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Microcystins and cylindrospermopsins molecular markers for the detection of toxic cyanobacteria: a case study of northeastern Brazilian reservoirs

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ABSTRACT

Microcystins and cylindrospermopsins molecular markers for the detection of toxic cyanobacteria: a case study of northeastern Brazilian reservoirs

The presence of potentially toxic cyanobacteria in 10 northeastern Brazilian reservoirs used for public water supply was evaluated with molecular markers specific to genes of microcystins (MC) and cylindrospermopsins (CYN). The detection of the MC genes *mcy*A and *mcy*B was determined using 3 different primer pairs. Genes of CYN were assessed by PCR amplification of PKS and PS genes. Amplicons of the different cyanotoxin genes were subjected to sequencing. Genes coding for MC (*mcy*A and/or *mcy*B) were found in all of the analysed samples. Despite the presence of the *mcy*B gene and the subsequent immunological detection of MC, the absence of *mcy*A was observed in several samples. In general, the primers targeting the PKS gene were more specific, while those for the PS gene gave uneven PCR amplification. In addition, the detection of CYN was only possible when the samples were positive for both the PKS and the PS genes. These findings show that, when working with environmental samples, the use of molecular markers for the detection of potentially toxic cyanobacteria may generate variable results, and consideration should be given to possible phytogeographic genetic variations.

Key words: Early warning, genotype composition, peptide synthetase, public water supply, water monitoring.

RESUMEN

Marcadores moleculares de microcistinas y cilindrospermopsinas para la detección de cianobacterias tóxicas: un caso de estudio en embalses del nordeste brasileño

Se evaluó la presencia de cianobacterias potencialmente tóxicas en diez embalses del nordeste brasileño utilizados para el abastecimiento público por medio del uso de marcadores moleculares específicos para genes codificadores de microcistinas y cilindrospermopsinas. La detección de los genes mcy*A y* mcy*B que codifican microcistinas sintetasas se realizó usando tres pares de cebadores diferentes. Se evaluaron genes de cilindrospermopsinas mediante la amplificación por PCR de los genes de PKS y PS. Los amplicones de los diferentes genes de cianotoxinas fueron sometidos a secuenciación. Los genes que codifican las microcistinas (*mcy*A y/o* mcy*B) se encontraron en todas las muestras analizadas. A pesar de la presencia del gen* mcy*B y de la detección inmunológica posterior de microcistinas, se observó la ausencia del gen* mcy*A en algunas muestras. En general, los cebadores dirigidos a genes codificadores de PKS presentaron resultados más específicos, mientras que aquellos para el gen PS resultaron en amplificaciones por PCR irregulares. Además, la detección de cilindrospermopsinas sólo se mostró posible cuando las muestras fueron positivas tanto para genes codificadores de PKS como de PS. Estos resultados sugieren que cuando se trabaja con muestras ambientales, el uso de marcadores moleculares para la detección de cianobacterias potencialmente tóxicas puede presentar datos variables, y que se deben hacer consideraciones en cuanto a posibles variaciones genéticas fitogeográficas.*

Palabras clave: *Sistemas de alerta temprana, composición genotípica, péptido sintetasa, abastecimiento público, monitoreo del agua.*

INTRODUCTION

Northeastern Brazil has many reservoirs that provide water to this region during extended periods of drought. In addition to supplying drinking water, most of these reservoirs are important for meeting the irrigation, fishing, ranching and bathing needs of people living in and around their catchments (Bouvy *et al.*, 2000). However, nutrient-enriched conditions, coupled with high water temperatures and extended water residence times, have resulted in excessive proliferation of potentially toxic cyanobacteria in these water bodies (Bittencourt-Oliveira *et al.*, 2011, 2012, 2014; Molica *et al.*, 2002; 2005). Among the toxins detected in these reservoirs are the hepatotoxins microcystins (MC) and cylindrospermopsins (CYN) (Bittencourt-Oliveira *et al.*, 2014).

MC are cyclic heptapeptides synthesized non-ribosomally by multifunctional enzymes that include polypeptide synthetase (PS) and polyketide synthase (PKS) modules (Nishizawa *et al.*, 1999; Tillett *et al.*, 2000). Over 80 structural variants of MC have been isolated and characterized to date that differ in the type of amino acids incorporated into the MC or by modifications to the peptide backbone (Dittmann *et al.*, 2013; Kaasalainen *et al.*, 2012; Meriluoto & Spoof, 2008; Sivonen, 2009; Welker & von Döhren, 2006). This group of toxins is encoded by the MC synthetase (*mcy*) gene cluster, which spans 55 kb of DNA (Rouhiainen *et al.*, 2004; Tillett *et al.*, 2000). Similarly, CYN are synthesized on large, modular, non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) enzyme complexes (Dittmann *et al.*, 2013). The *cyr* gene cluster for CYN biosynthesis contains 43 kb encoding 15 open-reading frames (*cyr*A-O) (Mihali *et al.*, 2008).

