Toxic *Microcystis novacekii* T20-3 from Phakalane Ponds, Botswana: PCR Amplifications of Microcystin Synthetase (*mcy*) Genes, Extraction and LC-ESI-MS Identification of Microcystins

Elbert Mbukwa1,2,3, Titus AM Msagati2, Bhekie B Mamba1, Sammy Boussiba3, Victor Wepener4, Stefan Leu3 and Yuval Kaye3

1University of Dar es Salaam, DUCE Campus, P.O. Box 3239, Dar es Salaam, United Republic of Tanzania
2University of South Africa, College of Science Engineering and Technology, Science Campbell, 1710 Roodepoort, Johannesburg, Republic of South Africa
3Microalgal Biotechnology Laboratory, Jacob Blaustein Institutes for Desert Research, Ben-Gurion university of the Negev, Sede-Boker Campus, 84990, Israel
4North-West University, Potchefstroom Campus, Faculty of Natural Sciences School of Biological Sciences, 2520, Potchefstroom, Republic of South Africa

Abstract

Treated water effluent from Phakalane waste water secondary maturation ponds in Gaborone City (Botswana) enters the Limpopo River via Notwane River. Effluent samples from these ponds were collected and investigated using molecular and analytical methods to determine presence of *mcy* genes and microcystins (MCs), respectively. It was observed that, potentially toxic algal blooms were present in this effluent and therefore algal toxins. Using Polymerase Chain Reaction (PCR) method; cyanobacterial 16S-rRNA and *mcy*A, -B, -C, -D, -E and -G genes were amplified and PCR products separated by gel electrophoresis and visualized after ethidium bromide staining. DNA sequence for *mcy*A gene was obtained using PCR products from a newly designed primer pair for the amplification of *mcy*A gene. BLAST results of the obtained DNA sequence were evaluated and aligned to NCBI database for species identification. The alignment gave the highest similarity (100%) in nucleotide sequence that was aligned to the DNA sequence of a toxigenic *M. novacekii* T20-3 based on the published data. Microcystin-RR, -YR, -LR and -WR were chromatographed, identified and quantified from *M. novacekii* T20-3 cell extracts using LC-ESI-MS technique after liquid-partitioning (LP) and solid-phase extraction (SPE) steps complementing PCR findings. Higher quantities of MC-RR, -LR and YR: 53.620 ± 0.063, 12.114 ± 0.024 and 5.280 ± 0.035 µg/g DW were observed, respectively.

Keywords: Phakalane ponds; PCR amplification; *mcy* genes; DNA sequence; Toxic *M. novacekii* T20-3; Liquid-partitioning; SPE; LC-ESI-MS; Microcystins

Introduction

Waste water treatment plants (WWTP) are among the major anthropogenic related sources of excessive nutrientous and phosphate (N/P) nutrient loads in fresh water systems leading to eutrophication favoring growths of toxic algal blooms [1-4]. Other N/P sources include the undue disposal of industrial, domestic and agricultural solid wastes that are later carried out by storm water run-offs into major water bodies including ponds, rivers, lakes, etc. [3-5,8]. Known fresh water toxic algal blooms include *Microcystis* spp, *Anabaena* spp, *Planktothrix* spp, *Aphanizomenon* spp and *Nostoc* spp [5,9-14]. However, toxic *Microcystis* spp producing potent microcystins are of particular health concern worldwide [5-7,15-18].

The Phakalane waste water secondary treatment ponds (GPS 25° 57.60 E, 24° 37.20 S) are secondary maturation sewer units for the Glen valley WWTP serving Gaborone city (capital of Botswana) residents [19]. The ponds situated about 20 Km from Gaborone City centre, occupy about 70 ha of area forming a supportive ecosystem of the Botswana’s important bird area (IBA) recognised internationally by the Bird Life International [20]. Treated water effluent from Phakalane ponds enters Notwane River [21,22] on its way to the Limpopo River. The later empties its water into the Indian Ocean at XaiXai in Mozambique. However, the water in the ponds and the effluent are used for irrigation on nearby golf grounds, horticultural fields downstream Notwane River and on the farms under the Glen valley irrigation scheme [23,24]. Higher N/P nutrient loading into these ponds and suitable pH were observed as of recent [4,24] and shown to favor growths of algal blooms [4], however their toxigenicity have not been characterized. Morphological appearances of the algal cells from the effluents were similar to those pertaining to *M. aeruginosa* [25]. But, morphological features have no relationship with algal toxicity [25] and thus little is known about its toxigenic properties as there were no documented cases of animal poisoning related to algal growths in the ponds. Cases of unlawful fishing from the ponds/effluent and subsequent fish sales on street markets have been reported [26,27]. However, there had been no detailed study to address any potential for MCs production from the pond algae and possible fish contaminations posing health hazards to fish, fish consumers as well as birdlife-aquatic life relationships in the IBA. Literature show that toxic *Microcystis* spp flourish well in eutrophic waters where higher temperatures above 20°C [28,29] are prevalent, hence, the average temperatures between 22°C from July to 33°C in January common prevalent in Botswana [30] are within the optimal values that would favor growths of toxic *M. aeruginosa* [28, 29] in the ponds.

