PCR amplification and DNA sequence of *mcy*A gene: The distribution profile of a toxigenic *Microcystis aeruginosa* in the Hartbeespoort Dam, South Africa

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ABSTRACT

Using new polymerase chain reaction (PCR) primers, a once known to be under-transcribed microcystin synthetase A (*mcy*A) gene from the only known toxigenic cyanobacterium *Microcystis aeruginosa* dominating the Hartbeespoort Dam was consistently amplified from genomic DNA extracted from a set of algal and cell free water samples collected across this dam. In addition to this, five more *mcy* genes (*mcy*BCDEG) were also amplified during this study. The resultant *mcy*A PCR products (518 bp) were purified and sequenced and gave nucleotide sequence segments of 408 bp sizes. The obtained sequence was aligned to the published *mcy*A gene sequence available online on the NCBI database and resulted in 100% similarity to a 408 bp *mcy*A gene sequence segment of *M. aeruginosa* UWOCC RID-1. Furthermore, it was found that the above sequence segment (408 bp) spans from a common base in *M. aeruginosa* PCC 7806 and *M. aeruginosa* PCC 7820 from 141 to 548 bp in the *N*-methyl transferase (NMT) region signifying their closer relatedness to *M. aeruginosa* UWOCC strains. This study has for the first time amplified *mcy*A gene consistently from both intracellular and extracellular DNA extracts obtained from algal and cell free water samples, respectively. Sequence data and the amplified *mcy* genes showed that *M. aeruginosa* is widely distributed and dominant in this dam.

Key words | DNA sequence, dominance profile, Hartbeespoort Dam, *M. aeruginosa*, *mcy*A gene, PCR amplification

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INTRODUCTION

The Hartbeespoort Dam is a man-made water impoundment located in North-West Province, South Africa. This dam occupies an area of about 20 km^2 with a water bank line of approximately 56 km around the Dam. The dam was basically constructed for irrigation scheme purposes and boasts approximately 10 m and 32 m of minimum and maximum depths, respectively (DWA 1991). Since its completion in the 1920s, the water quality in the dam has been deteriorating due to eutrophication. Recurrent growths of cyanobacterial blooms, in particular *Microcystis* spp., have been observed every year during summer seasons in records dating beyond the 1970s (Steyn & Toerien 1975; Hoffmann 1976; Harding & Paxton 2001; Owuor *et al*. 2007; Conradie & Barnard 2012). Excessive N/P nutrient enrichments from large wastewater treatment and industrial plants as well as farms located upstream of the dam have led to cases of increased and persistent toxic *M. aeruginosa* in the dam and downstream (DWA 1991; Harding & Paxton 2001; Taylor *et al.* 2005). The presence of water surface *Microcystis* scums (Zohary 1985) on the dam is characterised by unpleasant odours, mucilage, green colours and toxins which have compromised the quality of water used for irrigation, industrial productions, recreational and domestic purposes around and downstream of the dam (DWA 1991).

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One of the interesting and unique characteristic features of this dam is that besides its size (20 km^2) and ecological richness that supports growths of different aquatic organisms including non-toxic algal species, the dam is home to only one single known toxigenic cyanobacterium *M. aeruginosa* that has dominated the dam for decades as described in the literature (Scott 1991; Quibel *et al.* 1995; Downing & van Ginkel 2004; van Ginkel 2004; Masango 2007; Owuor *et al.* 2007; Oberholster *et al.* 2009; Oberholster & Botha 2010; Conradie & Barnard 2012). The successful overdominance of *M. aeruginosa* in this dam against other potentially toxic algal species found in other South African eutrophic waters is, however, not well understood. Some common non-toxic algal species inhabiting this same dam are *M. wesenbergii*, *Spirulina* spp., *Planktothrix* spp., *Melosira* spp. and *Nitzschia* spp. (Masango 2007; Owuor *et al.* 2007; Oberholster & Botha 2010; Conradie & Barnard 2012).

Different methods have been employed to evaluate the occurrence, distribution and, more importantly, the dominance profile of the toxigenic *M. aeruginosa* in the Hartbeespoort Dam. Such methods include microscopic cell enumeration and/or toxicity studies, as shown in some of the most recent studies of Owuor *et al.* (2007), Oberholster & Botha (2010) and Masango (2007). In addition, microcystin production from this toxic cyanobacterium (*M. aeruginosa*) has also been investigated and published based on different methods including ELISA (Masango 2007; Oberholster & Botha 2010; Conradie & Barnard 2012), mouse assay (Hoffmann 1976; Toerien *et al.* 1976; Masango 2007), fish liver cell culture (Masango 2007), phosphatase enzyme inhibition assay (PP1 & 2A) (Masango 2007), LC-ESI-MS (Mbukwa *et al.* 2012), etc. Microcystin congeners that have been reported from this dam include MC-LR, -RR, -YR and -WR (Zohary 1985; Wicks & Thiel 1990; Mbukwa *et al.* 2012). Others are MC-(H4)YR and (D-Asp3, Dha7) MC-RR (Mbukwa *et al.* 2012) and MC-LA (Wicks & Thiel 1990). The presence of highly toxic variants in this dam (MC-LR, -RR and -YR) are in particular of health concern for quality recreational and drinking water resources as well as aquatic life (Carmichael 1992; WHO 1999; Zhang *et al.* 2009a, 2009b; Laughinghouse *et al.* 2012).

