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Quantification of microcystin-producing *microcystis* in freshwater bodies in the Southern Mozambique using quantitative real time polymerase chain reaction

Olivia Pedro[1,2,4]*, Elisabeth Lie[1], Dacia Correia[2,4], Luis Neves[2,4], Janneche U. Skaare[1,3], Morten Sandvik[3] and Knut G. Berdal[3]

[1]Norwegian School of Veterinary Science, Ullevalsveien 72, P.O.Box 8146 Dep, NO-0033 Oslo, Norway.
[2]Faculdade de Veterinária, Universidade Eduardo Mondlane, Av. de Mozambique km 1.5, P.O.Box 257. Maputo, Mozambique.
[3]Norwegian Veterinary Institute, P.O. Box 750, Sentrum, 0106, Oslo, Norway
[4]Centro de Biotecnologia da Universidade Eduardo Mondlane, Av. de Mozambique km 1.5, P.O.Box 257. Maputo, Mozambique.

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In the last decades, large number of reported cases of illnesses in Mozambique is related to drinking water. However, only a limited number of studies have focused on aquatic pollution in this country. Cyanobacterial blooms dominated by *Microcystis* sp are regularly identified in freshwater bodies in Mozambique. *Microcystis* is known to proliferate in freshwater bodies and produce microcystins which have adverse effects on animals and humans. The aim of this study was to quantify microcystin-producing *Microcystis* in three different freshwater bodies in Southern Mozambique. TaqMan based real time polymerase chain reaction (PCR) (*Taq* Nuclease Assay) was used to quantify populations of *Microcystis* in three aquatic ecosystems in Southern Mozambique. Total *Microcystis* spp (microcystin-producing and non-producing strains) were quantified in the three selected study areas with the determination of the copy numbers of the phycocyanin (PC) operon. Microcystin-producing gene copy numbers were quantified using specific primer pair, amplifying the *mcy*B gene. *Microcystis mcy*B copy numbers varied from 4.2 x 10^6 to 1.6 x 10^9 gene copies /L in 2008, corresponding to 2.15 to 98.55% of total *Microcystis*, and from 9.6 x 10^7 to 4.5 x 10^9 gene copies /L in 2009, corresponding to 1.53 to 34.52% of total *Microcystis*. High copy numbers of *mcy*B occurred in Nhambavale Lake in June 2008, whereas in March 2009, high copy numbers of *mcy*B was observed in Chòkwé Irrigation Channels. Samples from Pequenos Libombos Dam had the lowest number of *mcy*B gene copies in both sampling periods. The findings of the present study show that microcystin-producing strains are common in Southern Mozambique, and that their absolute and relative numbers varies geographically and temporarily. The highest concentration of *Microcystis sp.* in the sampling areas occurred in samples collected in March 2009, which corresponds to the rain season with warm temperatures. To our knowledge, this is the first report of the quantification of microcystin-producing *Microcystis* in Mozambique using molecular techniques.

Key words: *Microcystis sp*, Taq nuclease assay, phycocyanin (PC), *mcy*B, Mozambique.

INTRODUCTION

Pollution in the form of sewage from humans or from livestock in agriculture operations, can lead to elevated resources. Such contaminations are a major cause of illness and death worldwide, particularly in developing

countries (WHO, 1998; Foulds et al*.,* 2002). Cyanobacteria are globally distributed in both fresh and marine water (Dor and Danin, 1996) and toxic cyanobacterial blooms have been reported in most parts of the world (Ochumba, 1990; WHO, 1999; Ballot et al*.,* 2004; Ndetei and Muhandiki, 2005). Some cyanobacterial strains are harmful to animals and humans because of the toxins they produce, known as cyanotoxins. Cyanotoxins are classified as neurotoxins, hepatotoxins and cytotoxins (Carmichael, 1992; Carmichael, 1994; Rinehart et al*.,* 1994; Sivonen and Jones, 1999; Li et al*.,* 2001). Microcystins are the most common hepatotoxins and are synthesized nonribosomally via multifunctional enzyme complexes that includes polypeptide synthetase (NRPS) and polyketide synthase (PKS) modules (Nishizawa et al., 2000; Tillett et al., 2000; Vaitomaa et al., 2003; Christiansen et al., 2003). These hepatotoxic peptides act by inhibiting protein phosphatases, and they have been directly associated with the production of liver cancer, constituting thus a health risk for mammals (Falconer and Buckley, 1989; Falconer, 1994; Van Halderen et al., 1995; Mez et al*.,* 1997; Foulds et al*.,* 2002).

