Preliminary Study to Find Occurrence and Dynamics of Toxic Cyanobacteria in Nuwara wewa and Nachchaduwa wewa, Two Man-made Reservoirs in Sri Lanka

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Abstract

Bloom of cyanobacteria in water bodies causes devastating problems to the ecosystem and humans when they depend on these waters for drinking purpose. Microcystin and nodularin, produced by some cyanobacteria, are toxic for human and many other aquatic organisms. Nuwara wewa and Nachchaduwa wewa are two important reservoirs supplying water for drinking purpose in addition to agricultural use. Therefore, preliminary study was conducted to evaluate these reservoirs for the presence of toxic cyanobacteria and their dynamics is of importance to take proper measures to control cyanobacterial growth and to ensure supply of quality water for human drinking purpose. Water samples were collected for 12 months from both reservoirs and physic-chemicals parameters of water were also recorded. Correlations between the physico-chemical parameters of water and the cyanobacterial density were established. Toxic cyanobacteria were isolated and identified using conventional and molecular methods. 16S rRNA gene and mcy-A gene were targeted for molecular identification using PCR. Cyanobacterial density had strong positive correlations with some physico-chemical parameters such as pH, temperature and dissolved oxygen in both reservoirs. These parameters can be used as good indicators to evaluate the condition these reservoirs with respect to cyanobacterial bloom. Some cyanobacterial isolates recovered from both reservoirs harboured mcy-A gene, showing the potential of microcystin production.

Keywords: Cyanobacteria, Cyanobacterial toxin, *Microcystis*, Eutrophication, Molecular identification

INTRODUCTION

Cyanobacteria are ubiquitous organisms in aquatic environments. Their blooms in public water supply reservoirs have become a matter of ever increasing concern throughout the world due to their potential for producing toxic compounds such as microcystins (MC) and nodularin (NOD). Acute exposure to MS is reported to be a one of the contributory factor to cause liver failure and death in humans (Carmichael et al., 2001). Similarly, chronic effects have been reported with low level of exposure to MS (Fitzgeorge *et al*., 1994; Chorus and Bartram, 1999). Moreover, the outbreaks of human poisoning due to exposure of toxic cyanobacteria through exposure of contaminated drinking water have been reported in Australia (WHO,

1999). However, the worst case involving a human population due to exposure to MS had been reported in city of Caruaru in northeastern Brazil (Jochimson *et al*., 1998).

Production of MC depends on the existence of genes, encoding for MC synthetase. The *mcy* gene cluster is reported as a responsible operon for MC synthetase production (Tillett *et al.*, 2000). It has been reported that some cyanobacteria are unable to produce toxins, but all those have genes associated to MC biosynthesis (Ouellette *et al*., 2006). A few strains of cyanobacteria in nature have no MC production, but contain all the genes for MC synthetase production (Kurmayer *et al*., 2004). The toxicity of cyanobacteria cannot be evaluated by morphological characters due to lack of morphological differences between toxic and non-toxic types. Several authors have used *mcy* genes as molecular markers to identify the MC producing cyanobacteria (Baker *et al*., 2002; Nonneman and Zimba, 2002). Polymerase chain reaction (PCR) amplification of a fragment of *mcyA* gene had been used to identify MC producing genotypes of cyanobacteria in water reservoirs. Thus, the early detection of toxic cyanobacteria in water resources is advisable in order to avoid contamination and public health problems.

Ionization and cupric treatments are the only promising measures to get rid of the cyanobacterial toxins (Lambert *et al*., 1996). Cupric treatment kills the toxic cyanobacteria and it seems to be highly potential. More than 0.05 mg/L of Copper can be toxic to algae. Copper is an essential nutrient for animals at low concentration while toxic at higher concentration (Boyd, 2005). However, frequent use of this treatment is not advisable as other types of organisms may also be executed. Nuwara wewa and Nachchaduwa wewa are two important man-made reservoirs supplying water for domestic and cultivation purposes. People take water either directly from these reservoirs or irrigations channels of these reservoirs or even as tap-borne water supplied by the National Water Supply and Drainage Board from these reservoirs.

This preliminary study evaluates toxic cyanobacteria that inhabit Nuwara wewa and Nachchaduwa wewa and their seasonal dynamics with the change of physico-chemical parameters of water. This will help in applying measures to prevent the growth of toxic cyanobacteria and the accumulation of cyanobacterial toxins in drinking water.

