *et al.* 2011).

Cyanobacterial strains and sampling

Sampling took place on 7th of June 2008 and 5th of March 2009. Thirteen water samples were collected during each sampling period (5 samples from Chòkwé Irrigation Channel, 5 from Nhambavale Lake and 3 from Pequenos Libombos dam). In 2008 water samples were collected directly into 1 liter bottles, submersed to about 1 meter below the surface without an additional filtration. In 2009, 30 liters of water from the lake were filtered by conical plankton net (20 µm mesh) to 500 ml bottles. Samples were stored at -20^{o}C until processing. *Anabaena circinalis* (NIVA-CYA82) and *Anabaena lemmermannii var. minor* (NIVA-CYA 83/1), both purchased at NIVA (Norwegian Institute for Water Research) were included as positive and negative control respectively.

Sample preparation for microcystins detection

Extraction of toxins from water samples

Ten ml of water samples and 2 ml of cell cultures were sonicated in an ultrasonic bath for 10 min. Microcystins were extracted from twenty three samples with methanol. Samples were passed through a Bond Elut C_{18} cartridge (Sample preparation sample, VARIAN^{VA}) previously activated by 1 ml of methanol and washed by distillated

Table 1. Microcystin variants and concentration in freshwater bodies in Mozambique. (PL) Pequenos Libombos Dam, (CH) Chòkwé irrigation channel, (NL) Nhambavale Lake. (08) water samples collected in 2008, (09) water samples collected in 2009. The numbers 1 to 5 indicate the sampling stations.

Sampling area	Samples name	Concentrations (µg/L)			
		MC-LR	MC-YR	MC-RR	Total MC
Pequenos Libombos Dam	1PL-09	bql	-	-	-
	2PL-09	bql	0.10	-	0.10
Chòkwé irrigation channel	4CH-09	0.67	0.06	-	0.73
	5CH-09	0.68	-	-	0.68
Nhambavale Lake	4NL-08	1.65	0.04	-	1.69
	5NL-08	2.6	-	bql	2.6
	3NL-09	2.17	-	-	2.17
	4NL-09	7.82	0.07	-	7.89
	5NL-09	0.86	-	-	0.86

(-) negative samples on the test.
bql- below quantification levels.

Table 2. Microcystins in freshwater bodies in Mozambique in 2008 and 2009.

Sampling area	No. of samples	MC positive samples (%)	MC conc in water (µg/L)	MC variants	pH
Pequenos Libombos Dam	6	2 (33.3)	0.10	LR; YR	8.55 – 8.65
Chòkwé irrigation channel	7	2 (28.5)	0.68 – 0.73	LR; YR	8.20 –8.56
Nhambavale Lake	10	5 (50)	0.86 – 7.89	LR; YR; RR	8.18 –8.40

water. Toxins were eluted from cartridge with 2 ml of methanol.

LC-MS analysis.

For analysis of the extracted toxin for detection of microcystins a modified method described by Neffling *et al.* (2010) was used. Liquid chromatography was performed on a XBridge C_{18} column (3.5 µm, 50 × 2.1 mm) (Waters, Milford, MA, USA), using a Surveyor HPLC system (Thermo Electron Corporation, Waltham, MA, USA). Separation was achieved using linear gradient elution at 0.3 mL/min starting with MeCN–water (30:70, both containing 0.1% formic acid) rising to 100% MeCN over 10 min. Isocratic elution with 100% MeCN was maintained for 5 min before the eluent was switched back to 30% MeCN. The HPLC system was coupled to an LTQ ion trap mass spectrometer operating with an electrospray ionization (ESI) interface (Thermo Electron Corporation, Waltham, MA, USA). Typical ESI parameters were spray voltage 4 kV, heated capillary temperature 250 °C and sheath gas 60 units (ca 60 L/h) of N2. The mass spectrometer was operated in scan mode (m/z 400–1300) and all samples were diluted to appropriate concentrations with 60% MeOH. For full scan MS/MS analyses, the selected precursor ions were isolated with an isolation width of 3 Da. The detection limit of the method was 9 ng/ml. Microcystins -LR, -YR and -RR were included as standards in the study. LC-MS was conducted at Norwegian Veterinary Institute (Norway).