Advances in polymerase chain reaction (PCR)-based methods have led to increased specificity and sensitivity in detection of toxin producing bacteria through gene amplifications (Heijnen & Medema 2006). Toxigenic cyanobacteria from the genus *Microcystis* spp. produce highly toxic water soluble toxins called microcystins (MCs). These species can only be accurately identified through amplifications of a group of genes called microcystin synthetase (*mcy*) that are involved in the biosynthesis of MCs (Nishizawa *et al.* 1999; Dittmann *et al.* 2001; Tillett *et al.* 2001; Kurmayer & Kutzenberger 2003; Vaitomaa *et al.* 2003; Rinta-Kanto *et al.* 2005; Furukawa *et al.* 2006; Saker *et al.* 2007). A review by Pearson *et al.* (2010) showed that about six *mcy* genes namely *mcy*A, -B, -C, -D, -E and -G are directly involved in the biosynthesis of MCs in toxigenic *Microcystis* spp. The absence of *mcy* genes in non-toxigenic species provides a quick tool to discriminate between toxic and non-toxic species. PCR amplifications of *mcy* genes can further be used to easily study species diversity, distribution or dominance either by quantifying *mcy* gene copy numbers, DNA sequencing, phylogenetic typing, etc. (Wilson *et al.* 2005; Rinta-Kanto & Wilhelm 2006; Conradie & Barnard 2012). In this case, therefore, PCR methods are potentially attractive in the monitoring of toxigenic *Microcystis* spp. in water, due to the fact that these methods are relatively cheaper compared with other analytical methods and require very small amounts of samples to accurately detect toxigenic species.

From the Hartbeespoort Dam, Oberholster & Botha (2010) reported the detection of a toxigenic *M. aeruginosa* by the PCR amplifications of *mcy*A, B and D genes, whereas recently, Conradie & Barnard (2012) amplified *mcy*B and E from the same species. However, Oberholster & Botha (2010) reported to have experienced difficulties in the amplification of *mcy*A gene, from which their survey showed that only one site out of four studied gave positive results in the formation of *mcy*A PCR products based on the primers used. Some of the explanations given regarding the absence of low transcription of *mcy*A gene were the existence of genetic distinctions for South African *M. aeruginosa* strains. Among the other factors that could have contributed to non-amplification of this gene could be lack of suitable PCR primers. However, if the literature showed that the dam is home to only one toxigenic *M. aeruginosa*, there would not probably be such a large inconsistency in the amplifications of this gene from DNA extracts obtained from the same species.

Thus, the aims of the present study were: (i) to extract *Microcystis* DNA from both algal and cell free water

samples and investigate the amplifications of *mcy*A gene using new PCR primers; (ii) to determine if all six major *mcy* genes (i.e., *mcy*A, -B, -C, -D, -E and -G) are consistently amplifiable from all samples in (i) above collected from different locations in the dam and relate the results to the distribution of the toxigenic *M. aeruginosa* in this dam; and (iii) to analyse the DNA sequence of the *mcy*A gene to be amplified from (i) above and relate the results to the existing knowledge about occurrence, distribution and dominance profiles of a toxigenic *M. aeruginosa* known to be the sole producer of MCs in the Hartbeespoort Dam.

MATERIALS AND METHODS

Sample collection and preparations

During mid-summer 2011, water in Hartbeespoort Dam (GPS: 25°43′44.56″ S, 27°51′30.35″ E) was fully covered with thick visible green floating algal scum (Figure 1). Surface algal cells (Figure 1(a)) and water samples (1 m below) (Figure 1(b)) were collected into 250 mL bottles from four GPS-guided locations designated as location 1: (25°43′38.76″ S, 27°50′57.21″ E); location 2: (25°46′14.09″ S, 27°51′57.85″E); location 3: (25°45′37.71″ S, 27°48′5.72″ E) and location 4: (25°44′56.87″ S, 27°50′0.05″ E). Sampling map details with regard to the above sites and physico-chemical conditions that prevailed during sampling were described in Mbukwa *et al.* (2012). Surface algal cells were captured using a beaker whereas water samples were collected using a van Don Water sampler (Figure 1(b)). All samples were kept in capped bottles and transported to the laboratory in cooler boxes containing packs of frozen gels/ice blocks. In the laboratory, all samples were filtered using G/FC (0.45 μM) glass fibre filters after which the filters were freeze dried for 24 h and stored at −20 °C until further analysis. Filtrates from water samples were further filtered using 0.220 μM filter syringes for complete removal of any debris from the first filtrate (0.45 μM). Aliquots (9 mL) of micro-filtrates (0.220 μM) were also stored at −20 °C until DNA extraction. *Microcystis* algal cells were identified as stated previously in Mbukwa *et al*. (2012).

DNA extraction and purification

Two sources were used to extract genomic DNA from environmental samples and these were algal cells and cell free water. The DNA extracts were used for PCR amplification and investigation of *mcy* genes in this study.

DNA extraction from algal cells

In each case, 10 mg of lyophilised algal cells were used in the extraction of genomic DNA following a modified method of Gaviria *et al*. (2006) as described in Mbukwa *et al.* (unpublished).

DNA extraction from cell free water

Short fragments of genomic cyanobacterial DNA known to exist as dissolved entities in water (Kumar *et al*. 2011) were recovered from micro-filtered water (9 mL) samples (see

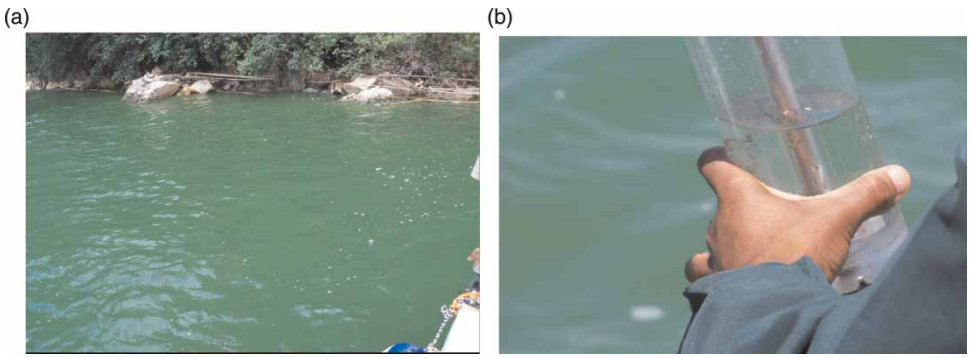

Figure 1 | Day time mid-summer water appearance in the Hartbeespoort Dam. (a) Greenish surface water near the dam wall. (b) Relatively clear water seen in the Van Don sampler collected at 1 m below the surface near the dam wall (February, 2011. Photos by EAM).