Due to the incident in Caruaru, in Pernambuco State, in 1996, in which the deaths of 76 patients at a dialysis centre were attributed to exposure to water contaminated with MC (Azevedo *et al.*, 2002; Jochimsen *et al.*, 1998), specific regulation (Brasil, 2011) for the control of cyanobacteria and cyanotoxins in water intended for public use was introduced in Brazil. Early detection of potentially toxic cyanobacterial blooms in these aquatic environments is of great interest in the monitoring of cyanobacteria as a public health risk.

Currently, many DNA-based detection methods, such as PCR and qPCR (quantitative real time PCR), that use primers specific to the genes involved in cyanotoxins biosynthesis have been widely employed. Thus, the detection of potentially toxic cyanobacteria can be achieved by amplifying several genetic markers, such as genes encoding for MC (Baker *et al.*, 2001, 2002; Fewer *et al.*, 2007, 2008; Hisbergues *et al.*, 2003; Neilan *et al.*, 1999; Nishizawa *et al.*, 1999; Nonneman & Zimba, 2002; Pan *et al.*, 2002; Pearson & Neilan, 2008; Rantala *et al.*, 2004; Tillett *et al.*, 2001), CYN (Kellmann *et al.*, 2006; Mihali *et al.*, 2008; Schembri *et al.*, 2001), saxitoxins (Kellmann *et al.*, 2008; Smith *et al.*, 2011), anatoxin-a and homoanatoxin-a (Méjean *et al.*, 2009), and nodularin (Kruger *et al.*, 2009).

Because testing efforts are geared toward the prediction of the occurrence of potentially toxic cyanobacterial blooms, the choice of the molecular marker is important. A number of studies using more than one genetic marker for isolated strains and/or environmental samples have shown that it is possible to find one or more of the *mcy* genes absent (Bittencourt-Oliveira *et al.*, 2012; Glowacka *et al.*, 2011; Mankiewicz-Boczek *et al.*, 2006; Mbedi *et al.*, 2005). Although DNA-based approaches for cyanotoxin detection are increasing, genetic diversity, notably of uncultured cyanobacteria, is rarely taken into account when designing PCR detection methods (Fewer *et al.*, 2009). This is crucial and should be considered when testing for the presence of toxin-producing cyanobateria in environmental samples. The primary aims of this study, therefore, were to: (1) evaluate the applicability of specific primers for detecting MC –(*mcy*A and *myc*B) and CYN– (PKS and PS) toxin genes in natural cyanobacterial populations of northeastern Brazilian reservoirs, and to (2) determine whether there was a correlation between the molecular detection of the cyanotoxin genes and their presence in environmental samples.

MATERIALS AND METHODS

Site description and sampling

Water samples were collected from the Alagoinha (08°27′31.9″S; 36°46′33.5″W); Arcoverde (08°33′32.5″S; 36°59′07.5″W); Carpina (07°53′03.8″S; 35°20′37.8″W); Duas Unas (08°05′02″S; 35°02′30.6″W); Ingazeira (08°36′41.2″S; 36°54′23.7″W); Ipojuca (08°20′43.7″S; 36°22′31.5″W); Jucazinho (07°59′00″S; 35°48′30″W); Mundaú (08°57′21.1″S; 36°30′07.3″W); Tapacurá (08°02′32.2″S; 35°11′46.7″W), and Venturosa (8°34′41.7″S; 36°52′46.1″W) reservoirs in northeastern Brazil. These water bodies are located in different phytogeographic regions, with rainfall regimes ranging from regular (Zona da Mata, Agreste) to sparse (Sertão) and an average annual temperature of 37 °C. Important characteristics of these reservoirs, such as types of use, phytogeographic region specification, water capacity (m^3), maximum depth (m), target community and trophic state, can be found in Bittencourt-Oliveira *et al.* (2014). Surface water samples were collected from each of the reservoirs in 2 different seasons (rainy and dry) (Bittencourt-Oliveira *et al.*, 2014) using van Dorn bottles, which were packed in ice in cooling boxes and transported to the laboratory. The Jucazinho reservoir was sampled 4 times (Feb 17, Mar 24, Apr 28 and Oct 27, 2009), and the Tapacurá reservoir was sampled 3 times (Mar 10, May 04 and Oct 05, 2009). Samples were collected twice from the Alagoinha (Apr 14 and Oct 13, 2009), Arco Verde (May 10 and Nov 30, 2009), Carpina (Apr 06 and Oct 06, 2009), Ingazeira (Apr 14 and Oct 13, 2009), and Mundaú (Mar 17 and Nov 09, 2009) reservoirs. The Duas Unas, Ipojuca and Venturosa reservoirs were sampled once, on Mar 10, Nov 10, and Oct 13, 2009, respectively.

