

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

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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 *mcyA*, -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 *mcyA* gene was obtained using PCR products from a newly designed primer pair for the amplification of *mcyA* 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 nitrogenous 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

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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 C₁₈ 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 C₁₈ 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 toxigenicity 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 proteinase 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 in 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 # 9PIM712, 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→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 data base.

Gene	PCR primer pair	Gene sequence (5'- 3')	Annealing Temp. (°C)	PCR Product size (bp)	Reference
Common NPRS	MTF2 MTR	GCNCG(CT)GG(CT)GCNTA(CT)GTNCC CCNCG(AGT)AT(TC)TTNAC(TC)TG	50	1000	[79]
<i>mcyA</i>	MSF MSR	ATCCAGCAGTTGAGCAAGC TGCAGATAAATCCGCAAGTTG	50	1300	[25]
	u-102F u-620R	CGATGAACAAATCGGGCAATGGCA TGCAAGTTTCGCACATCTCCAAGG	55	518	This study
<i>mcyB</i>	P5102F P5853R	AGTCATCATCTTCCTTACCAGCGT TGTCTGCCATCCGTTCAATCGTA	55	751	This study
	2156-F 3111-R	ATCACTTCAATCTAACGACT AGTTGCTGCTGTAAGAAA	44	955	[39]
<i>mcyC</i>	PSCF1 PSCR1	GCAACATCCCAAGAGCAAAG CCGACAACATCACAAAGGC	49	674	[35]
<i>mcyD</i>	PKDF2 PKDR2	AGTTATTCTCCTCAAGCC CATTCGTTCCACTAAATCC	44	859	[35]
<i>mcyE</i>	PKEF1 PKER1	CGCAAACCCGATTTACAG CCCCTACCATCTTCATCTTC	48	755	[35]
<i>mcyG</i>	PKGF1 PKGR1	ACTCTCAAGTTATCCTCCCTC AATCGCTAAAACGCCACC	48	425	[35]

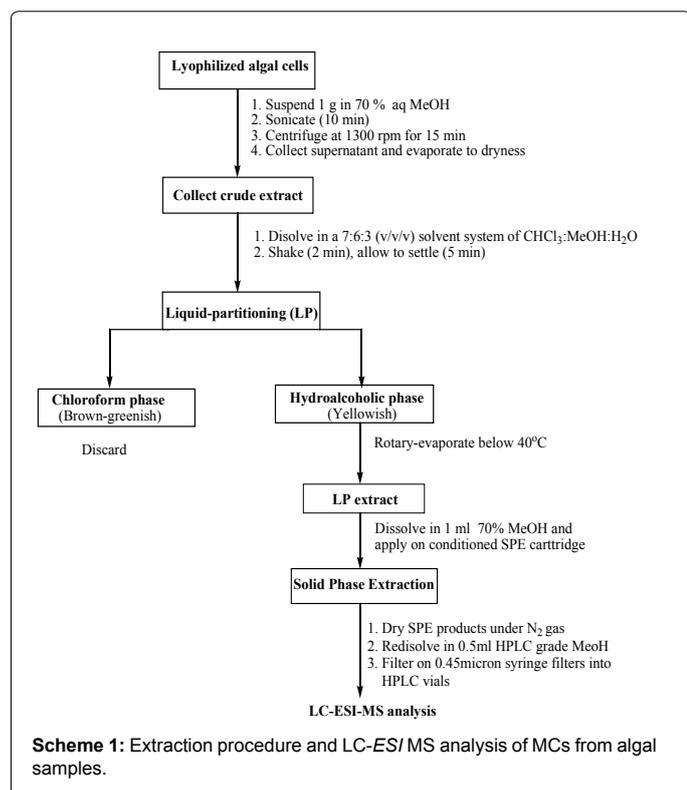
Table 1: Suitable PCR primers used in this study for the amplification of *mcy* genes.