Molecular tools to identify potentially toxigenic *Microcystis* spp in environmental samples have been developed and applied in PCR amplifications of microcystin synthetase (*mcy*) genes that are involved in the biosynthesis of MCs [3,25, 31-41]. From a recent review by Pearson et al., [42], it can be viewed that the research findings surveyed...
showed that non-toxic *Microcystis* species lack *mcy* genes and therefore they can be easily and more reliably differentiated from toxic species than using morphological methods. Besides the fact that molecular methods are versatile enough to detect potentially toxic species from either very complex (mixed species) or very small (e.g. single colony) sample sizes [3,33,34, 36,39,43] but they lack the capacity to identify individual MC congeners from potentially toxic species and therefore the methods serve as a screening tools only. Furthermore, lack of universal PCR primers [43] limits the PCR amplifications of *mcy* genes fitting the screening and detection of every potentially *Microcystis* species [40,41] due to regional, genetic, seasonal and environmental variations of the cyanobacteria [39,44-46]. Gene deletion and insertion are also common features such that not all species with *mcy* genes will actually produce MCs [47,48] or the once known to be non-toxic species may become toxic [25]. Therefore, it’s often recommended to complement PCR findings with analytical methods including the LC-ESI-MS [49] which have higher accuracy and precise capability in mass detection for the identification and confirmation of MC structures in algal samples [50]. However, for better LC-ESI-MS results in the analysis of environmental algal samples requires a prior clean-up step usually using solid-phase extraction (SPE), e.g. on C₈ cartridges [49,51] or liquid-partitioning (LP) using different solvent systems [52-56]. On the other hand when LP precedes SPE procedure it alleviates the drawbacks experienced by the later by reducing operational time and resulting in cleaner LC-MS signals [51] because LP removes pigments and co-eluting compounds that would have otherwise clogged SPE cartridges or co-eluted compounds masked LC-MS spectra [57-63]. While some SPE cartridges are renowned for their extraction efficiencies of MCs from complex matrices, for instance C₈ Oasis Waters™ HLB cartridges [62,64] but clogging remains inevitable during solid-phase extractions (SPE) due to aforementioned factors. Prior to SPE, the removal of pigments and other less polar organics involving an LP step using chloroform has proved useful in extraction of polar compounds including MCs from algal blooms [52,65] and mycosporine-like amino acids (MAAs) from algae [66].

To the best of our knowledge the toxigenicity, species identity and MCs produced from algal blooms occurring in Phakalane pond effluents have not been investigated in particular using molecular and analytical methods, respectively. Thus the objectives of this study were to: i) amplify and detect *mcy* genes in algal samples using PCR methods and determine the toxigenecity potential of this bloom ii) determine the species genetic identity through DNA sequencing of *mcyA* gene iii) extract MCs in algal samples using analytical methods incorporating LP-SPE followed by the identification and quantification using LC-ESI-MS technique.

**Experimental**

**Sampling**

Algal samples were collected from the pond effluent in February, 2010 by surface grab into 500 ml bottles and the capped bottles were packed into a cooler box containing ice blocks. Three batches of samples were collected from two separate points along the effluent. Samples for morphological cell identification were fixed with Lugol’s solution and kept in brown capped bottles and placed in the cooler box. Samples were then transported overnight to the University of Johannesburg and algal samples for MC and *mcy* genes analysis were stored in a -25°C freezer on arrival before filtration and freeze-drying.

**Microscopic identification of cyanobacterial cells**

Cellular morphologies were examined microscopically using Utermöhl method based on a standard procedure outlined in [67] at 10X or 40X object magnification of the microscope [67]. In cases where higher cell densities were observed in a counting chamber, a dilution factor of about 10X was applied for easy cell identifications [67]. Cell identifications were done at the Department of Zoology University of Johannesburg within the first 18hrs of sample collection.

**Extraction of genomic DNA**

A DNA extraction procedure of Gaviria et al., [68] was adopted albeit with some modifications: In this study: 10 mg of freeze-dried algal cells were weighed into 2 ml microcentrifuge tubes. Cells were then suspended in 0.7 ml of freshly prepared DNA extraction buffer (50 mM EDTA, 100 mM NaCl and 20 mM Tris.Cl pH 8.0). To this solution 100 µl of protease K (2 mg/ml) and 50 µl of 20% SDS were added and to facilitate cell lysis sterilized glass beads were also added and the microcentrifuge tubes mounted on a bead beater (rated at 60 sec/ cycle). Samples tubes were chilled on ice (1 min) after every cycle (the beating procedure was repeated 4X). After this step, microcentrifuge tubes were incubated at 55°C (2hrs), briefly chilled on ice followed by addition of 100 µl of 5M potassium acetate. The tubes were hand shaken to mix contents and incubated further on ice (30 min). The tubes were then centrifuged at 13000 rpm (15 min) and supernatants collected into new microcentrifuge tubes. Ten (10) µl of RNAase (10mg/ml) free of DNAase were added and tubes incubated at 37°C (30 min) prior to phenol/chloroform extraction. After incubation 1 volume containing phenol/chloroform/isoamylalcohol (25/24/1, v/v/v) solution was added, shaken well to mix and centrifuged at 13000 rpm for (5 min) to extract the DNA. For DNA re-extraction the upper phases were transferred into new microcentrifuge tubes and one volume of chloroform/isoamylalcohol (24/1, v/v) added and shaken well followed by centrifugation at 13000 rpm (5 min). For higher purity DNA the upper phases were transferred into a new tubes and the above procedure repeated (3X). Supernatants were transferred to 1.5 ml tubes followed by addition of 2.5 volumes of absolute ethanol and incubating at -20°C (60 min). After 1 hr, pure DNA was precipitated from each tube by centrifugation at 13000 rpm (25 min) and solvent removed by pipette. The pellets were washed once using ice cold ethanol (70%), the pellets were then dried under laminar hood and stored in a freezer at -25°C until further use.