The adverse effects of microcystins to human and animal populations have triggered the establishment of monitoring systems for cyanobacteria and microcystins in raw water sources and recreational water (Vaitomaa et al., 2003). Recognizing the effects of microcystin on health, the World Health Organization (WHO) has set a provisional guideline of 1 µg/L for MC-LR in drinking water (WHO, 1998). For recreational water, there are provisional guidelines that indicate low or moderate probabilities of adverse health effects, corresponding to densities of 2.0 x 10^4 and 2 x 10^5 cells/mL of toxic strains of cyanobacteria, respectively (Falconer et al., 1999).

The methods most commonly used to monitor microcystin concentrations are high-performance liquid chromategraphy (HPLC), protein phosphatase inhibition assay (PPIA) and enzyme-linked immunosorbent assay (ELISA) (Sivonen and Jones, 1999). However, such analyses do not indicate which genus of cyanobacteria is producing the toxins, since several genera of cyanobacteria may produce similar variants of microcystins (Sivonen and Jones, 1999). The quantification of microcystin-producers and non-microcystin-producers was for a long time limited because of the morphological similarity between strains, independent of microcystin production (Kurmayer and Kutzenberger, 2003). Since the genes involved in microcystin synthesis have been identified and sequenced (Dittmann et al*.,* 1997; Tillett et al., 2000) it became possible to study the occurrence of microcystin- producing genotypes directly from field samples (Rinta-Kanto et al*.,* 2005). The enzyme that produces microcystins, *Microcystin synthetase*, is encoded by the *mcy* operon (Nishizawa et al., 1999; 2000) found in toxic strains of *Microcystis aeruginosa* as well as *Anabaena, Nostoc* and *Oscillatoria* (Foulds et al*.,* 2002). Quantitative real time polymerase chain reaction (PCR) has been successfully applied to determine the quantities of microcystin-producing and non-producing *Microcystis* genotypes in natural populations (Foulds et al*.,* 2002; Kurmayer and Kutzenberger, 2003; Kurmayer et al*.,* 2003; Vaitomaa et al*.,* 2003; Rinta-kanto et al*.,* 2009). In this study, we employed the *Taq* Nuclease assay (*TNA*) using primers developed by Kurmayer and Kutzenberger (2003) to quantify Microcystin producing and non-producing *Microcystis.*

TNA was initially introduced to quantify specific genotypes of picocyanobacteria by Becker et al. (2000) or microcystin-producing cyanobacteria from field samples (Foulds et al*.,* 2002), calculated by the level of exponential amplicon accumulation. The level of exponential accumulation of the amplicon is monitored by the hydrolysis of the *TaqMan* probes, in which a fluorescent signal is generated during the amplification process (Kurmayer and Kutzenberger, 2003). Previous investigations in the same lakes in Mozambique, based on microscopy observation and ELISA technique, have identified the presence of cyanobacteria and cyanobacterial toxins (Bojcevska and Jergil, 2003) in water, however no study have been done to quantify the copy number abundances per liter in these aquatic ecosystems. The main focus in our study was to quantify the microcystin-producing and non-producing *Microcystis* in three lakes from the South of Mozambique using *TNA*.

