

Cylindrospermopsin in Water Supply Reservoirs in Brazil Determined by Immunochemical and Molecular Methods

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Abstract

It is reported for the first time in Brazil and South America the presence of cylindrospermopsin (CYN) in water supply reservoirs. CYN is a powerful hepatotoxic alkaloid implicated in outbreaks of human sicknesses. We detected CYN in different sources of water in Northeastern Brazil using molecular and immunological techniques. The highest concentrations of toxin occurred in the Jucazinho reservoir with the phytoplankton containing the potentially CYN-producing *Cylindrospermopsis raciborskii* and *Sphaerospermopsis aphanizomenoides* (previously known as *Aphanizomenon aphanizomenoides*). The polyketide synthase (PKS) and peptide synthetase (PS), which are directly related to the ability to produce CYN, were found in all the analyzed samples. The result of the present study emphasizes the need to improve monitoring of CYN in water bodies used for drinking and recreation, in order to avoid exposure of human populations to this toxin.

Keywords: *Aphanizomenon*, Cyanobacteria, *Cylindrospermopsis raciborskii*, CYN, *Sphaerospermopsis aphanizomenoides*, Toxin

1. Introduction

CYN, a powerful hepatotoxin, was responsible for a human intoxication occurred in Australia in 1979 after water ingestion from a reservoir. This *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju bloom was treated with an algaecide releasing the intracellular toxin [1,2].

CYN also affects kidneys, intestinal tract, thymic system, vascular system and muscles [3-6]. At low doses, CYN suppresses protein synthesis, perhaps through the inhibition of the ribosomal translation via binding to protein associated with the eukaryotic protein synthesis system [7]. There is also evidence that this toxin has genotoxic, carcinogenic and mutagenic effects [8]. Moreover, there are reports of its accumulation in organisms indicating a possible pathway for human exposure to the cyanotoxin [9-14].

Brazil has a history of human and mammal contamination by microcystin, another hepatotoxin, with tenths of patients deaths at a dialysis center [15,16]. Carmichael

et al. [10] has pointed out a possible CYN presence in liver samples of patients exposed in this outbreak [16]. However, because of the limited sample availability such a possibility has never been confirmed. On the other hand, there are not to date records for CYN occurrence in water bodies.

Brazilian legislation for water supply monitoring to the human population only recommends an upper limit of 15 µg. L⁻¹ for CYN [17]. Little attention was paid to this issue because CYN has never been recorded before in the country and South America. CYN production has been reported in others cyanobacteria [18-21]. When toxic, isolated Brazilian strains of *C. raciborskii* and *Aphanizomenon* spp, have been found to produce saxitoxins (SAX) [22-25].

No other previous study found CYN in public water supplies in Brazil. In view of this, it was our goal to investigate if CYN occurs in Brazil. The present paper reports the presence of CYN in public water supplies for the first time in Brazil and South America. A molecular method, based on detection of genes encoding polyketide

synthase (PKS) and peptide synthetase (PS) enzymes, plus immunoassay analysis (ELISA), were used to confirm the presence of this toxin. This finding highlights the importance of monitoring water quality with respect to CYN in public water supplies.

2. Material and Methods

2.1. Study Area

The Jucazinho, Duas Unas and Arcoverde reservoirs are located in Northeastern Brazil, supplying water for more than one million inhabitants. The climate of the region is low-latitude, semi-arid, with a mean annual temperature of 25°C, mean wind speed of 5.0 m. s⁻¹ and mean annual precipitation of 599 mm. There are two distinct seasons – one rainy (March to August) and the other dry (September to February).

The Jucazinho reservoir belongs to the *Agreste* region (transition between the moist coastal/rainforest zone and the semi-arid deep interior of the state) (08°56'41.1"S and 36°29'32.20"W) (**Figure 1(a)**), with a volume of 327,035,818 m³. Intensive breeding of “tilapia” (*Oreochromis niloticus* Linnaeus) has been carried out at this location, increasing thus cyanobacterial blooms. The Duas Unas reservoir (**Figure 1(b)**) (08°05'31"S and 35°02'19"W) is located in the coastal/rainforest zone and has an accumulation capacity of 28,548,500 m³. There are vast areas of sugarcane cultivation in its hydrographic basin, along with strips of native preserved Atlantic rainforest. The Arcoverde reservoir (08°33'33"S and 36°59'07"W) (**Figure 1(c)**) is located in the semi-arid

region and has a maximal capacity of 16,800,000 m³ in an area of 200 hectares.