**Quality and concentration of isolated DNA**

The amount and purity of crude DNA extracts were determined spectrophotometrically [69-71] using a Gen5 Nanodrop Spectrophotometer. Values for 260/280, 260/230 ratios and DNA concentrations of each sample were obtained in duplicate and mean values recorded. Pure DNA extracts were stored in freezers under -20°C until further use.

**Selections of suitable primer pairs**

Six PCR primer pairs for the amplification of *mcy* genes (Table 1) were selected from three literatures [25,35,39] and were synthesized at Integrated DNA Technologies (IDT) laboratory in Belgium. Based on geographical and regional weather similarities as well as the proximity between the Phakalane ponds (Botswana) and the Hartbeespoort Dam.
(South Africa) provided a starting platform in our genetic investigation for the identification of mcy genes from an unknown algal bloom that formed in the Phakalane pond effluents. Recently, Oberholster and Botha [38] published some mcy gene profiles from M. aeruginosa a cyanobacteria species that is well studied and established to be the only known microcystin producing species that has dominated the Hartbeespoort Dam for many decades [68]. Therefore, in this study, DNA extracted from algal samples collected from the Hartbeespoort Dam was used as our reference in the detection of mcy genes from Phakalane pond effluent samples.

In a preliminary study, the suitability of the selected primer pairs were therefore determined by investigating their consistency in the amplification of mcy genes and the formation of expected PCR products from DNA extracts of algal cells collected from both Phakalane pond effluent and Hartbeespoort Dam samples. Cellular morphological comparison was also determined (see Results and Discussion section).

**PCR protocol and conditions**

All PCR reactions were carried out in 25 µl reaction mixtures following procedures described in the manufacturer’s protocol for using a Promega polymerase GoTaq® Green Master Mix, 2x (Promega Part # 9PM712, Madison USA) albeit with modifications to suit different optimal primers’ conditions. Briefly, the reaction mixtures comprised of: i) GoTaq® Green Master Mix, 2X (12.5 µl); ii) Forward primer, 10 µM (1.0 µl); iii) Reverse primer, 10 µM (1.0 µl); iv) Genomic DNA template, <250 ng (1.0 µl) and v) PCR water (9.5 µl). Using primers enlisted on Table 1, a typical PCR reaction was performed using a 96 well MyCycler™ thermalcycler BIORAD (version 1.065) and the PCR machine was programmed as follows: Initial denaturation step at 95°C (2 min), followed by 36 cycles of 95°C (30 sec), gradient temperature (55 to 44°C) (30 sec), elongation 72°C (1 min) followed by final extension at 72°C (10 min).

**Primer designs for the amplification of mcyA and mcyB**

Different primer sets were sought and designed based on known Microcystis sp gene sequence found on NCBI database. The primers’ sequence for species specificity in detecting mcyA and B genes in Microcystis species were analysed using the basic local alignment search tool (BLAST), an online service of the National Centre for Biotechnology Information (NCBI). Suitable primers were then selected based on BLAST results and primer qualities assessed as described in a Promega Corporation (2008) brochure. The suitably designed primers (Table 1) were also synthesized at IDT laboratories (Belgium).

For real sample applications, different sets of new primer pairs (Forward/Reverse) were further tested to determine, i) formation of expected PCR products and sizes from genomic DNA samples and ii) consistence (repeatability) in the amplifications and formation of quality respective mcyA or mcyB PCR products from different batches of DNA samples from both known M. aeruginosa species (Hartbeespoort Dam) and unknown (Phakalane pond effluent).

**PCR-based identification of cyanobacterial DNA and the amplification of mcy genes**

Cyanobacterial specific 16S-rRNA gene was amplified on PCR using 16SF/R primer pairs whereas mcy genes (mcyA, -B, -C, -D, -E and -G) were amplified using PCR primers outlined on Table 1. PCR products were separated using 1% agarose gel electrophoresis (made of 50 or 80 ml of 20x TAE buffer) and visualized after staining with 0.5 µl ethidium bromide.