RESULTS AND DISCUSSION

LC-MS analysis revealed the presence of three variants of microcystins in water samples collected in Southern Mozambique (Table 1 and 2). MC-LR, -YR, and -RR were measured in the samples on the basis of both their mass spectra and retention time. Peaks at 995.6 *m/z* ($[M+H]^{1+}$), 1045.5 *m/z* ($[M+H]^{1+}$) and 1038.5 *m/z* ($[M+2H]^{2+}$) and retention times of 3.87, 3.72 and 3.07 min correspondent to MC- LR, -YR and –RR respectively were identified in some water samples (Table 1, Figure 2) as well as in positive control strain *Anabaena circinalis* (NIVA-CYA 82). Table 2 shows the summary of the concentration of microcystins in water samples collected in Nhambavale Lake, Chòkwé Irrigation Channel and Pequenos Libombos dam in June 7th 2008 and March 5th 2009. Microcystins were detected in 7 out of the 23 water samples analyzed (Table 2), with total concentration ranging from 0.10 µg/L to 7.89 µg/L. The highest concentration of total microcystins (7.89 µg/L) was detected in Nhambavale lake (Table 2) and was around 10 times higher compared with the microcystin concentrations measured in Chòkwé Irrigation Channel (the highest concentration was 0.73 µg/L) and around 7

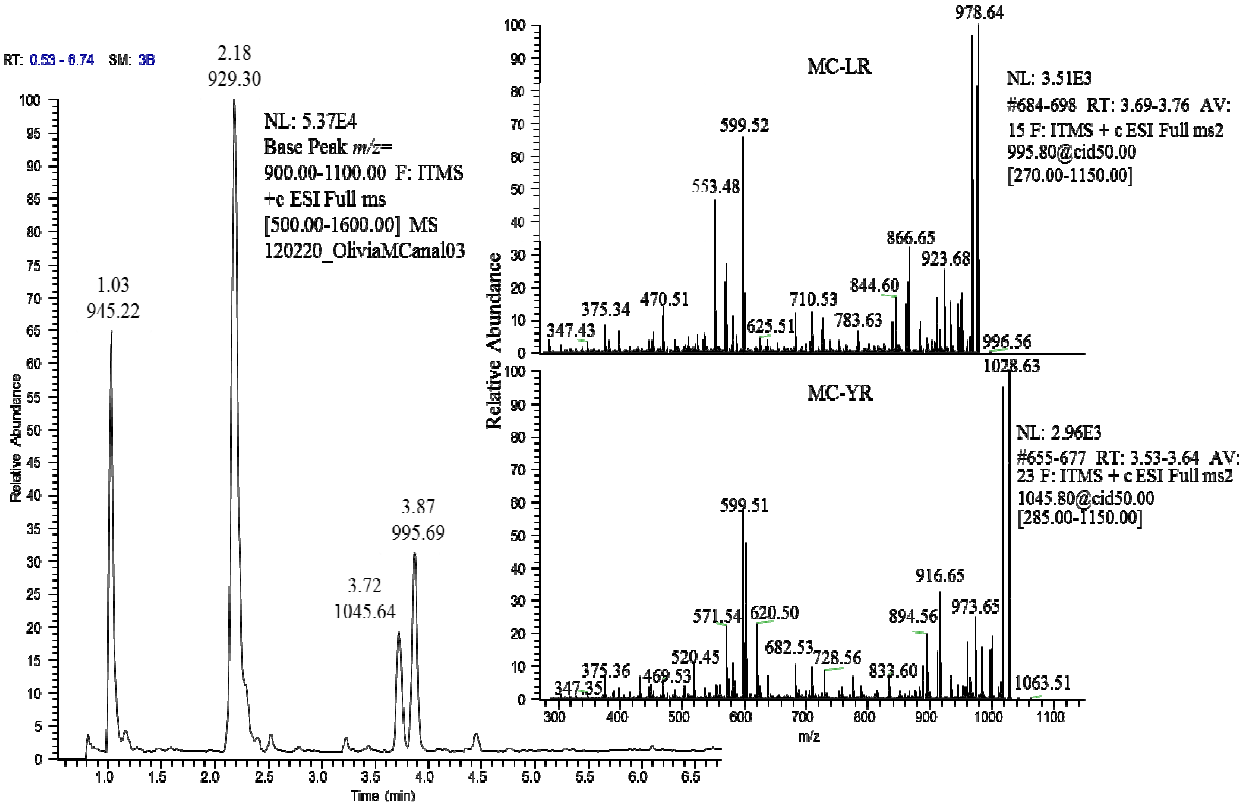

Figure 2. LC–MS chromatogram and MS^2 spectra of microcystins LR and YR of water sample collected in Nhambavale Lake, Mozambique.