2.2. Environmental Samples

Collections were carried out in 2009 at the Jucazinho (Feb 17, Mar 24, Apr 28 and Oct 27), Duas Unas (May 4) and Arcoverde (May 12) reservoirs, thereby totaling six samples. Samples for toxicological analyses and taxonomic identification were gathered from the surface of the reservoirs using a 20 µm mesh plankton net in the littoral zone. For quantitative analyses, water aliquots were collected using a van Dorn bottle and preserved in acetic Lugol's solution. Environmental samples were immediately concentrated by centrifugation, lyophilized and stored at -20°C until processing.

2.3. Identification and Quantification of Phytoplankton

Taxonomic identification was performed using a light microscope (Nikon YS100, Melville, NY, USA). Cyanobacteria were photographed with a light microscope (Nikon E200, Melville, NY, USA) equipped with a video camera system (Samsung SCC833, Tokyo, Japan) using the software Imagelab (Softium, São Paulo, SP, Brazil). Cell densities were determined using the Utermöhl method [26] expressed in cell. mL⁻¹ and in percentage. In order to express results for the species as percentage the number of cells of each taxon was multiplied by 100 and then divided by the total number of cells in

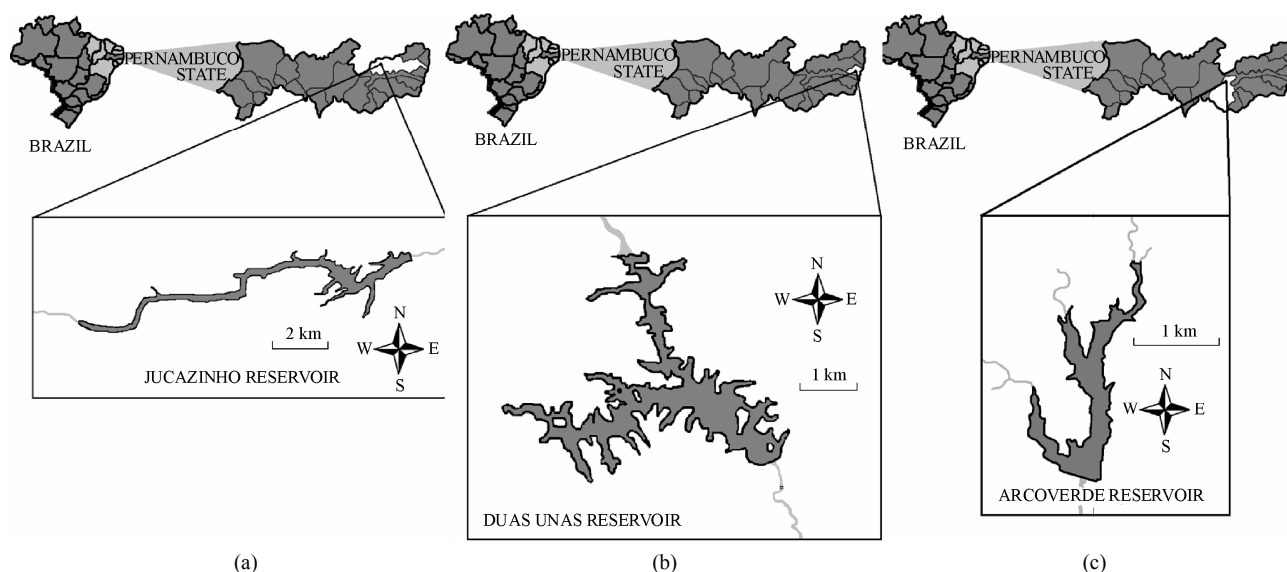


Figure 1. Location of reservoirs sampled in northeastern Brazil; (a) Jucazinho reservoir; (b) Duas Unas reservoir; (c) Arcoverde reservoir.

the sample.

2.4. DNA Extraction, Amplification and Sequencing

DNA was extracted from living cells from the environmental samples, according with [27]. For PCR amplification of partial PKS and PS genetic determinants, the oligonucleotide primer pairs (M4/M5 and M13/M14) described in [20] respectively, were used. Amplification of PKS (*aoaC* gene) and PS (*aoaB* gene) (650 to 725 bp) was performed using a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) and the pureTaq Ready-To-Go PCR Beads kit (GE Healthcare, Fairfield, CT, USA) according with [20]. Negative controls were carried out by using the same reaction conditions and primers, but without DNA. PCR reactions were performed at least in duplicate. Amplification products were visualized through electrophoresis on 1% agarose gels stained with ethidium bromide ($0.2 \mu\text{g. mL}^{-1}$). PCR products were purified using the Pure-link Kit (Invitrogen, Carlsbad, CA, USA), following manufacturer's instructions.