METHODS AND MATERIALS

Study site and sample collection

Two man-made reservoirs, Nuwara wewa and Nachchaduwa wewa in Anuradhapura were selected for sampling in this study. Water sampling was carried out at four sites by main four directions; North, South, East and West. The coordinates of North, South, East and West sampling sites in Nuwara wewa were 8^{o} 21’12.94’’ N & 80^{o} 25’ 36.44’’ E & 306 feet, 8^{o} 19’06.77’’ N & 80^{o} 26’ 10.21’’ E & 289 feet, 8^{o} 20’06.16’’ N & 80^{o} 26’ 11.55’’ E & 297 feet and 8^{o} 20’25.11’’ N & 80^{o} 25’ 00.23’’ E & 291 feet, respectively. North, South, East and West sampling sites of Nachchaduwa wewa had the coordinates of 8^{o} 16’ 29.87’’ N & 80^{o} 28’ 55.37’’ E & 346 feet, 8^{o} 14’03.17’’ N & 80^{o} 28’ 56.26’’ E & 361 feet, 8^{o} 15’13.73’’ N & 80^{o} 29’ 13.06’’ E & 345 feet and 8^{o} 15’07.60’’ N & 80^{o} 28’ 13.50’’ E & 335 feet, respectively. The samples were

collected from two different layers; surface layer and bottom layer (one meter below the surface), at a rate of three samples per each layer at a sampling site with a total of 24 samples per reservoir per month.

Water samples were collected in sterile brown glass bottles every month starting from January 2012 to December 2012. The samples were instantly tested for physico-chemical parameters within a few hours in addition to primary microscopic observations. Formalin solutions of 4% and 6% were added into aliquots of samples to avert the proliferation of cyanobacteria with time to preserve the water samples for short and long terms, respectively (Soumati *et al*., 2005). For the proliferation of microorganisms, aliquots of samples without formalin were kept under room temperature.

Physico-chemical parameters of water

Temperature, pH, dissolved oxygen in water and turbidity were measured as physico-chemical parameters of the collected water samples. The temperature was measured using a standard Celsius thermometer. Turbidity meter (WalkLab) and pH meter (PCE 228) were used to measure the turbidity and the pH of water samples, respectively. All these parameters were measured at the time of sampling. The turbidity of water was measured in the unit of Nephelometric Turbidity Units (NTU).

Generic identification of cyanobacteria

Collected water samples were concentrated by centrifugation at 3500 rpm for ten minutes. The resultant pellet was serially diluted and inoculated onto BG11 medium to isolate cyanobacteria (Stanier *et al*., 1971). The generic identification of cyanobacteria was carried out based on morphological criteria according to keys of identification (Lawton *et al*., 1999). Colour, size, shape of colonies and trichome, and the presence of akinete, gas vacuoles, heterocyst and gelatinous sheaths were taken into account (Soumati *et al*., 2005).

Maintenance of cyanobacterial cultures

BG11 medium (Table 1) was prepared to maintain the cyanobacterial cultures (Rippka *et al*., 1979). After inoculation, the cultures were incubated at room temperature with 12 hours light-dark cycling until the culture grew well. Sub sampling was done to obtain a pure sample of cyanobacteria for further investigations.

Table 1: Composition of BG 11 medium

Ingredients	Quantity/L
$NaNO_3$	1.500 g
$K_2HPO_4.3H_2O$	0.040 g
$MgSO_4.7H_2O$	0.075 g
$CaCl_2.2H_2O$	0.036 g
Citric acid	0.006 g
Ferric ammonium citrate	0.006 g

EDTA	0.001 g
Na_2CO_3	0.020 g
Trace metal mix A5+Co*	1.000 mL
Deionized water	1 L

*Trace metal mix should be consisted of H_3BO_3, 2.86 g/L; $MnCI_2.4H_2O$, 1.81 g/L; $ZnSO_4.7H_2O$, 0.222 g/L; $Na_2MoO_4.2H_2O$, 0.390 g/L; $CuSO_4$.$5H_2O$, 0.079 g/L; $Co(NO_3)_2.6$ H_2O, 0.0494 g/L

