

Full Length Research Paper

Polymerase chain reaction (PCR) detection of the predominant microcystin-producing genotype of cyanobacteria in Mozambican lakes

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Mozambique is a developing country with a wide range of aquatic ecosystems. Given the limited resources of the country, problems of aquatic pollution have not received the required consideration. The aim of the present study was to assess the presence of microcystins (MCs) and identify the genotypes of MC-producing cyanobacteria in Mozambique. Polymerase chain reaction (PCR) based detection methods were used to analyze samples from three study freshwater bodies which are used as sources of drinking water. The occurrence of cyanobacterial toxic genes in Nhambavale lake and Chòkwé irrigation channels is reported based on general and genus-specific PCR amplification of the *cpcB-cpcA*, *mcyA* and *mcyB* genes. The genera of MC-producing cyanobacteria were differentiated by restriction fragment length polymorphism (RFLPs) analysis. *Microcystis* was identified as the predominant potential MC-producing genera. Analysis for MCs in passive sampling devices (PSDs) by liquid chromatography-mass spectroscopy (LC-MS) revealed 3 MC variants (MC-LR, -YR and -RR) at concentrations of 2.1 to 159.4 ng/g of PSD. MC-LR was the dominant variant which was detected in all study sites. This study has established that *Microcystis* was the predominant genotype and it may be the genus responsible for the production of the MCs detected in water. Results from this study showed that the RFLPs method was able to differentiate MC-producing from the non- MC-producing cyanobacteria in Mozambique.

Key words: Cyanobacteria, microcystins, Mozambique, PCR, RFLP.

INTRODUCTION

Freshwater bodies are the primary sources of drinking

water for most of the world's human populations. Increased eutrophication in freshwater bodies has led to an increased occurrence of potential toxic cyanobacterial blooms (Phelan and Downing, 2007). The frequency and global distribution of potential toxic cyanobacterial incidents appear to have increased over recent years, with associated human intoxications (Codd et al., 1999; Duy et al., 2000). The number of identified potential toxic cyanobacteria is still increasing as a result of detection of new strains. The most common cyanobacteria genera known for their potential ability to produce cyanotoxins

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Abbreviations: MC, Microcystins; PCR, polymerase chain reaction; PSDs, passive sampling devices; LC-MS, liquid chromatography-mass spectroscopy; RFLPs, restriction fragment length polymorphism.

are *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya*, *Microcystis*, *Nodularia*, *Nostoc* and *Planktothrix* (Sivonen and Jones, 1999; Sivonen, 2007; Dittmann and Börner, 2005; Lyra et al., 2001; Hummert et al., 2001; Vaitomaa et al., 2003). Microcystins (MC) are the most commonly encountered cyanobacterial toxins in freshwater bodies (Van Apeldoorn et al., 2007). MCs are hepatotoxins, members of a peptide family which have the common structure cyclo-(D-Ala¹-X²-D-MeAsp³-Z⁴-Adda⁵-D-Glu⁶-Mdha⁷) where X and Z are variable L-amino acids (for example, MC-LR refers to leucine and arginine in the variable positions of this peptide), D-MeAsp is D-erythro-β-methylaspartic acid, Adda is (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, and Mdha is N-methyldehydroalanine (van Apeldoorn et al., 2007).

The presence of potential toxic cyanobacteria and cyanotoxins in freshwater bodies used as sources for drinking water has received increasing attention worldwide (Sivonen and Jones, 1999; Codd et al., 1999). The World Health Organization (WHO) considers that freshwater contamination by cyanobacteria and the toxins they synthesize constitutes a worldwide threat to the public and set a guideline value of 1 µg/L of MC-LR in drinking water (WHO, 1998). Cases of cyanotoxin intoxication and animal mortality have been increasingly reported in many African countries. In South Africa for example, *Microcystis aeruginosa* is reported as the most common specie of cyanobacteria which has been the cause of livestock mortality (Oberholster et al., 2004). Frequent mass mortalities of lesser flamingo have been reported in Kenyan alkaline-saline lakes during the recent decades (Ballot et al., 2004; Ndetei and Muhandiki, 2005). Moreover, in 1984 massive fish mortality was observed in the Nyanza Gulf of Lake Victoria, Kenya, which coincided with the occurrence of heavy cyanobacterial blooms (Ochumba, 1990).

In Mozambique, there are no reports of intoxication or mortality in animal or human population which are related to cyanotoxins, although a number of cyanobacterial species have been reported in some aquatic ecosystems. A study by Bojcevska and Jergil (2003) in some freshwater bodies in Mozambique reported the occurrence of *Anabaena* spp., *Cyanodictyon imperfectum*, *Cylindrospermopsis raciborskii*, *Chroococcus* cf *dispersus* *Chroococcus* cf *minutes*, *Geitlerina unigranulatum*, *M. aeruginosa*, *M. botrys*, *M. flos-aquae*, *M. novacekii*, *M. wesenbergii* and *Oscillatoria* spp. In spite of the reports on the presence of potential toxic cyanobacterial species in a number of freshwater bodies in Mozambique (Bojcevska and Jergil, 2003; Mussagy, 1990), the information on the MC-producing genotypes and MC concentrations is limited. Bojcevska and Jergil (2003) reported the presence of MCs in the eutrophic Pequenos Libombos dam, Chòkwé irrigation channels and Chidenguele lake. In that study, Bojcevska and Jergil

(2003) reported that Pequenos Libombos dam had developed heavy blooms of potential toxic cyanobacteria in October, 2002. Considering the importance of these freshwater bodies to the public, an effort should be made to put in place a systematic monitoring system for potential toxic cyanobacteria and cyanotoxins.