Times above the WHO guideline of 1µg/L total microcystins in drinking water (WHO 2008).

Figure 3 shows that the LR variant was found to be the predominant microcystin in the all three sampling areas. The concentration of MC-YR varied from 0.04 µg/L to 0.1 µg/L of water and was detected in only 4 out of 23 water samples analyzed. MC-RR was detected only in Nhambavale Lake and was at below quantifiable levels. MC-LR was also detected at below quantifiable level at Pequenos Libombos Dam (Table 1). High concentration of microcystins was observed in March 2009, a period characterized by high temperatures (21–26ºC). Warm temperatures are normally associated with high concentrations of microcystins (Neilan *et al.* 1994; Wicks and Thiel 1990; Chorus and Bartram 1999; Jöhnk *et al.* 2008; Paerl and Huisman 2008). Additionally, different factors such as sampling collection method and environmental conditions in the two years may be contributed to the differences in the microcystin concentrations measured.

An ELISA assay has previously demonstrated the presence of microcystins in Nhambavale Lake, with concentration ranging from 0.5 to 6.8 µg/L (Bojcevska and Jergil 2003) which were almost the same as this study (7.89 µg/L) and 7 times above the WHO guideline of 1µg/L total microcystins (WHO 2008). Previous studies in Chòkwé, have indicated concentration of microcystins below 0.1 µg/L (Bojcevska and Jergil 2003). In the present study MC-LR varied from 0.67 to 0.68 µg/L, indicating that microcystin concentration levels in this area usually may stay below the WHO threshold. Analysis for microcystins in passive sampling devices (PSDs) by LC-MS revealed 3 microcystin variants in Southern Mozambique (MC-LR, -YR and –RR) at the concentrations of 2.1– 159.4 ng/g of PSDs (Pedro *et al.* 2011). During the sampling no bloom formation was observed in the three areas, however Bojcevska and Jergil (2003) reported bloom formation in Pequenos Libombos Dam. This lack of consistent information strongly points out the urgent need for longitudinal screening studies in all relevant areas of Mozambique. ELISA method used previously to quantify microcystins in Mozambique is a good screening test, however it has the limitation of being expensive and not differentiates between variants of microcystins as the LC-MS method does.

Other studies in Africa reported high concentration of microcystins, with values above the WHO threshold. Microcystin concentrations ranging from 2 µg/L to 23.7 µg/L have been reported from different water bodies in