DNA extraction, PCR and molecular identification of toxic cyanobacteria

Five milliliter of exponentially growing cyanobacterial cells were harvested by centrifugation. Four isolates recovered from Nuwara wewa (NUW 01, NUW 02, NUW 03 and NUW 11) and three isolates from Nachchaduwa wewa (NCH 01, NCH 02 and NCH 09) exhibited exponential growth and were used for DNA extraction and PCR reaction. The cells were washed twice in wash buffer (Tris buffer, pH 8 – 100 mM, EDTA, pH 8 – 50 mM, NaCl 100 mM), and re-suspended in 200 µl of solution I (Tris buffer, pH 8 – 25 mM, Glucose – 50 mM, EDTA, pH 8 – 10 mM) followed by the addition of 2 mg of lysozyme and incubated at 37 ^oC for one hour.

SDS was added to a final concentration of 2 % and mixed vigorously and 100 µl of 5M NaCl was added and mixed. Mixture was left at 20^oC for 10 min. The mixture was then centrifuged at 12000 rpm for 5 min and to the supernatant, equal volume of phenol: chloroform (1:1) was added and the contents were mixed well and centrifuged at 12000 rpm for 5 min. DNA was re-suspended in 0.1X TE buffer for further use (Perumal, *et al.,* 2009).

The extracted DNA samples were quantified using Spectro UV - VIS double beam PC Scanning Spectrophotometer (Model: Labomed, UVD – 2950). Reaction mixtures of 25 µL were prepared for PCR. Each 25 µL reaction volume consisted of 12.5 µL of 2x PCR Master mix (Dream Taq Green of Life Sciences), 2.0 µL of 10 µM forward primer, 2.0 µL of 10 µM reverse primer, 5 µL of DNA template and 3.5 µL of nuclease free water. For the PCR amplification of cyanobacterial DNA, seven primer sequences were used (Table 2). Lambda DNA (λ DNA) with specific primer pairs designed to amplify a part of the λ DNA. PCR was performed in Veriti Thermal Cycler (USA) with initial denaturation temperature of 94 ^oC for 5 minutes, 35 cycles of template denaturation at 94 ^oC for 45 seconds, primer annealing at 35 ^oC for 45 seconds & primer extension at 72 ^oC for one minute and the final extension at 72 ^oC for 10 minutes.

Table 2: Primers used for PCR reactions

Primers used with cyanobacterial DNA	Sequence
*Cya781*RA	5’ - GAC TAC TGG GGT ATC TAA TCC CAT T - 3’
*Cya781*RB	5’ - GAC TAC AGG GGT ATC TAA TCC CTT T - 3’
*Cya781*RC	5’ - GAC TAC TGG GGT ATC TAA TCC CTT T - 3’
*Cya781*RD	5’ - GAC TAC AGG GGT ATC TAA TCC CAT T - 3’
*Cya359*F	5’ - GGG GAA TYT TCC GCA ATG GG - 3’

*mcyA*F	5’ - AAA AGT GTT TTA TTA GCG GCT CAT - 3'
*mcyA*R	5’ - AAA ATT AAA AGC CGT ATC AAA - 3'
Primers used with λ DNA	
Ent_Lam_F	5’ - CAA ACT GCG CAA CTC GTG AA - 3'
Ent_Lam_R	5’ - GAC AGG CGA ATC GCA ATC AC - 3'

The PCR products were resolved in 0.8% Agarose gel with 0.5X TBE buffer at 60 V for 2 hours. Gels were stained in Ethidium bromide and the gel profiles were observed in a gel documentation system (Bio - imaging Systems, Model: MiniLumi).

Statistical analysis

Descriptive statistics and Pearson’s correlations between cyanobacterial density and different physico-chemical parameters of water were established using SPSS (version 17.0) statistical software.

RESULTS AND DISCUSSION

Dynamics of cyanobacteria

Bottom and the water column of each reservoir had been always changing with the changes of the environment and weather parameters. The correlation analysis revealed that a strong positive correlation existed between the pH of the water in the study reservoirs and the cyanobacterial density (Tables 3 and 4). The highest cyanobacterial density was observed at a pH range of 8.00 – 8.50 (Table 5 and 6). This result is in line with the findings by Soumati *et al*., 2005 in Cheffia dam in the North-East of Algeria.