above, Sample collection and preparations) using DNeasy Plant Mini Kit following the manufacturer's protocol (Qiagen, Germany), albeit with slight modifications. Briefly, 4.25 mL of absolute ethanol was added to 8.5 mL aliquots of micro-filtered water samples and shaken well to mix. Following step 14 of DNeasy Plant Mini Kit protocol, 500 μL of the above mixture was pipetted and transferred into 2 mL DNeasy min columns and centrifuged at 8,000 rpm for 1 min. The flow-through (filtrate) was discarded and the DNeasy kit placed in a new collection tube (2 mL). Then step 16 was followed and all procedures through step 18 of the protocol. The extracted DNA was eluted with 100 μL of buffer AW and dried under laminar flow. The above procedure was repeated for the remaining water samples. The quality and quantity of genomic DNA extracts were determined after Sambrook *et al.* (1989) and stored at −25 °C.

PCR reaction and primers for amplification of *mcy* genes

PCR primers for the amplification of *mcy*A and *mcy*B genes were adopted from Mbukwa *et al.* (unpublished) whereas primers for the amplification of *mcy*C, D, E and G were adopted from Ouahid *et al.* (2005). All primers were synthesised at Integrated DNA Technologies (IDT, Leuven, Belgium). The primers and their sequences were as follows: *mcy*A: (u-102F) 5′-CGATGAACAAATCGGGCAATGGCA-3′, (u-620R) 5′-TGCAAGTTTCGCACATCTCCAAGG-3′; *mcy*B: (P5102F) 5′-AGTCATCATCTTCCTTCACCGCGT-3′, (P5853R) 5′-TGTCCTGCCATCCGTTCAATCGTA-3′; *mcy*C: (PSCF1) 5′-GCAACATCCCAAGAGCAAAG-3′, (PSCR1) 5′-CCGACAACATCACAAAGGC-3′; *mcy*D: (PKDF2) 5′-AGTTATTCTCCTCAAGCC-3′, (PKDR2) 5′-CATTCGTTCCACTAAATCC-3′; *mcy*E (PKEF1) 5′-CGCAAACCCGATTTACAG-3′, (PKER1) 5′-CCCCTACCATCTTCATCTTC-3′; *mcy*G: (PKGF1) 5′-ACTCTCAAGTTATCCTCCCTC-3′, (PKGR1) 5′-AATCGCTAAAACGCCACC-3′.

PCR reactions were performed in volumes of 25 μL containing GoTaq™Green Master Mix 2× polymerase (Promega, USA) (12.5 μL), primers (F/R) (1.0 μL, 10 μM each), DNA template (1.0 μL, <250 ng/μL) and nuclease-free PCR water (9.5 μL). The PCR machine (MyCycler™thermalcycler BIORAD; Applied Biosystems version 1.065, Spain) was programmed to run under gradient temperature. The initial denaturation step was 95 °C (2 min), followed by 95 °C (30 s) and elongation period of 1 min at 72 °C for 36 cycles. The final extension was performed at 72 °C (10 min) (Metcalf *et al.* 2012). The appropriate primers annealing temperatures for the PCR reaction were adopted from Mbukwa *et al.* (unpublished).

RESULTS AND DISCUSSION

Molecular identification of cyanobacterial DNA extracts

Light microscope observations showed the presence of cyanobacterial cells pertaining to *M. aeruginosa* as reported earlier (Mbukwa *et al.* 2012). From these cells genomic DNA was extracted and used in the amplification of *mcy* genes. In addition to this source, Kumar *et al.* (2011) reported that free short fragments of cyanobacterial DNA do exist in water, thus in order to further investigate the distribution and dominance of a toxigenic *M. aeruginosa* in the Hartbeespoort Dam some of the cyanobacterial DNA was extracted directly from cell free water samples. All DNA extraction methods yielded high quality cyanobacterial genomic DNA (260/280 ratio > 1.8; 260/230 ratio = 2) suitable for PCR reactions (Sambrook *et al.* 1989). This implies that cell free water samples containing cyanobacterial genomic DNA offer a reliable alternative source of *Microcystis* DNA for PCR investigation of the formation of potentially toxic species in water, especially at the early growth stages of blooming. Molecular identification and confirmation of cyanobacterial DNA extracted was done through the amplification of 16S-rRNA gene using a 16SF/R primer pair (16SF): 5′-CTGAAGAAGAGCTTGCGTC-3′ and (16SR): 5′-CCCAGTAGCACGCTTTCG-3′) as reported in Mbukwa *et al.* (unpublished). A PCR product of the expected size for 16S-rRNA amplicon (500 bp) was observed as indicated in lane 2 (Figures 2(a) and 2(b)).

