

Prevalence of paralytic shellfish poison-producing *Planktothrix agardhii* and *Cylindrospermopsis raciborskii* in a Brazilian semi-arid reservoir

Dominância de cianobactérias *Planktothrix agardhii* e *Cylindrospermopsis raciborskii*, produtoras de toxina paralisante de molusco, em reservatório na região semiárida brasileira

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Abstract: Aim: The present study aimed to investigate the composition and toxicity of the phytoplankton in Sítios Novos reservoir, used mainly as drinking water supply for approximately 30,000 people. **Methods:** Samples were collected between January 2010 and June 2011. **Results:** During this period 19 taxa of cyanobacteria and 22 of algae were identified. Out of 45 samples collected, algae accounted for no more than 10% of the quantified organisms in 44 samples. Cyanobacteria accounted for 100% of the organisms quantified in three samples and for 99% in other 29 samples. Among the cyanobacteria group, *Planktothrix agardhii* (Gomont) Anagnostidis & Komárek and *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya & Subbaraju prevailed and both strains were isolated and identified as paralytic shellfish poison (PSP) producers. *C. raciborskii* strain has shown to produce SXT and dcSXT while *P. agardhii* strain has shown to produce dcGTX2 or 3. **Conclusions:** To the author's knowledge, this is the first report of PSP-producer cyanobacteria species isolated in Northeastern Brazil and the first reported of a *P. agardhii* synthesizing dcGTX2/3.

Keywords: phytoplankton; saxitoxins; HPLC; ELISA; water quality.

Resumo: Objetivo: O presente estudo objetivou investigar a composição e toxicidade do fitoplâncton no açude Sítios Novos, usado como fonte de abastecimento para aproximadamente 30.000 pessoas, dentre outros usos. **Métodos:** As amostras foram coletadas mensalmente entre janeiro de 2010 e junho de 2011. **Resultados:** Durante esse período foram identificados 19 táxons de cianobactérias e 22 de algas. Das 45 amostragens realizadas, em 44 as algas não constituíram mais do que 10% dos organismos quantificados. As cianobactérias constituíram 100% dos organismos quantificados em três amostras e em outras 29 foram maiores que 99%. Dentre as espécies de cianobactérias, *Planktothrix agardhii* (Gomont) Anagnostidis & Komárek e *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya & Subbaraju dominaram. Ambas as espécies foram isoladas e identificadas como produtoras de toxina paralisante de molusco. A cepa de *C. raciborskii* mostrou-se produtora de STX e dcSTX, e a cepa de *P. agardhii* mostrou-se produtora de dcGTX2 ou 3. **Conclusões:** Ao conhecimento dos autores, este é o primeiro relato de espécies de cianobactérias produtoras de PSP isoladas no Nordeste Brasileiro além do primeiro relato da espécie *P. agardhii* como produtora de dcGTX 3/2.

Palavras-chave: fitoplâncton; saxitoxinas; HPLC; ELISA; qualidade da água.

1. Introduction

In the semi-arid Northeastern Brazil, due to irregular rainfall, climate and soil characteristics, the main sources of drinking water supply are the artificial surface reservoirs. Uncontrolled occupation and exploitation of natural resources on the hydrographic basins, alongside with high evaporation and solar radiation can have a negative impact on stored water quality. As a consequence, the reservoirs are prone to accelerated eutrophication, often leading to phytoplankton blooms, predominantly dominated by cyanobacteria. These microorganisms are of great concern since they have been shown to produce various metabolites, including taste and odor compounds and especially toxins.

One of the cyanobacterial toxins of concern is the paralytic shellfish poison (PSP), also known as saxitoxins. PSPs are carbamate alkaloids which prevent the communication between neuron and muscle cells by blocking sodium channels (Roset et al., 2001). In total, 57 analogues of PSPs have been identified, being subdivided into four groups: Carbamoyl, decarbamoyl, N-sulfocarbamoyl, and deoxydecarbamoyl toxins (Wiese et al., 2010). PSPs are globally widespread toxins, causing detrimental effects in public health and substantial losses in the aquaculture and fishing industries (Al-Tebrineh et al., 2010). In the U.S. alone harmful algal blooms have been estimated to cost at least \$ 82 million per year (Hoagland & Scatista, 2006).