MATERIALS AND METHODS

Study area and sampling

Water samples were collected from Pequenos Libombos Dam (PL), Nhambavale Lake (NL) and Chòkwé Irrigation Channels (CH), located in South of Mozambique (Figure 1). Pequenos Libombos Dam is a manmade impoundment located 35 km west of Maputo at 26°05'S and 32°14'E and is the main drinking-water source to Maputo, the capital of Mozambique. The dam has a mean depth of approximately 10 m, a surface area of 38 km^2, and a gross storage capacity of 400*10^6 m^3(ARA-Sul, 2011). This reservoir has been classified as mesotrophic (Mussagy, 1990). Nhambavale Lake is located North of Gaza province, in Chidenguele Village, at 24°54'S and 34°17'E and approximately 270 km south of Maputo. The lake is used as source of drinking water, as well as for recreational activities such as fishing and Olympic games. It is one of the lakes in the South of Mozambique that is used for tourism with a 35 km freshwater extension. Chòkwé Irrigation Channels, located in West of Gaza Province (220 km from Maputo) at 24°22'S and 33°00'E, is

*Corresponding author. E-mail: oliviapedro@uem.mz; olypedro76@gmail.com. Tel/Fax: +258 21 477227.

Abbreviations: NRPS, Polypeptide synthetase; **PKS,** polyketide synthase; **HPLC,** high-performance liquid chromatography; **PPIA,** protein phosphatase inhibition assay; **ELISA,** enzyme-linked immunosorbent assay; **PCR,** polymerase chain reaction; PL, Pequenos Libombos Dam; **NL,** Nhambavale lake; **CH,** Chòkwé irrigation channels, **TNA**, *Taq* nuclease assay; **SIMQUANT**, single molecule quantification.