Amplification of *mcy* genes

The PCR products (amplicons) for *mcy*A, B, C, D, E and G genes (as well as that for 16S-rRNA gene) presented in Figures 2(a) and (b) were of high quality and as-expected sizes as reported elsewhere (Ouahid *et al.* 2005; Mbukwa *et al.* (unpublished). Since universal PCR primers are very

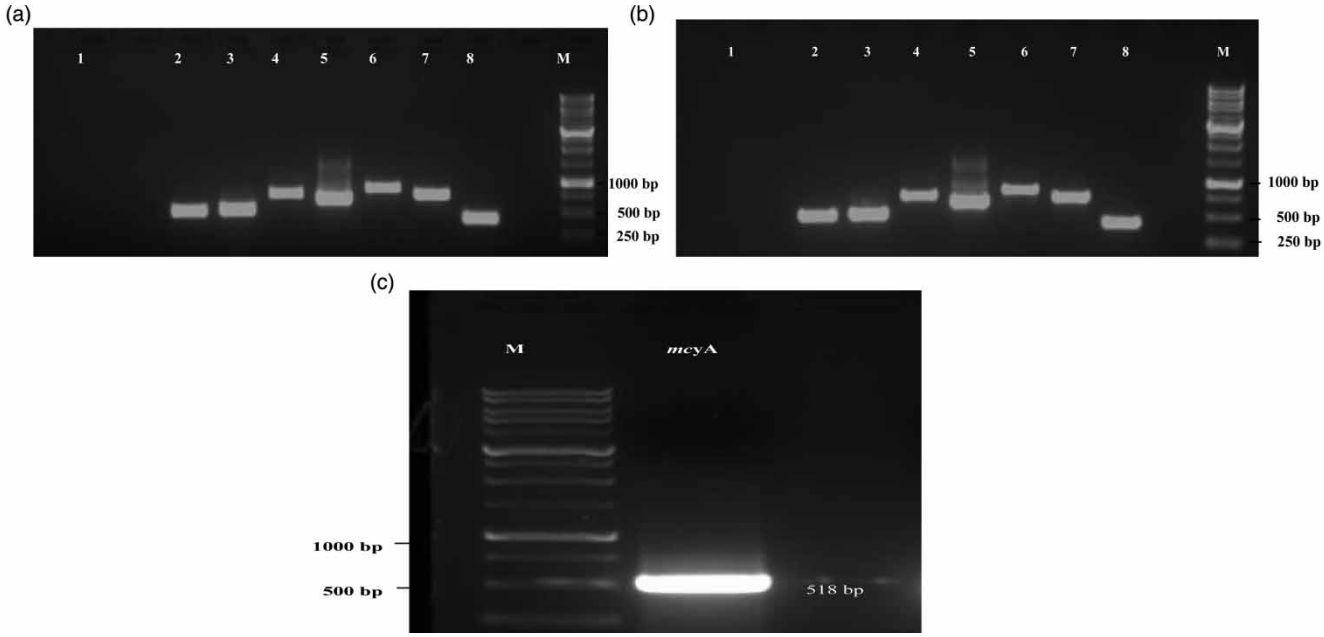

Figure 2 | Typical images of 1% agarose gel electrophoresis of amplified *mcy* gene PCR products from DNA extracts of a toxigenic *M. aeruginosa* from the Hartbeespoort Dam. (a) From algal cells; (b) from cell free water; (c) *mcy*A PCR product (518 bp) submitted for DNA sequencing. [Lane 1 = –ve control; lane 2 = 16SrRNA (+ve control); lane 3 = *mcy*A (518 bp); lane 4 = *mcy*B (751 bp); lane 5 = *mcy*C (674 bp); lane 6 = *mcy*D (859 bp); lane 7 = *mcy*E (755 bp); lane 8 = *mcy*G (425 bp); lane M = Standard DNA ladder (1000 kb)].

limited (Valério *et al*. 2010), primers used in this study were chosen based on previous success gleaned from the literature regarding their consistency in the amplification of *mcy* genes and also evaluated during preliminary PCR screening. Lack of universal primers is a consequence of characteristic genetic diversity (variation) and a wider distribution of various toxigenic *Microcystis* strains that have adapted to different environmental conditions producing genetic differences among *Microcystis* spp. (Kurmayer *et al.* 2002; Christiansen *et al.* 2003; Via-Ordorika *et al*. 2004; Wilson *et al.* 2005; Rinta-Kanto & Wilhelm 2006). All six *mcy* genes (*mcy*A, B, C, D, E, G) were consistently amplified from all studied samples (Table 1) indicating that both algal cells and cell free water samples are good sources of cyanobacterial genomic DNA for PCR amplifications of *mcy* genes.

DNA sequencing, evaluation of *mcy*A gene sequence and nucleotide alignment

The DNA sequence for the *mcy*A gene was carried out to further strengthen molecular findings on the occurrence and distribution of a toxigenic *M. aeruginosa* in the Hartbeespoort Dam following a wider range of successful and consistent amplifications of all six *mcy* genes and, in particular, *mcy*A gene from all DNA extracts studied (Table 1). Figure 3 shows a typical alignment of sequenced *mcy*A gene from a PCR product purified first on 1% agarose gel electrophoresis (Figure 2(c)), followed by Gel Purification kit (AccuPrep®, Bioneer Corporation, Korea). The sequenced *mcy*A gene comprised 408 bp nucleotides spanning from 141 to 548 bp in the N-methyl transferase (NMT) region (1,319 bp) of the nucleotide sequence of *mcy*A gene found in *M. aeruginosa* strains UWOCC (Figure 3), PCC 7806 and PCC 7820. A consistent amplification of *mcy*A gene and BLASTN results showing 100% similarities in the sequence alignments of a 408 bp nucleotides segment for *mcy*A gene sequenced from the studied genomic DNA was shown to be similar to *M. aeruginosa* UWOCC RID-1 without any nucleotide base mismatch or gaps (Figure 3).