Amplified fragments from the environmental samples were directly sequenced using the forward and reverse primers with the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Pittsburgh, PA, USA) in the 3100 ABI Sequencer (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. The sequences were checked by visual inspection using the BioEdit routine version 7.0.9.0 [28], analyzed by a similarity search using BLAST (Basic Local Alignment Search Tool) and stored for nucleotide sequences at the GenBank database.

2.5. Cyindrospermopsin Analysis by Immunoassay Method

The lyophilized cells from environmental samples were broken by sonication and used directly for analysis of CYN. Toxin quantification was carried out using a commercial ELISA kit (Beacon Analytical Systems Inc., Portland, ME, USA), following the manufacturer's protocols. The detection limit for CYN by ELISA was 0.1 ppb. The absorbance was measured using an ELISA plate reader (Asys Expert Plus, Cambs, UK) at a wavelength of 450 nm. The negative and positive controls of ELISA analysis are included in the commercial kit.

3. Results

Our results demonstrated the presence of CYN in envi-

ronmental samples from Jucazinho, Duas Unas and Arcoverde reservoirs (Table 1). The highest concentrations of CYN occurred in the Jucazinho reservoir ($2,718.0 \text{ ng.g}^{-1}$ freeze-dried cells). The phytoplankton community contains the potentially CYN-producing species *Sphaerospermopsis aphanizomenoides* (previously denominated *Aphanizomenon aphanizomenoides* Forti) (Figure 2 (a)-(b)) and *C. raciborskii* (straight and coiled) (Figure 2 (c)-(e)). It was not possible to determine which was the CYN-producing cyanobacteria.

In all of the three studied reservoirs the phytoplankton community was constituted nearly exclusively by potentially toxin-producing cyanobacteria (Table 2, Figure 2), with percentages ranging from 99.2 to 100%.

All samples were positive for the PKS (*aoaC* gene), and PS (*aoaB* gene), which are directly related to the ability to produce CYN (Figure 3). The partial PKS (457 - 521 bp) and PS (406 - 505 bp) nucleotide sequences of three environmental samples from the Jucazinho reservoir (Feb 17, 2009, Mar 24, 2009 and Apr 28, 2009) were identical. The partial sequences of the PS shared 100% identity with published PS from *Aphanizomenon ovalisporum* Forti (accession number EU076460 and AF395828), and PKS (*aoaC* gene) shared 100% identity with *Aph. ovalisporum* (EU076461 and AF395828) in the GenBank database.

4. Discussion

Falconer & Humpage [29] and [30] mentioned CYN presence in South America only vaguely in articles abstracts, without proving any kind of published literature, or data set, all along the text. In 2006, Falconer & Humpage [31] stated that (sic) "CYN identified in water

Table 1. Concentrations of CYN (ng. g^{-1} freeze-dried cells) in the environmental samples obtained by ELISA (mean of three replicates); (+) presence of molecular markers. PS. peptide synthetase. PKS. polyketide synthase.

Reservoir	Sample	CYN	Molecular markers	
			PKS (M4/M5)	PS (M13/M14)
Jucazinho	Feb 17, 2009	76.3 (± 10.7)	+ ^a	+ ^d
	Mar 24, 2009	2,718.0 (± 279.0)	+ ^b	+ ^e
	Apr 28, 2009	201.4 (± 90.0)	+ ^c	+ ^f
	Oct 27, 2009	0.5 (± 0.1)	+	+
Duas Unas	May 4, 2009	143.0 (± 14.0)	+	+
Arcoverde	May 12, 2009	33.3 (± 4.0)	+	+

^aAccession number: JF930684; ^bAccession number: JF930683; ^cAccession number: JF930685; ^dAccession number: JF930687; ^eAccession number: JF930686; ^fAccession number: JF930688.