**DNA sequencing**

The DNA sequence for mcyA gene was performed using combined PCR products amplified from separate DNA batches using a newly designed mcyA primers (Table 1). To obtain pure bands for mcyA gene sequencing, PCR products for mcyA gene (see PCR protocol above) were purified using 1% agarose gel electrophoresis and visualized after ethidium bromide staining. Bands were then precisely cut from the gel under minimum possible exposure time to external environment to avoid DNA degradation. The DNAs were recovered from the gel using Bioneer AccuPrep® Gel Purification kit (Bioneer Corporation, Korea) following the manufacture’s protocol without any modification. The quality and quantity of recovered DNA was checked accordingly as stated above. DNA sequencing was performed using a HITACHI Genetic Analyzer (Model 3500XL, Applied Biosystems) housed at the Ben-Gurion University of the Negev, Israel. The sequence identity was performed on BLAST and aligned to NCBI database.

<table>
<thead>
<tr>
<th>Gene</th>
<th>PCR primer pair</th>
<th>Gene sequence (5’-3’)</th>
<th>Annealing Temp. (°C)</th>
<th>PCR Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common NPRS</td>
<td>MTF2/ MTR</td>
<td>GCNGG(CT)GG(CT)GCNTA(CT)GTNCC CCNCG(AGTAT)(CT)TTNAC(CT)GTGG</td>
<td>50</td>
<td>1000</td>
<td>[79]</td>
</tr>
<tr>
<td>mcyA</td>
<td>MSF/MSR</td>
<td>ATTCAGCAGTGTAGCGAAGTGCAGAATAACTCGCCAGTTGG</td>
<td>50</td>
<td>1300</td>
<td>[25]</td>
</tr>
<tr>
<td></td>
<td>u-102F/u-620R</td>
<td>CGTAGAACAAATCGGCAGTAGCGAACATGCGAATAACTCGCCAGTGA</td>
<td>55</td>
<td>518</td>
<td>This study</td>
</tr>
<tr>
<td>mcyB</td>
<td>P5102F/P5853R</td>
<td>AGTCTAGTCTTCCTTCACCGGCTGTCCTGCTGGCATCGTTCAATCTGTA</td>
<td>55</td>
<td>751</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>2156-F/3111-R</td>
<td>ATGACTGTTGCTCTGGTAAAGAA</td>
<td>44</td>
<td>955</td>
<td>[39]</td>
</tr>
<tr>
<td>mcyC</td>
<td>PSCF1/PSCR1</td>
<td>GCAACATCCCAAGGCAAAGCG</td>
<td>49</td>
<td>674</td>
<td>[35]</td>
</tr>
<tr>
<td>mcyD</td>
<td>PKDF2/PKD2</td>
<td>AGTTTTGCTCTCCAGGCGCATTTGCTTCCCGAATAATC CCC</td>
<td>44</td>
<td>859</td>
<td>[35]</td>
</tr>
<tr>
<td>mcyE</td>
<td>PKEF1/PKER1</td>
<td>CGCAAGCCCAGGTATACCCAACC</td>
<td>48</td>
<td>755</td>
<td>[35]</td>
</tr>
<tr>
<td>mcyG</td>
<td>PKGF1/PKG1</td>
<td>ATCTCAAGTTATCCTCCTCTCAATGCTTAAACGGCACC</td>
<td>48</td>
<td>425</td>
<td>[35]</td>
</tr>
</tbody>
</table>

Table 1: Suitable PCR primers used in this study for the amplification of mcy genes.
Extraction and LC-ESI-MS detection of MCs

Sample clean-up, MCs extraction and identification: Freeze-dried algal samples (1.0 g) were extracted with 70% aqueous MeOH. Sample clean-up was performed by liquid partitioning and SPE using an optimized ratio of CHCl$_3$: MeOH: H$_2$O (7:6.3: v/v/v) mixture and C$_{18}$ Oasis Waters’ HLB SPE cartridges (60 mg, 3 ml), respectively, (Scheme 1). Solid Phase Extraction (SPE) products were then chromatographed and separated MCs using a Waters’ LC-MS instrument (with a Waters’ 3100 Mass Detector) coupled with an electrospray ionisation source (ESI). The instrument was conditioned for 10 min with the mobile phase shown below. Microcystins were separated on a C18 column (Waters Symmetry300TM, 4.6 mm × 75 mm, 3.5 μm) using the Alliance Waters e2695 separation module. The mobile phase consisted of a mixture of 0.5% (FA) in Milli-Q water (A) and 100% acetonitrile (B) and the column was operated at room temperature. Microcystins were eluted within a gradient window of 20 min using a reverse phase system consisting of 25% B (10 min), 70% B (10 min), 25% B (11.1 min), 25% B (20 min). Flow rate and sample injection volumes were set at 0.5 ml/min and 0.5 μl, respectively. The ion source was operated on both positive and negative ESI modes for all experiments. However, the positive mode gave better results, and therefore it was used throughout this study. Structural identification of microcystins in samples was based on retention time and fragment ion products relative to respective elution window/fragmentation pattern of authentic analytical standards. Furthermore, results were complemented with literature values reported elsewhere.