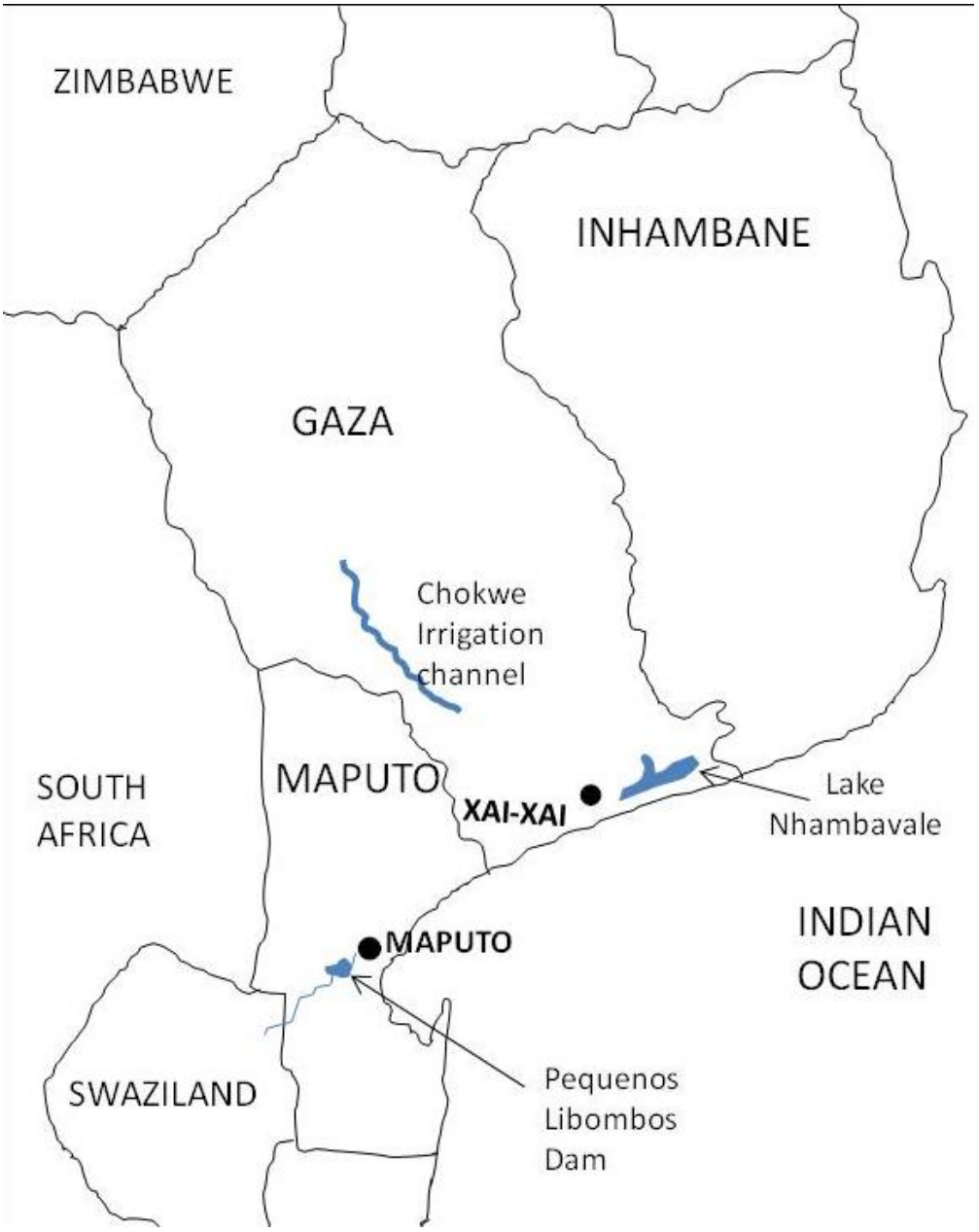

Figure 1. Map of South of Mozambique indicating the three sampling areas; Pequenos Libombos Dam, Nhambavale Lake and Chòkwé Irrigation Channels.

used mainly as a source of water for irrigation with the potential forirrigation of up to approximately 32000 ha of agriculture land in Chòkwé district. The current water consumption is about 21500m^3 /ha /year (HICEP, 2001; FAEF, 2001). The channel is also used as a source of drinking water and for fishing. At each sampling area, water was randomly collected at different sampling stations (NL 1 to 5; CH 1 to 5; PL 1 to 3), with distances between them varying from 1.5 to 5 km, depending on the accessibility. Sampling was conducted on June 7th 2008 and March 5th 2009. Thirteen (13) water samples were collected during each sampling period. In June 2008, water samples were collected directly into1 L bottles, submersed to about 1 m below the surface without an additional filtration. In March 2009, 30 L of water from the lake were filtered by conical plankton net (20 µm mesh) to 500 mL bottles. All field samples and standards were stored at -20°C until processing.

DNA extraction and quantification

Cultures of microcystin-producing and non-producing strains of *M. aeruginosa* (NIVA-CYA 228/1 and NIVA-CYA 144, respectively) were purchased at NIVA (Norwegian Institute for Water Research), and were used as positive and negative control, respectively. 2 mL of each culture and 30 mL of each water sample was pelleted by centrifugation and DNA extracted using a protocol described by Hisbergues et al. (2003), except that after the addition of lysis buffer(TES), the mixture was incubated in ice for 1 h (Kurmayer et al., 2003). DNA concentration in ng/µL was estimated by spectro-

photometric measurements on a NanoDrop ND-1000 V3.1.0 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All water samples were diluted before analysis. An initial inhi-bition test was performed by comparing three different dilutions (1x2; $1x10^1$ and $1x10^2$), mixed with 2.0 x 10^2 copies/5 µL of standard DNA (from microcystin-producing *Microcystis aeruginosa*)
and analyzed by *TNA*.