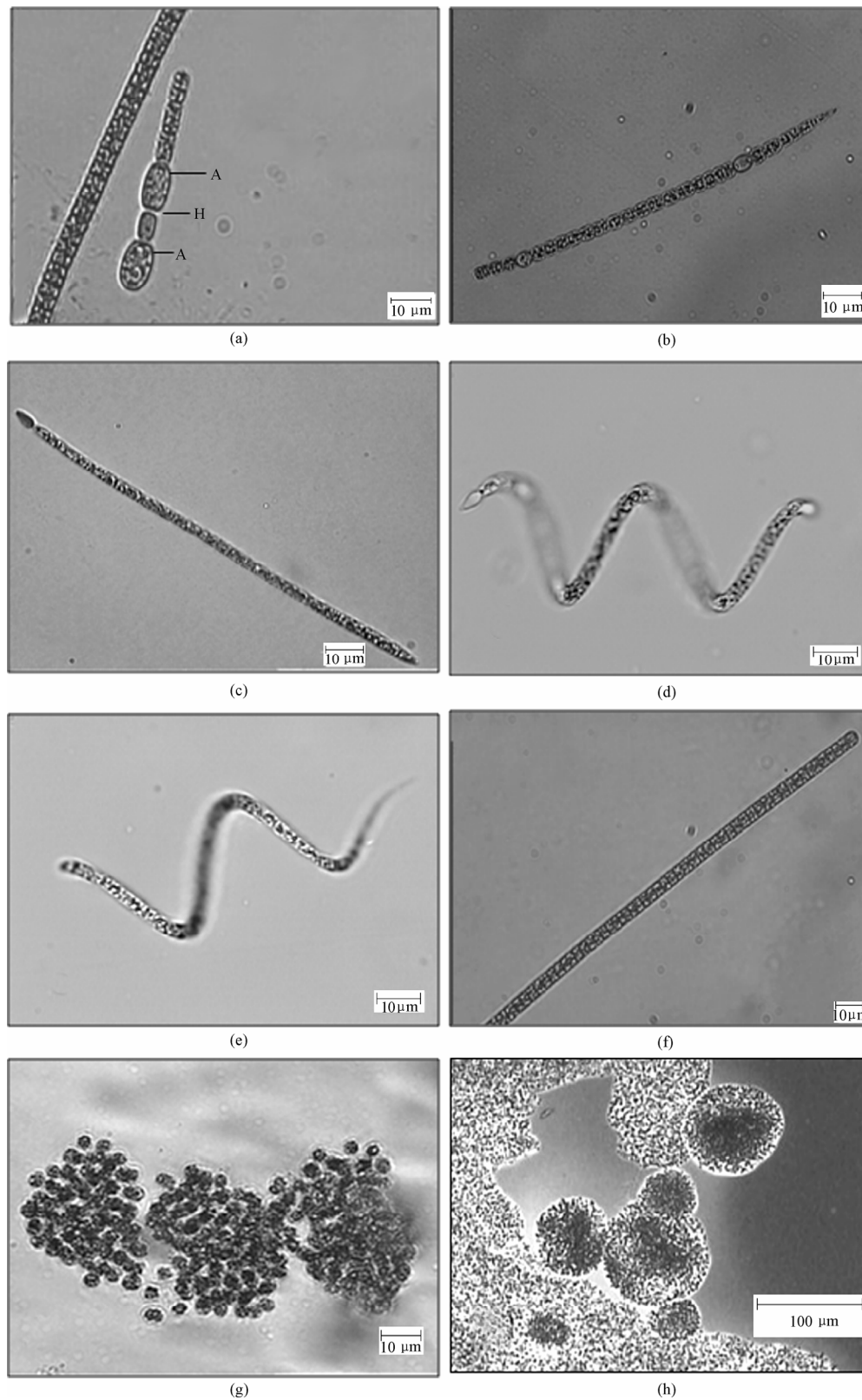


Figure 2. Bloom-forming and potential toxin-producing cyanobacteria found in samples; (a)-(b) *S. aphanizomenoides*; c. *C. raciborskii* (straight); d-e. *C. raciborskii* (coiled); f. *Planktothrix agardhii*; g. *M. novacekii*, h. *M. panniformis*. Jucazinho Reservoir: a-e, Duas Unas reservoir: f-h. A: akinete; H: heterocyte.

Table 2. Diversity and density of cyanobacteria and microalgae (10^6 cell. mL^{-1}); corresponding percentages of cell. mL^{-1} in phytoplankton are in parentheses; *n*: mean number of cells per organism; (*) Potential cylindrospermopsin-producing; (**) Potential microcystin-producing; (▼) Potential saxitoxin-producing; *Sphaerospermopsis aphanizomenoides* (*n* = 40); *Cylindrospermopsis raciborskii* (straight) (*n* = 20); *C. raciborskii* (coiled) (*n* = 25); *Geitlerinema amphibium* (*n* = 60); *Planktothrix agardhii* (*n* = 100); *Pseudanabaena catenata* (*n* = 20); *Merismopedia tenuissima* (*n* = 10); *Microcystis* spp.: *M. panniformis* and *M. novacekii*.