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 CHCl₃: MeOH: H₂O (7:6:3, v/v/v) mixture and C₁₈ 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 Symmetry300[™], 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 C₁₈ 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 CHCl₃: MeOH: ddH₂O (7:6:3, v/v/v). After partitioning, the phases were separated and MC-LR re-extracted from the hydroalcoholic phase using C₁₈ 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 microscopy 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

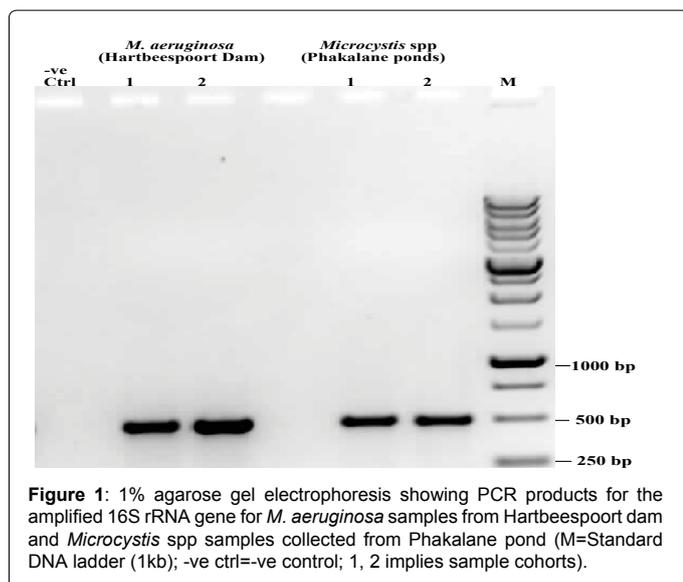


Figure 1: 1% agarose gel electrophoresis showing PCR products for the amplified 16S rRNA gene for *M. aeruginosa* samples from Hartbeespoort dam and *Microcystis* spp samples collected from Phakalane pond (M=Standard DNA ladder (1kb); -ve ctrl=-ve control; 1, 2 implies sample cohorts).

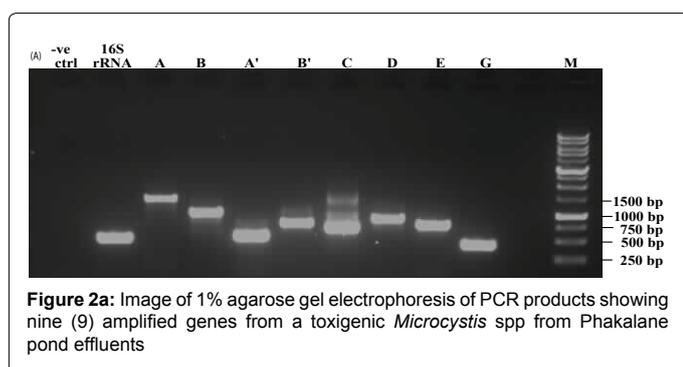


Figure 2a: Image of 1% agarose gel electrophoresis of PCR products showing nine (9) amplified genes from a toxicogenic *Microcystis* spp from Phakalane pond effluents