DNA extraction and PCR amplification

Total genomic DNA was extracted from living cells using a cetyltrimethyl-ammonium bromide (CTAB)-based extraction method (Rogers & Bendich, 1985), as described previously in Bittencourt-Oliveira *et al.* (2012). PCR amplification was performed using 2 independent DNA extractions taken from environmental samples, with 2 independent PCR reactions conducted per extracted DNA. To investigate the distribution of MC synthetase genes from water samples, the *mcy*A and *mcy*B genes were chosen. The 2 genes were amplified using the specific oligonucleotide primers *mcy*A-Cd/FR (297 bp) for *mcy*A (Hisbergues *et al.*, 2003), and the primer sets *mcy*B-F/R-A (315 bp) and *mcy*B-F/R-B (195 bp) for *mcy*B (Bittencourt-Oliveira *et al.*, 2012). Amplification of partial CYN PKS and PS genes was performed using the primer pairs M4/M5 (650 bp) and M13/M14 (597 bp), respectively (Schembri *et al.*, 2001). PCR reactions were performed using 10 ng of DNA and the pureTaq Ready-To-Go PCR Beads kit (GE Healthcare, Fairfield, CT, USA) in a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The following cycling parameters were used for the *mcy*B gene: 94 °C for 2 min, 35 cycles at 94 °C for 10 s, 50 °C for 20 s, and 72 °C for 1 min, followed by final extension at 72 °C for 5 min. The thermocycling conditions we used for the *mcy*A gene followed those outlined by Hisbergues *et al.* (2003), and for the CYN-genes were in accordance to those specified by Schembri *et al.* (2001). Negative controls were prepared using the same reaction conditions and primers, but without DNA. Amplicons were analysed on 0.7 % agarose gels stained with ethidium bromide (0.2 μg/mL) after electrophoresis in 1 × TBE running buffer. Gels were viewed on an Electrophoresis Documentation and Analysis System 290 (EDAS 290) (Kodak, Melville, NY, USA) and recorded. PCR products were purified using the Purelink Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions.

Sequencing

PCR fragments of MC (*mcy*A and *mcy*B) and CYN (PKS and PS) genes were directly sequenced according to Bittencourt-Oliveira *et al.* (2012) with forward and reverse primers, using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Pittsburgh, PA, USA)

and a 3100 ABI Sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were checked by visual inspection using the BioEdit routine version 7.0.9.0 (Hall, 1999) and analysed by a similarity search using BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990). All sequence data were stored for nucleotide sequences at the GenBank database.

Cyanotoxins analyses

Toxins analyses were performed on lyophilized cells collected from water samples, the results of which were published in Bittencourt-Oliveira *et al.* (2014). This information was used to verify the reliability of the molecular data. MC and CYN detection was performed using commercial ELISA kits (Beacon Analytical Systems Inc., Portland, ME, USA), following the manufacturer's protocols. The ELISA method has been extensively used for MC detection in field and laboratory samples, and the results correlate ($R = 0.96$; $p < 1 \cdot 10^{-10}$) well with those obtained using HPLC-DAD, which is the traditional method used for the detection of this toxin (Babica *et al.*, 2006; Metcalf *et al.*, 2000). Similarly, a 100 % correlation between the ELISA and LC/MS methods has been ob-

Table 1. Comparison of PCR amplification of the *mcy*A and *mcy*B genes, and the presence of microcystins as determined by the ELISA assay. *Comparación entre la amplificación por PCR de los genes* mcy*A y* mcy*B, y la presencia de microcistinas determinada por el ensayo ELISA.*

Reservoir	Sampling date (2009)	PCR products (%)												ELISA assay[a]
		*mcy*A-Cd/FR		*mcy*B-F/R-A					*mcy*B-F/R-B					
		+	–	+	–	*	**	#	+	–	*	**	#	
Alagoinha	Apr 14	100[b]	.	100[e]	.	.	.	.	75	.	25	.	.	+
	Oct 13	75	25	75	.	25	.	.	.	.	100	.	.	N.A.
Arcoverde	May 10	50	50	50	.	.	.	50	25	.	.	25	50	N.A.
	Nov 30	100	.	100[f]	.	.	.	.	100	.	.	.	.	N.A.
Carpina	Apr 06	.	100	.	.	.	75	25	.	50	.	.	50	+
	Oct 06	.	100	.	.	.	.	100	.	.	.	.	100	+
Duas Unas	Mar 10	.	100	50	.	50	.	.	.	.	.	.	100	+
Ingazeira	Apr 14	100	.	.	.	50	50	.	100	.	.	.	.	+
	Oct 13	100[c]	.	100[g]	.	.	.	.	.	.	100	.	.	+
Ipojuca	Nov 10	.	100	100	.	.	.	.	50	.	25	.	25	N.A.
Jucazinho	Feb 17	50	50	.	.	100	.	.	.	.	.	.	100	+
	Mar 24	.	100	50	50	.	.	.	.	25	.	.	75	+
	Apr 28	.	100	.	.	.	.	100	.	100	.	.	.	+
	Oct 27	.	100	25	.	50	.	.	.	.	.	.	100	+
Mundaú	Mar 17	100	.	100[h]	.	.	.	.	.	.	100	.	.	+
	Nov 09	.	100	100	.	.	.	.	.	.	.	.	100	+
Tapacurá	Mar 10	.	100	50	.	.	50	.	.	.	.	.	100	+
	May 04	75	25	100	.	.	.	.	50	.	.	.	50	+
	Oct 05	25[d]	75	100	.	.	.	.	.	.	.	.	100	+
Venturosa	Oct 13	.	100	100	.	.	.	.	.	.	.	.	100	+