Table 3: Pearson’s correlation coefficients between different parameters of water in Nuwara wewa

	Cyanobacterial density	pH	Temperature	Water Level	Turbidity	Dissolved Oxygen
Cyanobacterial Density	1.00	0.69*	0.65*	-0.67*	0.67*	-0.54
pH	0.69*	1.00	0.67*	-0.66*	0.57	-0.43
Temperature	0.65*	0.67*	1.00	-0.88**	0.92**	-0.87**
Water Level	-0.66*	-0.66*	-0.88**	1.00	-0.88**	0.85**
Turbidity	0.67*	0.57	0.92**	-0.88**	1.00	-0.93**
Dissolved Oxygen	-0.54	-0.43	-0.87**	0.85**	-0.93**	1.00

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 4: Pearson's correlation coefficients between different parameters of water in Nachchaduwa wewa

	Cyanobacterial Density	pH	Temperature	Water Level	Turbidity	Dissolved Oxygen
Cyanobacterial Density	1.00	0.68*	0.74**	-0.85**	0.85**	-0.61*
pH	0.68*	1.00	0.56	-0.77**	0.67*	-0.40
Temperature	0.74**	0.56	1.00	-0.84**	0.86**	-0.93**
Water Level	-0.85**	-0.77**	-0.84**	1.00	-0.94**	0.74**
Turbidity	0.85**	0.67*	0.86**	-0.94**	1.00	-0.77**
Dissolved Oxygen	-0.61*	-0.37	-0.93**	0.74**	-0.77**	1.00

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Temperature also had a strong positive correlation with the cyanobacterial density within a particular range (Tables 3 and 4). It was stated that the presence of toxic cyanobacteria correlates with a moderate temperature (15 – 30 ^{o}C) (Prentice, 2008). However, the cyanobacteria density and the dissolved oxygen in water exhibited a modest negative correlation (Tables 3 and 4). Bloom of cyanobacteria and their elevated biological activities may be depleting the available dissolved oxygen in water. Temperature, pH and dissolved oxygen can therefore be used good indicators to evaluate the condition of a water body in terms of cyanobacterial density and distribution. In both reservoirs, the cyanobacterial density diminished with the increase in water level. This may be due to dilution of the cyanobacterial population as well as the unfavorable condition of water in the reservoirs for cyanobacterial proliferation.

Table 5: Temporal dynamics of physiochemical parameters of water in Nuwara wewa

Month	Avg. Density (x10^3)	Mean pH	Temperature (^{o}C)	Water level (feet)	Turbidity (NTU)	Dissolved O_2 (mg/L)
January	0.17	6.96±0.07	27.5	9	3	12
February	0.33	7.21±0.09	28	9.5	2	11
March	0.08	7.34±0.02	28	9.5	2	11.5
April	0.25	7.78±0.09	30	8	2.5	10
May	0.08	7.09±0.18	30.5	5	5	10
June	0.17	8.36±0.09	31	1	6	8.5
July	0.92	8.35±0.11	31	-1	7	7
August	2.08	8.35±0.12	33	-2	10	5.5
September	1.25	8.78±0.09	33	-1	12	4
October	0.42	7.10±0.23	31	-1.5	8	4.5

November	0.08	7.22±0.27	32	3	8.5	5
December	0.08	6.71±0.04	29	8	5	8

Chorus and Bartram (1999) stated that when temperature, light and nutrient status are favorable, surface water may host increased growth of cyanobacteria. The period from July to September was a dry spell and the temperature was also slightly high, favoring the proliferation of cyanobacteria. Toxic cyanobacteria produce cyanobacterial toxins like microcystin during high growth and metabolism (Ye, et al., 2009). Therefore, measures should be taken to reduce the cyanobacterial population or completely eliminate them during this period.