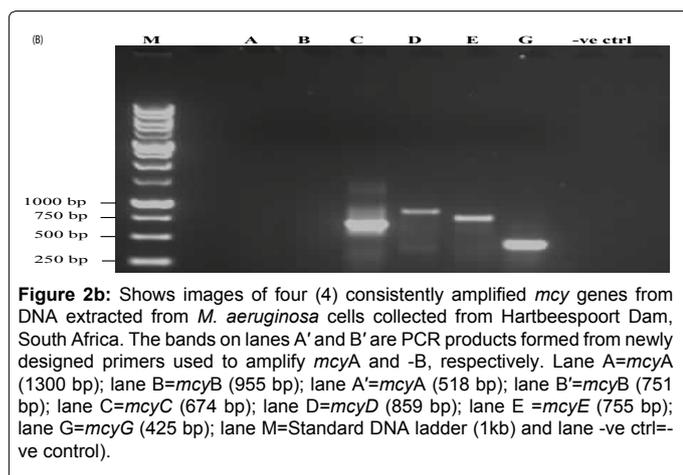


Figure 2b: Shows images of four (4) consistently amplified *mcy* genes from DNA extracted from *M. aeruginosa* cells collected from Hartbeespoort Dam, South Africa. The bands on lanes A' and B' are PCR products formed from newly designed primers used to amplify *mcyA* and -B, respectively. Lane A=*mcyA* (1300 bp); lane B=*mcyB* (955 bp); lane A'=*mcyA* (518 bp); lane B'=*mcyB* (751 bp); lane C=*mcyC* (674 bp); lane D=*mcyD* (859 bp); lane E =*mcyE* (755 bp); lane G=*mcyG* (425 bp); lane M=Standard DNA ladder (1kb) and lane -ve ctrl=-ve control).

products were observed, fresh DNA was re-diluted from stock and re-examined spectrophotometrically to obtain desired OD values and appropriate amounts suitable for PCR reactions.

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'-CTGAAGAAGAGCTTGCGTC-3' and 16SR:

5'-CCCAGTAGCACGCTTTCG-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* genes 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 inconsistency 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 inconsistency 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* Utex 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 for the expected (calculated) PCR product sizes for *mcyA* (518 bp) and *mcyB* (751 bp) genes (Figure 2a: lanes A' and B' and Figure 3). Interestingly, images for *mcyA* (518 bp) PCR products were of high quality for various genomic DNA batches from the reference *M. aeruginosa* thus being more consistent than what

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/or 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/ul) 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 microscopic 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

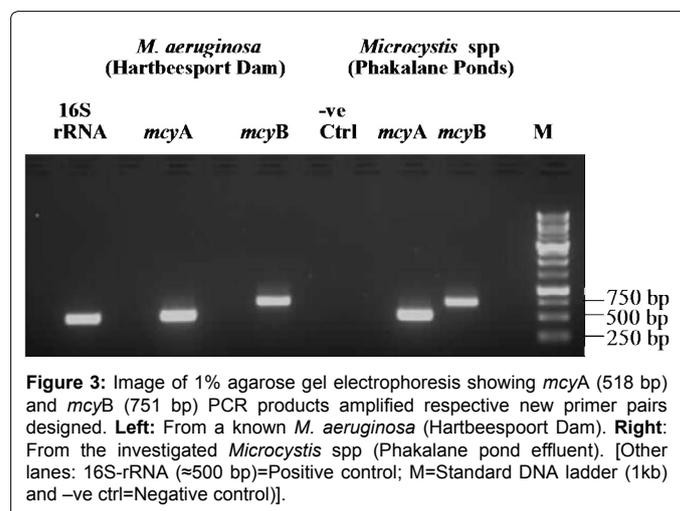


Figure 3: Image of 1% agarose gel electrophoresis showing *mcyA* (518 bp) and *mcyB* (751 bp) PCR products amplified respective new primer pairs designed. **Left:** From a known *M. aeruginosa* (Hartbeespoort Dam). **Right:** From the investigated *Microcystis* spp (Phakalane pond effluent). [Other lanes: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