DNA for calibration

DNA from toxic strain of *M. aeruginosa* (NIVA-CYA 228/1) was used as standard. The genome size of *M. aeruginosa* was estimated to be 5.8 Mbp (Kaneko et al*.,* 2007). A DNA calibration curve (standard curve) was used to determine the concentrations of the DNA extracted from water samples. Calibration curves for the amplicons were established with 10 fold serial dilutions of the DNA of the strain NIVA-CYA 228/1 (20 to 2.0 X 10^4 copies per 5µL), prepared in 0.1 mg/mL bovine serum albumin (BSA) (MBI fermentas) to protect the diluted DNA from sticking to the walls of the PCR tubes. The phycocyanin (PC) and *mcy*B copy numbers were calculated assuming that each genome has only one gene and that the molecular weight of 1 bp was 660 g/mol (Vaiotomaa et al*.*, 2003). Diluted standard DNA was analyzed by *TNA*. The sensitivity of this assay was tested with standard and water samples diluted to a single DNA copy as described by Berdal and Holst-Jensen (2001) and also using the Single Molecule Quantification (SIMQUANT) methodology (Berdal et al*.,* 2008). SIMQUANT is an approach developed for quantification of samples with extremely low amounts of DNA, which is based on statistics and application of multiple quailtative parallel PCR. With this method, the template DNA typically contains very few target copies (for example, one copy) per volume, and the quantity is estimated on basis of observed ratio between positive and negative individual PCRs. The approach results in a statistical estimate of the relative concentration based on the probability that one or more amplifiable template copies are present in discrete volumes. The number of target molecules (MPN-most probable number) per µL DNA solution (*E*) and 95% confidence interval (Y) were estimated using the following equation: Where, *V* is the volume (µL) of the template DNA solution added to the PCR in each test; *n* is the number of tests and *q* is the number of negative results (NMKL, 2002).

***Taq* Nuclease assay**

TaqMan based real time PCR was used to quantify two specific gene regions, the *mcy*B, which encode one step in microcystin biosynthesis and the intergenic spacer region within the PC operon. Primers 188F and 245R were used for PC and 30F and 108R for *mcy*B gene, together with specific *TaqMan* probes for each gene (Kurmayer and Kutzenberger, 2003). Each *TaqMan* probes had a fluorescent reporter dye covalently attached to the 5' end (5'-FAM) and a TAMRA fluorescent quencher dye at the 3' end. All primers and probes were purchased at DNA technology (Denmark). All real time PCR reactions were performed in duplicate on the ABI Prism® 7900HT (Applied Biosystems, Foster City, CA, USA), using software ABI SDS (Sequence Detection System) version 2.2.1. *TNA* was carried out in a volume of 25 µL in MicroAmp optical reaction plates (Applied Biosystems), containing 1X *TaqMan* Universal Master Mix (Applied Biosystems), and 5 µL of DNA. For *mcy*B, 0.9 µM of each primer and 0.25 µM of *TaqMan* probes were used and for PC 0.3 µM of each primer and 0.1 µM of probes were used. All PCR reactions were initiated with two holds, one for 2 min at 50°C and one for 10 min at 95°C. Subsequently, a 95°C denaturation step for 15 s was followed by 60°C annealing and extension step for 1 min (45 cycles).

RESULTS AND DISCUSSION

Both in June 2008 and in March 2009, the *Microcystis* PC gene was detected in all three study areas. In June 2008, the PC gene copy numbers varied from 7.9 x 10^6 to 2.4 x 10^9 gene copies/L (Table 1), with the highest number observed in NL and lowest in PL. In March 2009, PC gene copy numbers varied from 8.0 x 10^5 to 1.9 x 10^{10} gene copies/L (Table 1) with the highest number observed in CH and the lowest in PL. This shows that *Microcystis* strains (determined from PC amplification) were present in all locations, and that their copy numbers varied in the order of thousand-fold in both years.

To determine the amount of microcystin-producing *Microcystis spp.* with the genetic potential to produce microcystins, we used the *Microcystis* specific primer pair amplifying the *mcy*B region. The number of *mcy*B gene copies varied from 4.2 x 10^6 to 1.6 x 10^9 gene copies/L in June 2008, and from 9.6 x 10^7 to 4.5 x 10^9 gene copies/L in March 2009 (Table 1). The highest number of *mcy*B genes copies in June 2008 was observed in NL and was 5.4 to 9.9 times higher than the amount observed in CH and 47 to 115 times higher than PL in the same sampling period. Conversely, in March 2009 *mcy*B copy numbers were quite similar in CH and NL. The highest number of *mcy*B gene copies was observed in CH (Table 1). The difference in gene copy numbers between CH and NL in March 2009 was very low, varying from 1.4 to 1.7 times. The overall abundance of *mcy*B gene copy number in all locations was 2.7 times more abundant in March 2009 than in June 2008. DNA samples from Pequenos Libombos Dam yielded the lowest number of *mcy*B gene copies in June 2008 (Table 1 and Figure 2) and in March 2009 no *mcy*B gene copies were detected, and this can indicate either that the *Microcystis* spp. cells present during the sampling dates did not contain *mcy*B genes and were thus non-microcystin-producing or that there were present in very low amount not detectable by the method. Previously, analysis for microcystins dissolved in water conducted by Pedro et al*.* (2011) in the same sampling areas revealed the presence of MC-LR, -YR and –RR with concentrations varying from 2.1 to 159.4 ng/g of Passive sampling devices (PSDs). Furthermore, the highest concentration of MC was observed in NL and the lowest in PL (Pedro et al*.,* 2011). Based on these studies, we can deduce that Nhambavale Lake has a high potentiality of producing toxic microcystin-producing strains and microcystins that may constitute a risk for public health.