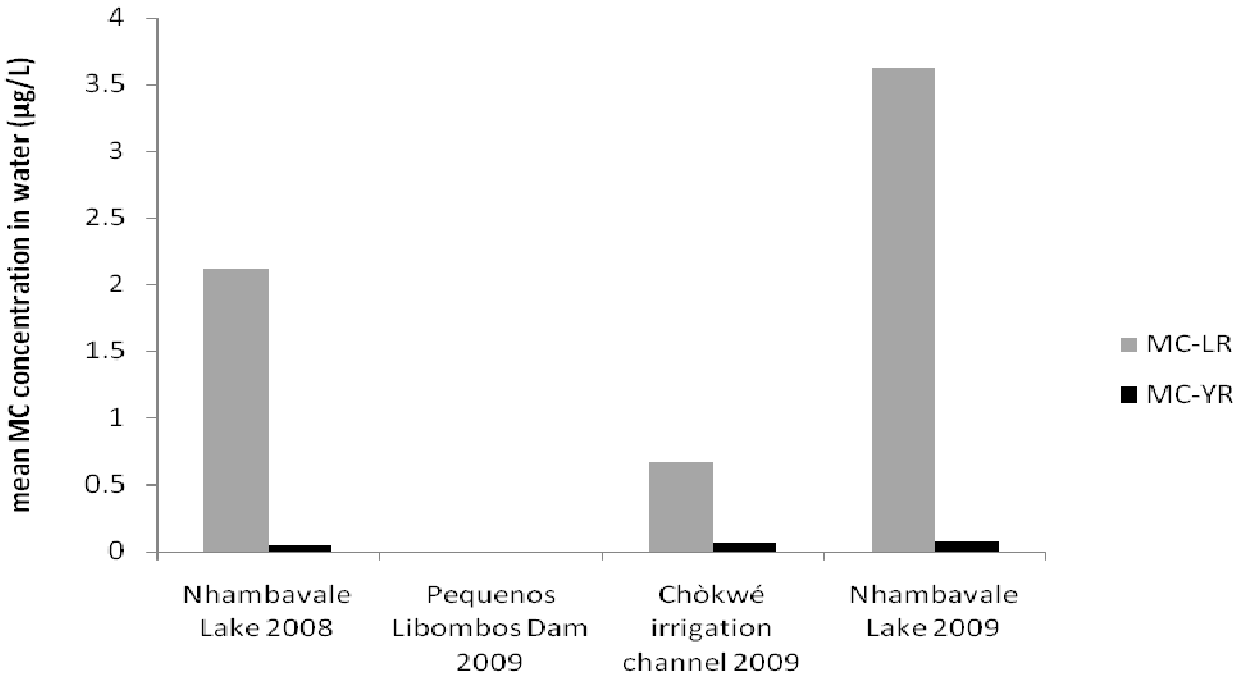

Figure 3. Microcystin concentrations (mean) in samples collected in 2008 (Nhambavale Lake) and 2009 (Pequenos Libombos Dam, Chòkwé Irrigation Schemes, and Nhambavale Lake). Pequenos Libombos Dam in 2009 showed MC-LR concentration at below quantifiable levels.

Southern Africa (Makhera *et al.* 2011; Oberholster *et al.* 2009). Okello *et al.* (2011) reported concentration of microcystins varying from 0.02 µg/L to 10 µg/L of water in Uganda. Microcystin concentrations found in the present study are within the range detected in different studies in Africa, which indicate the great variability of this parameter. Furthermore, microcystin concentrations are likely to vary substantially within the same water collection during the year and over a period of several years. Drinking water may be the major source of microcystins at the present time, based on the water intake of people every day for a long time. However, the possibility of exposure to microcystins through the food chain cannot be excluded (Ueno *et al.* 1996). Programs for monitoring water bodies should be considered by the responsible authorities in Mozambique.

The concentration of microcystins in water samples from the three areas varied from non-detectable levels in Pequenos Libombos dam to high levels (7.89 µg/L) and above the WHO guideline of total microcystins in water in Nhambavale Lake. Nhambavale Lake is a touristic area and populations from Chidenguele Village take untreated water for consumption and do hand washing into the lake. The lake is also used to practice recreation activities such as sailing, canoeing and fishing. Our results show that populations from Nhambavale Lake may be consuming surface water containing cyanotoxins that potentially could increase the risk of liver cancer. Carmichael (1994) and Juković *et al.* (2008) found that the incidence of primary liver cancer is higher in peoples who commonly use pond or river water for consumption. Ueno *et al.* (1996) also suggest that the consumption of microcystins at low level for long term may result also in liver damage. Conversely, Pequenos Libombos Dam is a source of water which supply Maputo city, after water treatment. However, rural populations have access to untreated water from the Incomati River that supply this dam. The conventional methods for water treatment are not effective for elimination of microcystins. Volterra *et al.* (1990) reported that trichomes of cyanobacteria may be disrupted during drinking water treatment, and their hepatotoxins released into the drinking water (Himberg *et al.* 1989; Brittain *et al.* 2000).

In conclusion, the presence of hepatotoxic microcystins in freshwater from Mozambique may constitute a health risk especially for rural communities that may be in contact with the contaminated water without any form of treatment. Taking into consideration the highest concentration of microcystins detected in the lakes (7.89 µg/L), it is important that drinking water supplies in Mozambique are monitored for the presence of microcystins to manage the risk of intoxication.

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