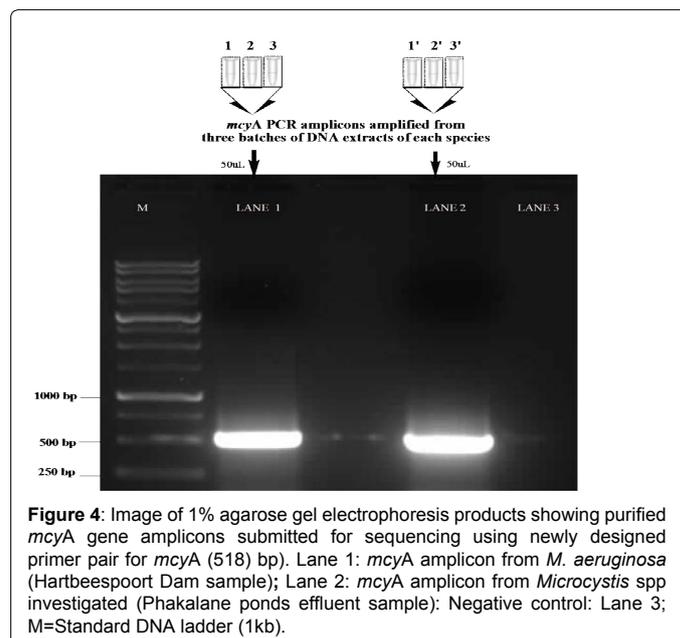


Figure 4: Image of 1% agarose gel electrophoresis products showing purified *mcyA* gene amplicons submitted for sequencing using newly designed primer pair for *mcyA* (518 bp). Lane 1: *mcyA* amplicon from *M. aeruginosa* (Hartbeespoort Dam sample); Lane 2: *mcyA* amplicon from *Microcystis* spp investigated (Phakalane ponds effluent sample); Negative control: Lane 3; M=Standard DNA ladder (1kb).

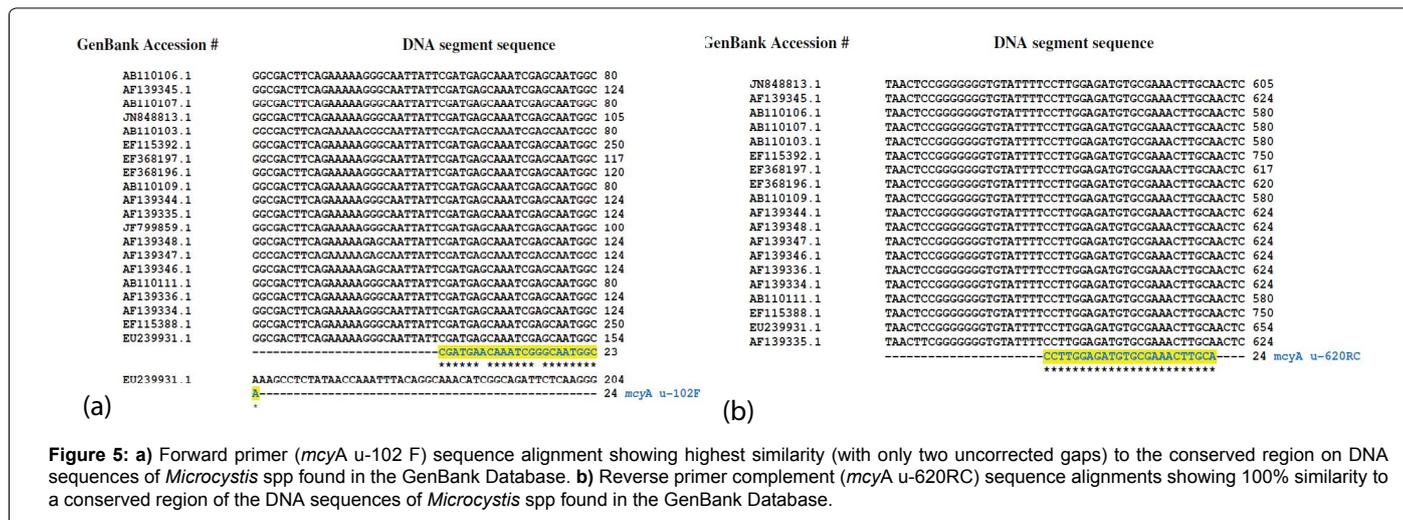


Figure 5: a) Forward primer (*mcyA* u-102 F) sequence alignment showing highest similarity (with only two uncorrected gaps) to the conserved region on DNA sequences of *Microcystis* spp found in the GenBank Database. b) Reverse primer complement (*mcyA* u-620RC) sequence alignments showing 100% similarity to a conserved region of the DNA sequences of *Microcystis* spp found in the GenBank Database.