Microcystins recoveries (effect of liquid-partitioning on MCs recovery by C$_{18}$ HLB SPE cartridges)

Standard solutions with serial concentrations of 2.5, 5.0, 10.0, 15.0 mg/L for a model microcystin-LR congener (MC-LR) were spiked in a solvent system making a total volume 15ml containing CHCl$_3$: MeOH: ddH$_2$O (7:6.3, v/v/v). After partitioning, the phases were separated and MC-LR re-extracted from the hydroalcoholic phase using C$_{18}$ Oasis Waters’ HLB SPE cartridges (Scheme 1). The recovered amounts of MC-LR (Table 1) were determined and quantified by LC-ESI-MS technique as reported previously [65].

Quantification of MCs from field samples

Microcystins from algal samples were extracted and quantified using peak areas as described in [65] based on the procedures described in the previous section.

Results and Discussion

Morphological information

Irregular shaped cells observed under the light microscopy were the most abundant and their morphologies were comparable to that of Microcystis cells [67] and toxic M. aeruginosa cells collected from a eutrophic Hartbeespoort Dam (HBD) in South Africa as studied earlier by our group [65]. The toxic M. aeruginosa from HBD is a well studied and known to be the only microcystin producing cyanobacteria dominating the HBD for several decades dating back in the 1970s [68], hence algal samples from this dam served as our reference toxic species for this study. While morphological information gives no reliable clues to the algal species identification [1] it essentially gives preliminary information on cellular abundance/or species dominance.

In Botswana Microcystis spp has been reported from Okavango Delta Northern Botswana (NRP report) [69], however, this species is known to be well distributed in other neighboring countries particularly in South African fresh water bodies [68]. From morphological point of view, since algal cells from Phakalane pond effluents displayed similar features under a light microscope to a known M. aeruginosa species from Hartbeespoort Dam, thus it was preliminarily considered that the collected blooms from Phakalane pond effluents belonged to Microcystis spp closely related to M. aeruginosa.

Cyanobacterial DNA identification and the amplification of mcy genes

Quality and quantity of isolated DNA: All DNA extracts were freshly prepared in sterilized conditions to avoid possibilities for DNA degrading conditions that would have otherwise led to low and poor DNA yields unsuitable for PCR reactions [70]. Following our extraction method, DNA extracts obtained were of high purity based on observed optical density (OD) values (260/280 ratio=1.8; 260/230 ratio=2) [70,71] and all DNA yields obtained were in the range of 400–1300 ng/μl. Poorly extracted or degraded DNA is spectrophotometrically characterized by lower OD values (260/280 ratio<1.8; 260/230 ratio<2) indicating presence of large amounts of contaminants including phenols, phenolates, etc [71,72] which render the DNA extract unsuitable for PCR reactions. In this case whenever poor quality and/or yields of DNA extract were observed, fresh DNA was re-extracted from algal cells and re-checked for its quality and quantity.

For PCR reactions, however, the concentrations of the high purity DNA (260/280 ratio>1.8) obtained in this study were adjusted by diluting the crude DNA extract with nuclease free PCR water to obtain acceptable DNA concentrations of no more than 250 ng/μl based on the instructions found a Promega PCR protocol adopted. Concentrations between 50 and 250 ng/μl of genomic DNA was used in all experiments resulting in quality PCR products (Figures 1-3). In cases were very poor quality DNA (260/280<1.7) or degradation...
Amplification of mcy genes

In this study, six mcy genes (i.e., mcyA, -B, -C, -D, -E and -G) located on the large open reading frames (ORF) of the mcy gene cluster [42] were amplified from all samples using primers has been discussed in this work and the PCR products were visualized after ethidium staining (Figure 2). PCR primer pairs for the amplification of mcyA [25], mcyB [39] and mcyC, -D, -E and G [35] were found to be suitable for a wide range detection of mcy genes from all Phakalane pond effluent samples (Figure 2a) studied. However, under the same PCR conditions, positive and consistent results were only obtained for mcyC, -D, -E and -G genes (Figure 2b) for the reference genomic DNA from M. aeruginosa that is dominant in the Hartbeespoort Dam, South Africa. There was inconsistency in the results for the PCR amplifications of mcyA and mcyB genes (data not shown) and in particular there was an overly inconsistency in the amplifications of mcyA gene. The inconsistence in the amplifications of mcyA gene from M. aeruginosa in the Hartbeespoort Dam was observed earlier [38] when TOX3P/2M and MSR/MSF primer pairs were used and led the authors to conclude that mcyA gene in M. aeruginosa strains found in South African fresh waters are highly under expressed. But, it is however widely accepted that universal primers are limited [43] for the amplifications of a particular mcy gene across the globe. This could also have been the reason for the observed low or non-detection of the said mcyA gene due to regional disparity and genetic diversity among Microcystis species [39,44-46].