[a] Bittencourt-Oliveira *et al.* (2014). (+) Positive; (–) Negative; (*) Positive with other bands, but there is an identification of the expected band based on band intensity and base pairs (bp) number; (**) Positive with other bands, but there is an identification of the expected band based only on bp; (#) Multiple bands, but the expected band could not be detected; (.) Not applicable; (N.A.) Not analyzed. [b] Accession number: KJ883531; [c] Accession number: KJ883524; [d] Accession number: KJ883530; [e] Accession number: KJ883525; [f] Accession number: KJ883526; [g] Accession number: KJ883529; [h] Accession number: KJ883527.

served for CYN detection in laboratory samples, whereas with environmental samples, the ELISA method gave a tenfold concentration of that detected by LC/MS method (Bláhová *et al*., 2009).

Data analyses

Data obtained from the detection of different molecular markers for MC and CYN were subjected to homogeneity of variance with the Levene's test and analysis of variance (ANOVA) using a repeated general linear model (GML). In the presence of significant interactions between the primers and PCR product types, slicing was employed across the occurrence of the following PCR products: positive (single band), negative (no band), positive with many bands but identifiable using band intensity and number of base pairs, positive with many bands but identifiable with only the number of base pairs, and many bands with no detection. The correlation between the detected molecular markers for MC and CYN and the presence of these toxins in the reservoirs was determined using a correlation-based principal component analysis (PCA). All analyses were performed using a 5 % significance level. ANOVA was performed using the SAS version 9.2 (Cary, NC, USA) software for Windows,

Table 2. The distribution of PKS and PS genes (PCR amplification), and cylindrospermopsin production in cyanobacterial populations from northeastern Brazilian reservoirs. *Distribución de genes de PKS y PS (amplificación por PCR), y la producción de cilindrospermopsinas en poblaciones de cianobacterias de los embalses del nordeste brasileño.*

Reservoir	Sampling date (2009)	PCR products (%)										ELISA assay[a]
		M4/M5					M13/M14					
		+	–	*	**	#	+	–	*	**	#	
Alagoinha	Apr 14	50	50	.	.	.	.	.	50	25	25	–
	Oct 13	.	100	.	.	.	.	.	.	.	100	–
Arcoverde	May 10	75	.	25	.	.	.	.	.	.	100	N.A
	Nov 30	.	100	.	.	.	.	.	.	.	100	N.A.
Carpina	Apr 06	.	100	.	.	.	.	25	.	.	75	–
	Oct 06	.	100	.	.	.	.	25	.	.	75	–
Duas Unas	Mar 10	100	.	.	.	.	.	.	.	.	100	–
Ingazeira	Apr 14	.	100	.	.	.	.	.	.	.	100	–
	Oct 13	.	.	75	25	.	.	.	.	.	100	–
Ipojuca	Nov 10	100	.	.	.	.	.	.	.	.	100	N.A.
Jucazinho[b]	Feb 17	100[c]	.	.	.	.	.	.	100[f]	.	.	+
	Mar 24	100[d]	.	.	.	.	.	.	100[g]	.	.	+
	Apr 28	.	.	100[e]	.	.	.	.	100[h]	.	.	+
	Oct 27	.	.	75	25	.	.	.	100	.	.	+
Mundaú	Mar 17	100	.	.	.	.	50	.	50	.	.	–
	Nov 09	.	50	50	.	.	.	.	50	.	50	–
Tapacurá	Mar 10	50	.	50	.	.	.	.	50	.	50	–
	May 04	50	50	.	.	.	50	25	.	.	25	–
	Oct 05	.	100	.	.	.	.	.	.	.	100	-
Venturosa	Oct 13	.	50	.	50	.	.	25	.	.	75	–

[a] Bittencourt-Oliveira *et al*. (2014). [b] Bittencourt-Oliveira *et al*. (2011). (+) Positive; (–) Negative; (*) Positive with other bands, but there is an identification of the expected band based on band intensity and base pairs (bp) number; (**) Positive with other bands, but there is an identification of the expected band based only on bp; (#) Multiple bands, but the expected band could not be detected; (.) Not applicable; (N.A.) Not analyzed. [c] Accession number: JF930684; [d] Accession number: JF930683; [e] Accession number: JF930685; [f] Accession number: JF930687; [g] Accession number: JF930686; [h] Accession number: JF930688.

while PCA was performed using Statistica 8.0 (Stat Soft. Inc., Tulsa, OK, USA) software.