LC signal (Figure 6)	Microcystin Congener	Retention time (min)	Major LC-ESI-MS ions (m/z)	Standard curve linearity (r ²)	Amount extracted (µg/g DW)
i	MC-RR	6.61	134.7 (ADDA); 1039.3 [M+H] ⁺	0.9963	53.620 ± 0.063
ii	MC-YR	7.50	135.4 (ADDA); 1046.2 [M+H] ⁺ ; 523.3 [M+2H] ²⁺	0.9948	5.280 ± 0.035
iii	MC-LR	7.77	135.8 (ADDA); 996.3 [M+H] ⁺	0.9948	12.114 ± 0.024
iv	MC-WR	8.37	135.0 (ADDA); 1069.3 [M+H] ⁺	n.a	*1.714 ± 0.001

Key: *MC-LR equivalent concentration; n.a: Not Applicable; MC amounts are average values of n=3

Table 2a: LC-ESI-MS identification and quantification of MCs extracted from toxigenic *M. novacekii* cells collected from Phakalane pond effluent. The MCs were pre-concentrated using SPE Oasis Waters™ HLB cartridges after the removal of pigments and less polar compounds by a liquid partitioning step in methanolic aqueous CHCl₃.

MC-LR spiked in DD H ₂ O (µg/L) (9 ml)	Blank (DD H ₂ O) (9 ml)	MC-LR Recovery without liquid partitioning (µg/L)	MC-LR Recovery after liquid partitioning (µg/L)	Percentage difference in recovery efficiencies (%)
15.0	ND	13.739 ± 0.125 (90.83%)	13.486 ± 0.107 (89.90%)	0.93
10.0	ND	9.185 ± 0.116 (91.85%)	9.468 ± 0.095 (94.68%)	2.83
5.0	ND	4.178 ± 0.086 (83.56%)	4.117 ± 0.099 (82.34%)	1.22
2.5	ND	2.199 ± 0.027 (87.95%)	2.141 ± 0.112 (85.65%)	2.30

Key: ND: Not Detected

Table 2b: Comparative recovery of spiked MC-LR in double distilled water using Oasis Waters® HLB SPE cartridges with and without liquid partitioning (Recoveries are mean values of n=3 and the average % recoveries are shown in brackets).