PCR amplifications of mcyA and mcyB using new sets of primer pairs

To overcome the observed inconsistence in the formation of mcyA and mcyB PCR products using respective literature primers as stated on the above Section, new suitable primer pairs for the PCR amplifications of mcyA and mcyB were designed (for new primer pairs and sequences see Table 1). Out of six new primers designed (list not shown), only four primers forming respective complementary pairs i.e. mcyA u-102F/u-620R and mcyB P5102F/P5853R were found to be suitably useful in the PCR amplifications of mcyA and mcyB genes from all cyanobacterial DNA samples studied, respectively. The primer pair mcyA-u-102F/u-620R (size of 518 bp) was developed based on a DNA sequence of M. aeruginosa Utx 2666 strain [35] to amplify a region between 102 and 620 bp on the mcyA gene sequence in Microcystis spp. On the other hand the mcyB primer pair (751 bp) was developed based on the DNA sequence of M. aeruginosa strain PCC 7806 [35,38] amplifying a region between 5102 and 5853 bp of the cyanobacterial DNA sequence.

Both Phakalane pond effluents and Hartbeespoort dam samples consistently showed clear bands after ethidium staining of the amplified 16S-rRNA gene and the expected size of about 500 bp was obtained using cyanobacterial specific primer pair (16SF: 5'-CTGAAAGAGAGCTTGCAGC-3' and 16SR: 5'-CCGATTACCGCTTTCG-3'). The detected 16S-rRNA band was observable from both DNA samples extracted from known toxigenic cyanobacteria M. aeruginosa cells (sourced from a eutrophic Hartbeespoort Dam) and the unknown (sourced from Phakalane pond effluents) confirmed that the algal bloom investigated was of cyanobacterial origin [43]. However, at this stage it was difficult to determine the potential toxicity of the unknown algae sample based on the amplified 16S-rRNA gene [73]. There was no formation of PCR products for the 16S-rRNA gene from all negative controls from either of the DNA isolates studied (Figure 1).

Amplification of cyanobacterial 16S-rRNA

From Figure 1 gel electropherogram showed clear bands after ethidium staining of the amplified 16S-rRNA gene and the expected size of about 500 bp was obtained using cyanobacterial specific primer pair (16SF: 5'-CTGAAAGAGAGCTTGCAGC-3' and 16SR: 5'-CCGATTACCGCTTTCG-3'). The detected 16S-rRNA band was observable from both DNA samples extracted from known toxigenic cyanobacteria M. aeruginosa cells (sourced from a eutrophic Hartbeespoort Dam) and the unknown (sourced from Phakalane pond effluents) confirmed that the algal bloom investigated was of cyanobacterial origin [43]. However, at this stage it was difficult to determine the potential toxicity of the unknown algae sample based on the amplified 16S-rRNA gene [73]. There was no formation of PCR products for the 16S-rRNA gene from all negative controls from either of the DNA isolates studied (Figure 1).

Amplification of mcy genes

In this study, six mcy genes (i.e., mcyA, -B, -C, -D, -E and -G) located on the large open reading frames (ORF) of the mcy gene cluster [42] were amplified from all samples using primers has been discussed in this work and the PCR products were visualized after ethidium staining (Figure 2). PCR primer pairs for the amplification of mcyA [25], mcyB [39] and mcyC, -D, -E and G [35] were found to be suitable for a wide range detection of mcy genes from all Phakalane pond effluent samples (Figure 2a) studied. However, under the same PCR conditions, positive and consistent results were only obtained for mcyC, -D, -E and -G genes (Figure 2b) for the reference genomic DNA from M. aeruginosa that is dominant in the Hartbeespoort Dam, South Africa. There was inconsistency in the results for the PCR amplifications of mcyA and mcyB genes (data not shown) and in particular there was an overly inconsistence in the amplifications of mcyA gene. The inconsistence in the amplifications of mcyA gene from M. aeruginosa in the Hartbeespoort Dam was observed earlier [38] when TOX3P/2M and MSR/MSF primer pairs were used and led the authors to conclude that mcyA gene in M. aeruginosa strains found in South African fresh waters are highly under expressed. But, it is however widely accepted that universal primers are limited [43] for the amplifications of a particular mcy gene across the globe. This could also have been the reason for the observed low or non-detection of the said mcyA gene due to regional disparity and genetic diversity among Microcystis species [39,44-46].

PCR amplifications of mcyA and mcyB using new sets of primer pairs

To overcome the observed inconsistence in the formation of mcyA and mcyB PCR products using respective literature primers as stated on the above Section, new suitable primer pairs for the PCR amplifications of mcyA and mcyB were designed (for new primer pairs and sequences see Table 1). Out of six new primers designed (list not shown), only four primers forming respective complementary pairs i.e. mcyA u-102F/u-620R and mcyB P5102F/P5853R were found to be suitably useful in the PCR amplifications of mcyA and mcyB genes from all cyanobacterial DNA samples studied, respectively. The primer pair mcyA-u-102F/u-620R (size of 518 bp) was developed based on a DNA sequence of M. aeruginosa Utx 2666 strain [35] to amplify a region between 102 and 620 bp on the mcyA gene sequence in Microcystis spp. On the other hand the mcyB primer pair (751 bp) was developed based on the DNA sequence of M. aeruginosa strain PCC 7806 [35,38] amplifying a region between 5102 and 5853 bp of the cyanobacterial DNA sequence.