According to Smith et al. (2011) cyanobacteria known as saxitoxin producer are *Dolichospermium circinalis* (formally described as *Anabaena circinalis*) in Australia (Humpage et al., 1994), *Cylindrospermopsis raciborskii* in Brazil (Lagos et al., 1999), *Lyngbya wollei* in North America (Yin et al., 1997; Camacho & Thacker, 2006), *Planktothrix* sp. in Italy (Pomati et al., 2000), *Aphanizomenon* spp. in Portugal (Ferreira et al., 2001), North America (Mahmood & Carmichael, 1986) and in China (Liu et al., 2006).

Therefore, public health agencies have major concerns regarding the presence of these compounds in human water supplies. In Brazil, since the episode of several renal patients' deaths reported by Azevedo et al. (2002), the Brazilian Federal Ministry of Health established maximum allowable concentration for cyanotoxins in the drinking water potability standard. Currently, the Ordinance 2914 of 2011 (Brasil, 2011) establishes maximum concentration limits for microcystin and saxitoxins in drinking water of 3 $\mu\text{g}\cdot\text{L}^{-1}$ and 1 $\mu\text{g}\cdot\text{L}^{-1}$,

respectively. In the previous legislation regarding this matter (BRASIL, 2004), monitoring of saxitoxin was just recommended.

For that reason, it is extremely important to monitor phytoplankton communities in superficial reservoirs used for drinking water supply and to investigate the toxicity of these organisms. Consideration of the local phytoplankton communities is especially important in a country like Brazil with its large extension and climatic diversity, as dominant species might change with geographic position. The present study aimed to investigate the phytoplanktonic community in a eutrophic reservoir of multiple uses located in a semi-arid tropical region in order to identify and isolate the dominant cyanobacterial strains and identify the toxins produced by these organisms.

2. Material and Methods

2.1. Sampling location

Sítios Novos reservoir (Figure 1a) is located in the county of Caucaia, in the metropolitan region of Fortaleza, Ceará, Brazil. The reservoir is part of the integrated system of Sítios Novos, composed of a transposition channel, a water treatment plant (WTP) that produces drinking water for approximately 30,000 people, an irrigation project and a fish farm (Figure 1b) (COGERH, 2008). It is located in a watershed of 446 km² and has a hydraulic basin of 2010 ha. The weir (504.470E; 9.583.122N) stores water from São Gonçalo River, providing a maximum storage capacity of 126 million m³.

The region experiences a mean annual rainfall of about 950 mm concentrated from January to May, the temperature ranges between 26 and 30 °C and has an average annual evaporation rate of 959.5 mm (COGERH, 2008). The reservoir is used for watering animals, drinking water supply, primary and secondary contact recreation, irrigation, aquaculture, intensive farming, industry and agriculture ebb. As a result of this intensive use, the reservoir is classified as eutrophic.

2.2. Sampling procedure

For phytoplankton quantitative analysis, samples were collected fortnightly from a depth of 30 cm at the WTP intake, between January 2010 and June 2011. A volume of raw water (1000 mL) was collected in amber glass vials containing 5 mL of Lugol's solution for sample preservation. Subsequently, the samples were stored at 4 °C in the dark until use.

2.3. Cell enumeration

Before analysis, the samples were concentrated by sedimentation in a 1000 mL beaker for 24 h. The cell density was estimated using a Sedgewick-Rafter chamber and an inverted optical microscope (Zeiss Axio Vert.A1), calibrated according to APHA (2005). Counting was performed using tracks or fields, according to the Poisson distribution with a confidence interval of $95\% \pm 20\%$.

2.4. Isolation of cyanobacteria

For cyanobacteria isolation, samples of raw water (1000 mL) were collected, concentrated using a plankton net (20 μm mesh size), and stored in amber glass bottles at 4 °C. Cyanobacteria and algae were identified using classification keys based on the following bibliographies: Bicudo & Menezes (2006), Komárek & Anagnostidis (2005), and Sant'Anna et al. (2006). Measures of cell morphology, colony morphology, sheaths and mucilaginous envelopes helped in the identification, since some species are similar and cannot be differentiated by cell or colony characteristics alone.

The dominant and potentially PSP producing strains were isolated from the environmental samples using a glass capillary, slides, an inverted optical microscope (Zeiss Axio Vert.A1), and inoculated in ASM-1 culture medium (Gorham et al., 1964). The isolation procedure, adapted from Allen & Stanier (1968) and Ferris & Hirsch (1991), required at least two replicates of this separation methodology. To inhibit the growth of eukaryotic organisms, cyclohexamide was added to the medium at a final concentration of 70 $\mu\text{g}\cdot\text{mL}^{-1}$. The isolated strains were cultured in ASM-1 under continuous aeration and illumination at a light intensity of 50 $\mu\text{mol}\cdot\text{photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 24 ± 2 °C, for 2 weeks, until a concentration of approximately 10^7 cells $\cdot\text{mL}^{-1}$ was achieved.