Other observed close similarities in *mcy*A gene sequence ranging from 95 to 99% pertaining to the same spanning region (from 141 to 548 bp in a 1,319 bp *mcy*A gene length) were also related to other *Microcystis* spp. UWOCC strains; however, there were several base mismatches and/or gaps in their nucleotide sequences (Table 2). In addition, the

Table 1 | List of locations, sampling locations and sample sets used to amplify *mcy*A and others correlating to the occurrence and distribution of *M. aeruginosa* in the Hartbeespoort Dam

	DNA extract from							
	Location 1		Location 2		Location 3		Location 4	
PCR product	Algal cells	Cell free water	Algal cells	Cell free water	Algal cells	Cell free water	Algal cells	Cell free water
16S rRNA	+	+	+	+	+	+	+	+
*mcy*A	+	+	+	+	+	+	+	+
*mcy*B	+	+	+	+	+	+	+	+
*mcy*C	+	+	+	+	+	+	+	+
*mcy*D	+	+	+	+	+	+	+	+
*mcy*E	+	+	+	+	+	+	+	+
*mcy*G	+	+	+	+	+	+	+	+
*mcy*A gene sequence alignment and similarity to a 408 bp nucleotide segment[a]	100%	n.d.	100%	n.d.	100%	n.d.	n.d.	n.d.

+ = Amplified and clearly observed PCR products after 1% agarose gel electrophoresis and ethidium bromide staining.
[a] = BLASTN alignment results similar to published *mcy*A gene sequence segment of *M. aeruginosa* (GenBank Accession number AF139335.1), see Figure 3.
n.d. = not determined.

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GenBank Accession # AF139335.1 Microcystis aeruginosa strain UWOCC RID-1
microcystin synthetase (mcyA) gene, partial cds
Length=1319

Score = 754 bits (408), Expect = 0.0
Identities = 408/408 (100%), Gaps = 0/408 (0%)
Strand=Plus/Plus

Query  1    AATTTACAGGCAAACATCGGCAGATTCTCAAGGGATATTTAATATTGTTGGCTGGAATAG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  141  AATTTACAGGCAAACATCGGCAGATTCTCAAGGGATATTTAATATTGTTGGCTGGAATAG  200

Query  61   TAGTTACACGGGGGAACCTATCCCGGTTGCTCAGATGCGAGAATGGCTAGATGATAAAGT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201  TAGTTACACGGGGGAACCTATCCCGGTTGCTCAGATGCGAGAATGGCTAGATGATAAAGT  260

Query  121  TAAGGTTATTCTCGCTCAAAAACCGAAAAAAGTTCTGGAAATAGGTTGTGGAACCGGGTT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261  TAAGGTTATTCTCGCTCAAAAACCGAAAAAAGTTCTGGAAATAGGTTGTGGAACCGGGTT  320

Query  181  AATATTATTCCAAGTTGCTCCCCATTGCCAGTGTTATTGGGGAACCGATATTTCATCAGT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  321  AATATTATTCCAAGTTGCTCCCCATTGCCAGTGTTATTGGGGAACCGATATTTCATCAGT  380

Query  241  AGCCTTAGACCATATTCAGCGAATTAATCAAGAAGGGCCTCAGCTAGAGCAAGTCAGGCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  381  AGCCTTAGACCATATTCAGCGAATTAATCAAGAAGGGCCTCAGCTAGAGCAAGTCAGGCT  440

Query  301  ATTGCATAGCACAGCCGATAATTTTGAGGGTTTGGAGTCAGAAGGATTCGATACAATTAT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  441  ATTGCATAGCACAGCCGATAATTTTGAGGGTTTGGAGTCAGAAGGATTCGATACAATTAT  500

Query  361  CCTTAACTCGGTTGTGCAGTATTTCCCCCATATAGATTACTTACTGAG  408
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  501  CCTTAACTCGGTTGTGCAGTATTTCCCCCATATAGATTACTTACTGAG  548
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Figure 3 | A typical DNA sequence alignment and similarity analysis of the sequenced *mcy*A gene segment (408 bp) from a toxigenic *M. aeruginosa* from the Hartbeespoort Dam, South Africa.

Table 2 | Some *Microcystis* spp. strain UWOCC, whose published *mcy*A gene sequence segments spanning from 141 to 548 bp in a 1,319 bp NMT nucleotide length showed higher similarities to the sequenced *mcy*A gene from the DNA of *M. aeruginosa* collected from the Hartbeespoort Dam

***Microcystis* spp.**	**GenBank Accession #**	**Mismatch**	**Gaps**	**% Similarity**	**Presence of NMT domain and toxicity[a]**
M. aeruginosa strain UWOCC RID-1	AF139335.1	0	0	100	+
Microcystis spp. UWOCC AK (GV-)	AF139348.1	1	0	99	+
Microcystis spp. UWOCC AK-1	AF139347.1	1	0	99	+
M. aeruginosa strain UWOCC S-15-b	AF139336.1	2	0	99	+
M. aeruginosa strain UWOCC 84/1	AF139334.1	2	0	99	+
M. aeruginosa strain UWOCC BauldE	AF139346.1	3	0	99	+
Microcystis spp. strain UWOCC F	AF139345.1	4	0	99	+
M. aeruginosa strain UWOCC MR-A	AF139337.1	5	0	99	+
M. aeruginosa strain UWOCC MR-B	AF139338.1	5	0	99	+
M. aeruginosa strain UWOCC MR-C	AF139339.1	5	0	99	* NMT (+) Toxicity (−)
M. aeruginosa strain UWOCC MR-D	AF139340.1	5	0	99	+
Microcystis spp. strain UWOCC CBS	AF139344.1	5	0	99	* NMT (+) Toxicity (−)
M. *aeruginosa strain* UWOCC 019	AF139332.1	12	0	97	+
M. aeruginosa strain UWOCC 017	AF139343.1	13	2	97	+
M. aeruginosa strain UWOCC 001	AF139330.1	13	2	97	+
M. aeruginosa strain UWOCC 023	AF139333.1	14	2	97	+
M. aeruginosa strain UWOCC E7	AF139341.1	22	2	95	+