RESULTS

The distribution of genes involved in the biosynthesis of MC and CYN was evaluated in 20 samples from 10 reservoirs located in the northeastern region of Brazil (Tables 1 and 2). The presence of potential MC-producing cyanobacteria was detected by PCR amplification of the *mcy*A gene using the oligonucleotide primers *mcy*A-Cd/FR, while that of the *mcy*B gene was performed with the primer sets *mcy*B-F/R-A and *mcy*B-F/R-B. Both genes were simultaneously detected in 6 of the 10 reservoirs investigated (Table 1). However, detection was only recorded for samples collected on Feb 17 in the Jacuzinho reservoir, Mar 17 in the Mundaú reservoir, and May 04 and Oct 25 in the Tapacurá reservoir; while the *mcy*A gene was not detected in the samples taken from the Carpina (Apr 06 and Oct 06), Duas Unas (Mar 10), Ipojuca (Nov 10), Jucazinho (Mar 24, Apr 28 and Oct 27), Mundaú (Nov 09), Tapacurá (Mar 10) and Venturosa (Oct 13) reservoirs. Although MC were detected in Carpina (Apr 06 and Oct 06), Duas Unas (Mar 10), Jucazinho (Mar 24, Apr 28 and Oct 27), Mundaú (Nov 09), Tapacurá (Mar 10) and Venturosa (Oct 13) samples, the *mcy*A gene was absent in their respective samples. Comparing the sets of primers used to target the *mcy*B gene, the *mcy*B-F/R-A primers appeared to be more useful than the *mcy*B-F/R-B primers because the former returned positives for the samples from Alagoinha (Apr 14), Ingazeira (Oct 13), Mundaú (Mar 17 and Nov 09), Tapacurá (May 04 and Oct 05) and Venturosa (Oct 13) reservoirs, in agreement with the ELISA results, whereas the latter showed multiple bands for Carpina (Oct 06), Duas Unas (Mar 10), Jucazinho (Feb 17 and Oct 27), Mundaú (Nov 09), Tapacurá (Mar 10, Oct 05), and Venturosa (Oct 13). Therefore, the expected band could not be reliably detected with the *mcy*B-F/R-B primers. Sequenced PCR products for *mcy*A and *mcy*B gene fragments were very similar (95 % to 100 % identity, 100 % coverage) to published sequences of MC-producing *Microcystis* spp. (AJ515459, AJ515460, AJ515456, EU009869, EU009866, AJ492552, HQ852449, EU009868, AJ492554, JX965438, HQ852443, AF183408, AY147796) (Table 1). Interestingly, the sample collected from the Alagoinha reservoir (Apr 14) that was amplified using the primer set *mcy*B-F/R-B matched (95 % identity, 100 % coverage) MC synthetase C (*mcy*C gene, EU009885 and EU009886) sequences that were published in Tooming-Klunderud *et al.* (2008). Moreover, the BLAST analy-

Table 3. ANOVA summary results for different microcystin and cylindrospermopsin molecular markers used for cyanobacteria from different reservoirs in northeastern Brazil. *Resumen de los resultados del ANOVA para diferentes marcadores moleculares de microcistinas y cilindrospermopsinas utilizados para cianobacterias en diferentes embalses de la región nordeste de Brasil.*

Factor	Cyanotoxins			
	Microcystins		Cylindrospermopsins	
	F value	*p* value	F value	*p* value
Model	7.89	0.00	9.11	0.00
Primers	0.23	0.80	0.17	0.68
PCR Product type	16.58	0.00	4.42	0.00
Primers vs PCR Product type	5.52	0.00	16.08	0.00
Positive (+)	9.82	0.00	10.84	0.00
Negative (–)	0.55	0.58	13.60	0.00
Many bands with intense band and base pairs detection (*)	0.15	0.86	1.41	0.24
Many bands with base pairs only detection (**)	0.32	0.73	0.16	0.69
Many bands with no detection (#)	11.23	0.00	38.32	0.00

sis also revealed slightly lower similarities (94 % identity, 100 % coverage) with *mcy*B gene sequences, but matched uncultured *Microcystis* (Jiang *et al*., 2013).