Both Phakalane pond effluents and Hartbeespoort dam samples consistently showed clear bands after ethidium staining of the amplified 16S-rRNA gene and the expected size of about 500 bp was obtained using cyanobacterial specific primer pair (16SF: 5'-CTGAAAGAGAGCTTGCAGC-3' and 16SR: 5'-CCGATTACCGCTTTCG-3'). The detected 16S-rRNA band was observable from both DNA samples extracted from known toxigenic cyanobacteria M. aeruginosa cells (sourced from a eutrophic Hartbeespoort Dam) and the unknown (sourced from Phakalane pond effluents) confirmed that the algal bloom investigated was of cyanobacterial origin [43]. However, at this stage it was difficult to determine the potential toxicity of the unknown algae sample based on the amplified 16S-rRNA gene [73]. There was no formation of PCR products for the 16S-rRNA gene from all negative controls from either of the DNA isolates studied (Figure 1).
were described in the work of [38] who amplified mcyA gene using TOX3P/2M and MSR/MSF primer pairs. In this study, when the primer pair MSR/MSF was used to amplify mcyA for different batches of DNA samples there was either an inconsistency in/no formation of PCR products for mcyA gene (Figure 2b) as reported earlier [38], besides that M. aeruginosa is a highly distributed and the only dominant MC producing cyanobacterial species in the Hartbeespoort dam [68].

**McyA gene sequence and species identification**

Morphologically, under a light microscope the two samples displayed higher similarities forming irregular shaped colonies pertaining to M. aeruginosa. However, on a PCR reaction, as stated earlier there was either no formation or inconsistency in the formation of mcyA and mcyB PCR products for samples from Hartbeespoort Dam when respective literature primers were used in a PCR reaction (Table 1). On the other hand there were positive results for Phakalane pond effluent samples under the same conditions (compare Figures 2a and 2b; excluding lanes A’, B’ and 16SrRNA on 2a). Such a disparity in the formation of mcyA and mcyB PCR products indicated that despite of the observed cellular morphological similarities, yet the two blooms were actually genetically distinct, probably speaking at strain level [39,44-46], hence the two subjects responded differently during genotoxicity screening in particular for mcyA gene. Furthermore, when newly designed primers were used to amplify mcyA and mcyB genes from both DNA sources there were clear and consistent formation of PCR products in a wide range of samples tested (see typical bands on Figure 3).

PCR products used for mcyA gene sequence were of high quality DNA sample (260/280=1.98; yield>180 ng/μl) obtained after gel purification (Figure 4). The specificity of the designed mcyA primer pair (Forward: mcyA-u-102F) and Reverse: mcyA-u-620R) were evaluated using Clustal W program on BLAST results and their sequences were found to be highly conserved in a common region from a number of Microcystis spp (Figure 5) including a well known toxigenic cyanobacteria M. aeruginosa PCC 7806, a reference strain once used in [38]. The results from Figure 5 indicated that the primer pair was of high quality and powerful enough to give PCR products suitable for DNA sequencing needed for the identification of unknown Microcystis species from environmental samples after mcyA DNA sequencing. The DNA sequence from purified mcyA PCR amplicons (518 bp) from lanes 1 and 2 (Figure 4) showed highest similarities of 100% (without any gap between aligned nucleotide base pairs) to the DNA sequence segments of M. aeruginosa (GenBank Accession # AF139335.1) and M. novacekii (GenBank Accession # AB110113.1), respectively. Thus, based on the observed nucleotide sequence homologues between mcyA genes of a Microcystis spp from the Phakalane pond algal DNA extracts (Figure 4, lane 2) and that of a toxic M. novacekii T20-3 (GenBank Accession No AB110113.1) it was therefore concluded that, the toxigenic Microcystis species collected from the studied effluent in Phakalane Ponds, Botswana belonged to M. novacekii T20-3. The morphological shapes of M. novacekii T20-3 cells were of recently found to be virtually similar to that of M. aeruginosa, such that it is practically impossible to differentiate the two species based on morphometric methods [36]. In another advent Otsuka et al., [48] showed that the two species in question cannot be genetically differentiated using 16S-rDNA sequence as were shown in this study that both species responded equally when the 16SrRNA gene was amplified. However, using the mcyA gene sequence it was discovered that they are genetically distinct even though they microscopically looked similar.

Microcystis novacekii comprises of both toxic and non-toxic cyanobacterial strains [48] most of which are native to Asian countries [36]. However, El Herry et al [36] reported that these species are worldwide spread and some of them exhibit highly conserved genotypes. For instance, a toxigenic MC producing M. novacekii T20-3 strain was of recently reported to occur in Cheffia Dam, Algeria (Northern Africa) [36]; however, no data existed on its occurrence in the waters from Southern Africa prior to this study.

**MC separation, structural identification, recovery and quantification**

From Figure 6a, four signals corresponding to Microcystin-RR, -YR, -LR and –WR were observed and their respective masses (m/z) (Figure 6b) and retention times (RT) correlated to those of their respective authentic standards and/or literature values [65]. Table 2a shows the observed chromatographic and spectrometric information from Figure 6 pertaining to LC signal (column I), MC congener (column II), retention time (column III) and MS masses (m/z) column (column IV).