[a]According to Neilan (2000).

literature showed close *mcy*A gene sequence similarities (97%) to those of *M. aeruginosa* strains PCC 7806 (from 141 to 546 bp in a 1,319 bp *mcy*A gene length), PCC 7806 (from 114 to 519 bp in a 1261 bp *mcyA* gene length) and PCC 7820 (from 141 to 548 bp in a 1,319 bp *mcyA* gene length). The gene sequences and lengths of the above PCC strains are accessible on GenBank Accession #AF139342.1, JN848810.1 and AF139343.1, respectively. The closer relatedness of the *mcy*A gene segment spanning in a similar region in *M. aeruginosa* PCC 7806 strains and *M. aeruginosa* UWOCC strains is of particular interest for future studies. Neilan (2000) had earlier reported that the *mcy*A gene sequence of South African UWOCC strain (017) is closely related to *M. aeruginosa* PCC 7806 (the Netherlands) and UWOCC 001 (Canada). In the studies of Oberholster & Botha (2010) and Conradie & Barnard (2012), they employed *M. aeruginosa* strain PCC 7806 as a reference toxic species when studying *mcy*A, B and D, and *mcy*B and E genes from a toxigenic *M. aeruginosa* in the Hartbeespoort Dam, respectively. The two above reports therefore corroborate previous observations made by Mbukwa *et al.* (unpublished) that the new pair of the designed PCR primers (u-102F/u-620R) had the capacity to amplify *mcy*A gene from a large group of *Microcystis* spp. due to the conserved nucleotide sequence found in a number of *Microcystis* spp. including *M. aeruginosa* strain PCC 7806. In the present study, however, the observed DNA sequence alignments on the PCC 7806 strain showed the presence of two gaps and 13 mismatches in the *mcy*A nucleotide sequences obtained using a u-102F/u-620R primer pair during DNA sequencing (data not shown). Thus, probably it would be correct to suggest that the toxigenic *Microcystis aeruginosa* dominating the Hartbeespoort Dam does not actually belong to the *M. aeruginosa* PCC 7086 strain, even though the latter has been used as reference strain in several studies from this dam. Positive results were probably due to such close similarities in the NMT region (Neilan 2000). Thus, with the advent of the discovered 408 bp *mcy*A gene sequence from this study, an investigation would be needed to deepen the understanding regarding the dominance of *M. aeruginosa* from this dam; and also to expand the study to other water reservoirs from which it has been difficult to amplify this

gene for South African *M. aeruginosa* strains as reported in the literature (Oberholster & Botha 2010).

Table 2 indicates that overall results from the *mcy*A gene sequence showed that all genomic DNA used originated from a toxigenic *Microcystis* spp., in particular from the *M. aeruginosa* UWOCC strain; and, furthermore, these results suggest that the responsible species is widely distributed across the dam. However, while we are conscious that not all UWOCC strains listed in Table 2 were present during sampling or exist in this dam, it is of particular importance that the greatest similarity in the nucleotide sequence of *mcy*A gene is 100% matched to a known toxic *M. aeruginosa* strain, UWOCC RD-1, capable of producing MCs (Kappers 1982; Tillett *et al.* 2001). From BLASTN and *mcy*A gene nucleotide alignment results, this strain showed 100% matching to the NCBI database for all DNA samples analysed. These findings, together with the identification of the conserved region of the gene sequence and the consistent expression (amplification) of the *mcy*A gene, support the idea about the wider occurrence, distribution and dominance profiles of a single toxigenic *M. aeruginosa* in this dam. Moreover, a recent report by Conradie & Barnard (2012), whose findings correlated microcystin production to the distribution and quantity of *mcy*E copies, showed that all *mcy*E genes were amplified from toxigenic *Microcystis* spp. only found in this dam; thus, their findings corroborate positively with this current study.

LC-ESI-MS confirmation on the biosynthesis of microcystins in the Hartbeespoort Dam

The presence and ability of the toxigenic *M. aeruginosa* strain to produce cyanobacterial toxins in the dam were confirmed by LC-MS findings. The production and distribution of six microcystin congeners (MC-RR, -LR, -YR, -WR, -(H_4)-YR and (*D-Asp*^{3}, *Dha*^{7}) MC-RR) from all locations shown in Table 2 were reported recently from the Hartbeespoort Dam (Mbukwa *et al.* 2012). Some recent studies conducted elsewhere (Pašková *et al.* 2008; Zhang *et al.* 2009a, 2009b, 2012; Lance *et al.* 2010) have shown that MCs accumulate in various organs in a number of aquatic organisms posing health risks to aquatic food resources through food web and food chain relationships. Apart from irrigation schemes, large-scale fishing activities and sales are practised in the Hartbeespoort Dam, thus, biomonitoring of MCs' accumulation in harvested fish products would be essential.

CONCLUSIONS

In an effort to further understand the occurrence and dominance profiles of a commonly encountered and known to be the only single toxigenic *M. aeruginosa* existing in the Hartbeespoort Dam, molecular-based explanations were necessary to describe its distribution among other indicators. Consistent amplifications of all six *mcy* genes from both algal cells and cell-free water samples sourced from different locations in this dam provided a list of evidence that this species is well distributed in the dam. The results of a *mcy*A gene sequence demonstrated with higher molecular certainty that the dam is home to one dominant toxigenic *M. aeruginosa* strain UWOCC RD-1. Furthermore, this study successfully identified a conserved region of the *mcy*A gene sequence from this species. This gene (*mcy*A) was previously shown to be difficult to amplify and was therefore considered to be under-expressed in a similar way to other *M. aeruginosa* strains found in South African eutrophic waters are considered. Therefore, from this discovery further analysis would be needed with a possible extension of the study to include investigations of the *mcy*A gene from other South African *M. aeruginosa* strains from which there have been difficulties in the amplification of this gene, and also DNA sequencing of other genes would be necessarily important to study species/strain diversity.