Potential CYN-producing cyanobacteria were analysed by PCR amplification of the PKS and PS genes using the oligonucleotide primers M4/M5 and M13/M14, respectively. The primer pair M4/M5 appeared to be more specific than the M13/M14 because the PCR product was less attributable to non-specific bands, and the expected PCR product could be assigned based on band intensity and number of base pairs (bp) (Table 2). The detection of single bands, several bands and no bands was found to be significant, thereby confirming the advantage of the M4/M5 primers over the M13/M14 primers (Table 3). Although PCR fragments could be associated with the PKS gene for samples taken from the Alagoinha (Apr 14), Duas Unas (Mar 10), Mundaú (Mar 17) and Tapacurá (Mar 10 and May 04) reservoirs, no CYN production was detected by the ELISA test. However, correlation between CYN production and the presence of both genes in all of the samples collected from the Jucazinho reservoir was previously reported by Bittencourt-Oliveira *et al*. (2012). Sequenced PCR fragments of partial PKS and PS genes in 3 of the water samples collected from this reservoir (Feb 17, Mar 24, and Apr 28, 2009) were shown to have identical sequences to the respective genes. The BLAST sequence analyses performed for the PKS gene revealed a very high degree of similarity (100 % identity, 100 %

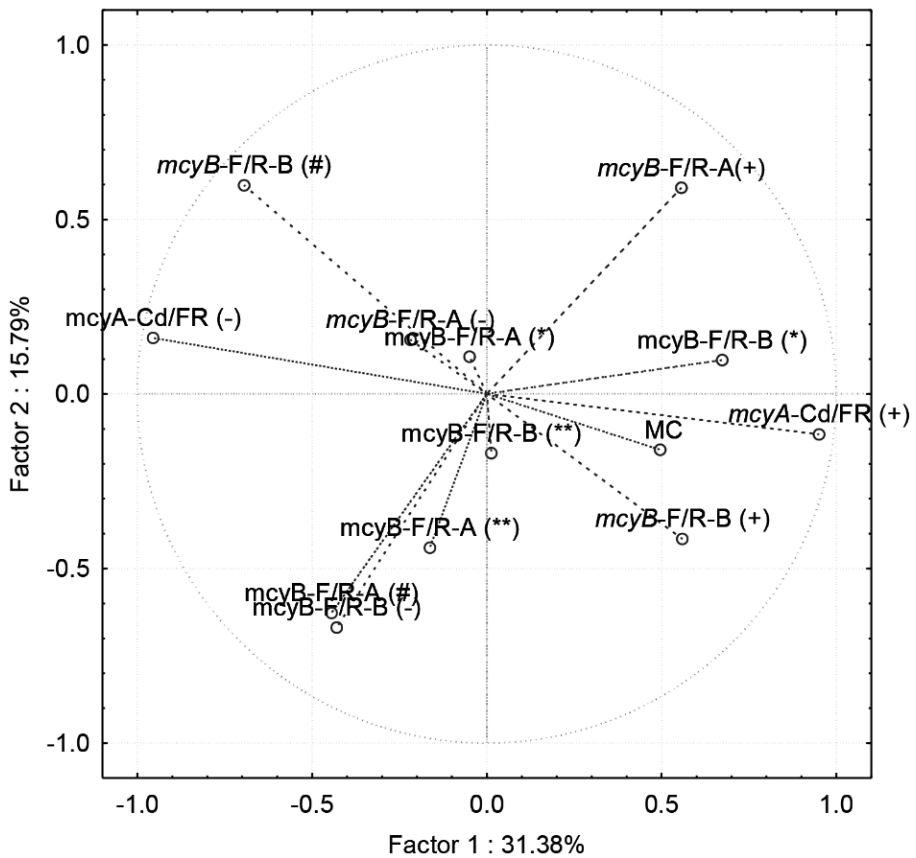

Figure 1. PCA biplot showing the association between the detection of microcystin molecular markers using *mcy*A-Cd/FR, *mcy*B-F/R-A and *mcy*B-F/R-B primers and its presence in different reservoirs in northeastern Brazil. *Biplot PCA que muestra la asociación entre la detección de marcadores moleculares de microcistinas, utilizando los cebadores* mcy*A-Cd/FR,* mcy*B-F/R-A y* mcy*B-F/R-B, y su presencia en diferentes embalses de la región nordeste de Brasil.*

coverage) with toxic strains of *Aphanizomenon ovalisporum* (EU076461 and AF395828). Sequences of PS gene from this same genus have matched to the PS sequences from the Jucazinho reservoir (EU076460 and AF395828) with 100 % identity (100 % coverage) (Bittencourt-Oliveira *et al.*, 2012). In the case of the primer pair M13/M14, multiple bands were generated as demonstrated in Table 2, and the expected band could not be detected in most of the analysed reservoirs except Jucazinho.

Principal component analysis (PCA) revealed a significant correlation between the production of MC in the reservoirs and the detection of molecular markers using *mcy*A-Cd/FR, *mcy*B-F/R-A and *mcyB*-F/R-B primers (Fig. 1). The PCA showed that the first 3 principal components accounted for over 46 % of the total variation. The presence of CYN was significantly associated with the detection of its molecular marker using the M4/M5 primer pair (Fig. 2). However, detection with the M13/M14 primer pair combination only showed a significant positive correlation with the presence of CYN when it was detected in the presence of several other bands. The first 2 principal components of the PCA were responsible for over 50 % of the total variation. As expected, the detection of no bands and many bands with no identifiable band for MC and CYN molecular markers showed significant negative association with the presence of either cyanotoxins, respectively.