No studies have been conducted to quantitatively determine cyanobacteria in freshwater bodies including their possible seasonal fluctuation in Mozambique. To address the lack of knowledge, a polymerase chain reaction (PCR) based on the detection of genes encoding for biosynthesis of MCs and c-phycoerythrin and Liquid Chromatography-Mass Spectrometry (LC-MS) have been employed. DNA-based detection methods have become popular because of their potential specificity (targeting genes involved in toxin biosynthesis), sensitivity, and speed which may provide rapid and sensitive diagnosis of toxic and toxigenic cyanobacteria (Ouellette and Wilhelm, 2003). Many studies are currently using molecular methods to detect the potential toxic cyanobacteria in the aquatic ecosystems (Neilan, 1995; Rudi et al., 1998a, b; Neilan et al., 1999; Ouellette and Wilhelm, 2003; Fathalli et al., 2011); most of them targeting the MC synthetase (*mcy*) gene cluster (Dittmann et al., 1997; Tillett et al., 2000). Recently, sequenced MC biosynthesis genes in *Microcystis*, *Planktothrix* and *Anabaena* (Nishizawa et al., 1999, 2000; Tillett et al., 2000) are being used throughout the world for the design and construction of primer sets for PCR-based toxin gene detection (Baker et al., 2001; Tillett et al., 2001; Nonneman and Zimba, 2002; Pan et al., 2002; Baker et al., 2002). The availability of a sensitive, specific and robust method for detection of toxic or non-toxic cyanobacteria is important for water management and for studies of geographical distribution of these organisms in water bodies. The aim of this present study was to identify MC-producing genotypes of cyanobacteria by using PCR-based approach and establish the presence of MCs in selected freshwater bodies in Mozambique.

MATERIALS AND METHODS

Study area

This study was carried out in three different areas in the South of Mozambique (Figure 1). Pequenos Libombos dam (PL) which is located 35 km west of Maputo serves as the main source of drinking-water for Maputo city. Nhambavale lake (NL) is located at North of Gaza province in the Chidenguele village. The lake is a tourist area and is used as a source of drinking water, for fishing and recreational activities. The third study area was Chòkwé irrigation channels (CH), located in the center of Gaza province and used as source of water for irrigation.

Sampling and cyanobacterial control strains

The sampling process was conducted in June 2008 and March 2009. Thirteen water samples (5 samples from Chòkwé irrigation channels, 5 from Nhambavale lake and 3 from Pequenos Libombos



Figure 1. Map of south of Mozambique showing the three study areas: Pequenos Libombos dam; Chòkwé irrigation channels and Nhambavale lake.

dam) were collected during each sampling period. In June 2008, water samples were collected directly into 1 L bottles, submerged to about 1 m without an additional filtration. In March 2009, 30 L of water was drawn from each sampling sites and filtered through a plankton net (20 μm mesh size), to a final volume of 500 ml. The samples were subsequently stored frozen (-20°C) for analysis. Toxic (NIVA-CYA 228/1) and non-toxic (NIVA-CYA 144) strains of

M. aeruginosa were purchased at NIVA (Norwegian Institute for Water Research), and were used as positive and negative controls of *mcy* gene in MC-producing and non MC-producing strains. Toxic strain of *M. aeruginosa* PCC7806 which was also used as a positive control was kindly provided by The Pasteur Culture Collection of Cyanobacteria (Institut Pasteur). An unknown algae sample from South Africa and *Arthrospira sp.* were also included in the analysis.

Table 1. PCR primers sets used in this study.

Gene	Name	Sequence 5'- 3'	Fragment size (bp)	Targeting strains	Reference
mcyA	mcyA-Cd1F	5'- AAAATTAAGCCGATATCAAA-3'	297	Cyanobacterial genotypes	Hisbergues et al. (2003)
	mcyA-Cd1R	5'- AAAAGTGTTTTATTAGCGGCTCAT -3'			
	MISYf	5'- CGACCGAGGAATTTCAAGCT-3'	122	<i>M. aeruginosa</i>	
	MISYr	5'- AGTATCCGACCAAGTTACCCAAAC-3'			
mcyB	30F	5'- CCTACCGAGCGCTTGGG-3'	78	<i>Microcystis sp.</i>	Kurmayer and Kutzenberger (2003)
	108R	5'-GAAAATCCCCTAAAGATTCTGAGT-3'			
cpcB-cpcA	188F	5'- GCTACTTCGACCGCGCC -3'	66	<i>Microcystis sp.</i> (toxic and non toxic)	Kurmayer and Kutzenberger (2003)
	245R	5'- TCCTACGGTTTAATTGAGACTAGCC-3'			

DNA extraction

2 ml of each cyanobacterial culture (controls), and 30 ml of each water sample were pelleted by centrifugation and DNA extraction was done using a protocol modified from the methods described by Hysbergues et al. (2003) and Kurmayer et al. (2003). Briefly, the pellets were re-suspended in 0.75 ml of lysis buffer (TES = 50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 25% Sucrose) and incubated in ice for 1 h. The cells were treated with lysozyme (final concentration of 5 mg/ml) for 1 h at 37°C. Proteinase K and SDS were added to a final concentration of 150 µg/ml and 2%, respectively and incubated for 2 h at 50°C (completed lysis). The DNA was extracted three times with equal volumes of chloroform: isoamyl alcohol (24:1). The DNA was precipitated by the addition of one volume of ethanol 96%, washed with 70% ethanol, dried and re-suspended in TE pH 7.5. Additional treatment with *RNase A* was done for 1 h at 37°C. The pellets were re-suspended in 50 µl of TE pH 7.5 and stored at -20°C until further analyses. DNA from unknown algae sample from South Africa and *Arthrospira sp.* sample from Norway were extracted using a CTAB based as described by van den Eede et al. (2000).