IV). 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

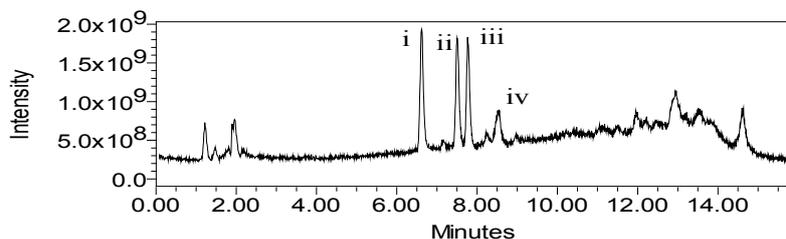
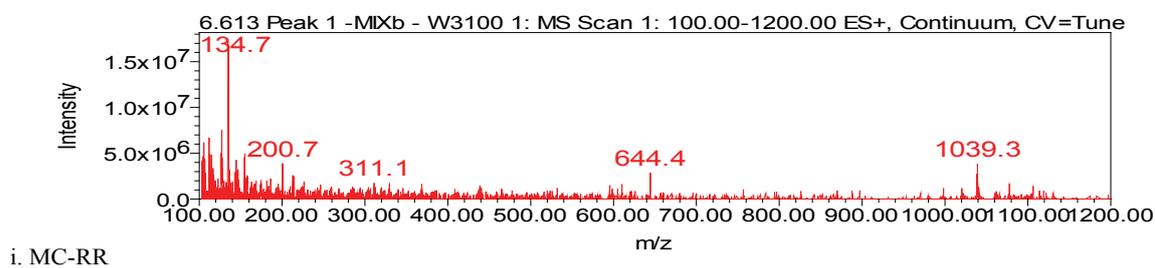
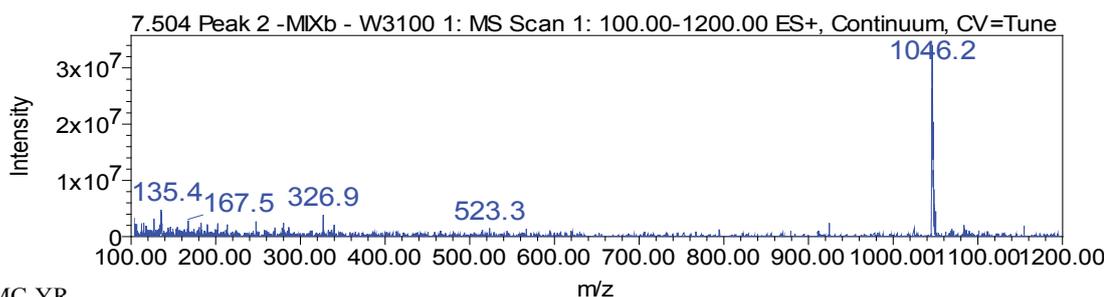


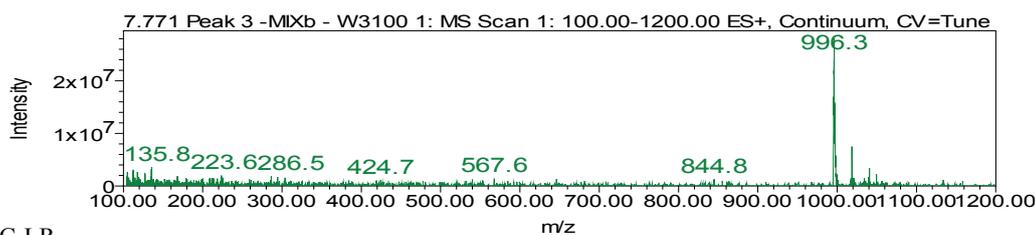
Figure 6: a) LC chromatograms showing extracted and separated MCs from algal samples after liquid-partitioning and SPE steps: (i) MC-RR (ii) MC-YR (iii) MC-LR and (iv) MC-WR.



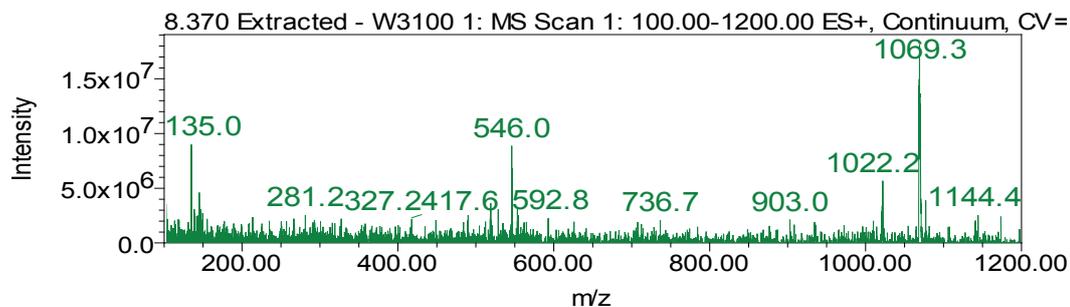
i. MC-RR



ii. MC-YR



iii. MC-LR



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 $\mu\text{g/g}$ 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 *mcyA* 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 effluent 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.

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