2.5. Cyanotoxin extraction

To perform the extraction of intracellular toxins, the culture's biomass was concentrated by centrifugation at 2,700 G for 15 min (25 °C), the supernatant was discarded, and the pellet was collected. The pelleted material was subjected to three freeze-thaw cycles in order to promote cells lysis and release intracellular contents. After this

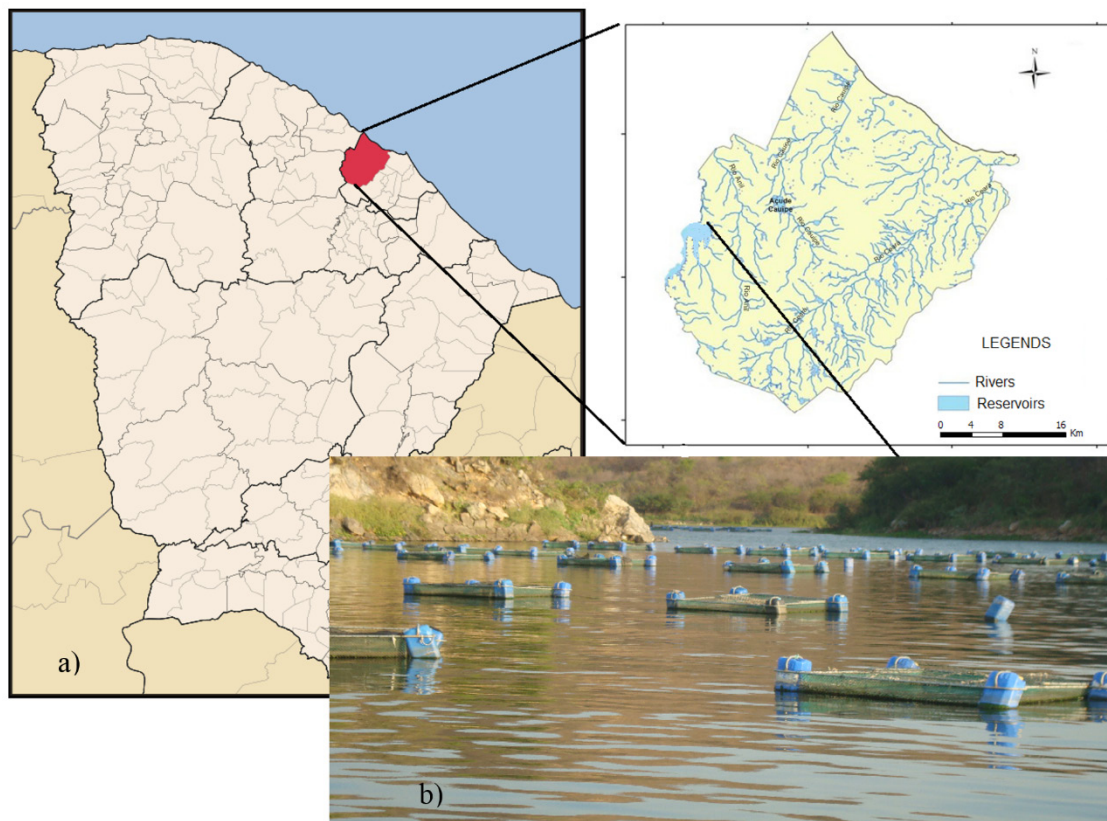


Figure 1. a) Geographic location of Sítios Novos reservoir in Caucaia, Ceará, Brazil; b) fish farming in Sítios Novos reservoir.

step, the material was filtered through a fiberglass membrane (0.45 μm ; Macherey-Nagel) and the filtrate was acidified with 0.1 M acetic acid until a pH of approximately 4.0 was achieved. This was done in order to guarantee the toxins' stability (Indrasena & Gill, 2000). The sample was then subjected to a pre-purification step by solid phase extraction (SPE) on C18 cartridges (Supelco). Separation of the different PSPs potentially present was performed by SPE with a 3mL carboxylic acid cartridges (Supelco), according to Lawrence et al. (2005).