PCR amplification and PCR-RFLP

To determine the presence of cyanobacterial genotype and

the toxin genes *mcyA* and *mcyB*, four different pair of primers were used (Table 1). PCR amplification of a fragment region of the gene encoding c-phycoyanin (*cpcB-cpcA*) was carried out using 188F/245R pair of primers (Kurmayer and Kutzenberger, 2003). For MC-producing cyanobacteria, the PCR was performed with specific pairs of primers (MISYf/MISYr, *mcyA*-Cd1F/R) designed to target regions of the *mcyA* gene (Foulds et al., 2002; Hisbergues et al., 2003) and the primers 108R/30F were used for *mcyB* gene (Kurmayer and Kutzenberger, 2003). The target cyanobacterial strains for each pair of primers used is shown in Table 1.

The subsequent RFLP analysis of the amplified *mcyA*-Cd fragments allowed discriminating between *Microcystis*, *Anabaena* and *Planktothrix*. PCR was performed in 0.2 µl thin-walled PCR tubes. PCR reaction (25 µl) contained 1 × PCR buffer, 2 mM MgCl₂, 200 µM dNTPs, 0.6 µM of each primer, 5 µl of DNA template (15 to 28 ng) and 2U Taq polymerase. Thermal cycling was carried out in thermocycler (TETRAD) using the following program: initial denaturation at 95°C for 10 min; 45 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 5 min. All primers used were synthesized by DNA Technology A/S (Denmark).

For RFLP analyses, the PCR products of the *mcyA* gene were digested with *HindIII* and *EcoRV* restriction enzymes according to the manufacturer's protocol. All the amplifications and digested products were visualized by electrophoresis on 2.5% agarose gels together with pUC

Mix Marker, 8, ready-to-use DNA ladder (Fermentas). The gel was stained with ethidium bromide, and run for 2 h in 1X TBE running buffer. The fragment pattern of one unique band indicates the presence of *Anabaena* if digested by *HindIII* or *Planktothrix* if digested by *EcoRV* restriction enzymes. *Microcystis* is evidenced by two bands of different sizes when digested either by *HindIII* or *EcoRV* (Hisbergues et al., 2003) (Table 2).

Sampling of dissolved MCs in water using passive samplers

Screening of MCs dissolved in water at each sampling sites was done by the use of passive sampling devices (PSDs) as described by Rundberget et al. (2009). A 40 µm nylon mesh (Sefar AG, Switzerland) folded in half, a 75 mm diameter plastic embroidery frame (Permin, Denmark) and 3.0 g of HP-20 resin (polystyrene divinylbenzene resin) (Diaion HP-20, Mitsubishi Chemical Corporation, Japan) were used as adsorbent materials for each PSDs. The resin was activated by soaking the packed device in MeOH for 15 min and washing in distilled water, as described in the resin-manufacturer's instructions. MeOH-activated PSDs were submerged in the lake at one meter depth for 12 days. After they were withdrawn from the lake, the PSDs were washed in distilled water and placed in air-tight plastic bags and stored in a fridge at 4°C prior to toxin extraction. In June, 2008, a total of 26 PSDs (5 samples

Table 2. Theoretical restriction fragment lengths of the *mcyA*-Cd fragments from three cyanobacterial strains digested with the indicated restriction enzymes (Extracted from Hisbergues et al. (2003).

Species	<i>HindIII</i> (bp)	<i>EcoRV</i> (bp)
<i>Anabaena</i>	297	232
		65
<i>Microcystis</i>	191	232
		59
<i>Planktothrix</i>	261	267

from Chòkwé irrigation Channels, 8 from Nhambavale Lake and 13 from Pequenos Libombos Dam) were collected while 19 PSD (6 samples from Chòkwé irrigation Channels, 7 from Nhambavale lake, and 6 from Pequenos Libombos Dam) were collected in March, 2009.

To extract the toxins, the embroidery ring was opened, and the used resin was quantitatively transferred to a 25 ml Varian Bond-elute reservoir fitted with a 20 µM nylon frit (Varian, Palo Alto, CA) and washed with 30 to 50 ml deionized water. Excess water was drawn from the column by application of a vacuum. Up to 10 ml of MeOH was added to the column and the resin was stirred gently then left to stand for 15 min. The column was then eluted slowly (0.5 to 1 drop/sec) and when finished, the process was repeated with another 10 ml MeOH. Finally, an additional 3 ml MeOH was pushed through to flush the column, and the combined eluate evaporated to dryness in vacuo. The residue was dissolved in 1.0 ml 80% MeOH, centrifuged through 0.2 µM Spin-X filters (COASTER®), and the supernatant analyzed by LC-MS analysis.

Liquid chromatography was performed on a XBridge C18 column (3.5 µM, 50 × 2.1 mm) (Waters, Milford, MA, USA), using a Surveyor HPLC system (Thermo Electron Corporation, Waltham, MA, USA). MC-LR, -YR and -RR were included as standard and *Anabaena circinalis* (NIVA-CYA82) as positive control. 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.