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REFERENCES

Carmichael, W. W. 1992 Cyanobacteria secondary metabolites – the cyanotoxins. *J. Appl. Bacteriol.* **7**, 445–459.

Christiansen, G., Fastner, J., Erhard, M., Börner, T. & Dittmann, E. 2003 Microcystin biosynthesis in *planktothrix*: genes, evolution, and manipulation. *J. Bacteriol.* **185**, 564–572.

Conradie, K. R. & Barnard, S. 2012 The dynamics of toxic *Microcystis* strains and microcystin production in two hypertrofic South African reservoirs. *Harmful Algae* **20**, 1–10

Dittmann, E., Neilan, B. A. & Börner, T. 2001 Molecular biology of peptide and polyketide biosynthesis in cyanobacteria. *Microbiol. Biotechnol.* **57**, 467–473.

Downing, T. G. & van Ginkel, C. E. 2004 Cyanobacterial Monitoring 1990–2000: Evaluation of SA data. WRC Report No. 1288/1/04. Water Research Commission, Pretoria.

DWA (Department of Water Affairs) 1991 *Hartbeespoort Government Water Scheme.* Department of Water Affairs, Pretoria. Available at: http://www.harties.org.za/hartiesdev/documents/HartbeespoortWaterScheme1991.pdf.

Furukawa, K., Noda, N., Tsuneda, S., Saito, T., Itayama, T. & Inamori, Y. 2006 Highly sensitive real-time PCR assay for quantification of toxic cyanobacteria based on microcystin synthetase a gene. *J. Biosci. Bioeng.* **102**, 90–96.

Gaviria, D. A., Aguilar, E., Serrano, H. J. & Alegria, A. H. 2006 DNA fingerprinting using AFLP markers to search for markers associated with yield attributes in the silkworm, *Bombyx mori*. *J. Insect. Sci.* **6** (15), 1–10.

Harding, W. R. & Paxton, B. R. 2001 Cyanobacteria in South Africa – A Review, WRC Report No. TT 153/01. Water Research Commission, Pretoria.

Heijnen, L. & Medema, G. 2006 Quantitative detection of *E. coli*, *E. coli* O157 and other Shiga toxin producing *E. coli* in water samples using a culture method combined with real-time PCR. *J. Water Health.* **4** (4), 487–498.

Hoffmann, J. R. H. 1976 Removal of *Microcystis* toxins in water purification processes. *Water SA* **2** (2), 58–60.

Kappers, F. I. 1982 Toxic cyanobacteria in the Netherlands. *Hydrolog. Bull.* **16**, 291.

Kumar, A., Kumar, A., Rai, A. K. & Tyagi, M. B. 2011 PCR based detection of *mcy* genes in blooms of *Microcystis* and extracellular DNA of pond water. *Afr. J. Microbiol. Res.* **5** (4), 374–381.

Kurmayer, R., Dittmann, E., Fastner, J. & Chorus, I. 2002 Diversity of microcystin genes within a population of the toxic cyanobacterium *Microcystis* spp. in Lake Wannsee (Berlin, Germany). *Microb. Ecol.* **43**, 107–118.

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**, 6723–6730.

Lance, E., Brient, L., Carpentier, A., Acou, A., Marion, L., Bormans, M. & Gérard, C. 2010 Impact of toxic cyanobacteria on gastropods and microcystin accumulation in a eutrophic lake (Grand-Lieu, France) with special reference to *Physa* (=*Physella*) *acuta*. *Sci. Total. Environ.* **408**, 3560–3568.

Laughinghouse IV, H. D., Prá, D., Silva-Stenico, M. E., Rieger, A., Frescura, V. D.-S., Fiore, M. F. & Tedesco, S. B. 2012 Biomonitoring genotoxicity and cytotoxicity of *Microcystis aeruginosa* (Chroococcales, Cyanobacteria) using the *Allium cepa* test. *Sci. Total Environ.* **432**, 180–188.

Masango, M. G. 2007 A Comparative Analysis of the Cytotoxicity of Cyanotoxins using In-Vitro (Cell Culture) and In-Vivo (Mouse) Assays. M.Sc. Thesis, University of Pretoria, Pretoria, South Africa.

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

Metcalf, J. S., Richer, R., Cox, P. A. & Codd, G. A. 2012 Cyanotoxins in desert environments may present a risk to human health. *Sci. Total Environ.* **421–422**, 118–123.

Neilan, B. A. 2000 Genetics of *Microcystis* toxicity. Identification of the genetic pathway for microcystin production in *Microcystis aeruginosa*. Cooperative Research Centre for Water Quality and Treatment Research Report No. 6, July 2000. CRC for Water Quality and Treatment, Salisbury, South Australia, pp 1–27.

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.

Oberholster, P. J. & Botha, A.-M. 2010 Use of remote sensing and molecular markers to detect toxic cyanobacterial hyperscum crust: a case study on Lake Hartbeespoort, South Africa. *Afr. J. Biotechnol.* **9** (51), 8791–8799.

Oberholster, P. J., Botha, A.-M. & Myburgh, J. G. 2009 Linking climate change and progressive eutrophication to incidents of clustered animal mortalities in different geographical regions of South Africa. *Afr. J. Biotechnol.* **8** (21), 5825–5832.