2.6. Analysis of cyanotoxins

Prior to chromatographic analysis of the cyanobacterial pre-purified extracts, a cyanotoxin screening was conducted in triplicate by enzyme linked immuno sorbent assay (ELISA) using microplate kits (Abraxis®) for cylindrospermopsin, microcystin, and saxitoxin. Following this analyses, high performance liquid chromatography (HPLC) was performed using an Agilent 1260 equipped with quaternary pump, C18 chromatography column (250 X 4 mm, 5 μm) maintained at 30 °C in the thermostated column chamber, automatic mobile phases gradient controller, manual injection device with a loop of 20 μL , degasser and fluorescence detector (FLD) (excitation at 340 nm and emission at 390 nm). As mobile phase, a 0.05 M ammonium formate aqueous solution with 5% HPLC grade acetonitrile (phase A) and a 0.1 M ammonium formate aqueous solution (phase B)

with a total flow rate of 1.5 ml.min⁻¹ were applied. The process began with 100% mobile phase A. From 0 to 7.5 min, phase B increased from 0 to 20%. From 7.5 to 11 min, phase B increased from 20 to 80%, remaining unchanged until min 13. From 13 to 15 min, flow returned to 100% of A. The above methodology was adapted from Lawrence et al. (2005).

3. Results

Throughout the sampling period, 19 taxa of cyanobacteria (46% of the total organisms) and 22 taxa of algae were identified (Table 1). Algae were classified into five classes: Bacillariophyceae, Chlorophyceae, Cryptophyceae, Euglenophyceae and Zygnemaphyceae (Figure 2). The most representative algae class was Chlorophyceae (32%),

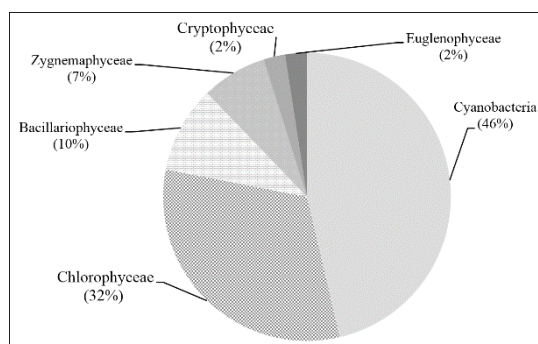


Figure 2. Distribution of phytoplankton taxonomic classes in Sítios Novos Reservoir (Ceará, Brazil) over the course of 18 months (from January 2010 to June 2011).

Table 1. Identified phytoplankton in Sítios Novos Reservoir (from January 2010 to June 2011).

CYANOBACTERIA	CHLOROPHYCEAE	BACILLARIOPHYCEAE
<i>Aphanizomenon</i> sp.	<i>Actinastrum</i> sp.	<i>Aulacoseira granulata</i>
<i>Anabaena</i> sp.	<i>Actinochloris sphaerica</i>	<i>Aulacoseira</i> sp.
<i>Aphanizomenon</i> sp.	<i>Ankyra</i> sp.	Bacillariophyta 1
Chroococcales 1	<i>Botryococcus</i> sp.	<i>Cyclotella</i> sp.
<i>Chroococcus</i> sp.	Chlorococcales 1	EUGLENOPHYCEAE
<i>Coelomoron</i> sp.	<i>Coelastrum</i> sp.	<i>Trachelomonas</i> sp.
<i>Cylindrospermopsis catemaco</i>	<i>Crucigenia</i> sp.	
<i>Cylindrospermopsis phillippinensis</i>	<i>Desmodesmus</i> sp.	
<i>Cylindrospermopsis raciborskii</i>	<i>Dictyosphaerium</i> sp.	
<i>Geitlerinema</i> sp.	<i>Micractinium</i> sp.	
<i>Lyngbya</i> sp.	<i>Monoraphidium contortum</i>	
<i>Merismopedia</i> sp.	<i>Oocystis</i> sp.	
<i>Microcystis aeruginosa</i>	<i>Scenedesmus</i> sp.	
Oscillatoriales 1	ZIGNEMAPHYCEAE	
<i>Planktolyngbia</i> sp.	<i>Closterium</i> sp.	
<i>Planktothrix agardhii</i>	Desmidiaceae 1	
<i>Planktothrix</i> sp.	<i>Staurastrum</i> sp.	
<i>Pseudanabaena</i> sp.	CRYPTOPHYCEAE	
<i>Raphidiopsis</i> sp.	<i>Cryptomonas</i> sp.	

followed by Bacillariophyceae (10%). From the total of 45 samples collected, algae accounted for no more than 10% of the quantified organisms in 44 samples. Cyanobacteria accounted for 100% of organisms quantified in three samples and 99% in other 29 samples.