RESULTS

Amplification of the *cpcB-cpcA*, *mcyA* and *mcyB* region

The PC primer pair amplified the intergenic spacer region (*cpcB-cpcA*) within the phycocyanin operon and allowed the detection of total populations of *Microcystis* (MC-producing and non-producing strains) in all samples

collected from Nhambavale lake, Pequenos Libombos dam and Chòkwé irrigation channels in Mozambique (Table 3). Most of the samples from Nhambavale lake and Chòkwé irrigation channels contained detectable *mcyA* and *mcyB* genes encoding for MC production (Figure 2). The fragment size of various *mcy* amplicons matched with the size of *M. aeruginosa* (NIVA-CYA 228/1) positive control (Figure 2). The *mcyB* gene was detected in 60% of the samples from Nhambavale lake and in all samples from Chòkwé irrigation channels collected in March 2009. For the June 2008 sampling, the *mcyB* gene was detected in 75% of the samples from Nhambavale lake and 25% in Chòkwé irrigation channels. The *mcyA* gene was present in all the samples from Chòkwé irrigation and 20% of the samples from Nhambavale lake in March 2009. Similarly, 50% of the samples from Nhambavale lake had *mcyA* gene while 25% of the samples from Chòkwé irrigation channels were also positive (Table 3). No positive results were found for MC-producing genes in all the samples from Pequenos Libombos dam collected in 2008 and 2009. Non-toxic strains of *M. aeruginosa* NIVA-CYA 144, used as negative control in all PCR yielded no detectable signal.

Amplification of the *mcyA*-Cd1 region

A fragment corresponding to the *mcyA*-Cd gene region (300 bp) was amplified by PCR from samples collected in June 2008 and March 2009 in Chòkwé irrigation channels and Nhambavale lake. In March 2009, all the samples that were collected from Chòkwé irrigation channels had gene fragment corresponding to *mcyA*-Cd gene region while 40% of the samples from Nhambavale lake were positive to *mcyA*-Cd gene region. Differently, in June 2008, 75% of samples from Nhambavale lake were positive to *mcyA*-Cd gene region while only 25% of the samples in Chòkwé irrigation channels were found to be positive. In both years, no positive band was observed in DNA samples from Pequenos Libombos dam. Digestion of the PCR product from water samples with *HindIII* and *EcoRV* restriction enzymes identified one restriction site for each restriction enzyme, with fragment patterns corresponding to *Microcystis* both in Nhambavale lake and Chòkwé irrigation channels (Table 3 and Figure 2).

Results of the MCs analysis in PSDs

Both in June, 2008 and March, 2009, three MCs variants (MC-LR *m/z* 995.5, YR *m/z* 1045.5 and RR *m/z* 1038) were detected in all the studied freshwater bodies (Table 4; Figure 3). The main peak of LC–MS chromatograms in most of the samples corresponded with that of MC-LR standard. In June, 2008, high concentrations of MCs (159.4 ng/g of PSD) were observed in Nhambavale lake while low concentrations of MCs (2.1 ng/g of PSD) were

Table 3. Results from analysis of water samples from Mozambique using different PCR methods. NL1-5 (samples from Nhambavale Lake), CH1-5 (Samples from Chòkwé irrigation Channels), PL1-2 (samples from Pequenos Libombos Dam).

Samples	PC gene		mcyA-MISY gene		mcyB gene		RFLP (<i>Microcystis</i> +)	
	2008	2009	2008	2009	2008	2009	2008	2009
NL1	/	+	/	-	/	-	/	-
NL2	+	+	+	-	+	+	+	-
NL3	+	+	-	-	+	-	+	-
NL4	+	+	-	+	-	+	-	-
NL5	+	-	+	-	+	-	+	-
CH1	+	+	+	+	+	+	+	+
CH2	/	+	/	+	/	+	/	-
CH3	-	/	-	/	-	/	-	/
CH4	+	+	-	+	-	+	-	+
CH5	-	+	-	+	-	+	-	+
PL1	+	+	-	-	-	-	-	-
PL2	-	-	-	-	-	-	-	-
PL3	+	-	-	-	-	-	-	-

(+) Positive results, (-) negative results, (/) not tested.