Ouahid, Y., Pérez-Silva, G. & del Campo, F. F. 2005 Identification of potentially toxic environmental *Microcystis* by individual and multiple PCR amplification of specific microcystin synthetase gene regions. *Environ. Toxicol.* **20**, 235–242.

Owuor, K., Okonkwo, J., Van Ginkel, C. & Scott, W. 2007 Environmental factors affecting the persistence of toxic phytoplankton in the Hartbeespoort Dam. WRC Report No. 1401/3/07. Water Research Commission, Pretoria.

Pašková, V., Adamovský, O., Pikula, J., Skocovská, B., Band'ouchová, H., Horáková, J., Babica, P., Maršálek, B. &

Hilscherová, K. 2008 Detoxification and oxidative stress responses along with microcystins accumulation in Japanese quail exposed to cyanobacterial biomass. *Sci. Total Environ.* **398**, 34–37.

Pearson, L., Mihali, T., Moffitt, M., Kellmann, R. & Neilan, B. 2010 Review on the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. *Mar. Drugs* **8**, 1650–1680.

Quibel, G., Badenhorst, J. E. & Careisen, C. I. C. 1995 A national toxic blue-green algal surveillance programme for South Africa. Internal Report N/0000/DPQ/1094. Department of Water Affairs and Forestry, Pretoria.

Rinta-Kanto, J. M. & Wilhelm, S. W. 2006 Diversity of microcystin-producing cyanobacteria in spatially isolated regions of Lake Erie. *Appl. Environ. Microbiol.* **72**, 5083–5085.

Rinta-Kanto, J. M., Ouellette, A. J. A., Boyer, G. L., Twiss, M. R., Bridgeman, T. B. & Wilhelm, S. W. 2005 Quantification of toxic *Microcystis* spp during the 2003 and 2004 blooms in western Lake Erie using quantitative real-time PCR. *Environ. Sci. Technol.* **39**, 4198–4205.

Saker, M. L., Vale, M., Kramer, D. & Vasconcelos, V. M. 2007 Molecular techniques for the early warning of toxic cyanobacteria blooms in fresh water lakes and rivers. *Appl. Microbiol. Biotechnol.* **75**, 441–449.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989 *Molecular Cloning: A Laboratory Manual*, 2nd edn, Cold Spring Harbor Laboratory, New York.

Scott, W. E. 1991 Occurrence and significance of toxic cyanobacteria in Southern Africa. *Water Sci. Technol.* **23**, 175–180.

Steyn, D. J. & Toerien, D. F. 1975 Eutrophication levels of some South African impoundments II. Hartbeespoort Dam. *Water SA* **1** (3), 93–101.

Taylor, J. C., Harding, W. R., Archibald, C. G. M. & van Rensburg, L. 2005 Diatoms as indicators of water quality in the Jukskei-Crocodile river system in 1956 and 1957, a re-analysis of diatom count data generated by BJ Cholnoky. *Water SA* **31** (2), 237–246.

Tillett, D., Parker, D. & Neilan, B. A. 2001 Detection of toxigenicity by a probe for the microcystin synthetase A gene (*mcy*A) 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.

Toerien, D. F., Scott, W. E. & Pitout, M. J. 1976 *Microcystis* toxins: isolation, identification, implications. *Water SA* **2** (4), 160–163.

Vaitomaa, J., Rantala, A., Halinen, K., Rouhiainen, L., Tallberg, P., Mokelke, 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.

Valério, E., Chambel, L., Paulino, S., Faria, N., Pereira, P. & Tenreiro, R. 2010 Multiplex PCR for detection of microcystin-producing cyanobacteria from fresh water samples. *Environ. Toxicol.* **25**, 251–260.

van Ginkel, C. E. 2004 A National Survey of the Incidence of Cyanobacterial Blooms and Toxin Production in Major Impoundments. Internal Report No. N/0000/00/ DEQ/ 0503. Resource Quality Services, Department of Water Affairs and Forestry, Pretoria.

Via-Ordorika, L., Fastner, J., Kurmayer, R., Hisbergues, M., Dittmann, E., Komárek, 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**, 592–602.

WHO (World Health Organization). 1999 *Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring and Management* (I. Chorus & J. Bartram, eds). E&FN Spon, London and New York.

Wicks, R. J. & Thiel, P. G. 1990 Environmental factors affecting the production of peptide toxins in floating scums of the cyanobacterium *Microcystis aeruginosa* in a hypertrophic African reservoir. *Environ. Sci. Technol.* **24**, 1413–1418.

Wilson, A. E., Sarnelle, O., Neilan, B. A., Salmon, T. P., Gehringer, M. M. & Hay, M. E 2005 Genetic variation of cyanobacterium *Microcystis aeruginosa* within and among lakes: implications for harmful algal blooms. *Appl. Environ. Microbiol.* **71** (10), 6126–6133.

Zhang, D., Xie, P., Liu, Y., Chen, J. & Wen, Z. 2009a Spatial and temporal variations of microcystins in hepatopancreas of a freshwater snail from Lake Taihu. *Ecotoxicol. Environ. Saf.* **72**, 466–472.

Zhang, D., Xie, P., Liu, Y. & Qiu, T. 2009b Transfer, distribution and bioaccumulation of microcystins in the aquatic food web in Lake Taihu, China, with potential risks to human health. *Sci. Total Environ.* **407**, 2191–2199.

Zhang, J., Wang, Z., Song, Z., Xie, Z., Li, L. & Song, L. 2012 Bioaccumulation of microcystins in two freshwater gastropods from a cyanobacteria-bloom plateau lake, Lake Dianchi. *Environ. Pollut.* **164**, 227–234.

Zohary, T. 1985 Hyperscum of the cyanobacterium microcystis aeruginosa in a hypertrophic lake (Hartbeespoort Dam, South Africa). *J. Plankton. Res.* **7**, 399–409.

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