Table 6: Temporal dynamics of physiochemical parameters of water in Nachchaduwa wewa

Month	Avg. Density ($x10^3$)	Mean pH	Temperature (^oC)	Water level feet	Turbidity (NTU)	Dissolved O_2 (mg/L)
January	0.25	6.88±0.12	27	10	5	11
February	0.25	7.10±0.09	27.4	9.5	3	12
March	0.17	7.20±0.09	28	9.5	2	11.5
April	0.08	8.04±0.16	29	8	7	10
May	0.17	7.03±0.13	29	5	8	10
June	0.42	8.65±0.11	30	1	10	8.5
July	1.50	8.41±0.20	31	-3	12	6.5
August	2.92	8.36±0.13	33	-5	18	5
September	2.08	8.89±0.08	33	-1	12	4.5
October	0.83	7.59±0.30	31	2	10	4
November	0.17	7.01±0.13	32	4	9	5
December	0.08	6.44±0.16	30	9	6	7

Morphological identification of cyanobacteria

In most of the eutrophic and hypertrophic lakes, *Microcystis*, *Planktothrix* and *Anabaena* are the major microcystin producing cyanobacterial genera (Carmichael, 1994; Carmichael and Falconer, 1993; Chorus and Bartram, 1999, Fleming and Stephan, 2001and Ye., *et al.*, 2009). According to the standard identification keys, many of the isolates recovered in this study were identified as *Chroococcidiopsis* and *Cylindrospermopsis* isolates through microscopic observations. Magana-Arachchi and Wanigatunge (2013) also revealed the prevalence of the genus *Chroococcidiopsis* in waters in the dry zone of Sri Lanka.

Molecular identification of toxic cyanobacteria

The classical method for examination of cyanobacterial taxonomic composition is based on the use of an inverted microscopic analysis of cyanobacterial samples (Bukowska *et. al*., 2014). However, it is

impossible to distinguish potentially toxic and non-toxic strains of cyanobacteria solely based on phenotypic traits. Therefore, use of molecular methods becomes indispensable for this purpose. Molecular methods are now widely used in environmental studies and they support the assessment of taxonomic composition of cyanobacteria and also the identification of toxic cyanobacteria that possess *mcy*-gene cluster in their genome (Bukowska *et al*., 2014). Therefore, DNA amplification of 16S rRNA gene and *mcy*A gene was performed using PCR to identify toxic cyanobacteria at molecular level.

The results revealed that most of the isolates recovered from Nuwara wewa and Nachchaduwa wewa produced PCR products of ~450 bp length with 16S rRNA gene specific primers (Figure 1). In a separate study, Magana-Arachchi and Wanigatunge (2013) also obtained a unique PCR product of similar size in a PCR reaction with cyanobacterial genomic DNA and cyanobacteria specific oligonucleotides as used in the present study. No band was observed for the isolate NUW1 with *Cya*RA + *Cya*F primers though all the other primer combinations yielded either clear or little faint bands (Figure 1). This may be due to any mutation in the annealing site of the gene for these primer pairs or low concentration of DNA in the sample used for PCR.

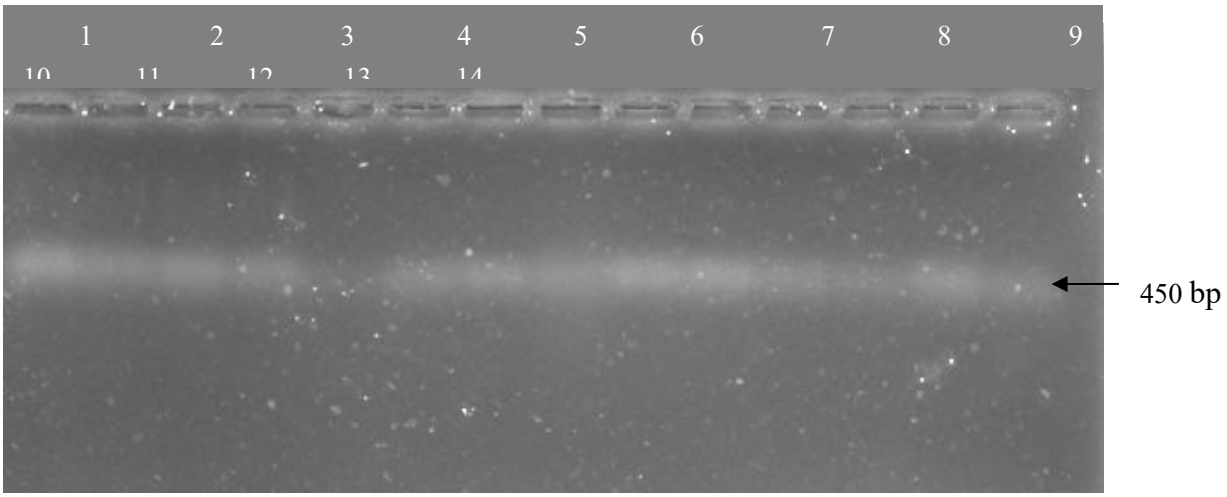

Figure 1: Agarose gel profile of PCR amplified products of the samples 1, 2, 3 and 11 with rRNA gene specific primers