It is reasonable to estimate that one gene copy corresponds to a single cell (Vaitomaa et al*.,* 2003). Given this, samples with more than 10^8 copies of cyanobacterial cells per liter (corresponding to 50 µg of chlorophyll-a/L with dominance of cyanobacteria) may cause adverse effects in long or short-term of exposure, according to the WHO provisional guidelines (WHO, 1998). Such concentrations were observed in NH and CH in both sampling periods (Table 1). The relative abundance of MC-producing

Table 1. Copy number of total *Microcystis* (PC), microcystin-producing *Microcystis* (*mcy*B) genes

Sample Name	PC gene (gene copies/L)	mcyB gene (gene copies/L)
Sampling date: 07/06/2008		
NL2	2.4×10^9	1.6×10^9
NL3	1.1×10^9	7.2×10^8
NL4	2.1×10^8	2.0×10^8
NL5	6.7×10^8	4.3×10^8
CH1	2.1×10^8	1.6×10^8
CH3	ND	ND
CH4	8.7×10^7	3.7×10^7
CH5	ND	ND
PL1	1.9×10^8	4.2×10^6
PL2	7.9×10^6	ND
PL3	1.6×10^8	1.4×10^7
NK	2.9×10^7	ND
NT	ND	ND
Sampling date: 05/03/2009		
NL1	3.9×10^8	7.8×10^7
NL2	1.6×10^8	5.6×10^7
NL3	6.7×10^8	2.3×10^8
NL4	8.9×10^9	3.1×10^9
NL5	3.4×10^9	6.5×10^8
CH3	1.9×10^{10}	4.5×10^9
CH4	1.4×10^{10}	3.3×10^9
CH5	2.5×10^9	1.4×10^8
CH6	6.3×10^9	9.6×10^7
PL1	ND	ND
PL2	1.4×10^7	ND
PL3	8.0×10^5	ND
NK	2.8×10^7	ND
NT	ND	ND

NL, Nhambavale Lake; CH, Chòkwé Irrigation Channel; PL, Pequenos Libombos Dam; NK, *M. aeruginosa* NIVA-CYA 144 – non-toxic strain; NT, non-template-MQ water; ND, not detected.

Microcystis carrying the *mcy*B genes are shown in Figure 2. Microcystin-producing *mcy*B gene copies comprised 2.15 to 98.55 % of total *Microcystis* (PC copy numbers) in 2008. The relative abundance is similar to the findings reported by Davis et al. (2009) in Lake Ronkonkoma in New York (12 to 100%) and by Kurmayer and Kutzenberger (2003) in Lake Wannsee in Berlin (1.7 to 71%). In 2009, the proportions of Microcystis containing the mcyB gene were below 50% in all lakes, varying from 1.53 to 34.52%. Several studies have revealed similar findings (Rita-Kanto et al., 2009; Baxa et al., 2010; Yoshida et al., 2007; Kurmayer & Kutzenberger, 2003).