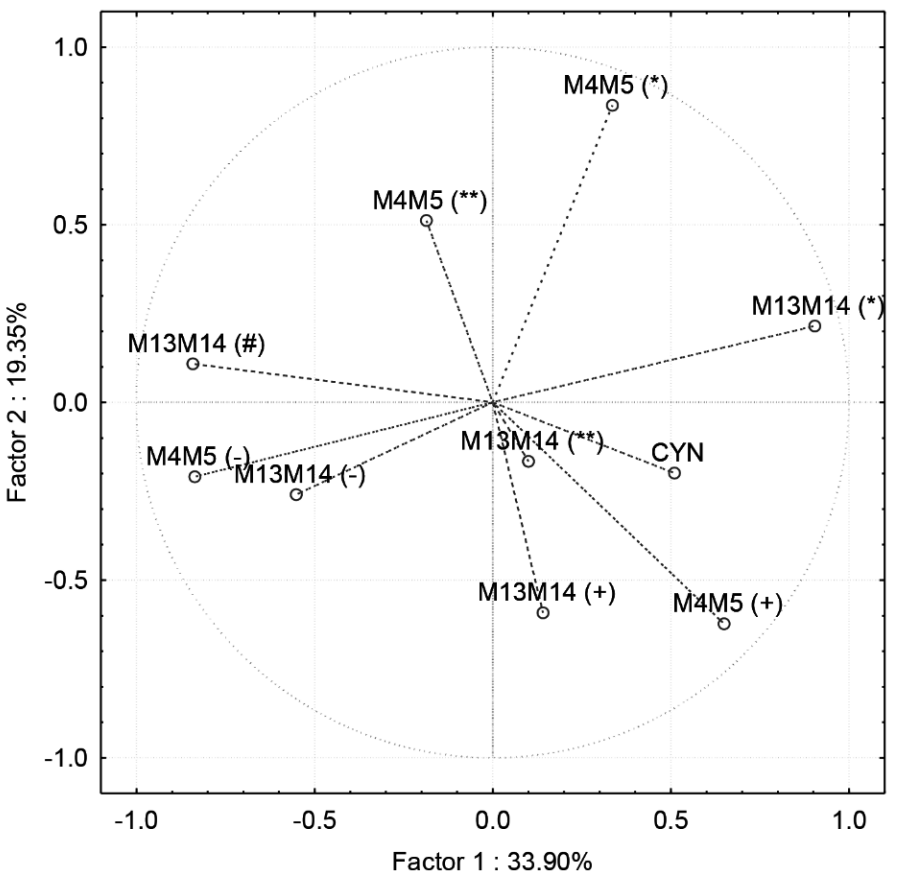

Figure 2. PCA biplot showing the relationship between the detection of cylindrospermopsin molecular markers using M4/M5 and M13/M14 primers and its presence in different reservoirs in northeastern Brazil. *Biplot PCA que muestra la relación entre la detección de marcadores moleculares de cilindrospermopsinas, utilizando los cebadores M4/M5 y M13/M14, y su presencia en diferentes embalses de la región nordeste de Brasil.*

DISCUSSION

Due to the health risks associated with MC and CYN toxins, their presence in reservoirs used mainly for public water supply in the northeastern region of Brazil was evaluated using molecular markers. Despite the positive results for the presence of MC, the lack of PCR amplification of the *mcy*A gene could be observed in 3 of the 10 studied reservoirs. The absence of *mcy*A gene amplification most likely occurred as result of a partially deleted *mcy* operon (Kim *et al*., 2010; Tooming-Klunderud *et al*., 2008) that caused faulty PCR results. The *mcy* genes are diverse, with very few universal priming sites (Fewer *et al*., 2009), which may sometimes present variations in natural cyanobacterial populations and affect the usability of some primers. The *mcy* regions with the highest site variation are the *mcy*A (Kurmayer *et al*., 2005; Tanabe *et al*., 2004) and *mcy*B (Kurmayer & Gumpenberger, 2006; Mikalsen *et al*., 2003), which may result in non-amplification of target genes due to non-annealing of the oligonucleotide primers (Kurmayer *et al*., 2005; Mbedi *et al*., 2005).

Natural variations in the cyanobacterial *mcy*B gene may explain why the primer pair *mcy*B-F/R-A outperformed the *mcy*B-F/R-B, as the former were more specific to the detection of MC-producing cyanobacteria in the reservoirs. This variation affected the annealing potential of the oligonucleotide primers used in this study (Bittencourt-Oliveira *et al*., 2012). It is important to note that single PCR products were produced in most samples amplified using the primers *mcy*B-F/R-A, which also had good correlation with detected MC data, as shown by the PCA results. In addition, the ANOVA results showed that the tendency to produce single-band PCR products by this primer pair was significant.