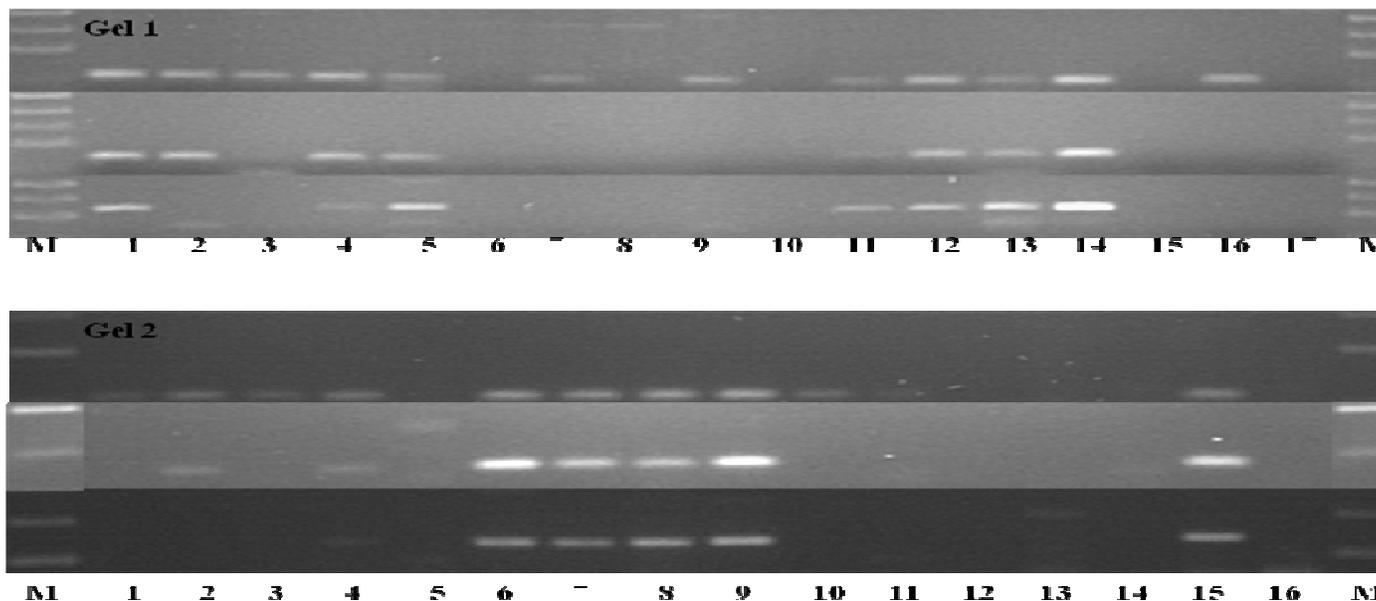


Figure 2. 2% agarose gels of the DNA from water samples collected in Mozambique. C-phycoyanin gene amplification products using *cpcB-cpcA* method (A) and microcystin synthetase gene amplification products using *mcyB* (B) and *mcyA* (C) methods. Gel 1- samples collected in June, 2008; Nhambavale lake (Lanes 1-4), Chòkwé irrigation channels (Lanes 5- 8), non-toxic strain of *Microcystis* NIVA-CYA 144 (Lane 9), non template- water (Lane 10), algae sample from South Africa (Lane 11), toxic strain of *Microcystis* NIVA-CYA 228/1 (Lane 12), Toxic strain of *Microcystis* PCC7806 (Lanes 13-14) Pequenos Libombos Dam (Lanes 15- 17). Gel 2- Samples collected in March, 2009; Nhambavale lake (Lanes 1- 5), Chòkwé irrigation channels (Lanes 6- 9), Pequenos Libombos dam (Lanes 10- 12), non template-water (Lane 14), toxic strain of *Microcystis* NIVA-CYA 228/1 (Lane 15), non template- water (Lane 16).

Table 4. Microcystins in the study freshwater bodies in Mozambique in 2008 and 2009.

Study area	In 2008				In 2009			
	Number of samples	MC positive samples (%)	MC conc in PSDs (ng/g)	MC variants	Number of samples	MC positive samples (%)	MC conc in PSDs (ng/g)	MC variants
Pequenos Libombos Dam	13	2 (15.4)	2.1–2.3	LR, YR	6	3 (50.0)	3.5–3.9	LR, YR
Nhambavale Lake	8	8 (100.0)	7.3–159.4	LR, YR, RR	7	4 (57.1)	3.2–7.2	LR, YR
Chôkwé irrigation channels	5	5 (100.0)	bql	LR	6	4 (66.7)	2.4–2.7	LR

bql, Below quantifiable level.

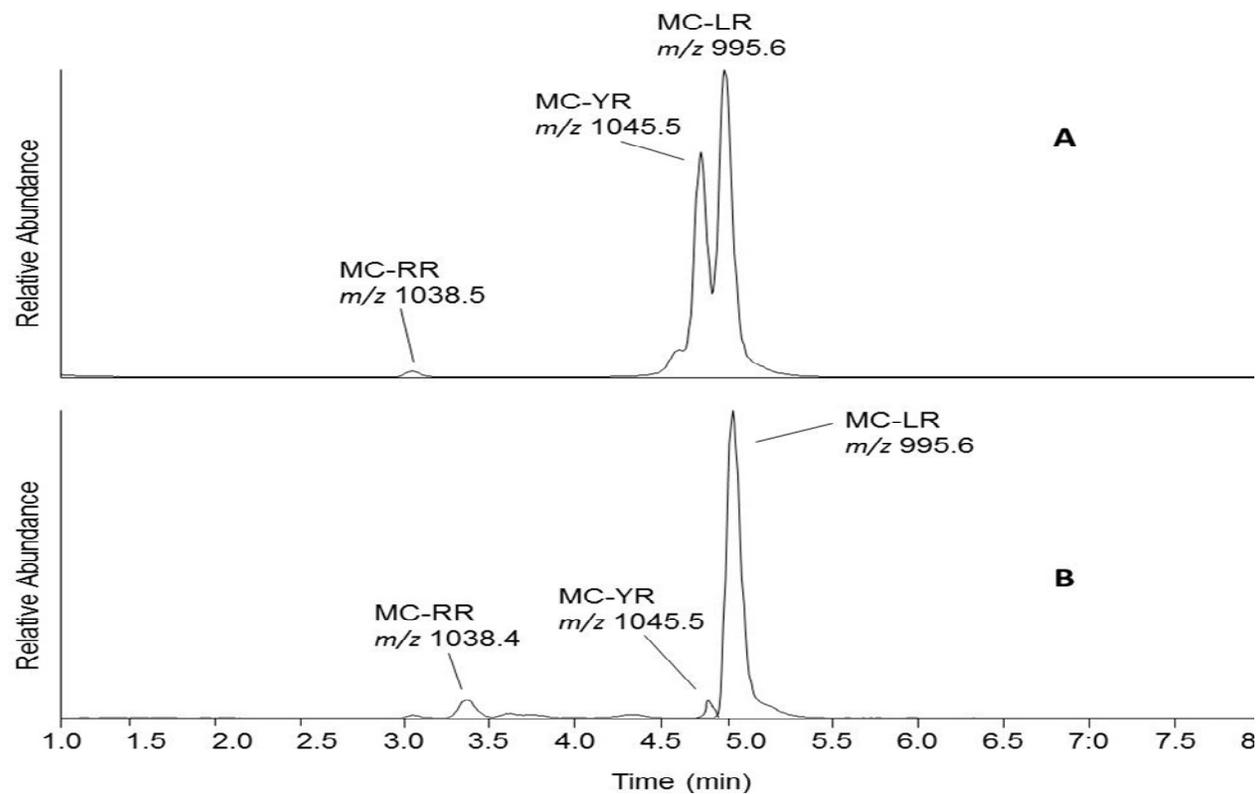


Figure 3. Selected ions of LC-MS chromatograms: (A) Three MC standards (MC-RR, -YR and -LR); (B) Passive sampling device extract sample from Nhambavale Lake with 3 MC variants namely MC-RR, MC-YR and MC-LR.

recorded in Pequenos Libombos dam. MC-LR was the only variant detected at below quantifiable level in all the samples collected in June, 2008 from Chòkwé irrigation channels. In March, 2009, high concentrations of MCs (7.2 ng/g of PSD) were observed in Nhambavale lake and low concentrations (2.4 ng/g of PSD) were detected in Chòkwé irrigation channels.