Taxa	Jucazinho				Duas Unas	Arcoverde
	Feb 17, 2009	Mar 24, 2009	Apr 28, 2009	Oct 27, 2009	May 4, 2009	May 12, 2009
*▼ <i>S. aphanizomenoides</i>	19.5 (36.5%)	28.5 (14.6%)	29.3 (6.5%)	2.2 (2.9%)	0	0
*▼ <i>C. raciborskii</i> (straight)	4.1 (7.7%)	5.7 (2.9%)	6.9 (1.5%)	4.2 (5.5%)	28.8 (0.1%)	95.7 (77.5%)
*▼ <i>C. raciborskii</i> (coiled)	4.3 (8.1%)	9.5 (4.9%)	10.5 (2.3%)	0.5 (0.6%)	0.0%	0.8 (0.6%)
** <i>P. agardhii</i>	22.0 (41.3%)	101.6 (52.1%)	333.4 (74.4%)	61.9 (81.5%)	0.0%	0.0%
** <i>Microcystis</i> spp.	0.0%	0.0%	0.0%	0.0%	3356.7 (99.1%)	0.0%
<i>G. amphibium</i>	0.2 (0.4%)	41.2 (21.1%)	63.7 (14.2%)	6.9 (9.0%)	0.0%	24.2 (19.6%)
<i>P. catenata</i>	3.1 (5.8%)	8.4 (4.3%)	4.2 (1.0%)	0.2 (0.3%)	0.0%	1.0 (0.8%)
<i>M. tenuissima</i>	0.0%	0.0%	0.0%	0.0%	0.0%	1.9 (1.5%)
Microalgae	0.2%	0.1%	0.1%	0.2%	0.8%	0.0%

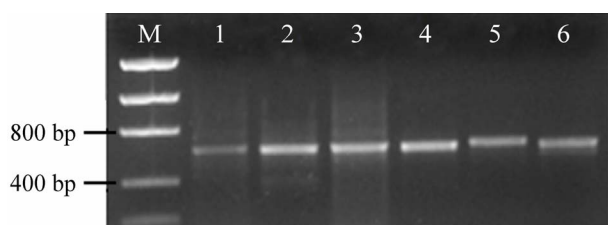


Figure 3. Presence of cylindrospermopsin biosynthesis genes in environmental samples by PCR amplification from Jucazinho reservoir. M. Molecular marker (Low DNA Mass™); Lanes 1, 3 and 5: PCR products for partial PKS genes (M4/M5 primers); Lanes 2, 4 and 6: PCR products for partial PS genes (M13/14 primers); Lanes 1-2: Feb 17, 2009 sample; Lanes 3-4: Mar 24, 2009 sample; Lanes 5-6: Apr 28, 2009 sample. Ethidium bromide-stained 1% agarose gel.

in Brazilian drinking water reservoir has not yet been attributed to any particular species of cyanobacterium”, without mentioning even a single reference of literature, or by simply showing a new finding.

We have detected CYN in cyanobacterial blooms in three reservoirs in Northeastern Brazil belonging to geographic basins located at different phyto-geographic areas (coastal/forest zone, *Agreste* and semi-arid region). However, these hydrographic basins are not isolated from each other, which can explain the dispersion of the toxin and of its producing organisms.

The PKS and PS genetic determinants are located within the *aoaC* and *aoaB* genes, respectively [32]. The genes encoding polyketide synthase (PKS) and peptide synthetase (PS) enzymes have been found to be related to the production of CYN. The presence of these genes in-

icates the ability to produce CYN [20].

The present findings show the 100% homology between sequences of PCR fragments amplified from DNA samples from Brazilian reservoirs and those from in the GenBank database for the *aoaB* and *aoaC* genes indicating that molecular method was specific to detect genes which are directly related to the production of CYN.

Our results report detection of CYN in water supply reservoirs in Brazil using molecular and immunological assays. Brazilian legislation imposes the monitoring of water bodies for public supply. This monitoring should involve density of cyanobacteria (cell. mL^{-1}) and an upper microcystin concentration of $1 \mu\text{g L}^{-1}$ in drinking water. This bylaw, on the other hand, only recommends a maximal limit of $15 \mu\text{g L}^{-1}$ for CYN [17], but this is not mandatory.

Because of the occurrence of CYN in water public supply, we stress the monitoring necessity of water bodies used for drinking and leisure, in order to avoid exposure of humans to this toxin.

5. Acknowledgments

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6. Abbreviations

CYN, cylindrospermopsin, PKS, polyketide synthase, PS, Peptide synthetase.

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