Standard curves were established using four serial dilution (ranging from 2.0×10^4 to 20 copies per PCR reaction) of the standard genomic DNA isolated from *M. aeruginosa* NIVA-CYA 228/1. Figure 3 shows the standard curve parameters of the two genes (PC and *mcy*B) determined by the standard sample. Some of the tested water samples had DNA copy numbers below that of the standard with the lowest concentration (20 copies per 5 µL) which will increase the measurement uncertainty. The amplification efficiencies were 0.96 for *Microcystis* PC and 0.92 for *Microcystis mcy*B (Figure 3) which is within the range found in other studies (Vaitomaa et al., 2003; Rinta-Kanto et al., 2005). The sensitivity of this assay was tested with samples diluted to approximately a single DNA copy (Table 2). The method was able to detect a single DNA molecule. Table 3 shows the mean Ct-values of the two genes and the SIMQUANT estimates after dilution to approximately a single DNA copy gene per PCR assay. The confidence interval is influenced by the ratio of positive and negative parallels. The observed positive: negative ratios are very similar for both genes and close to the theoretically predicted ratio. Twenty two (22) parallels of standard genomic DNA isolated from *M. aeruginosa* NIVA-CYA 228/1 were tested

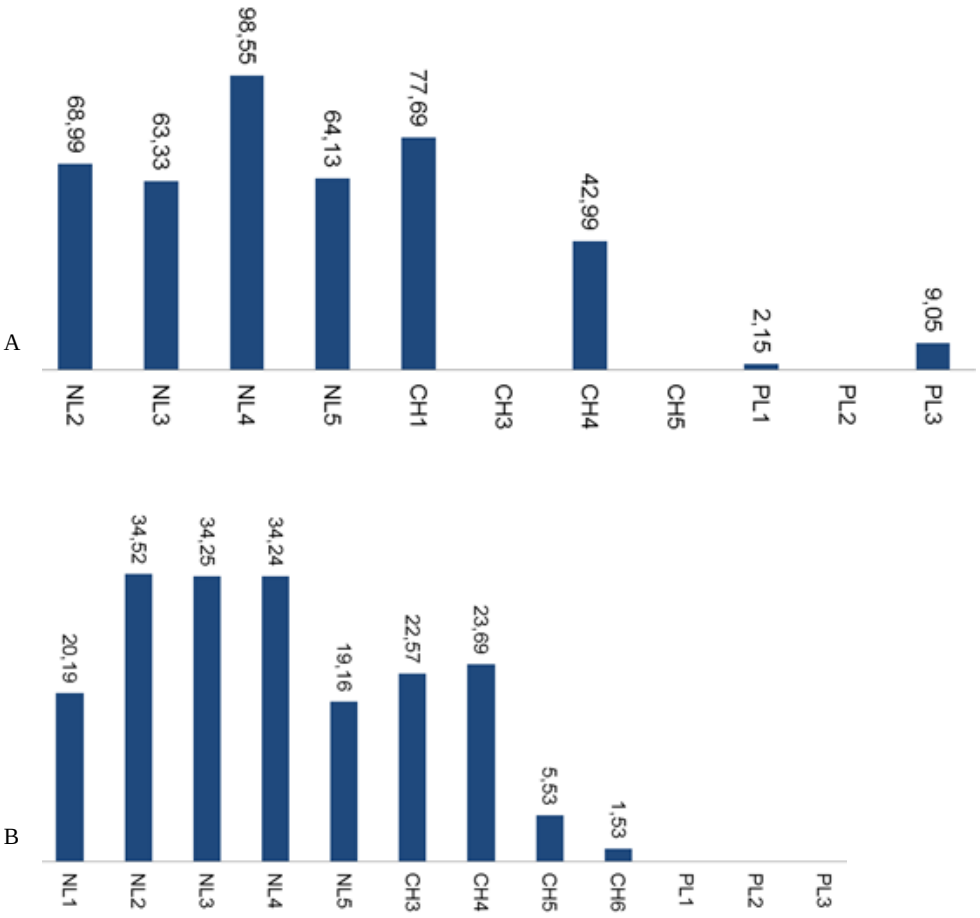

Figure 2. Relative abundance (%) of *mcy*B genes at Nhambavale Lake (NL), Chòkwé Irrigation Channels (CH) and Pequenos Libombos Dam (PL) in June 2008 (A) and March 2009 (B). Sampling stations were identified by numbers.