DISCUSSION

The detection of toxic or non-toxic cyanobacteria is important for water management and for geographical distribution studies (Ouellette et al., 2006). To our knowledge, this is the first report that employed PCR detection methods for MC-producing cyanobacteria in Mozambique. Samples from the three sampling sites tested positive for c-phycoerythrin gene (66 bp), but almost 63% of these samples tested negative in June 2008 and 50% in March 2009, when amplified with specific primer to detect MC-producing cyanobacteria (*mcyA*-MISY, *mcyB* and *mcyA*-Cd genes). As described by Kurmayer and Kutzenberger (2003), the PCR method targeting phycoerythrin gene detects both toxic and non-toxic strains of *Microcystis* sp. A study by Neilan et al. (1995) also reported that the phycoerythrin gene is found in freshwater environment almost exclusively in cyanobacteria and it provides a rapid and direct identification of cyanobacterial strains in samples containing complex microbial community.

Comparing the level of detection of the specific primer sets used, it was observed that *mcyB* method detected more target gene than *mcyA*-MISY. In an experimental study, Foulds et al. (2002) reported that MISY set primers were specific for toxic *M. aeruginosa* strains. However, it is worth mentioning that Blast analysis of the MISY primer pair during this current study showed 100% match with *M. botrys*, *M. novacekii*, *M. viridis* which suggests that these primers are not as specific as previously reported. Conversely, the primers targeting *mcyB* gene amplify specific sequences in a wide range of *Microcystis* toxic strains (Kurmayer and Kutzenberger, 2003), which supports the high detection level of these primers.

The RFLP analysis demonstrated that the predominant cyanobacteria genus present in the sampling sites from Mozambique was *Microcystis*. Members of the genus *Microcystis* are known worldwide for the production of MCs (Codd et al., 1999; van Apeldoorn et al., 2007). The presence of potentially toxic *Microcystis* in freshwater bodies in Mozambique which are used as sources of drinking water is of greater public importance. Indeed, the LC-MS/MS analysis of dissolved MCs revealed presence of MCs predominated by MC-LR in Nhambavale lake. An ELISA analysis by Bojcevska and Jergil (2003) reported the presence of MCs in different freshwater bodies in Mozambique. MC-LR is the most common MC variant in

freshwater bodies and is potentially toxic among the MC variants (Yoshida et al., 1997). The LD₅₀ for MC-LR by the intraperitoneal route is approximately 25 to 150 µg/kg of body weight in mice and the oral LD₅₀ is 5000 µg/kg of body weight (Yoshida et al., 1997). Human exposure to MCs is mainly through ingestion and the toxins mediate their toxicity by inhibition of serine protein phosphatases 1 and 2A particularly in the liver which causes cytoskeleton damage, hepatic haemorrhages, necrosis and hepatomegaly (Codd et al., 1999). Therefore, since this study freshwater bodies is used as the main source of drinking water, fishing, agriculture and recreational activities, the community may be at risk of MC intoxications.

Apart from *Microcystis*, several studies have demonstrated synthesis of MCs by a range of cyanobacterial species including *Anabaena*, *Anabaenopsis*, *Nostoc*, *Planktothrix*, *Hapalosiphon* and *Aphanocapsa* (Codd et al., 1999; Sivonen and Jones, 1999; van Apeldoorn et al., 2007). During our study, none of the three freshwater bodies (Nhambavale lake, Pequenos Libombos dam and Chòkwé irrigation channels) contained PCR products indicative of *Anabaena* and *Planktothrix* genotypes. Based on the results of this study, it may be concluded that *Microcystis* is the sole toxicogenic species in all the three freshwater bodies studied. However, previous study by Bojcevska and Jergil (2003) reported the occurrence of *Anabaena* in the freshwater bodies in Mozambique.

The detected *Microcystis* during this current study was found to contain *mcyA* and *mcyB* encoding for MC production. This result is in agreement with other studies showing that *Microcystis* populations always contain the *mcy* genotype. For example, in seven Tunisian water reservoirs survey of the *Microcystis* population, *mcyA* and *mcyB* were the common genes always detected in samples with detectable MCs (Fathalli et al., 2011). Other studies in freshwater bodies reported *mcyA* and *mcyB* genes in MC-producing *Microcystis* samples (Kumar et al., 2011; Via-Ordorika et al., 2004; Sivonen and Börner, 2008). However, Okello et al. (2009) detected only *mcyE* gene in the MC-producing *Microcystis* cultures from different freshwater bodies in Uganda. Several workers have reported that populations of *M. aeruginosa* may contain genotypes with and without one of the genes responsible for microcystin production (Wilson et al., 2005).

Problems with false positives/negatives were not experienced in this study. This can be evidenced by the expected results obtained from the amplification and non-amplification of positive and negative controls, respectively.