Among the cyanobacteria group, *P. agardhii* and *C. raciborskii* were the most common species encountered. They were present during almost the entire sampling period (Figure 3), reaching up to 98% of the phytoplankton cell density. In eight other samples, their presence exceeded 80% of the organisms quantified in the reservoir.

P. agardhii abundance along the entire sampling period ranged from 0 to 5.83×10^5 cells.mL⁻¹, which accounted for 94% of the total phytoplankton abundance. The average density of *P. agardhii* was 7.54×10^4 cells.mL⁻¹. The *C. raciborskii* abundance in the same period ranged from 0 to 3.32×10^4 cell.mL⁻¹, accounting for 19% of total phytoplankton community. The average abundance of *C. raciborskii* was 8.21×10^3 cells.mL⁻¹.

Due to the predominance of *C. raciborskii* and *P. agardhii* and their potential to synthesize toxins (Williams et al., 2001; Hisbergues et al., 2003; Chonudomkul et al., 2004; Christiansen et al., 2006; Kosol et al., 2009; Manganelli et al., 2010), we decided to isolate them. The isolation attempts were successful for both species and they were named IRA02 (Figure 4a) and IRA07 (Figure 4b) respectively.

Five separate ELISA screenings were used to verify for the presence of microcystins, cylindrospermopsin, and saxitoxins. Saxitoxins were the only toxin identified in both isolated cyanobacterial species, therefore chromatographic analysis was directed to the PSP group. The production of SXT and dcSXT by IRA02 was detected by HPLC analysis, based on the retention times obtained using analytical standards (12.6 and 7.2 min respectively, Figure 5a). Furthermore, the fact that SXT demonstrates a more intense response with peroxide than with periodate oxidation and that dcSTX demonstrates a byproduct peak just after its own peak, both with periodate and with peroxide oxidation, were used to uniquely identify the two PSP analogues.

In the IRA07 extracts, no STX, NEO dcSTX, GTX or C-toxins were detected. However, one peak was observed at 2.86 min in the sample oxidized with peroxide (Figure 5b). The detected peak might represent dcGTX2 or dcGTX3 based on the peak detection sequence proposed by Lawrence et al. (2005).

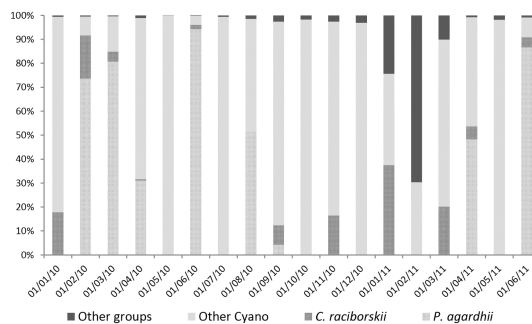


Figure 3. Distribution of phytoplankton groups in Sítios Novos Reservoir (Ceará, Brazil) from January 2010 to June 2011.

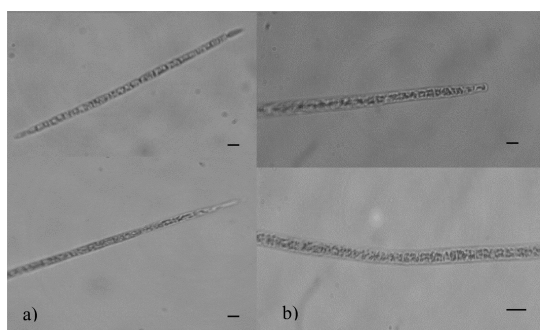


Figure 4. Isolated species: a) *Cylindrospermopsis raciborskii* (IRA02); b) *Planktothrix agardhii* (IRA07). Scale bar: 10 μ m.

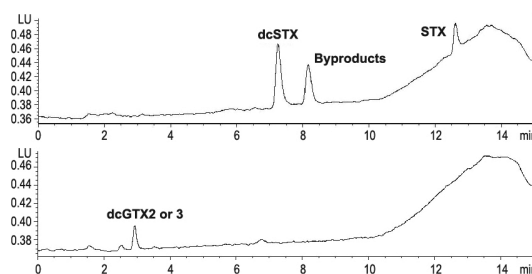


Figure 5. Chromatogram of a) *Cylindrospermopsis raciborskii* (IRA02) extract indicating the presence of STX and dcSTX and b) *Planktothrix agardhii* (IRA07) extract indicating the presence of dcGTX 2 or 3.