The undesired results produced by the primer set *mcy*B-F/R-B that targeted *mcy*C gene sequences may have been caused by recombination. Multiple recombination events giving rise to a 'phylogenetic mosaic' between the *mcy*B and *mcy*C regions encoding adenylation domains in modules McyB1 and McyC have been reported previously (Tooming-Klunderud *et al*., 2008). These results suggest that the genetic diversity of MC synthetase genes from natural cyanobacteria should be taken into account when designing methods to detect MC-producing cyanobacteria. This genetic variation is supported by the diversity of potential microcystins producers (*Plankthothrix agardhii*, *Microcystis aeruginosa*, *M. panniformis, Merismopedia tenuissima*) in these reservoirs, as reported by Bittencourt-Oliveira *et al*. (2014).

To evaluate the distribution of genes involved in CYN biosynthesis, the PKS and PS genes were PCR amplified. The PKS and PS genetic components have been described by Schembri *et al*. (2001) and assigned to subsequences located within the *aoa*C and *aoa*B genes, respectively (Rasmussen *et al*., 2008; Shalev-Alon *et al*., 2002). In the present study, the best PCR results were obtained with the primers set for the *aoa*C (PKS) gene, while the primers for the *aoa*B (PS) gene gave poor/patchy/and no PCR products and in most cases made the identification of the expected band impossible. These results imply that *aoa*B gene sequences from the northeastern Brazilian population differ slightly from those of CYN-producing populations found in other geographic regions. The absence of *aoa*B (PS) gene amplification suggests that this sequence was probably absent or slightly different in the cyanobacterial population investigated in this study, which would explain the positive correlation of poor or no amplification of the PS gene with the absence of CYN in most of the reservoirs. The high sequence identity found for the *aoa*B and *aoa*C genes in this study when compared with those reported by Yilmaz *et al*. (2008) suggests that PCR amplification was specific for CYN-producing cyanobacteria. CYN-producing cyanobacteria must possess the gene homologues of *aoa*A, *aoa*B and *aoa*C (*cyr*A, *cyr*B and *cyr*C, respectively –Mihali *et al*., 2008) for the production of the toxin to be possible (Rasmussen *et al*., 2008). Thus, the absence of any of the genes will result in the lack of PCR-amplification for it and the cyanobacterium/cyanobacteria will most likely be non-toxic. Therefore, the patchy distribution or no amplification of either *aoa*B (PS) or *aoa*C (PKS) molecular markers observed

in this study implies that the cyanobacterial population was not toxic, which correlated with the absence of the toxin in most of the reservoirs we investigated. However, Ballot *et al*. (2011) reported that non-CYN-producing strains of *A. ovalisporum* and *A. bergii* collected from Israel's Lake Kinneret contained *aoa*A/*cyr*A, *aoa*B/*cyr*B and *aoa*C/*cyr*C segments of the CYN gene cluster, but lacked the *cyr*J gene. The *cyr*J gene is only present in the genome of CYN-producing cyanobacteria (Mihali *et al*., 2008) and is therefore considered to be a good molecular marker. Although the *cyr*A gene has been associated with false positive results (Ballot *et al*. 2011), the rates of misidentification for *cyr*A and *cyr*J were similar (Kokociński *et al*., 2013). Based on the results of the present study together with those of other studies, further research is required to clarify this discrepancy. In addition, the detection of both *aoa*B (PS) and *aoa*C (PKS) molecular markers in the samples taken from the Jucazinho reservoir correlated with the presence of CYN in the reservoir.

In conclusion, our results show that potential toxin-producing cyanobacteria occur widely in the northeastern reservoirs of Brazil, as demonstrated by the distribution of genes involved in the biosynthesis of MC and CYN. Our data suggest that the primer sets *mcy*B-F/R-A and M4/M5 targeting the *mcy*B and *aoa*C (PKS) genes can be useful for the detection of potential MC and CYN producers, respectively, in aquatic environments in Brazil. The detection of the molecular markers using these primers showed a significant positive correlation with the presence of the toxins in the reservoirs, as confirmed by the PCA results. In addition, the tendency for the primers used in this study to produce particular PCR products was also significant, suggesting that molecular markers should be employed in tandem and that possible genetic variability that may occur in natural cyanobacterial populations of different regions be taken into consideration when selecting molecular markers. Finally, special attention should be given to the *mcy* genes because they presented high variability.

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AUTHOR CONTRIBUTIONS

Piccin-Santos, V. and Bittencourt-Oliveira, M.C. designed and performed the experiments. Lorenzi, A.S. and Chia, M.A. analysed the results/data. Piccin-Santos, V., Bittencourt-Oliveira, M.C., Lorenzi, A.S. and Chia, M.A. wrote the manuscript.

ABBREVIATIONS

MC, microcystins;

CYN, cylindrospermopsins;

PKS, polyketide synthase;

PS, peptide synthetase.

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