These results obtained in this study were consistent between different PCR methods (Table 3) and indicated the presence of cyanobacteria in all sampling sites during this study periods. Therefore, these results suggest that the molecular tools used in this study can be used for monitoring freshwater collections for the presence of *mcy* genes. It would be important to use these tools to

determine seasonal variation of the cyanobacteria populations and to perform sequencing analyses to better understand the phylogeography of these organisms.

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REFERENCES

- Baker JA, Entsch B, Neilan BA, McKay DB (2002). Monitoring changing toxigenicity bloom by molecular methods. *Appl. Environ. Microbiol.* 68: 6070-6076.
- Baker JA, Neilan BA, Entsch B, McKay DB (2001). Identification of cyanobacteria and their toxigenicity in environmental samples by rapid molecular analysis. *Environ. Toxicol.* 16: 472-482.
- Ballot A, Krienitz L, Kotut K, Wiegand C, Metcalf JS, Codd GA, Pflugmacher S (2004). Cyanobacteria and Cyanobacterial Toxins in three Alkaline Rift Valley Lakes of Kenya-Lakes Bogoria, Nakuru and Elmenteita. *J. Plank. Res.* 26 (8): 925-935.
- Bojcevska H, Jergil E (2003). Master Degree Projec. Removal of cyanobacterial toxins (LPS endotoxin and microcystin) in drinking-water using the BioSand household water filter. *Uppsala University, Sweden*, pp. 1-44.
- Codd GA, Bell SG, Kaya K, Ward CJ, Beattie KA and Metcalf JS (1999). Cyanobacterial toxins, exposure routes and human health. *Eur. J. Phycol.* 34: 405-415.
- Dittman E, Börner T (2005). Genetic contributions to the risk assessment of microcystin in the environment. *Toxicol. Appl. Pharmacol.* 3: 192-200.
- Dittmann E, Neilan BA, Erhard M, von Döhren H, Börner T (1997). Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. *Mol. Microbiol.* 26: 779-787.
- Duy N, Lam PKS, Shaw GR, Connell DW (2000). Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water. *Rev. Environ. Contam. Toxicol.* 163: 113-186.
- Fathalli A, Jenhania ABR, Moreira C, Welker M, Romdhane M, Antunes A and Vasconcelos V (2011). Molecular and phylogenetic characterization of potentially toxic cyanobacteria in Tunisian freshwaters. *Syst. Appl. Microbiol.* 34: 303-310.
- Foulds IV, Granacki A, Xiao C, Krull UJ, Castle A, Horgen PA (2002). Quantification of microcystin-producing cyanobacteria and *E. coli* in water by 5- nuclease PCR. *J. Appl. Microbiol.* 93: 825-834.
- Hisbergues M, Christiansen G, Rouhiainen L, Sivonen K, Börner T (2003). PCR-based identification of microcystin-producing genotypes of different cyanobacterial genera. *Arch. Microbiol.* 180: 402-410.
- Hummert C, Dahlmann J, Reichel M, Luckas B, (2001). Analytical techniques for monitoring harmful cyanobacteria in lakes. *Lakes Reserv Res Manage.* 6: 159-168.
- Kumar A, Kumar A, Rai AK, Tyagi MB (2011). PCR-based detection of *mcy* genes in blooms of *Microcystis* and extracellular DNA of pond water. *Afr. J. Microbiol. Res.* 5: 74-381.
- Kurmayer R, Kutzenberger T (2003). Application of Real-Time PCR for Quantification of Microcystin Genotypes in a Population of the Toxic Cyanobacterium *Microcystis* sp. *Appl. Environ. Microbiol.* 69(11): 6723-6730.
- Kurmayer R, Christiansen G, Chorus I (2003). The abundance of microcystin- producing genotypes correlates positively with colony size in *Microcystis* sp. and determines its microcystin net production in Lake Wannsee. *Appl. Environ. Microbiol.* 69: 787-795.
- Lyra C, Suomalainen S, Gugger M, Vezie C, Sundman P, Paulin L, Sivonen K (2001). Molecular characterization of planktic cyanobacteria of Anabaena, Aphanizomenon, Microcystis and Planktothrix genera. *Int. J. Syst. Evol. Microbiol.* 51: 513- 526.
- Mussagy A (1990). A preliminary study of the physical, chemical and biological components of the Pequenos Libombos reservoir. *Tese de Licenciatura. Maputo, Eduardo Mondlane University.* p. 41.
- Ndetei R, Muhandiki VS (2005). Mortalities of lesser flamingos in Kenyan Rift Valley saline lakes and the implications for sustainable management of the lakes. *Lakes Reserv. Res Manage.* 10: 51-58.
- Neilan BA, Dittmann E, Rouhiainen L, Bass RA, Schaub V, Sivonen K, Börner T (1999). Nonribosomal peptide synthesis and toxigenicity of cyanobacteria. *J. Bacteriol.* 181: 4089-4097.
- Neilan BA, Jacobs D, Goodman AE (1995). Genetic diversity and phylogeny of toxic cyanobacteria determined by DNA polymorphisms within the phycocyanin locus. *Appl. Environ. Microbiol.* 61: 3875-3883.
- Neilan BA (1995). Identification and phylogenetic analysis of toxigenic cyanobacteria by multiplex randomly amplified polymorphic DNA PCR. *Appl. Environ. Microbiol.* 61 (6): 2286-2291.
- Nishizawa T, Ueda A, Asayama M, Fujii K, Harada KI, Ochi K, Shirai M (2000). Polyketide synthase gene coupled to the peptide synthetase module involved in the biosynthesis of the cyclic heptapeptide microcystin. *J. Biochem.* 127: 779-789.
- Nishizawa T, Asayama M, Fujii K, Harada K, Shirai M (1999). Genetic analysis of the peptide synthetase genes for a cyclic heptapeptide microcystin in *Microcystis* spp. *J. Biochem.* 126: 520-529.
- Nonneman D, Zimba PV (2002). A PCR-based test to assess the potential for microcystin occurrence in channel catfish production ponds. *J. Phycol.* 38: 230-233.
- Oberholster PJ, Botha AM, Grobbelaar JU (2004). *Review. Microcystis aeruginosa*: source of toxic microcystins in drinking water. *Afr. J. Biotechnol.* 3(3): 159-168.
- Ochumba PBO (1990). Massive fish kills within the Nyanza gulf of lake Victoria, Kenya. *Hydrobiologia*, 208: 93-99.
- Okello W, Portmann C, Erhard M, Gademann K, Kurmayer R (2009). Occurrence of microcystin-producing cyanobacteria in Ugandan freshwater habitats. *Environ. Toxicol.* 25: 367-80.
- Ouellette AJ, Handy SM, Wilhelm SW (2006). Toxic *Microcystis* is widespread in Lake Erie: PCR detection of toxin genes and molecular characterization of associated cyanobacterial communities. *Microb. Ecol.* 51: 154-165.
- Ouellette AJ, Wilhelm SW (2003). Toxic cyanobacteria: the evolving molecular Toolbox. *Reviews. Front. Ecol. Environ.* 1(7): 359-366.
- Pan H, Song L, Liu Y, Börner T (2002). Detection of hepatotoxic *Microcystis* strains by PCR with intact cells from both culture and environmental samples. *Arch. Microbiol.* 178: 421-427.
- Phelan RR, Downing TG (2007). Optimization of laboratory scale production and purification of microcystin-LR from pure cultures of *Microcystis aeruginosa*. *Afric. J. Biotechnol.* 6(21): 2451-2457.
- Rudi K, Skulberg OM, Jakobsen KS (1998a). Evolution of cyanobacteria by exchange of genetic material among phylogenetically related strains. *J. Bacteriol.* 180: 3453-3461.
- Rudi K, Skulberg OM, Larsen F, Jakobsen KS (1998b). Quantification of toxic cyanobacteria in water by use of competitive PCR followed by sequence-specific labeling of oligonucleotide probes. *Appl. Environ. Microbiol.* 64(7): 2639-2643.
- Rundberget T, Gustad E, Samdal IA, Sandvik M, Miles CO (2009). A convenient and cost-effective method for monitoring marine algal