Lanes 1-4: NUW 11, 5-8: NUW 01, 9-12: NUW 02, & 13-14: NUW 03

Lanes 1,5,9&13: CyaRA + CyaF; Lanes 2,6,10&14: CyaRB + CyaF; Lanes 3,7,&11: CyaRC + CyaF; Lanes 4,8&12: CyaRD + CyaF

Although several phycologists have used the 16S rRNA gene for the identification and phylogenetic studies of cyanobacteria, the identity of 16S rRNA sequence data is not sufficient for establishing species identity. Therefore, researches have increasingly moved into 16S-23S internal transcribed spacer (ITS) region (Boyer *et al*., 2001). The ITS is a fragment located between the 16S and 23S rRNA genes. It possess greater degree of sequence heterogeneity that 16S rRNA sequence and therefore permit many genera of

cyanobacteria to be distinguished (Janse *et al*., 2003). However, the present study was aimed at assessing the presence of toxic cyanobacteria and therefore, the 16S rRNA gene was targeted.

Many species of cyanobacteria can produce toxins such as hepatotoxins, neurotoxins and dermatotoxins. Fresh water cyanobacteria belonging to the genera Microcystis, Anabaena, Dolichospermus, Planktothrix and Nostoc produce hepatotoxins known as microcystins, which are the most common yanobacterial toxins in fresh waters (Sivonen and Jones, 1999). The aquatic cyanobacterial community can comprise both toxic and non-toxic strains of cyanobacteria. The presence of *mcy*-gene cluster correlates with the production of cyanobacterial toxins (Pearson and Neilan, 2008). The *mcy*-gene cluster contains seven genes such as *mcy*A, *mcy*B, *mcy*C, *mcy*D, *mcy*E, *mcy*G, and *mcy*I (Christiansen *et al*., 2008). Therefore, *mcy*A gene specific primers were used to check the presence of toxic cyanobacteria as the presence of this gene shows potential microcystin producers (Hisbergues *et al*., 2003).

In the present study, the isolates, which showed positive results in a PCR with 16S rRNA gene specific primers, were subjected to PCR with *mcy*A-gene specific primers. Only a few isolates (NUW 11, NUW 02 and NCH 09) revealed to produce a PCR product with a size of ~300 bp with *mcy*A-gene specific primers. Very faint bands were observed in the Agarose gel (gel photo is not included). This is in line with the finding that Hotto *et at*. (2007). They obtained *Microcystis mcy*A sequence with the size of 291 bp. Richardson *et at*. (2007) revealed in a separate study that direct sequencing of the *mcy*A gene using the *mcy*A-Cd 1F primer yielded partial sequences of 267 and 285 bp for *M. aeruginosa* and BBD *Leptolyngbya*, respectively. This clearly shows that the study reservoirs harbor a few strains of toxic cyanobacteria. However, denaturing gradient gel electrophoresis analysis of PCR amplified ITS regions with sequencing of the same and the mcyA gene are important for further assessment of the presence of toxic cyanobacteria and their identification to species level in these reservoirs. Moreover, proper mechanism should be implemented to get rid of the toxic cyanobacteria when their growth is elevated unsure cyanobacteria-toxin-free water for domestic use.

CONCLUSION

Cyanobacterial density had strong positive correlations with some physico-chemical parameters such as pH, temperature and dissolved oxygen in Nuwara wewa and Nachchaduwa wewa. These parameters can be used as good indicators to assess the status these reservoirs in terms of cyanobacterial density and distribution. Some cyanobacterial isolates recovered from both reservoirs harboured mcy-A gene, showing the potential for microcystin production. However, denaturing gradient gel electrophoresis analysis of PCR amplified ITS regions with sequencing of the PCR amplified product and the mcy-A gene are important to further assess the presence of toxic cyanobacteria and their identification to species level.

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