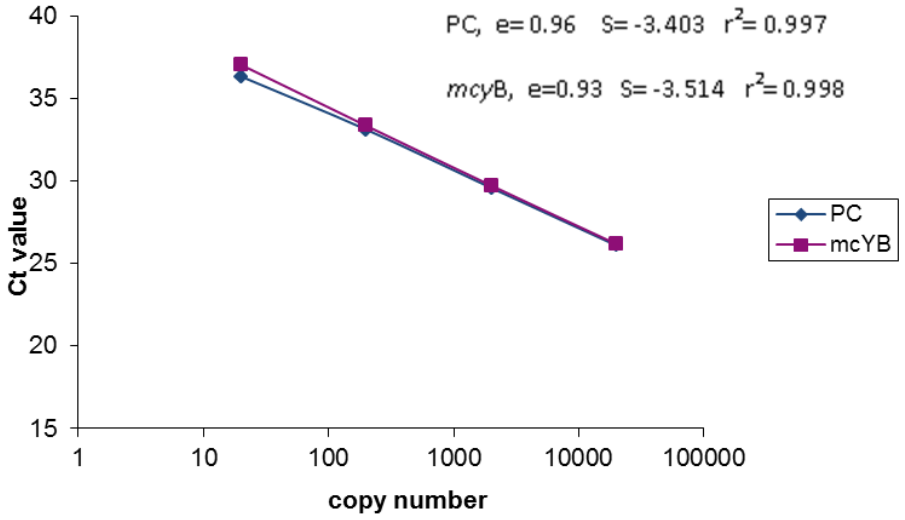

Figure 3. Ct Values obtained by *TNA* of *c-phycocyanin* gene (PC) and microcystin-synthetase gene *mcy*B with the strain *Microcystis* NIVA-CYA 228/1. "e" is the amplification efficiency calculated with the equation $e=10^{-1/s}-1$ (Vaitomaa et al. 2003) and "S" is the slope of the linear regression).

Table 2. Amplification data used to determine the absolute detection limit of the *mcy*B gene with the two fold dilution series of *M. aeruginosa* NIVA-CYA 228/1.

Estimate number of template molecule	50	25	12.5	6.25	3.125	1.56	0.78
Signal rate (number of positives)	6/6	6/6	6/6	6/6	5/6	4/6	3/6
Mean Ct-Value (of positives)	33.6	34.7	35.7	37.3	38.3	38.4	38.6
ΔCt (differences between two successive dilutions)	NA*	1.1	1	1.6	1.3	0.1	0.2

* Not applied

Table 3. Calculation of the most probable number (MPN) of the standard genomic DNA isolated from *M. aeruginosa* NIVA-CYA 228/1 using SIMQUANT. Confidence interval as a function of the ratio of negative samples out of 22 analyzed.

Gene	Ratio negative PCR	Average Ct	MPN	Confidence interval (95%)	
				Lower	Upper
PC	7/22	39.5	1.145	0.780	1.728
McyB	8/22	41.75	1.012	0.676	1.521

and the optimal ratio was found to be approximately 31% negative parallels (15 positives for 7 negatives). This approach proves the ability of the method to detect a single copy. A previous study in Mozambique based on PCR-restriction fragment length polymorphism (RFLP) analysis of the same samples confirmed the predominance of genera *Microcystis* (Pedro et al*.,* 2011). The findings of the present study show that in Southern Mozambique, microcystin-producing *Microcystis* are abundant and that their concentration varies temporary and geographically. The highest concentration of *Microcystis sp.* at the sampling areas occurred in samples collected in March 2009, which corresponds to the rain season with warm temperatures. However, as the samples were collected in only one time point in each year it was not possible to determine the seasonal abundance of *Microcystis sp.* In order to clarify the dynamics of *Microcystis* populations and determine the different levels of microcystin- production, a longitudinal study is required.

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