- toxins with passive samplers. *Toxicon*, 53(5): 543-550.
- Sivonen K, Börner T (2008). Bioactive compounds produced by cyanobacteria. In: *The Cyanobacteria: Molecular Biology, Genomics and Evolution* (eds A. Herrero and E. Flores), Academic Press, Norfolk, UK. pp. 159–97.
- Sivonen K (2007). Emerging high throughput analyses of cyanobacterial toxins and toxic cyanobacteria. *Proceedings of the Interagency, International Symposium on Cyanobacterial Harmful Alga Blooms. Adv. Exp. Med. Biol.* pp. 523-542.
- Sivonen K, Jones G (1999). Cyanobacterial toxins. In I. Chorus and J. Bartram (ed.), *Toxic cyanobacteria in water. A guide to their public health consequences, monitoring and management*. E and FN Spon, London, United Kingdom. pp. 41–111.
- Tillett D, Parker DL, Neilan BA (2001). Detection of Toxicity by a Probe for the Microcystin Synthetase A Gene (*mcyA*) of the Cyanobacterial Genus *Microcystis*: Comparison of Toxicities with 16S rRNA and Phycocyanin Operon (Phycocyanin Intergenic Spacer) Phylogenies. *Appl. Environ. Microbiol.* 67(6): 2810-2818.
- Tillett D, Dittmann E, Erhard M, von Döhren H, Börner T, Neilan BA (2000). Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system. *Chem. Biol.* 7: 753-764.
- Vaitomaa J, Rantala A, Halinen K, Rouhiainen L, Tallberg P, Mokolke L, Sivonen K (2003). Quantitative real-time PCR for determination of microcystin synthetase e copy numbers for *Microcystis* and *Anabaena* in lakes. *Appl. Environ. Microbiol.* 69: 7289-7297.
- Van Den Eede G, Lipp M, Eyquem F, Anklam E (2000). Report from the EC-JRC, Ispra, Italy, Eur. 19: 677.
- Van Apeldoorn ME, Van Egmond HP, Speijers GJA, Bakker GJL (2007). Toxins of cyanobacteria. *Mol. Nutr. Food Res.* 51: 7-60.
- Via-Ordorika L, Fastner J, Kurmayer R, Hisbergues M, Dittmann E, Komarek J, Erhard M, Chorus I (2004). Distribution of microcystin-producing and non-microcystin-producing *Microcystis* sp. in European freshwater bodies: Detection of microcystins and microcystin genes in individual colonies. *Syst. Appl. Microbiol.* 27: 592–603.
- WHO (1998). Guidelines for drinking water quality, Second Edition, Addendum, Health Criteria and other supporting information. World Health Organization, Geneva. Vol. 2
- Wilson AE, Sarnelle O, Neilan BA, Salmon TP, Gehringer MM, Hay ME (2005). Genetic variation of the bloom-forming cyanobacterium *Microcystis aeruginosa* within and among lakes: implications for harmful algal blooms. *Appl. Environ. Microbiol.* 71: 6126-6133.
- Yoshida T, Makita Y, Nagata S, Tsutsumi T, Yoshida F, Sekijima M, Tamura SI and Ueno Y (1997). Acute oral toxicity of microcystin-LR, a cyanobacterial hepatotoxin, in mice. *Nat. Toxins*, 5: 91-95.