4. Discussion

The results of phytoplankton enumeration and identification demonstrate that the reservoir may be undergoing nutrient input above its carrying capacity as a result of intense aquaculture activity, for example. An internal report by the Water Resources Management Company of Ceará (COGERH, 2008) supports that theory, showing that Sítios Novos reservoir has been receiving twice as much phosphorus than its maximum nutrient carrying capacity of 9,553 kgP.a⁻¹. According

to Azevedo (1998), the increase in the number of cyanobacteria with concomitant decrease in phytoplankton diversity, is a good indicator of high eutrophication.

Cyanobacteria are widespread in reservoirs used for public supply in Brazil. Bittencourt-Oliveira et al. (2011), studying seven reservoirs in Northern and Southern Brazil, reported the presence of potentially toxic cyanobacteria in 21 out of 27 samples collected. Costa et al. (2006) noted the predominance of cyanobacteria at the expense of other phytoplankton groups in Ribeiro Gonçalves Reservoir, state of Rio Grande do Norte, accounting from 90 to 100% of the total phytoplankton density and with mixed blooms of *Aphanizomenon* sp., *C. raciborskii* and *Microcystis* sp.

Saker et al. (2003), in Portugal, reported concentrations of *C. raciborskii* greater than 3.00×10^6 cells.mL⁻¹, but as co-dominant species with other cyanobacteria, in most cases, *Aphanizomenon* sp., *Merismopedia* sp. or Oscillatoriales. Fuentes et al. (2010) also reported the dominance of *C. raciborskii* in Fausse Pointe and Dauterive lakes in Louisiana (USA) with concentrations greater than 1.60×10^4 cells.mL⁻¹.

Although *C. raciborskii* has been found less abundant than *P. agardhii* in this investigation, it may still present a high risk for consumer since it does not form surface blooms and its maximum densities occur at 2-3 m below the water surface (Saker & Griffiths, 2001). Based on that, it is possible that the sampling methodology may have led to an underestimation of the real abundance of *C. raciborskii* in Sítios Novos. Therefore, sampling procedures modifications should be considered, with consequent adjustment of the water intake depth and the implementation of additional barriers to the current WTP, such as activated carbon or membrane filtration.

In Sítios Novos, the population of *P. agardhii* persisted during almost every month of the year, behavior also reported by Sas (1989) and Hašler & Poulíčková (2003). It has been previously demonstrated that *P. agardhii* has the capability to be the dominant specie in a cyanobacterial bloom (Mankiewicz-Boczek et al., 2011). Considering the high average temperatures of Ceara's reservoirs (28 °C), however, the dominance of *P. agardhii* in detriment of *C. raciborskii*, contradicts the results obtained by Bonilla et al. (2012), who observed that *C. raciborskii* was dominant over *P. agardhii* at temperatures higher than 20 °C, in eutrophic and hypereutrophic waters of Brazil, Uruguay and

Hungary. Furthermore, higher growth rates of *C. raciborskii* at higher temperature (25 °C) and high light intensity ($135 \mu\text{mol.photons m}^{-2}.\text{s}^{-1}$) were also observed by Carneiro et al. (2009).

Smith (1983), states that nitrogen-fixing cyanobacteria, including *C. raciborskii*, dominate in aquatic systems with low N:P ratios. In the case of nitrogen abundance however, those organisms lose their competitive advantage. Bezerra et al. (2014), studying the same reservoir (Sítios Novos) from October 2010 to July 2011, identified N:P ratios from 8 to 14 indicating that the limiting factor may be, in fact, nitrogen. In spite of that, *C. raciborskii* did not show dominance over *P. agardhii* in our study and therefore, other factors rather than temperature or N:P ratio may be driving their relationship in Sítios Novos Reservoir.

Costa et al. (2006), studying a reservoir in the Brazilian semi-arid region, detected the presence of dissolved saxitoxins in the raw water with an average concentration of $3.14 \mu\text{g.L}^{-1}$. However, the isolation of the toxin-producer strain was not reported and the inference that *C. raciborskii* was to blame was based on the observation that the higher saxitoxin concentrations coincided with higher *C. raciborskii* cell densities.

P. agardhii has often been reported as a microcystin producer (Marie et al., 2012). Pomati et al. (2000) observed saxitoxin synthesis by *Planktothrix* sp isolated from Varese Lake, in Italy. Pomati's group was unable, however, to identify the PSP-producing organism to the species level. Therefore, we believe that this is the first time cyanobacteria species have been identified as PSP-producers in Northeastern Brazil and the first report in the literature of dcGTX producing *P. agardhii*.

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