**Figure 3:** Image of 1% agarose gel electrophoresis showing mcyA (518 bp) and mcyB (751 bp) PCR products amplified respective new primer pairs designed. **Left:** From a known M. aeruginosa (Hartbeespoort Dam). **Right:** From the investigated Microcystis spp (Phakalane pond effluent). [Other lanes: 16S-rRNA (≈500 bp)=Positive control; M=Standard DNA ladder (1kb) and –ve ctrl=Negative control].

**Figure 4:** Image of 1% agarose gel electrophoresis products showing purified mcyA gene amplicons submitted for sequencing using newly designed primer pair for mcyA (518 bp). Lane 1: mcyA amplicon from M. aeruginosa (Hartbeespoort Dam sample); Lane 2: mcyA amplicon from Microcystis spp investigated (Phakalane pond effluent sample); Negative control: Lane 3; M=Standard DNA ladder (1kb).
These results complement the PCR findings for the identified mcy genes in the DNA extracts for the Phakalane pond effluent algal samples which indicated that the bloom was potentially toxic posing environmental health hazards [5]. The full list of mcy genes found on the large ORF gene cluster of Microcystis spp were elucidated during this study and there are those known to be the major mcy genes involved in the biosynthesis of MCs [42].

The separation spectrum (Figure 6a) for MCs was clean enough to enable subsequent quantification of individual MC congeners using a quantification method reported earlier [65]. The quantities of extracted MC from algal cells were shown on Table 2a (column VI).

The efficiency of the partitioning step in extraction of MC prior to SPE protocol was evaluated by studying the recovery of MC-LR from spiked distilled water. Table 2b shows that using Oasis Waters® HLB SPE cartridges the recovered MC ranged between 82.34 and 94.68% after partitioning step (column IV) which was in the same range (83.56 to 91.85%) without liquid partitioning step (column III). Table 2b, column V shows that there was very small % loss differences in the content of MC-LR recovered from the two approaches. However, it could be concluded that there was insignificant loss in MC-LR content after a partitioning step. The observed minor losses could probably be due to handling procedures and the HLB sorbent’s chemistry because there closeness in the amounts of MCs recovered. The observed MC-LR recoveries were comparable to those reported in literature using Isolute C18 trifunctional [44], C18 Oasis Waters® HLB [64] SPE cartridges (MCs are in principle insoluble in CHCl3 [and therefore the later has been
Figure 6: a) LC chromatograms showing extracted and separated MCs from algal samples after liquid-partitioning and SPE steps: (i) MC-RR (ii) MC-YR (iii) MC-LR and (iv) MC-WR.

Figure 6: b) LC-ESI-MS spectra of (i) MC-RR (ii) MC-YR (iii) MC-LR and (iv) MC-WR.
used as a partitioning solvent to remove pigments and other less polar compounds during MC extraction to alleviate SPE cartridge blockage as reported elsewhere [52] resulting in reduced SPE run time and cleaner LC-MS spectrum as was shown on Figure 6a.

The concentrations of MC-RR, -LR and YR quantified from toxigenic M. novacekii cells were in the range between 53.62 ± 0.063 and 1.714 ± 0.001 µg/g DW and in the order of MC-RR > MC-LR > MC-YR > MC-WR (Table 2a, column VI). Although the investigated water body is not directly used to supply drinking water but the observed MC concentrations (Table 2a, column VI) and in particular that of MC-LR were basically above the minimum recommended level [5]. Such higher MCs concentrations are of health concern especially when it is reported that this water is used for vegetable irrigations downstream [23,24]. The absence of strategic MC monitoring practice by the respective authority would pose dangers to vegetable consumers due to possibilities of MC bioaccumulation in the irrigated vegetables and other aquatic organisms including fish [2,53-63,74-80].

Conclusion

This work was presented using a bio-analytical approach involving among others a mcy DNA sequencing that led to the identification of a toxigenic cyanobacterial species, M. novacekii T20-3 from the effluent of waste water secondary maturation ponds in Gaborone, Botswana. Subsequent extraction, structural elucidation and quantification of MC congeners it produces were also achieved. The potential for MC production was shown based on the amplified mcy genes. Higher quantities of MCs observed (especially MC-LR and -RR) calls for the need for efficient water quality monitoring particularly with regard to its use on edible plant irrigations. Also, the authors recommend that fishing practices from the eutrophic ponds/effluents should be monitored and fish sales in the local markets regulated to avoid MC related health fatalities due to possible MC contaminations.

The occurrence of a toxigenic M. novacekii T20-3 is reported for the first time from Phakalane pond effluents in Gaborone, Botswana.

Acknowledgment

We are grateful to the WB-STHE project (Dar es Salaam, Tanzania) for their financial support to E.M. We further would like to thank Dr. D. Shushu (University of Botswana, Botswana) for her help during sample collection in Phakalane, Botswana and finally but not least we are very much grateful to Dr. M. Talyshinsky (Ben-Gurion University of the Negev, Israel) for DNA sequencing.

References


